STUDIES ON *IN VITRO* MORPHOGENETIC RESPONSE OF HORTICULTURAL CROP – CASHEW

A THESIS

SUBMITTED TO THE

UNIVERSITY OF PUNE

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IN

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BY

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dedicated to

my dear father……..

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(Shilpa S. Gogate)

CERTIFICATE

This is to certify that the work incorporated in this thesis titled **"Studies on *in vitro* morphogenetic response of horticultural crop – Cashew"** submitted by **Ms. Shilpa S. Gogate**, was carried out under my supervision at Tissue Culture Pilot Plant, National Chemical Laboratory, Pune. Such material, as has been obtained from other sources, has been duly acknowledged.

Pune
Date:

Dr. (Mrs.) R. S. Nadgauda
Research Guide

List of Abbreviations Used

Abbreviation	Full form
ABA	Abscissic acid
AC	Activated charcoal
Ad	Adenine
$AgNO_3$	*Silver nitrate*
ANOVA	Analysis of Variance
BA	Benzyl adenine
C 1 to C 9	Callus maintenance media
CH	Casein hydrolysate
CN	Cotyledonary node
CNSL	Cashewnut shell liquid
conc	Concentration
CW	Coconut water
DPX	DPX-4 1889 – (2 chloro-N-methoxy-6-methyl-1,3,5-triazin-2yl aminocarbonyl) benzene sulfonamide
°C	Degree Celsius
EDTA	Ethylene diamine tetra acetic acid
FAA	Formaldhyde: Acetic acid: Alcohol
Fig	Figure
GA_3	Gibberellic acid
g / l	Gram per litre
h	Hours
HCl	Hydrochloric acid
$HgCl_2$	*Mercuric chloride*
I 1 to I 11	Callus initiation media
IAA	Indole 3 acetic acid
IBA	Indole 3 butyric acid
IZE	Immature zygotic embryo
Kin	Kinetin
LSD	Least significant difference
mg	Milligram
mg/ l	Milligram per litre
mm	Millimetre
mM	Millimole
min	Minutes
MS	Murashige and Skoog
MZE	Mature zygotic embryo
MZEA	Mature zygotic embryo axis
N	Normal

NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
No.	Number
PEG	Polyethylene glycol
PGR	Plant growth regulator
rpm	Revolutions per minute
RT	Room temperature
s	Second
SD	Standard deviation
SH	Schenk and Hildebrandt
suc	Sucrose
TBA	Tertiary butyl alcohol
TDZ	Thidiazuron {1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea}
v/v	Volume by volume
WPM	Woody Plant Medium
w/ v	Weight by volume
Zea	Zeatin
2,4-D	2,4 - dichlorophenoxy acetic acid
2,4,5 - T	2,4, 5, trichlorophenoxy acetic acid
2ip	N^6-(3-methyl but-2-enyl) adenine
μE	Microeinstein
μm	Micrometer
μM	Micromole

ABSTRACT

Cashew (*Anacardium occidentale* L.) is an important cash crop of India, fetching foreign earnings. Cashewnut kernels are very popular dessert throughout the world. The corrosive viscous cashewnut shell liquid (CNSL) also finds commercial uses. India is a major cashew producing country and increasing attention is being given towards cultivation of this crop to reap maximum benefits.

Vegetative methods of propagation of the selected varieties are essential to retain the desirable traits. Vegetative methods presently being used for propagation of cashew are slow and unable to meet the existing demand for good quality planting material. Tissue culture technology can effectively supplement the conventional means of vegetative propagation in cashew, by being able to generate large number of planting material in short time. The micropropagation procedure could be further utilized in the future for genetic manipulation studies aimed at variety improvement.

Cashew is a woody tree species and has been recalcitrant to *in vitro* culture. Response has been obtained only from juvenile plant material. Exudation of phenolics in culture medium and presence of indigenous contaminants are the additional hurdles faced during *in vitro* culture. The work included in the present thesis titled “Studies on Morphogenetic response of Horticultural crop – Cashew” is an attempt to study the response of the different tissues of cashew to *in vitro* culture manipulations and, to develop a regeneration system through axillary bud proliferation and somatic embryogenesis. The objective of the study was to optimize the media and culture conditions to develop an efficient protocol of plantlet formation, which can be later utilized for improvement programs in this economically important nut- tree species.

THESIS ORGANIZATION

The thesis has been divided into five chapters followed by summary and bibliography.

Chapter 1: General Introduction

This chapter gives a general introduction about cashew (*Anacardium occidentale* L.), its position and importance in Indian horticulture, and the role of biotechnology in its improvement and propagation. The chapter includes a survey of tissue culture studies on cashew carried out over the years, giving more emphasis on axillary bud proliferation and somatic embryogenesis. It also includes the objectives with which the present studies were initiated and the approach with which the experiments have been planned and conducted.

Chapter 2: General Materials and Methods

In this chapter, general methodology followed throughout the course of study has been explained. The source of various chemicals, glassware, plasticware and other materials utilized during the course of work, glassware cleaning and preparation, basic media compositions and preparation, culture conditions etc. have been discussed. Materials and methodology related to specific experiments and studies have been included separately in chapters 3, 4 and 5.

The following chapters (3, 4 and 5) are further sub-divided into Introduction, specific Materials and Methods, Results and Discussion, Summary and Conclusions.

Chapter 3: Axillary bud proliferation in seedling tissue and studies on *in vitro* seed germination

Attempts to develop micropropagation protocol for this species using seedling tissues are discussed. To obtain seedling explants aseptically, a suitable method for *in*

vitro germination of cashew seeds was formulated. It was found that filter paper support partially immersed in distilled water was a convenient and effective substrate for *in vitro* germination of cashew seeds. Cotyledonary nodes and nodal explants of the *in vitro* germinated seedlings were then used for experiments.

Axillary shoot proliferation was obtained from cotyledonary nodes on MS medium containing BA. Response shown by the cotyledonary nodes was dependent on age of explant. Nodes collected from 4-5 weeks old seedlings produced maximum number of shoots. The optimal medium for shoot production from cotyledonary nodes was found to be Murashige and Skoog (MS) (full strength) + BA 10 μM + 3% sucrose. The shoots elongated on the shoot production medium itself. Liquid half strength MS medium with IAA 5 μM + IBA 2.5 μM + Kin 2.5 μM + sucrose 1% was optimal for *in vitro* root formation. Rooted shoots were transferred to greenhouse for hardening.

Attempts were made to induce axillary shoots from germinating seeds and intact seedlings by using BA in the germination medium. Cytokinin treatment was however, ineffective in inducing axillary shoots from seeds as well as intact seedlings. Seedlings grown in presence of high concentrations (250 and 500 μM) of BA showed a very poorly developed root and shoot system.

Chapter 4: Studies on embryogenic response of nucellar tissue

The importance of using nucellus as explant has been noted in the case of fruit trees. As it is a juvenile tissue, it is a valuable explant for recalcitrant species, where, plant regeneration is frequently obtained only from juvenile tissues. Being a maternal tissue, traits of the mother plant are retained in the plants regenerated from nucellus, which is the necessity of clonal multiplication. Also, this tissue is believed to be free of any pathogens, so the plants having nucellar origin would be disease-free.

This chapter includes studies to develop a propagation protocol using nucellar tissue. Initially, response of nucellus was observed on a variety of culture media having different concentrations and combinations of growth regulators. It was seen that nucellus formed callus on most of the media tested. The response of nucellar tissue was age-dependent. Nucellus obtained from immature fruits collected 3-4 weeks after fertilization formed embryogenic callus after culture on certain media combinations. Callus was

initiated on Murashige and Skoog (MS) medium + 2,4-D 5 μM + GA_3 15 μM + BA 5 μM + sucrose 3 % + AC 0.5%. This callus formed an embryogenic mass after 9 weeks on Maintenance Medium, which was MS + 2, 4-D 10 μM + GA_3 15 μM + CH 0.05% + CW 10% + sucrose 4% + AC 0.5%. The embryogenic mass, after transfer to embryo Expression medium (MS + 2,4-D 5 μM + GA_3 30 μM + CH 0.05% + CW 10% + sucrose 4% + AC 0.5%, differentiated into somatic embryos. Further development of somatic embryos occurred on MS + AC 0.5%. Experiments are being carried out for maturation of these somatic embryos and their subsequent conversion to plantlets. The development of somatic embryos was also studied by observing histological sections of embryogenic tissue, which showed that superficial cells of embryogenic callus gave rise to somatic embryos.

Chapter 5: Direct somatic embryogenesis from immature zygotic embryo

In woody tree species, mature explants are recalcitrant to culture conditions, and juvenile tissues like zygotic embryos have to be used to develop regeneration systems. This chapter describes experiments on the embryogenic response of the immature zygotic embryo (IZE). The immature zygotic embryos (IZEs) were collected at different stages of development and cultured *in vitro* on a variety of media with different combinations and concentrations of growth regulators. IZEs obtained from fruits at 3-5 weeks post-fertilization stage showed embryogenic response. The IZEs were inoculated on MS + 2,4-D 5 μM + BA 5 μM + GA_3 3 μM + sucrose 3% + AC 0.5%, and incubated in dark. After 4-5 weeks of incubation, somatic embryos were induced directly from the radicle tip of the precociously germinated IZEs. The somatic embryos were transferred to MS + ABA 20 μM + maltose 3% for maturation. Somatic embryos germinated on MS + AC 0.5%. Histological study of somatic embryo development was carried out. It was observed that somatic embryos arose directly from the superficial cell layer at or near the radicle tip of IZE. Studies are being carried out for efficient conversion of somatic embryos.

(Shilpa S. Gogate) {Dr. (Mrs.) R.S. Nadgauda}

Research student Research Guide

CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1.1 Introduction

Cashew (*Anacardium occidentale* L.) is an evergreen tropical nut-bearing tree. Cashewnuts enjoy worldwide popularity as dessert, due to their delectable flavor. Cashewnut ranks third in the world market after almonds and walnut in the tree nut category, and its trade is valued at more than U.S. $ 2 billion annually (Montealegre *et al*., 1999). As per a recent (1999) estimate by FAO, the total area under cashew cultivation in the world was more than 1 million acres and the total production about 470,000 metric tonnes (http://apps.fao.org/default.htm).

India is a major cashewnut producing country, and has a major share in the world cashew trade. During 2001-2002, cashew held the second position among agricultural commodities exported from India, amounting to 0.86% of the total export earnings. During 2001-2002, 97,550 metric tonnes of cashew kernels, and 1814 metric tonnes of cashew nut shell liquid were exported from India, bringing in foreign exchange worth 372 million U.S. dollars (Pillai, 2002).

1.1.1. Origin and Distribution

Cashew is believed to be a native of Central and South America, having its origin in eastern Brazil (Opeke, 1982). The Portuguese traders and seafarers introduced this species to the west coast of India in the 16th century. Cashew adapted very well in India, from where it probably was distributed to other parts of India, East Africa and South-East Asia (Johnson, 1973).

The Indian common name for cashewnut is *Caju*, derived from the Portuguese name *Caju*, which in turn has originated from the term *Acaju*, the name given to cashewnut by the native Indians of Brazil.

Cashew is tolerant to a wide range of soil conditions. It is believed that cashew was originally grown mainly for afforestration and soil conservation in the coastal areas. It was not until the beginning of the twentieth century that it gradually gained importance as a plantation crop.

Presently, cashew grows in the tropical regions, from 30º N to 30º S latitudes. The countries producing cashewnut are listed in Table 1.1.

Table 1.1: Distribution of cashewnut producing countries in different continents

Continents	Countries
Africa	Angola, Benin, Guinea Bissau*, Madagascar, Mozambique*, Nigeria*, Kenya*, Senegal, Tanzania*, Togo
Australia	Australia
Latin America	Brazil*, Barbados, Dominican Republic, El Salvador, Honduras, Peru
South East Asia	India*, Bangladesh, Indonesia*, Malaysia*, Phillipines, SriLanka, Thailand*, Vietnam*

*Major cashew producing countries

In India, cashew is naturalized, and grown, along the coastal regions. The main cashew growing states of India are Andhra Pradesh, Goa, Karnataka, Kerala, Maharashtra, Orissa, Tamilnadu and West Bengal. The states of Madhya Pradesh, Manipur, and the Union Territory of Andaman and Nicobar Islands have been identified as regions for introducing this crop. The cashew-growing regions in the different states are shown in Figure 1.1.

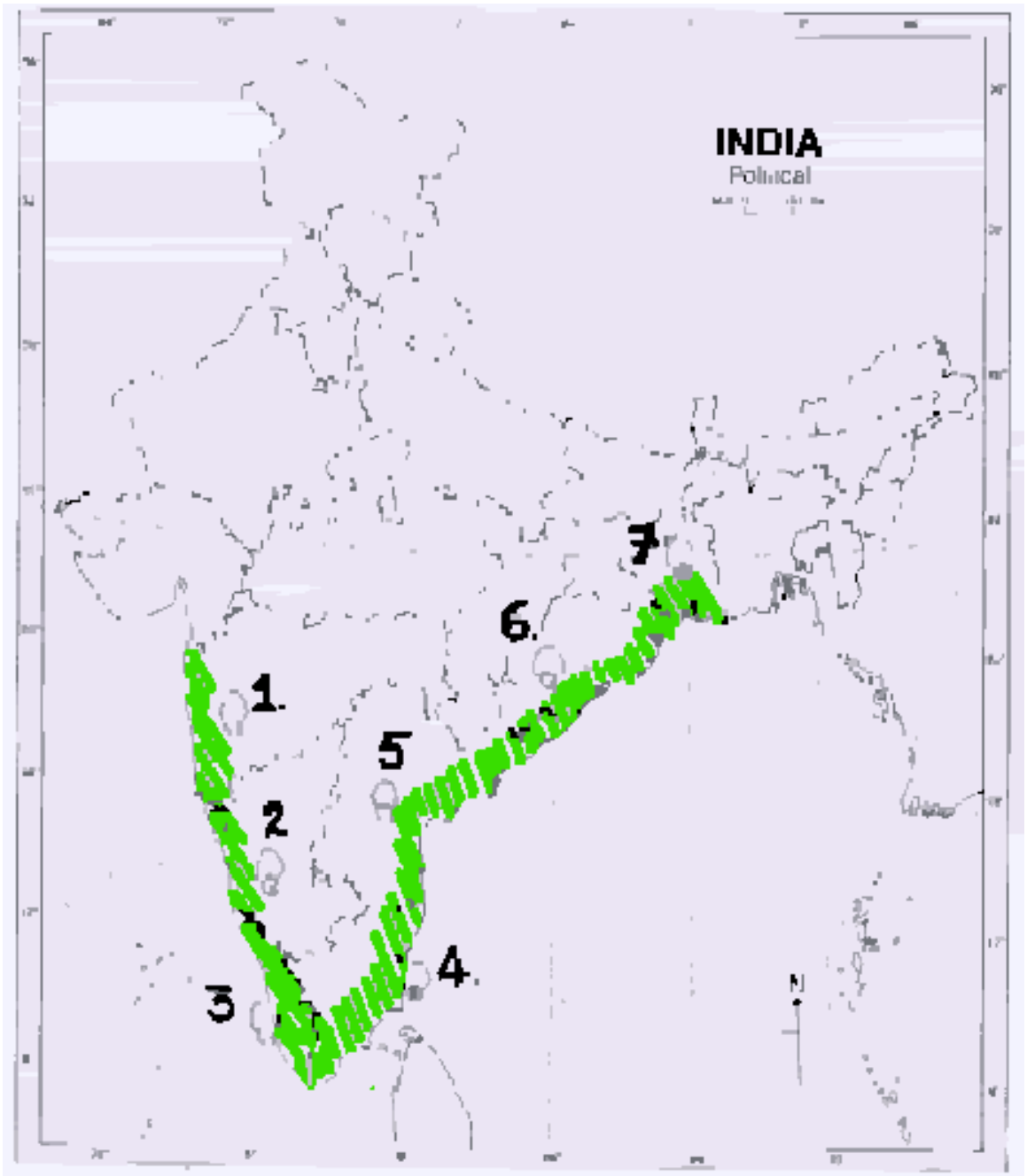


Figure 1.1: Cashew growing states in India

1 Maharashtra
2 Andhra Pradesh
3 Karnataka
4 Kerala
5 Tamilnadu
6 Orissa
7 West Bengal

1.1.2. Botany

Cashew belongs to family Anacardiaceae, (Order Sapindales, Class Dicotyledonae), which comprises about 60 genera and 400 species, including mostly tropical trees and shrubs (Purseglove, 1982). Cashew tree is low spreading, evergreen, having a dome shaped canopy that almost touches the ground (Fig. 1.2). The tree can grow up to a height of 10-12 metres. The branches are stout, many in number, and arise very low on the trunk. Some of them are drooping and trail on the ground. The bark is thick,

Figure 1.2: The cashew tree

resinous and scaly. The wood is yellow in colour, moderately soft, light, having a relative density of 0.5 (Tavares, 1959). The leaves are simple, alternate, obovate and entire, with prominent lateral veins. They are frequently notched at the apex. Petioles are short (1-2 cm long) and the leaves are crowded at the ends of branches. The root

system consists of a prominent tap root and a well-developed and extensive network of lateral roots.

The tree starts flowering at 3-5 years of age. It bears staminate and hermaphrodite flowers. Both types of flowers occur in the same inflorescence, which is an indeterminate panicle (Rao and Hassan, 1957) (Fig. 1.3a). The average length of the panicle ranges from 14-21 cm and the number of flowers per panicle varies from 200 to 1600 (Damodaran *et al*., 1966). Nearly 96% of the flowers in a panicle are staminate (male) (Fig. 1.3b), and the remaining are perfect (hermaphrodite) (Fig. 1.3c) flowers (Rao and Hassan, 1957). Only about 10% of the hermaphrodite flowers produce mature fruits (Damodaran *et al*., 1966).

The flowers are small, scented and have pentamerous symmetry (Copeland, 1961). They are white to light green at the time of opening. Pink stripes appear on the petals few hours after opening of flowers (Fig. 1.3a).

Figure 1.3: a: Inflorescence of cashew (arrowhead indicates a recently opened flower); b: single male/ staminate flower; c: single hermaphrodite/ perfect flower)

Both, hermaphrodite and male flowers have androecium, which consists of 1-2 fully developed fertile stamens, and 7-8 sterile staminodes. The staminodes have short filaments and are concealed in the lower half of the open flower (Fig. 1.4 a,b). The level of anthers of the fertile stamen is below the level of stigma in the perfect flower (Fig. 1.4b). Both, male and perfect flowers have gynoecium, but it is very rudimentary in the male flower. The perfect flower has well developed gynoecium

consisting of an ovary, style and stigma (Fig. 1.4c). The ovary is superior, laterally compressed and unilocular, with a single ovule. The style is long and slender, ending in a slightly expanded stigma (Nair *et al*., 1979). The flowers are cross-pollinated (Wait and Jamieson, 1986) and insects are the pollinating agents (Reddi, 1992).

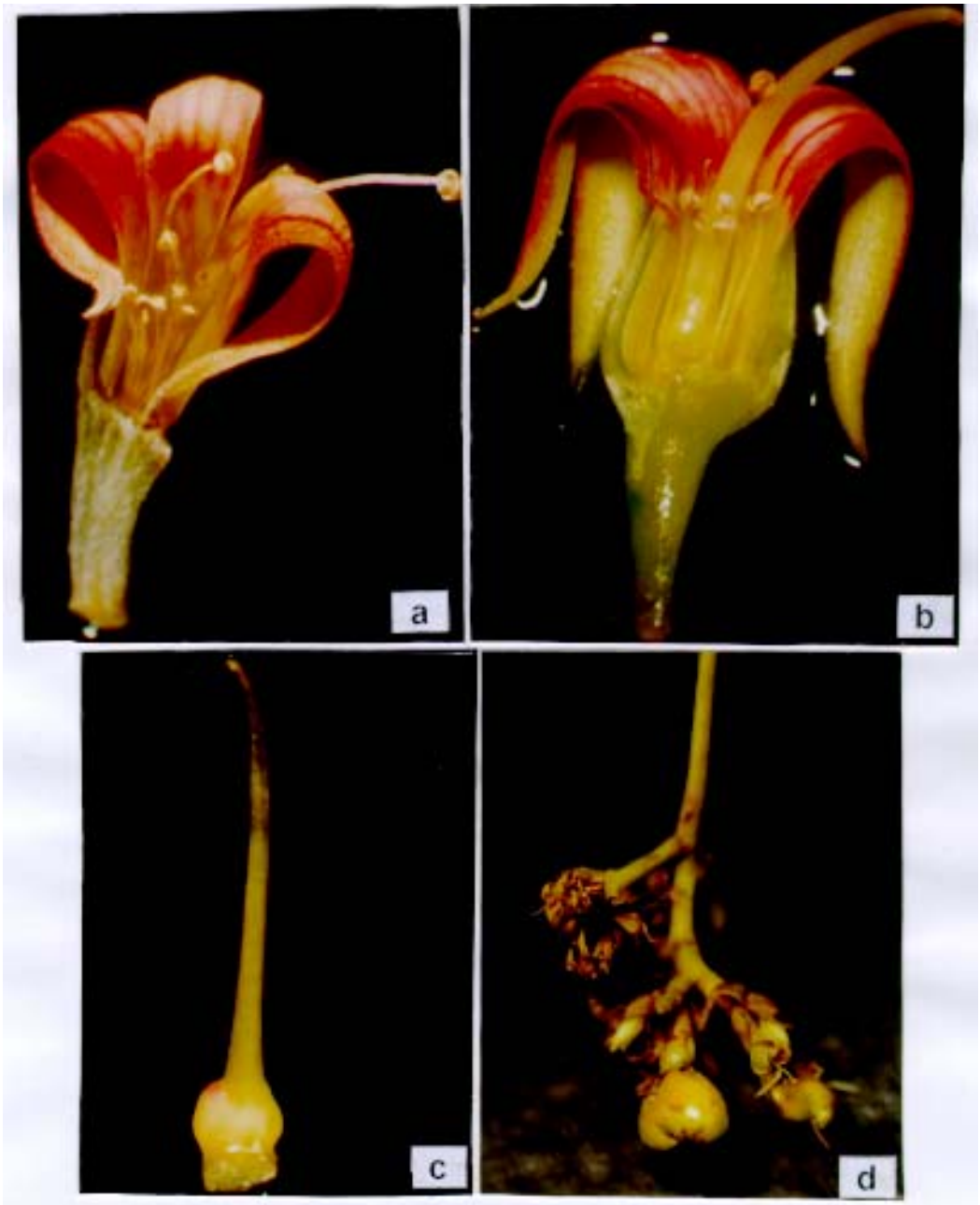


Figure 1.4: a: L.S. of male flower (sterile staminodes in lower half of flower); b: L.S. of hermaphrodite flower (short staminodes and longer fertile stamen with anthers below the level of stigma); c: gynoecium of hermaphrodite flower showing ovary, style and stigma; d: enlargement of ovary after fertilization (2 very young fruits are seen)

The fruit of the cashew tree is unusual. The ovary enlarges (Fig.1.4d) after fertilization, and develops into the fruit, which is a nut. This nut is the true fruit of cashew. Nut is grey-colored, kidney-shaped, about 3-5 cm in length and 2-3.5 cm in width (Fig.1.5a). The nut consists of three parts: outermost shell (seedcoat), middle testa and innermost seed (kernel). The shell is thick and very tough (Fig. 1.5 b), affording protection to the seed inside. It is formed of three layers, epicarp, mesocarp and endocarp. Epicarp is the outermost thin layer. Mesocarp is the thick middle layer with a honey-comb structure, filled with sticky, corrosive, resinous oil, the cashewnut shell liquid (CNSL). The endocarp is the innermost thin and hard layer of sclerenchymatous cells. The testa is a thin, papery, brown colored layer, which covers the seed (Fig. 1.5b). The innermost part of the nut is the seed. There is a single seed in each nut. The seed is kidney shaped, with two semi-lunar cotyledons and a small embryo. The seed forms the much sought-after kernel, or 'cashewnut' of commerce (Fig. 1.5c). The nut matures in 55-70 days.

As the nut matures (Fig. 1.6a-d), the receptacle and peduncle of the flower enlarge very rapidly. This enlarged structure is called cashew apple, and is the false fruit of cashew. The cashew apple is brightly colored, and may be yellow, red, orange, or varying shades (Fig. 1.6 e-i) depending on the variety. It is fleshy, fibrous with a very tender skin. The mature nut (true fruit) remains attached at the lower end of this massively enlarged cashew apple (false fruit) (Fig. 1.7).

1.1.3. Pests and Diseases

The main insect pests affecting the cashew plant in India are tea mosquito (*Helopeltis antoni*,), (Devasahayam and Nayar, 1986), stem and root borer (*Ploecederus ferrugineus*) (Rai, 1984), leaf miner (*Acrocercops syngramma*), and leaf and blossom webber (*Lamida moncusalis* and *Orthaga exvinacea*). Fungi like *Gloesporium mangiferae*, *Aspergillus niger*, *Corticium salmonicolor* and *Colletotrichum gleosporioides* can cause severe crop losses (Nair *et al.,* 1979; Nambiar *et al*., 1990; Bhaskara Rao *et al*., 1993).

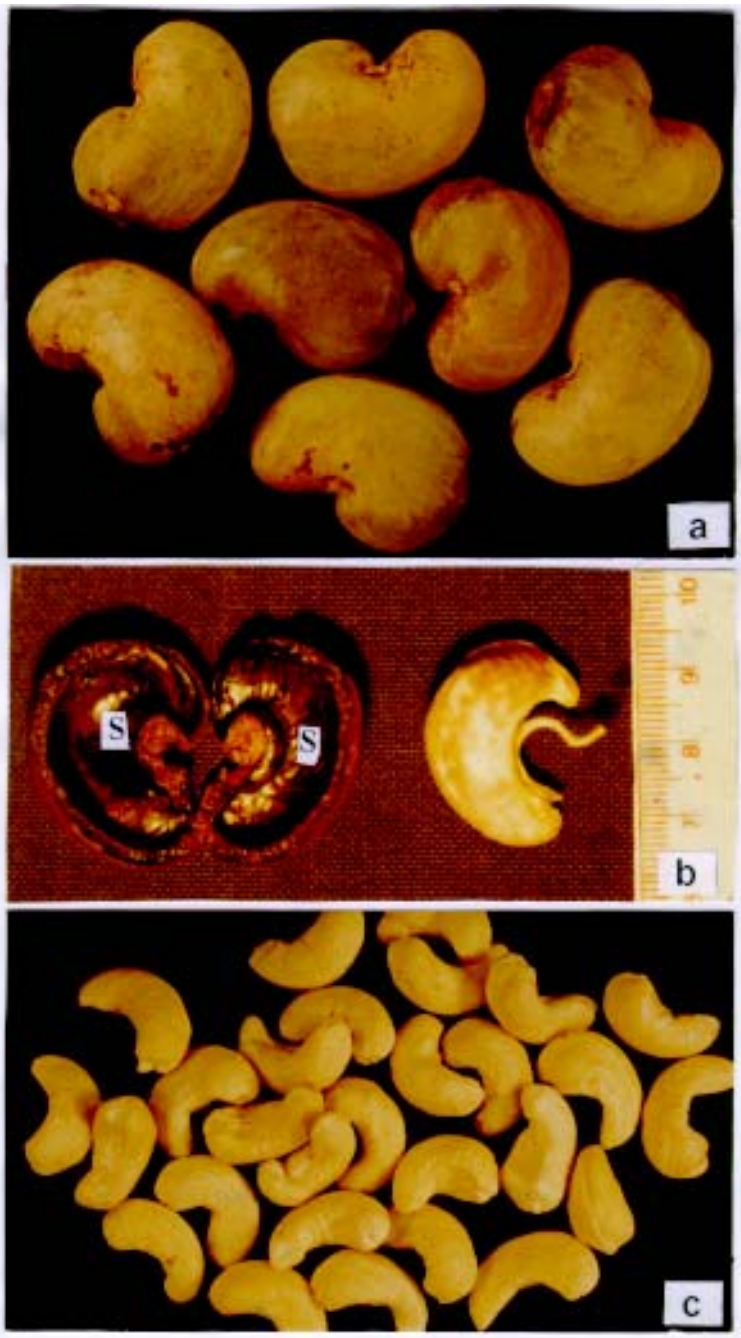


Figure 1.5: a: Mature nuts (seeds) of cashew (variety Vengurla 1) ; b: mature nut dissected to show shell halves (s) and kernel covered with testa; c: kernels without testa

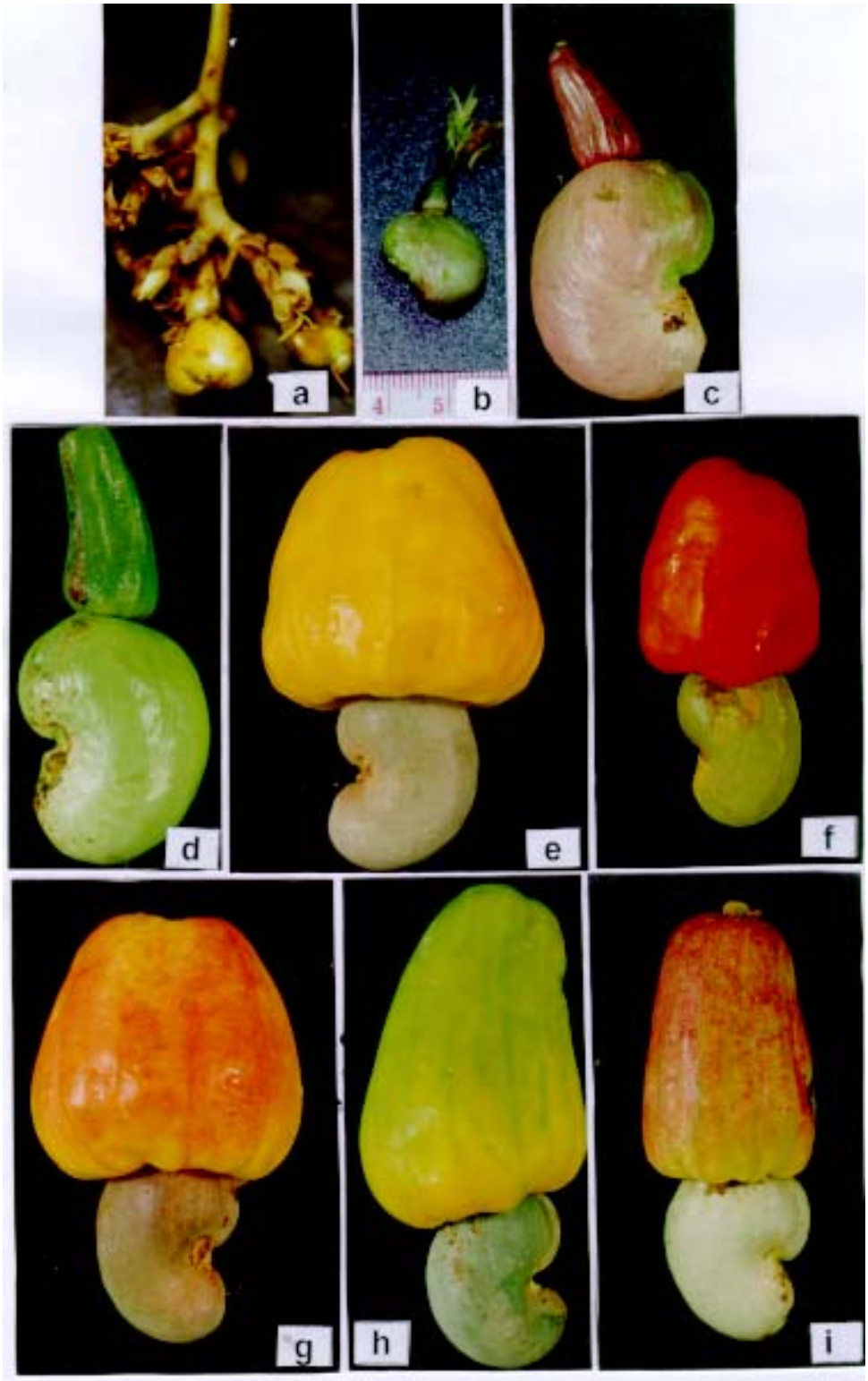


Figure 1.6: Development of nut and cashew apple (weeks post-fertilization) a: 1-2 weeks; b: 2-3 weeks; c, d: 5-8 weeks; e-i: Variation in color of ripe cashew apple

1.2. Economic importance

Cashew is of great socio-economic importance to the African, Latin American and Asian countries, which are the main producers of this crop (Balasubramanian, 1987). The commodity of commercial importance of this tropical tree is the nut. The cashew-producing countries gain valuable foreign exchange from export of processed cashewnuts. During processing, nuts are roasted mechanically (Tyman, 1980). Due to heat, the outer layers of the hard shell burst, and the corrosive CNSL is released, which is collected separately. The endocarp layer of the shell remains intact during roasting.

The roasted nuts are then shelled manually or mechanically. In India, manual shelling is preferred (Tyman, 1980) since this yields more than 90% of the kernels as intact wholes, which fetch premium price in the export market, as compared to broken halves and bits. Apart from the labour required during processing, grading the processed nuts and packaging, harvesting of raw nuts and manual shelling of roasted nuts provides much needed employment to unskilled labor in the rural sector. Cashew industry annually provides employment to nearly half million people, majority of whom are women from rural areas (Balasubramanian, 1997).

Cashewnuts (Fig. 1.8a) are used in various forms, such as plain, salted, spiced, and sugared. They are consumed directly as dessert, used in the baking and confectionery industry, or used as garnishing in various food preparations. Apart from being tasty, cashewnuts are also highly nutritious. They are rich in proteins, carbohydrates, unsaturated fats, calcium, phosphorus, iron and vitamins. Cashewnut proteins are complete having all the essential amino acids. The nuts supply about 6000 calories per kilogram, as compared to 3600 from cereals, 1800 from meat and 650 from fresh fruit (Nambiar *et al.*, 1990). As majority of the fatty acids present in the nuts are unsaturated, they are easy to digest, and can be consumed safely by young and old alike (Nambiar *et al.*, 1990). The kernels also have an antibacterial substance effective against tooth decay caused by *Streptococcus mutans* (Himejima and Kubo, 1991).

The shell (Fig. 1.8b) contains corrosive viscous oil, cashewnut shell liquid (CNSL), which is a natural protection to the kernel against insects. It is extracted from the shells after removal of kernels. It has proved to be a valuable by-product, with the main buyers being the United States, United Kingdom, many European countries and Japan. Many uses of CNSL are extensively patented (Tyman, 1980). It is chiefly used for production of friction dusts needed in brake linings and clutch facings. It finds use in various polymer-based industries, such as the paint and varnish industry, resins and laminations, and foundry chemicals (Kamath, 1956). It is also used in making waterproofing compounds, chemical-resistant cements, surfactants, and ion-exchange resins. Cardanol, cardol and anacardic acid in CNSL are used as dyestuff intermediates, pharmaceuticals and antioxidants (Tyman, 1980). Phenolics isolated from the oil show antimicrobial activity. The oil also exhibits anti-filarial, pesticidal and molluscicidal properties (Kubo *et al*., 1986; Suresh and Kaleysa Raj, 1990; Himejima and Kubo, 1991).

The cashew apple (Fig. 1.7) is edible and a rich source of vitamin C (261.5 mg/100 g). Due to very tender skin and juicy nature, it has a relatively short shelf life. The astringent taste and perishable nature are hurdles in wide marketing of this product. It is consumed locally as a fresh fruit, or processed and used in preparing variety of products like juice, vinegar, candy and soft drinks. In India, an alcoholic beverage ("Feni") is prepared by fermentation of cashew apple juice, which is well known for it's distinctive flavor. Cashew apple juice has been reported to serve as a good substrate in the production of single cell protein culture (Layokun, 1986).

The wood of cashew tree also finds uses as fuel (Vimal and Tyagi, 1986), and as raw material for furniture, fishing boats, and packing material. Wood pulp is used to fabricate corrugated and hardboard boxes. Indelible ink- pads are produced from the acrid sap. The cashew tree gum has antimicrobial and insecticidal properties (Marques *et al*., 1992) and is used for book-binding.

Figure 1.7: Mature nut attached to ripe, enlarged cashew apple

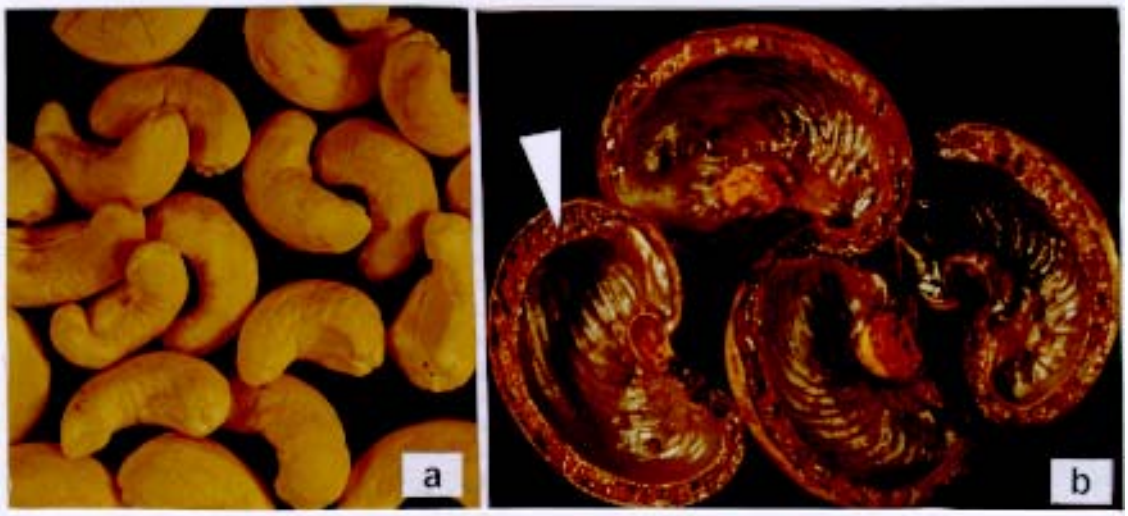


Figure 1.8: a: Roasted kernels – the chief commercial product; b: shells used for extracting CNSL, mesocarp layer (arrowhead) containing oil is seen

Cashew can be used for afforestration of degraded and waste lands, due to its ability to withstand poor soil conditions (Sastry and Kavathekar, 1990). The low trailing branches form an effective soil cover, helping to prevent soil erosion.

1.3. India's position in the international cashew trade

India has been exporting cashew kernels since 1950s (Bhaskara Rao and Nagaraja, 2000). India is the largest producer, processor and exporter of cashewnuts in the world (Nayar, 1999). The major customers for cashew kernels in the international market are United States of America, The Netherlands, Germany, United Kingdom, other European nations, and Japan. The world production of raw cashewnuts, and the share of major cashew-producing countries are given in Table 1.2.

Table 1.2: Cashewnut production in the world, and the share of major cashew-producing countries between 1965 till 2002

Year	World production (in 1000 MT)	Production (1000 MT) by the main cashew producing countries (figures in parentheses indicate percent of world production)				
		Brazil	India	Nigeria	Tanzania	Vietnam
1965	386.3	13.8	100.0 (25.9)	22	76.0	0.7
1970	511.9	20.4	123.3 (24.1)	25	107.4	2.1
1975	563.8	20.5	144.3 (25.6)	25	115.8	3.5
1980	464.2	75.0	180.3 (38.8)	25	41.4	5.6
1985	571.9	115.0	221.3 (38.7)	25	32.8	60.0
1990	730.1	107.7	285.6 (39.1)	30	17.1	140.0
1995	1126.5	185.2	321.6 (28.6)	95	63.4	202.4
2000	1667.9	138.6	520.0 (31.2)	184	121.2	270.4
2001	1591.0	124.1	450.0 (28.3)	185	121.9	272.0
2002	1516.9	183.7	460.0 (30.3)	186	121.9	127.4

(Source: FAO database FAOSTAT, http://www. apps.fao.org.; MT: metric tonnes)

It can be seen from Table 1.2 that India has been producing the highest quantity of raw cashewnuts consistently since 1965. It is also the major exporter of processed kernels. The export of cashew kernels from India and the resulting export earnings are shown in Table 1.3.

Table 1.3: Export of cashew kernels from India

Year	Quantity of kernels exported (in 1000 MT)	Foreign exchange earned (Million Rs)
1965	56	29.0
1970	60	57.4
1975	65	118.1
1980	38	118.0
1985	32	180.0
1990	45	3650.7
1995	77	12458.0
2000	95	25,000.0

(Source: Bhaskara Rao and Nagaraja, 2000; MT: metric tonnes)

1.3.1. Production of cashewnuts and processing capacity

In India, in the year 2000, cashew was cultivated over an area of 0.683 million hectares, with an annual production of about 0.52 million metric tonnes of raw nuts (Bhaskara Rao and Nagaraja, 2000). This production is however lower than the capacity of the installed nut processing units, which is about 0.7 million metric tonnes of raw cashewnuts per annum. Efficient processing by the mechanized units, confinement of cashew cultivation to marginal lands, non-availability of suitable high-yielding varieties, low yields due to improper crop management have also contributed to widen the gap between production and processing.

To make up for the deficit in production, raw nuts are imported from other countries, for continued functioning of the indigenous processing units. India has imported about 0.3 million metric tonnes of raw nuts from African and East Asian countries during the year 2002 (Unnikrishnan, 2003).

The import of raw nuts was easier till recently. Due to lack of infrastructure and developing economy, these countries preferred exporting their raw nut produce to India. But the scenario is changing rapidly.

1.4. Need for increased production of cashewnuts in India

Along with India, other cashewnut-producing countries have also realized the potential of cashewnut as a commodity of international trade, and its importance in improving national economy. Consequently, they are focusing their attention on increased productivity, and in-house processing of raw nuts. To prevent export, the levy on raw cashews has been increased. This increasing competition in cashew trade has resulted in decreased imports of raw nuts in India, affecting the working of the processing units due to shortage of raw nuts. Additionally, due to increased levy charges, importing raw nuts drains away almost 50% of the foreign earnings gained through export of processed nuts (Unnikrishnan, 2003).

This problem can be solved by increasing the indigenous raw nut production, which will not only meet the requirements of the processing industry, but will also consolidate India's leading position in the world cashewnut trade. Increased indigenous production of cashewnuts will end the necessity for import of raw nuts and strengthen the processing industry. Saving on imports will help in effective increase in the foreign exchange earnings.

1.4.1. Development and release of high yielding varieties for increased production

There is an urgent need to increase the production of cashewnuts in India. Production can be increased by: (i) increasing area under cashew cultivation, and (ii) increasing productivity (yield per hectare). These objectives can be achieved with the use of high yielding varieties, and improved crop management programs.

The National Commission on Agriculture and the Working Group constituted by the Planning Commission, have targeted a production of two metric tonnes of nuts per hectare of cultivated area.

Reaching this target will certainly eliminate the need for imports by providing sufficient raw material to the processing industry. It will also secure India's position in the increasingly competitive global market.

As a first step towards the targeted production, the previous method of cultivation has to be modified. Earlier, cashew was cultivated on marginal or wastelands. There was no systematic plantation of this crop, and most of the old plantations are seedling progenies of non-descript origin, growing in neglected condition. These plantations showed inconsistency in production, due to the high degree of variability arising from cross- pollinated nature of the crop. The variation in yield is highly undesirable for export-oriented production.

Realizing the tremendous export potential of this crop and the consequent need to increase production, the Indian Council of Agricultural Research initiated systematic research on this crop under the auspices of Central Plantation Crop Research Institute in 1970, and All-India Co-ordinated Research Project on Spices and Cashew. Cashew is also one of the priority crops identified by the Department of Biotechnology, Government of India (Mascarenhas, 1998). A further boost was given to cashew research with the launching of National Research Center for Cashew in 1986. Regional Research Stations/ Centers were established under the agricultural universities in all cashewnut growing states of India (Bhaskara Rao, 1997). Research is primarily aimed at improving productivity, and maintaining India's leading position in the global cashewnut market.

As a result of the concerted efforts of the various research centers, new high yielding varieties were developed through conventional breeding methods, and released for commercial plantation purpose. These varieties are listed in Table 1.4.

Table 1.4: Varieties released for commercial cultivation by different Cashew Research Stations in India

State	Research Station	Varieties released as	
		Selections	Hybrids
Andhra Pradesh	CRS, Bapatla	BPP-3, BPP-4, BPP-5, BPP-6	BPP-1, BPP-2, BPP-8
Karnataka	NRCC, Puttur, ARS, Ullal ARS Chintamani	NRCC Selection 1 & 2, Ullal-1, 2, 3, & UN-50, Chintamani - 1	
Kerala	CRS, Anakkayam CRS, Madakkathara	Anakkayam –1, Sulabha Madakkathara – 1, 2 K-22 / 1	Dhana, Kanaka, Priyanka, Dhanashree, Amrutha
Maharashtra	RFRS, Vengurla	V-1, V-2	V- 3, V-4, V-5, V- 6, V-7
Orissa	CRS, Bhubaneshwar	Bhubaneshwar-1	
Tamilnadu	RRS, Vridhachalam	VRI-1, 2, 3	
West Bengal	RRS, Jhargram	Jhargram-1	

CRS: Cashew Research Station, NRCC: National Research Centre for Cashew, RFRS: Regional Fruit Research Station, RRS: Regional Research Station

The superior high yielding varieties were developed at the regional level, and were suitable for cultivation under the agroclimatic conditions of that particular State. For reaping maximum benefits, it is essential to plant the variety most suited for growth in any given region. Specific varieties have been recommended for the different states in India, according to the prevalent climatic conditions, which are given in Table 1.5.

Table 1.5: Superior varieties of cashew recommended for different cashew growing states in India

State	Recommended varieties for cultivation
Andaman and Nicobar	Madakkathara 1, Ullal 1, VRI-2, Vengurla 1, 4
Andhra Pradesh	BPP-4, 6, 8, VRI-2
Karnataka	Selection 1 and 2, Ullal 1 2, 3, UN-50, VRI 1 and 2, Vengurla 1 and 4
Kerala	Madakkathara 1, 2, K-22-1, Dhana, Priyanka
Madhya Pradesh	T.No. 40, Vengurla 1, 4, BPP- 4, 6, 8, VRI-2
Maharashtra and Goa	Vengurla 1, 4 and 6
Manipur	Madakkathara 1, VRI-2
Orissa	VRI-2, Bhubaneshwar 1, BPP-1, 2
Tamilnadu	VRI-1, 2, 3
West Bengal	Jhargram 1, Madakkathara 1

With the availability of new and high yielding varieties, it is now possible for farmers to choose a suitable variety for raising plantations catering to demands of the international export market. Considering the low productivity and high variability of seedling orchards, it would be prudent to use planting material generated through vegetative propagation of the selected variety. There is increasing awareness among cashew-growers about the advantages of using improved varieties appropriate for the particular region, and the merits of planting only clonal material of the selected variety (Gunjate, 1997).

1.5. Propagation of high yielding varieties by conventional methods

To reach the targeted productivity of two metric tonnes of raw nuts per hectare, the important developmental support required is making available large numbers of suitable planting material (Bhaskara Rao, 1997).

The conventional method of propagation through seed is now discontinued due to high degree of heterozygosity in plants and low productivity. With the need to increase production, and in keeping with the demand of farmers, vegetative methods are now preferred for the propagation of selected varieties. Different methods of vegetative propagation like air layering, budding, veneer grafting have been followed for multiplying selected varieties, with varying degrees of success. However, observations over a number of years show softwood grafting (Bhaskara Rao *et al*., 1993) as the most useful method for vegetative propagation of cashew. It is now being commonly employed to multiply the chosen elite varieties.

Bringing new areas under cashew cultivation, replacing old unproductive plantations, and high density planting programs are the main strategies to be used for increased production. Superior quality planting material required for all these activities is very large. Generating so much planting material through grafting alone is practically impossible, and would be very time-consuming. A reliable method of rapid clonal propagation would be of immense use in producing sufficient amount of planting material of the desired elite cultivar.

1.5.1. Need for in vitro approach

In vitro plant production can effectively supplement the conventional means of vegetative propagation in cashew, because of the ability to generate very large numbers of planting material in a short time. It also has the added advantages of being independent of environmental conditions and requiring only small quantity of parent material. The system could be further exploited for variety improvement through modern techniques of gene manipulation. It can also be used as a method of germplasm conservation.

Organogenesis and somatic embryogenesis are the two morphogenic responses shown by plant tissues *in vitro*. Both processes involve different developmental events, and cells are committed to only a single pathway (Ammirato, 1985).

In organogenesis, adventitious shoot meristems develop from the explants either directly, or through a callus. In this pathway, the shoots can be formed from any part of the plant tissue, thus are adventitious. Axillary shoot proliferation is one more method of *in vitro* shoot formation. However, in this case, the shoots arise from shoot meristems pre-existing in explants, such as apical buds and axillary buds. Normally, only one shoot is produced from such explants. However, using the correct nutrient media and growth regulators, it has been possible to induce shoot meristems to form a large number of shoots *in vitro*. This method has been extensively used for *in vitro* plant propagation in many plant species (George, 1993).

Shoots formed adventitiously or from pre-existing meristems are not complete plants, and have to be treated further for induction of rooting. Generally, *in vitro* shoot formation is followed by shoot elongation, *in vitro* root induction, hardening of rooted shoots, and transplanting the hardened plantlets to soil. The shoots are also recycled for production of more shoots. This procedure can generate large amounts of clonal planting material in a relatively short period.

Somatic embryogenesis is the pathway involving the development of bipolar embryos from somatic cells or tissues. Although individual cells are totipotent and have all the genetic information necessary for the development of a whole plant, development of each cell is tightly regulated through spatial and temporal expression of genes (De Jong *et al*., 1993). Externally applied stimuli are necessary for conversion of a somatic cell to an embryogenic cell. This stimulus must be responsible for expression of genes involved in the embryogenic pathway (Wilde *et al*., 1995; Zimmerman, 1993).

As the somatic embryos are bipolar propagules, the step of root induction is not necessary. However, the somatic embryos formed *in vitro* frequently show precocious germination. Therefore, the somatic embryos have to undergo maturation

treatments, simulating the maturation phase in zygotic embryogenesis. Maturation step helps in conversion of somatic embryos to normal plantlets.

Regeneration of plants *in vitro* from cells or tissues either by organogenesis or somatic embryogenesis is an important pre-requisite for genetic manipulations of higher plants (Litz and Gray, 1995).

1.6. In vitro studies in cashew

All the high yielding superior varieties of cashew have been developed through breeding programs (Table 1.4). Cashew being a tree species, developing a single superior variety takes many years. Very small size of flowers, cross-pollinated nature, poor fruit set, and early fruit drop are other factors hampering the success of breeding programs in this species. To maintain the genetic integrity of these elite varieties, it is essential to propagate them through vegetative means.

In vitro clonal propagation of the selected trees would greatly improve the efficiency of plant production. It would also have considerable impact on the improvement programs in this tree species. Additionally, a suitable *in vitro* plant regeneration system would facilitate long-term storage and international exchange of germplasm.

Studies on *in vitro* culture of cashew were initiated in India and many other laboratories worldwide, not only for clonal propagation, but also to develop a system which can be used for variety improvement. In cashew, axillary shoot proliferation, adventitious shoot formation, and somatic embryogenesis are reported. The studies are summarized in Tables 1.6 a, b and c.

Table 1.6 a: *In vitro* studies in cashew: axillary shoot proliferation/ organogenesis

Explant	Basal medium	Growth regulators (μM) and supplements	Response	Reference
Cotyledon (mature seed)	LS	IAA 2.85 + Kin 2.32	Plantlets	Philip, 1984
Axillary buds (6-15 month old seedling)	L	2iP 9.8 + GA_3 1.44; BA 8.9 + BA 22.2 solution (two phase medium); 2iP 9.8 + $GA_3$1.44; IBA 9.8	Induction of sprouting; Development of sprouted buds Elongation Rooting	Lievens *et al*., 1989
Nodes (3-year-old seedling)	MS [a]	ZR 14.23 ZR 14.23 + $GA_3$1.44 NAA 26.85	Stimulation of bud growth Elongation and node formation Rooting	Falcone & Leva, 1990
Cotyledon (mature seed)	LS	NAA 2.69 + Kin 4.6	Multiple shoots and roots	Hegde *et al*., 1991
Cotyledonary node (3-week-old *in vitro* germinated seedling	MS	suc 117mM + mal 14.6 mM + BA 22.2; suc 146 mM + mal 14.6 mM + BA 4.4 + 10%CW; IAA 2.9 + IBA 4.9	Multiple shoots Elongation Rooting	D'Silva & D"Souza, 1992
Base of microcuttings	MS[b]	BA 4.44; IAA 2.85	Rooted shoots	Bessa & Sardinha, 1994
Cotyledonary node (20-day-old *in vitro* germinated seedling)	MS	BA 4.4 + Kin 2.32 + Zea 9.12; IBA 2460 for 2 h	Multiple shoots Rooting	Das *et al*., 1996

Continued…..

Table 1.6 a continued……

Explant	Basal medium	Growth regulators (μM) and supplements	Response	Reference
Nodes (1-month, 1-year, 5-year glasshouse grown plants)	MS[c]	suc 20 g/l + glu/ mal 20 g/l suc 40 g/l or GA_3 5-10 IBA 100	Sprouting of buds Elongation Rooting	Boggetti *et al*., 1999
Nodes (20-25-day-old *in vitro* germinated seedling)	MS 3/4th WPM (liq)	TDZ 0.45/ TDZ 0.45 + NAA 0.54/ IBA 0.49 NAA 13.43 + IBA 12.3	Multiple shoots Rooting	Thimmapaiah & Samuel, 1999
Mature cotyledons	MS MS liquid	BA 22.2 + suc 3%; IBA 122.6 (72 hr)/ IBA 4.9 + IAA 5.7	Adventitious shoots Rooting of shoots	Ananthakrishnan *et al*., 2002
In vitro produced shoots	WPM	IBA 100 for 5 days	Rooting of microshoots	Boggetti *et al*., 2001
Nodes from young grafts	MS[c]	TDZ 0.45; suc 116.8 mM; micrografting	Bud-break; elongation; rooting	Thimmapaiah *et al*., 2002

a**:** nitrogen half strength; b: with Morel's vitamins; c: half strength macro salts; glu: glucose; L: Lepoivre; LS: Lin & Staba (1967); mal: maltose; suc: sucrose; ZR: zeatin riboside;

Table 1.6 b: *In vitro* studies in cashew: Somatic embryogenesis

Explant	Basal medium	Growth regulators (μM) and supplements	Response	Reference
Zygotic embryo (1-month-old nuts)	MS	2,4-D 18.1 + NAA 21.48 + Kin 9.3 + PVP 250 mg/l	embryoids	Jha, 1988
Cotyledon pieces (6-8 weeks old seedling)	MS	NAA 26.85 + 2,4-D 4.52 + BA 4.44 + CW 10% v/v NAA 5.37 + BA 2.22 or Kin 2.32	callus embryoids	Laxmi Sita, 1989
Cotyledon (mature seeds)	SH	NAA 16.1 + BA 3.55 CH 250 mg/l + AdSO4 217.16	embryoids	Sy *et al*., 1991
Immature cotyledon	LS	Ca pantothenate 200 mg/l + IAA 11.42 + BA 8.9 + ascorbic acid 150 mg/l + AC 0.05 g/l AC 1 g/l	embryoids leafy shoots and roots	Hegde *et al*., 1994
Nucellus	MS MS liquid	suc 6% + gln 400 mg/l + CH 100 mg/l + CW 20% v/v + AC 2 g/l + Ascorbic acid 100 mg/l + 2,4-D 6.89 suc 6% + gln 400 mg/l + CH 200 mg/l + Ascorbic acid 100 mg/l + 2,4- D 4.52	callus somatic embryos	Ananthakrishnan *et al*., 1999
Nucellus	MS	2,4-D 5 + GA_3 15 + BA 5 2,4-D 10 + GA_3 15 + CH 0.05 % + CW 10% v/v + AC 0.5 % 2,4-D 5 + GA_3 30 + CH 0.05 % + CW 10% v/v + AC 0.5 % AC 0.5 %	callus embryogenic callus somatic embryos germination	Gogte & Nadgauda, 2000

Continued…

Table 1.6 b continued…..

Explant	Basal medium	Growth regulators (μM) and supplements	Response	Reference
Nucellus	MS	Pic 0.5 mg/l + suc 2% Pic 0.5 mg/l + Putrescine 1 mg/l ABA 0.5 mg/l + suc 3% MS	embryogenic callus somatic embryos embryo development germination	Cardoza & D'Souza, 2002
Immature zygotic embryo	MS	Pic 2.07 MS	somatic embryos germination	Cardoza & D'Souza, 2000
Immature zygotic embryo	MS	2,4-D 5 + GA_3 3 + BA 5 ABA 20 + mal 3% MS	somatic embryos maturation germination	Gogate & Nadgauda, 2003

AC: activated charcoal; $AdSO_4$: adenine sulfate; Gln: glutamine; LS: Lin & Staba (1967); mal: maltose; Pic: picloram; PVP: poly vinyl pyrrolidone; suc: sucrose

Table 1.6 c: *In vitro* studies in cashew: other reports

Explant	Basal medium	Growth regulators (μM) and supplements	Response	Reference
Pollen grains	-	PEG 30% + suc 20% + 20 mg/l each of Ca and B	pollen germination and pollen tube growth	Subbbaiah, 1984
Mature zygotic embryos	MS	NAA 10.74	germination	Hegde *et al*., 1991
Seedling and mature tree tissues	MS	Bavistin™ (carbendazim) (0-50 mg/l)	reduction in contamination	D'Silva & D'Souza, 1993
Zygotic embryos (1-2mm to 14 mm in size)	MS	BA 8.9 + GA_3 5.77	germination	Das *et al*., 1999
In vitro germinated rootstock & seedling scion	-	NAA	graft fusion and development	Ramanayake & Kovoor, 1999
seedling rootstocks ; seedling shoot tips (scions)	MS	suc 2% + Phytagel 0.3%	graft fusion and development	Mneyney & Mantell, 2001
seedling rootstocks; young shoot tips (scion)	MS (1/2 strength major salts)	glutamine 0.04% + AC 0.2% + suc 3 or 8%	graft fusion and development	Thimmappaiah *et al*., 2002

AC: activated charcoal; B: boron; Ca: calcium; PEG: polyethylene glycol; suc: sucrose

In cashew, developing cultures from mature explants is difficult. It can be seen from Table 1.6 a, b and c, that there are no reports on *in vitro* culture of mature tissues obtained from field grown trees. Recalcitrancy of mature explants has been

commonly observed in many tree species (McCown, 2000), and cashew is no exception. Response has been obtained only from juvenile plant material.

Exudation of phenolics in culture medium and presence of indigenous contaminants are the additional hurdles faced during *in vitro* culture of this species. Browning of media and tissues due to phenolic exudates is a serious problem in tree tissue culture (Preece and Compton, 1991), and has not been successfully eliminated (Wang and Charles, 1991).

In cashew, among the juvenile tissues, only cotyledons, nodes of seedlings, immature zygotic embryos, and nucellus have shown response. All other explants have failed to exhibit any morphogenic potential. Thus, even in the juvenile explants, morphogenic potential is restricted to only few tissues. This indicates the strongly differentiated state, and the intractable nature of most of the tissues in this species.

1.6.1. Axillary shoot proliferation/ Organogenesis in cashew

In cashew, adventitious shoot formation has been reported from proximal portions of mature cotyledons (Philip, 1984; Hegde *et al*., 1991; Ananthakrishnan *et al*., 2002). In all the studies, only the proximal ends of the cotyledons formed shoots. The distal portions of cotyledons did not respond to culture, indicating a morphogenic gradient along the length of cotyledons. Shoots were formed directly, without any callus formation.

Cotyledonary nodes of seedlings have been used for *in vitro* shoot production (D'Silva and D'Souza, 1992; Das *et al*., 1996) through axillary proliferation. As compared to nodal explants and apical shoot tips of seedlings, cotyledonary node was the most suitable explant for shoot production, since it produced the maximum number of (8-10) shoots *in vitro*. In case of nodal explants, sprouting of the existing axillary buds has been observed. Nodal explants from seedlings have been used for axillary bud sprouting (Lievens *et al*., 1989; Falcone and Leva, 1990; Boggetti *et al*., 1999; Thimmapaiah *et al*., 2002), where, the axillary buds formed a single shoot. Multiple shoots (4-5 per node) were formed from nodes of 25-day old seedlings using thidiazuron in the culture medium (Thimmapaiah and Samuel, 1999). The shoot tip,

an explant commonly used for *in vitro* shoot proliferation in many woody species, has yielded only two shoots per explant in one study (Das *et al*., 1996).

Different cytokinins were found to be effective for shoot formation, such as BA (Lievens *et al*., 1989; D'Silva and D'Souza, 1992; Bessa and Sardinha, 1994), 2iP (Boggetti *et al*., 1999), combination of 2iP, zeatin and kinetin (Das *et al*., 1996), and TDZ (Thimmapaiah and Samuel, 1999). Elongation of shoots was obtained with 2iP and GA_3 (Lievens *et al*., 1989), zeatin riboside and GA_3 (Falcone and Leva, 1990) or sucrose (Boggetti *et al*., 1999). Lowering the concentration of BA in the medium was necessary for proper shoot elongation (Lievens *et al*., 1989; D'Silva and D'Souza, 1992).

It was generally observed that root induction in the *in vitro* formed shoots occurred at low frequency, and IBA was most effective for root induction (Lievens *et al*., 1989; Bogetti *et al*., 1999 and 2001; Thimmapaiah and Samuel, 1999). Thimmapaiah and co-workers (2002) circumvented the difficulty faced during *in vitro* rooting of shoots by micrografting the *in vitro* formed shoots onto seedlings.

1.6.2. Somatic embryogenesis in cashew

Somatic embryogenesis in cashew has also been reported only from juvenile explants. Immature zygotic embryo formed embryoids and leafy structures (Jha, 1988). Embryoids were obtained also from cotyledon pieces of germinated seedling (Laxmi Sita, 1989), mature (Sy *et al*., 1991), and immature (Hegde *et al*., 1994) cotyledons. In all these studies, the embryoids developed abnormally and failed to regenerate plantlets. Somatic embryos were obtained from immature zygotic embryos in later studies (Cardoza and D'Souza, 2000), which germinated precociously, without developing into plantlets.

Somatic embryos have been obtained from the nucellar tissue (Ananthakrishnan *et al*., 1999; Cardoza and D'Souza, 2002). Auxins 2,4-D and picloram were effective in inducing embryogenesis from this tissue. Somatic embryos of nucellar origin have also not regenerated to plants. Since nucellus is a maternal

tissue, and considered to be free of pathogens, plantlets of nucellar origin would be of considerable importance in clonal propagation.

Among other *in vitro* studies, micrografting has been carried out using young seedling explants (Ramanayake and Kovoor, 1999; Thimmapaiah *et al*., 2002; Mneyney and Mantell, 2002). The system could be extrapolated for rejuvenation of mature explants. A nutrient medium containing polyethylene glycol, 20% sucrose, calcium and boron was found suitable for *in vitro* pollen germination (Subbaiah, 1984). *In vitro* germination of immature (Das *et al*., 1999) and mature zygotic embryos (Hegde *et al*., 1991) was studied. The presence of cotyledons was of utmost importance for successful germination of zygotic embryos (Hegde *et al*., 1991). Embryos without cotyledons failed to grow in culture.

Thus, in cashew, only the juvenile tissues have responded to *in vitro* culture. There are no reports of successful culture of mature explants. As compared to the number of reports in any other woody species, the success rate achieved in cashew appears to be low. But it only indicates the extremely recalcitrant nature of tissues of this species. With mature explants not responding to culture conditions, studies have been limited only to juvenile tissues. It is obvious that much more efforts are needed to develop a suitable regeneration system in this economically important crop. With an increase in the number of *in vitro* studies in cashew in the last decade (Table 1.6 a, b and c), and the success reported in other commercially important woody and fruit tree species (Jain *et al*., 1995 and 2000; Hammerschlag and Litz, 1992; Tzfira *et al*., 1998; Nadgauda *et al*., 2000), we can certainly hope to develop workable regeneration protocols in cashew in the near future.

1.7. Purpose and objectives of the present study

Developing a reliable regeneration system in cashew is an urgent necessity. It would be of tremendous use for clonal multiplication as well as for variety improvement. With the success reported from our laboratory of *in vitro* clonal propagation of various woody species (Muralidharan and Mascarenhas 1987; Jana *et al*., 1994; Kendurkar *et al*., 1995; Nadgauda *et al*., 1997; Mascarenhas, 1998; Nadgauda *et al*., 1998; Mathur and Nadgauda, 1999) it was decided to initiate studies in cashew.

Experiments were conducted to study the *in vitro* morphogenic response in cashew, with the following objectives:

1. To develop a regeneration protocol for cashew through organogenesis using juvenile tissues. Seedlings were chosen as source of explants because of the extreme recalcitrance of mature explants. The steps involved for devising a regeneration system were:
    - Devising method for *in vitro* germination of seeds to have a convenient source of seedling explants
    - Selecting seedling explants at a suitable stage of development to obtain maximum shoot proliferation
    - Optimization of culture medium and growth regulators for shoot proliferation
    - Elongation of shoots and *in vitro* root induction
    - Hardening of shoots

2. To develop a regeneration system through somatic embryogenesis using nucellar tissue as explant. Nucellus is a maternal tissue and therefore important for clonal propagation of selected varieties. The studies were conducted for:
    - Choosing the correct developmental stage of nucellar explant for induction of somatic embryogenesis

- Optimizing culture medium and growth regulator requirements for proliferation of nucellar tissue, formation of embryogenic callus and induction of somatic embryos
- Maturation and conversion of somatic embryos
- Maintenance of embryogenic callus on semi-solid medium and as suspension culture

3. To develop a regeneration system through somatic embryogenesis using zygotic embryos. Such a system would be helpful in assisting the breeding programs.

   Following studies were conducted:

   - Identification of proper developmental stage for induction of somatic embryogenesis
   - Standardization of nutrient medium and growth regulators for induction of somatic embryogenesis
   - Optimization of medium for maturation and germination of somatic embryos

CHAPTER 2

GENERAL MATERIALS AND METHODS

General materials and methods are described in this chapter. The specific materials and methodologies employed for various experiments are described separately in chapters 3 - 5.

2.1. Glassware and plasticware

Test tubes (25 x 150 mm), conical flasks (125, 150, 250, 500, 1000 ml capacity), pipettes (1, 2, 5 and 10 ml capacity), measuring cylinders, funnels, etc. used in various experiments were of Corning or Borosil make. Locally manufactured glass jars with polypropylene screw caps were used as culture vessels. All glassware was cleaned using liquid soap and thoroughly washed in running tap water. Washed glassware was rinsed with distilled water and oven dried before use. Culture tubes and flasks were plugged with absorbent cotton prior to autoclaving. Flasks and bottles containing infected cultures were decontaminated by sterilization for 1 hour, prior to washing.

Pre-sterilized disposable transparent polypropylene petri-plates (55 X 15 mm and 85 X 15 mm) from Laxbro®, India Ltd. were also used in the studies. Auto-pipettes from Tarsons Accupipet®, India (0-20 µl, 20–200 µl and 200–1000 µl) and autoclavable micro-pipette tips (20, 200 and 1000 µl capacities) were used for accurate addition of fine chemicals in the medium. Autoclavable filter assembly units (Laxbro®, India) were used with 0.22 µm MF Millipore™ filter membranes (mixed cellulose acetate and nitrate) for filter sterilization of heat labile chemicals like L-glutamine, ABA, IAA and IBA, 2iP, TDZ and Zea. For experiments regarding effect of BA on axillary shoot production from germinating seeds and intact seedlings, BA was used as filter sterilized solution.

2.2. Glassware sterilization

All the empty glassware to be used for media preparation (flasks, bottles, test tubes, pipette tips) and instruments needed for sterile dissection and subculturing (forceps, scalpel, filter paper pads) were autoclaved at 121°C and 1.1 kg/cm^2 pressure for 1 hour.

2.3. Chemicals

Various chemicals used and their sources are listed in the Table 2.1 below. Any other specific chemical used in the present investigation has been mentioned separately in chapters 3-5.

Table 2.1: Chemicals used and their source

Chemical	Source
Activated charcoal	Sigma Chemical Co., USA.
Casein hydrolysate	Sigma Chemical Co., USA.
Labolene ®	Qualigens, India
Macro - and Micro –nutrients (AR grade)	Qualigens, India.
Maltose	Hi-Media, India
Myo-Inositol	Sigma Chemical Co., USA.
Phytagel (gellan gum)	Sigma Chemical Co., USA.
Polyethlene Glycol (PEG)	Hi-Media, India.
Plant Growth Regulators	Sigma Chemical Co., USA.
Sucrose (ExelAR grade)	Qualigens, India
Vitamins and amino acids.	Sigma Chemical Co., USA.

Coconut water was collected from tender coconuts and autoclaved at 121°C at 1.1 kg/cm^2 pressure for 20 minutes. After autoclaving, it was filtered through Whatman Number 1 filter paper, and stored at –20°C for routine use.

2.4. Tissue culture media

For all the experiments during the investigations, MS basal medium (Murashige and Skoog, 1962) was fortified with different auxins and/or cytokinins or any other test chemicals as mentioned in the following chapters. Other basal media used in the studies are SH (Schenk and Hildebrandt, 1972) and WPM (Lloyd and McCown, 1981). The concentrations of the macro- and microelement salts, organic constituents and amino acids of the basal media used are given below in Table 2.2.

Table 2.2: Composition of basal media: MS (Murashige and Skoog, 1962), SH (Shenck and Hildebrandt, 1972) and WPM (Lloyd and McCown, 1981)

(mg/l)	MS	SH	WPM
Macro-nutrients			
KNO_3	1900	2500	400
NH_4NO_3	1650	-	-
$Ca(NO_3)_2.4H_2O$	-	-	556
$CaCl_2.\ 2H_2O$	440	200	96
$MgSO_4.\ 7H_20$	370	400	370
KH_2PO_4	170	300	170
K_2SO_4	-	-	990
Micro-nutrients			
$MnSO_4.\ 4H_2O$	22.3	10.0	22.3
$ZnSO_4.\ 7H_2O$	8.6	1.0	8.6
H_3BO_3	6.2	5.0	6.2
KI	0.83	1.0	-
$Na_2MoO_4.\ 2H_2O$	0.25	0.2	0.25
$CuSO_4.\ 5H_2O$	0.025	0.1	0.25
$CoCl_2$	0.025	0.1	-
$FeSO_4.\ 7H_2O$	27.8	15	27.8
Na_2. EDTA	37.3	20	37.3
Vitamins			
Nicotinic acid	0.5	5.0	0.5
Pyridoxine – HCl	0.5	0.5	0.5
Thiamine – HCl	0.1	5.0	1.0
Glycine	2.0	-	2.0

2.4.1. Stock and media preparation

All the stock solutions and media were prepared in glass distilled water or Milli-Q (Millipore water purification system) water. Stock solutions of major

elements were prepared as 10X and stock solutions of minor elements as 100X (Table 2.3.). Sucrose, myo-inositol, CH, L-glutamine were always weighed and added at the time of preparation of the medium. Growth regulators were added at the time of media preparation. Distilled water was used as diluant for all growth regulator solutions (Table 2.4). Growth regulators like IAA, IBA, Thidiazuron and Zeatin were added to nutrient media after autoclaving.

Table 2.3: Stock solutions of basal medium

Stock	Concentration	Storage
Major salts	10 X	Refrigeration
Minor salts and vitamins	100 X	Refrigeration
$Na_2FeEDTA$*	100 X	Refrigeration

* The $Na_2FeEDTA$ stock (total volume 500 ml) was prepared by dissolving Na_2EDTA (100X) in 200 ml of hot water and to this, 200 ml solution of $FeSO_4$ (100X) was added drop-wise. The resultant solution was mixed thoroughly and the final volume was made to 500 ml.

Table 2.4: Stock solutions of growth regulators used

Growth regulator	Solvent	Concentration of stock	Storage	Addition before (B) / after (A) autoclaving
ABA	1N NaOH	500 µM	0 ºC	A
BA	"	2 mM	RT	B & A
2,4-D	Ethanol	"	"	B
GA_3	"	"	4 ºC	B & A
IAA	1N NaOH	"	0ºC	A
IBA	"	"	0 ºC	A
Kin	"	"	RT	B & A
NAA	"	"	"	B
2iP	"	500 µM	0 ºC	B & A
TDZ	Ethanol	"	4 ºC	A
Zea	1N NaOH	"	0 ºC	A

After dissolving all the ingredients except for solidifying agent, the pH of the medium was adjusted between 5.8-6.0 (unless otherwise stated) using 1.0 N HCl/ NaOH. The final volume was adjusted and gelling agent was added for semi-solid media before sterilization. If the media were to be poured in test tubes, bottles or flasks before autoclaving, gelling agent was melted by steaming the medium and then dispensed in the appropriate container. Media was dispensed in petri-plates in laminar airflow after sterilization.

2.4.2. Media sterilisation

All nutrient media were autoclaved at 1.1 kg/cm^2 pressure at 121°C for 20 minutes. Heat labile chemicals were added in the required quantity, after filter sterilization, into the autoclaved media.

2.5. Culture incubation conditions

Cultures were incubated in the dark or under illumination of cool white fluorescent light for 16 hours, depending on the experiment. The intensity of the light was 10 to 20 μE $m^{-2}s^{-1}$. The temperature of incubation was 22±2°C or 25±2°C. Suspension cultures were incubated on a rotary shaker at 100 rpm in dark.

2.6. Statistical Analysis

All the experiments were repeated two to three times (depending on the availability of the plant material) with a minimum of 90 explants per treatment before conducting the statistical analysis. The data was subjected to analysis of variance (ANOVA) for comparison between different treatments. Standard deviation for each treatment was also calculated. The separation of means was tested by Fisher's Least Significant Difference (LSD) test.

CHAPTER 3

AXILLARY BUD PROLIFERATION IN SEEDLING TISSUE AND STUDIES ON *IN VITRO* SEED GERMINATION

CHAPTER 3
AXILLARY BUD PROLIFERATION IN SEEDLING TISSUE AND STUDIES ON *IN VITRO* SEED GERMINATION

Production of multiple shoots from axillary and apical buds is the most widely used method of *in vitro* propagation of plants (George, 1993). Using appropriate nutrient media and growth regulators, multiple shoots can be produced from a shoot tip or axillary bud. These shoots can be rooted to produce plantlets, and also used for further multiplication cycles. A single explant can give rise to a large number of plantlets in a short period. This method has been successfully employed in several fruit trees, listed in Table 3.1. Table 3.2 covers the literature pertaining to micropropagation of woody species using cotyledonary nodes of seedlings.

In cashew, when attempts were made to induce multiple shoots from axillary and apical buds, it was observed that mature tissues are extremely recalcitrant to *in vitro* culture. Shoots were formed only from explants obtained from seedlings or very young plants (Das *et al*., 1996; D'Souza *et al*., 1996; Boggetti *et al*., 1999; Nadgauda and Gogte, 2003).

Obtaining seedlings aseptically is an important step in the micropropagation of cashew. A convenient method for *in vitro* germination of seeds would certainly be of great help in obtaining aseptic seedling tissues.

In tissue culture studies in India, cashew varieties cultivated in the particular geographical location (Table 1.5 in Chapter 1: Introduction) were used. There are no reports on varieties from Maharashtra State, which has emerged as a leading cashew-producing state in India, with potential for further growth (Unnikrishnan, 2003). Such studies will undoubtedly benefit the on-going research aimed at improvement in yield and quality of cashewnuts.

Table 3.1: Axillary shoot proliferation from nodal explants in fruit and nut species

Species	Basal medium	PGR used for shoot production	PGR used for rooting	Reference
Aegle marmalos	MS	BA +IAA	IAA/ IBA	Ajithkumar & Seeni, 1998
Annona hybrid	MS	BA + Kin	IBA or AC	Nair *et al*., 1984
Annona hybrid (atemoya)	MS	BA + Kin	IBA	Rasai *et al*., 1994
Annona muricata	WPM	BA + NAA	NAA	Lemos & Blake, 1996
Artocarpus heterophyllus	MS	BA	IBA	Rahman & Blake 1988
		BA + Kin	IBA + NAA	Roy *et al*., 1990
		BA + IBA	IBA	Singh & Tiwari, 1998
Carya illinoensis	MS	BA + IBA	IBA	Wood, 1982
		BA + GA_3	IBA	Hansen & Lazarte, 1984
Castanea sativa	MS	BA	-	Chevre *et al*., 1983
Castanea mollissima	MS	BA	IBA	Qi-Guang *et al*., 1986
Capparis decidua	MS	BA + NAA; BA + IAA	IBA	Deora & Shekhawat, 1995
Citrus sinensis, C. medica, C. aurantifolia	MS	BA	NAA	Duran-Vila *et al*., 1989
Citrus aurantifolia	MS	BA + Kin + NAA	IBA + NAA	Al-Bahrany, 2002
Corylus avellana	K (h) ½	BA	-	Perez *et al*., 1985
Feronia limonia	MS	BA + Kin + NAA	IBA	Purohit & Tak, 1992
Juglans regia L.	K (h)	BA +IBA	-	Rodriguez *et al*., 1989
	DKW	BA + IBA	vermiculite	Tetsumara *et al*., 2002
Malus domestica	MS	BA	NAA + IBA	Sriskandarajah *et al*., 1982
Malus cultivar	MS	BA + GA_3 + IBA; BA + Kin	Phg + IBA	Modgil *et al*., 1999
Morus indica	MS	BA	-	Mhatre *et al*., 1985
M. cathayana, M. ihou, M. serrata	MS	BA + GA_3	IAA+IBA +IPA	Pattnaik & Chand,1997
M. indica L. cv M-5	MS	2,4-D; BA + GA_3	2,4-D	Vijaya Chitra & Padmaja, 1999
M. latifolia Poilet	MS	BA	IBA	Lu, 2002

continued….

Table 3.1 continued……

Species	Basal medium	PGR used for shoot production	PGR used for rooting	Reference
Olea europaea	MS ½	ZR + IBA + GA_3	NAA	Rugini & Fontanazza, 1981
	OM	BA/ 2ip/ ZR + IAA + GA_3	NAA	Rama & Pontikis 1990
	DKW	BA + IBA	IBA	Santos *et al*., 2003
Pistacia vera L	MS	BA	IBA	Barghchi & Alderson, 1983a,b, 1985; Onay, 2000
Punica granatum L. cv. Ganesh	MS	Zea riboside		Naik *et al.,* 1999
Psidium guajava L.	MS	BA	IBA + NAA	Amin & Jaiswal, 1987
	MS	BA	IBA	Mohamed-Yasseen *et al*., 1995.
Prunus armeniaca L	WPM	2iP	MS½	Snir, 1980
Prunus mume	WPM	BA + sorbitol	NAA	Harada & Murai, 1996
Prunus avium L.	MS	BA + IBA	NAA/ IBA	Muna A-S *et al*., 2000
Ribes magellanicum Poiret	MS	BA	MS ½	Arena & Guillermo, 1995
Rubus glaucus	MS	BA+GA_3	IAA	Hernandez *et al*.,1999
Rubus idaeus	MS ½	BA	IAA	Welander, 1985
Syzygium alternifolium	MS	BA + NAA	IBA	Sha Valli Khan *et al*., 1997
Syzygium cuminii	MS	BA + NAA	NAA	Yadav *et al*., 1990
Vaccinium corymbosum	WPM	Zea; 2iP	*Ex vitro*	
Rubus spp.,	MS	BA + IBA	-do-	Gonzalez *et al*., 2000
	MS	BA + IBA	-do-	
Vitis rotundifolia,	MS	BA		Lee & Wetzstein, 1990.
Vitis vinifera	NN,LS; WP,B5; MS	BA + NAA; BA; BA + IAA	IBA	Mhatre *et al*., 2000
Vitis aestivalis	MS	BA	-	Norton & Skirvin, 2001
Zizyphus nummularia, Zizyphus mauritiana	MS	BA + IAA	IBA	Rathore *et al* 1992

ZR: Zeatin riboside

Table 3.2: Axillary shoot proliferation from cotyledonary nodes, in tree and fruit tree species

Species	Basal Medium	Growth regulators used for shoot initiation	Growth regulators used for rooting of shoots	Reference
Acacia albida	MS (N6 vit)	BA + NAA	Sucrose	Duboux & Davis, 1985
Acacia mangium	DKW	BA + TDZ		Douglas & Mcnamara, 2000
Acacia nilotica	B5	BA	IAA	Dewan *et al*., 1992
Achras sapota	SH	BA	IBA	Purohit & Singhvi, 1998
Anogeissus acumulata	MS	BA + IAA	IBA	Rathore *et al*., 1993
Azadirachta	MS	BA + IAA	IBA	Salvi *et al*., 2001
Bauhinia vahlii	MS MS	TDZ TDZ + Kin	NAA NAA	Upreti & Dhar, 1996 Bhatt & Dhar, 2000
Castanea sativa	MS	BA; TDZ		San-Jose *et al*., 2001
Cercis canadensis	DKW	BA+ TDZ; BA	IBA	Distabanjong & Geneve, 1997
Cinnamomum zeylanicum	MS	BA+ Kin /NAA	IAA + IBA + IPA	Rai & Jagdish Chandra, 1987
Coryllus avellana	K(h) ½	BA	IBA	Perez *et al*., 1985

Continued…

Table 3.2 continued……

Species	Basal Medium	Growth regulators used for shoot initiation	Growth regulators used for rooting of shoots	Reference
Dalbergia latifolia	MS	BA	IAA + IBA + IPA	Pradhan *et al*., 1998
Dalbergia sissoo	MS	BA	IAA + IBA + IPA	Pradhan *et al*., 1998
Fraxinus excelsior	DKW	BA	IBA	Hammat & Ridout, 1992
Fraxinus pennsylvanica	MS (B5 vit)	BA+ TDZ+ IBA	*Ex vitro*	Kim *et al*., 1997
Litchi sinensis	MS	BA	IBA	Das *et al*., 1999
Melissa officinalis	MS	BA	IBA or NAA	Tavares *et al*., 1996
Prosopis cineraria	MS	Kin + IAA	IBA	Nandwani & Ramawat, 1993
Prosopis tamarugo	MS	BA	IBA	Nandwani & Ramawat, 1992
Punica granatum	B5 MS	NAA + Kin + BA; BA	IAA NAA	Sharon & Sinha, 2000 Naik *et al*., 2000
Sterculia urens	MS	BA	IBA	Purohit & Dave, 1996
Tecomella undulata	MS	BA + IAA; BA	NAA + IAA + IBA	Nandwani *et al*., 1995
Wrightia tinctoria	MS	BA+ NAA	IBA	Purohit & Kukda, 1994

The present study was undertaken with the following objectives:

1. Devising a convenient method for *in vitro* germination of cashew seeds

2. Developing a regeneration system using the seedling tissues from *in vitro* germinated seeds. The variety selected was Vengurla 1, one of the varieties recommended for cultivation under the agro-climatic conditions of Maharashtra State.
3. Studying the effect of BA on multiple shoot induction from germinating seeds and intact seedlings.

{Note: In cashew, mature nut consists of the outer seed-coat, middle papery thin testa, and the innermost seed (kernel). In the present study, the word 'seed' means mature nut (seed-coat + testa + seed), and 'embryo axis' means only the isolated embryo axis, without cotyledons}.

The experiments were carried out as outlined below:

1. Devising suitable method for *in vitro* germination of mature seeds: Seeds were germinated on different substrates. Seeds and isolated embryo axes were also compared for their germination pattern. Based on the observations, a suitable germination method was devised.

2. Designing regeneration system for variety Vengurla 1: *In vitro* germinated seeds were used as source of different explants. Experiments were carried out to induce multiple shoots from these explants.

3. Studying the effect of BA on multiple shoot induction from germinating seeds and intact seedlings: Seeds were germinated in presence of varying concentrations of BA. Effect of BA on induction of multiple shoots from the germinating seeds was studied. The germinated seeds were further allowed to develop into seedlings in BA containing medium to see whether BA induced axillary shoot production in intact seedlings. Cotyledonary nodes and epicotyledonary nodes were taken from 4-5 week old seedlings and inoculated on MS medium to study if pre-treatment of BA enhanced axillary shoot production from these explants.

The present chapter describes the experiments conducted, results obtained and discussion, in Sections 3.1 to 3.5, as follows:

3.1. Experimental - collection of plant material and surface sterilization procedure; **3.2:** Culture conditions for (i) devising suitable method for *in vitro* germination of mature seeds using different substrates, (ii) axillary shoot proliferation from seedling explants, and (iii) effect of BA on multiple shoot induction from germinating seed and intact seedling are mentioned. Particulars for each factor studied are described under separate heading ; **3.3:** Results and discussion. Results for each factor studied are discussed under separate headings; **3.4:** Summary; **3.5:** Conclusions.

3.1: Experimental

Collection of plant material: Mature seeds of cashew (variety Vengurla 1, Refer Fig. 1.5a) were procured from Balasaheb Sawant College of Agriculture, Dapoli, Maharashtra. The seeds were collected from 8-15 years old open pollinated field grown trees. Seeds were bought to the laboratory and were dusted with fungicide Bavistin®. They were stored in polythene bags at 4-8°C.

Surface sterilization of seeds

The seeds were washed thoroughly under running tap water. Any remnants of the cashew apple when present, were removed with blade/ scalpel. The seeds were surface sterilized in the following stepwise manner:

1. Thorough wash with laboratory detergent Labolene® (1% solution), 20-30 minutes.
2. Rinsing with water.
3. Treatment with 70% alcohol, 15-20 minutes.

Remaining procedure was carried out in laminar airflow cabinet:

4. Soaking of seeds in sterilized distilled water for 3 hours
5. Surface-sterilization with 0.1% $HgCl_2$, 45 minutes
6. Rinsing 4-5 times with sterile distilled water.
7. Soaking in sterile distilled water, 24 hours.

8. Surface-sterilization with 0.1% $HgCl_2$, 20 minutes.
9. Rinsing 4-5 times with sterile distilled water.

3.2: Culture conditions

3.2.1. Devising suitable method for *in vitro* germination of mature seeds: For germination studies, mature nuts were used in two forms – as whole seeds (seedcoat + testa + kernel), and as decoated seeds (only kernels). To obtain decoated seeds, the seedcoat, which was hard and difficult to crack open, had to be removed. For this purpose, mature nuts were soaked for 72 hours in sterile distilled water to soften the seedcoat. Softening of seedcoat facilitated dissection. Keeping the notched side of the nuts down, a longitudinal incision was made along the convex surface of the nut, to bisect the seedcoat. Intact kernels were carefully extracted. The thin brown testa covering the kernels was removed gently. Such decoated seeds (kernels) were used for germination.

Whole and decoated seeds were cultured on three substrates: (i) water + agar 0.6%, (ii) wet cotton, and (iii) filter paper supports in distilled water.

On agar and cotton substrates, there was heavy leaching of phenolic substances from whole and decoated seeds, causing intense browning of the substrates. Therefore these explants had to be transferred frequently to fresh substrate for proper growth, as the accumulated phenolics hampered seedling growth. Additionally, cotton proved to be a hindrance to the developing taproot. When distilled water was used as substrate and the explants were placed on filter paper supports, no exudation was observed from these explants. Therefore transferring seeds to fresh substrate was not required. Root system also showed healthy development in distilled water. Thus, between agar, cotton and distilled water, distilled water proved to be the most suitable substrate for seed germination.

Between whole seeds and decoated seeds, whole seeds were preferred as explants. The kernels (decoated seeds) were prone to mutilation and damage during dissection of seeds. Due to the presence of corrosive CNSL in seed coat, the process is hazardous and has to be performed very cautiously. Therefore, it

was decided to use whole seeds placed on filter paper supports in distilled water, for *in vitro* germination.

To decide the optimal liquid medium for germination, distilled water, quarter-strength, half-strength and full-strength liquid MS medium were compared. Whole seeds were placed on filter paper supports in the different liquid media. Seeds were cultured in bottles, each having 50 ml of liquid media. For all substrates, one seed was used per culture bottle. Thirty to thirty five seeds were used for each treatment and the treatment was repeated three times. All cultures were incubated at 25±2°C in the dark. Percent germination on the four media was noted after four weeks of incubation. Emergence of radicle was taken as indication of germination.

Two weeks after germination, germinated seeds were transferred to 16-hour photoperiod conditions. The germinated seeds developed further into seedlings on the same substrates. Four to five weeks after germination, seedlings were removed from the culture bottles, and cotyledonary nodes and nodal explants were obtained from seedlings grown on the four substrates. These explants were cultured on semi-solid MS medium containing BA 10 μM. Percent explants showing induction of multiple shoots, and average shoots produced per explant were noted six weeks after inoculation.

In another experiment, whole seeds and isolated mature zygotic embryo axes were used as explants, and compared for germination and growth of seedlings. Whole seeds were used as explained above. Mature zygotic embryo axes were obtained from kernels (decoated seeds) by carefully separating the embryo axis from the cotyledons, with scalpel. The embryo axes were germinated on identical substrates as used for whole seeds (on filter paper supports in distilled water, quarter, half, and full strength MS medium). Thirty to thirty five explants were used per treatment and each treatment was repeated three times.

3.2.2. Designing regeneration system for variety Vengurla 1: As the starting step of developing regeneration system, seeds were germinated *in vitro*, as per the

method devised in part I. The seedlings obtained were used as source of different explants (apical bud/ primary shoot tip, nodes, cotyledonary nodes, cotyledons, leaves, petioles, internodal segments, hypocotyls, and roots).

In preliminary experiments, all the explants were inoculated on semi-solid MS + BA 10 μM + sucrose 2%, since BA is a widely used cytokinin for shoot formation. In repeated attempts, only cotyledonary nodes and nodal explants responded to culture, and formed axillary shoots on the BA-containing medium.

For all further experiments to develop a regeneration system, only cotyledonary node and nodal explants from seedlings were used. Cotyledonary nodes were obtained by making transverse cuts, 0.5-1 cm above, and 1-2 cm below the point of attachment of cotyledons, to remove the primary shoot and roots respectively. Cotyledonary nodes were used keeping both the cotyledons intact. Occasionally, distal ends of the cotyledons were excised, just enough to fit the explant inside the culture bottle. Nodal explants were obtained by cutting about 0.5 cm above, and 1-2 cm below the node. Nodes were defoliated before culturing them.

Effect of different factors, such as age of explants, basal medium, cytokinins, and presence of cotyledons (only for cotyledonary node), were studied, in attempts to standardize the optimal conditions for axillary shoot proliferation from cotyledonary nodes and nodal explants. Axillary shoots obtained from these explants were elongated and used for *in vitro* root induction. Rooted shoots were acclimatized in greenhouse.

For all the studies, thirty to forty explants were used for each treatment and all treatments were repeated three times. Cultures were incubated in 16-hour photoperiod. For *in vitro* root induction, all cultures were incubated in the dark. Culture conditions for each of these factors are described separately below.

3.2.2.1. Effect of explant age on axillary shoot proliferation: The effect of increasing explant age on axillary shoot proliferation from cotyledonary and nodal explants was studied. Cotyledonary nodes were collected from seedlings 3-week post-germination through 8-week post-germination stages. Nodal

explants were obtained from seedlings from 4 through 8 weeks post-germination stages (nodes were formed on the primary shoot only 3-4 weeks after germination). For all experiments, full strength MS medium containing 10 μM BA, 3% sucrose and 0.6% agar (Qualigens, India) was used. Since maximum shoots were produced at 4-5 week post-germination stage, cotyledonary nodes and nodal explants from this stage were used for further experiments.

3.2.2.2. Effect of basal media on axillary shoot proliferation: Along with choosing the explants at appropriate stage, using a suitable nutrient medium is also necessary for ensuring production of maximum number of axillary shoots. Therefore, cotyledonary nodes and nodal explants from 4-5 weeks old seedlings were inoculated on three different basal media (MS, SH and WPM) to select a suitable basal medium for axillary shoot proliferation. Axillary shoot production from cotyledonary and nodal explants was observed on the three media. As MS medium was found optimal for axillary shoot proliferation, it was tested at different strengths (full, half and only major salts half). All media contained 10 μM BA, 3% sucrose and 0.6% agar (Qualigens, India).

3.2.2.3. Effect of cytokinins on axillary shoot proliferation: Cytokinins are frequently used for *in vitro* shoot production. Hence, different cytokinins (BA, Kin, Zea, 2iP and TDZ) were added to nutrient medium and their effect on axillary shoot proliferation was observed. Each cytokinin was used separately. Basal medium was MS (full strength) + sucrose 3% + agar 0.6% (Qualigens, India). As BA (10 μM) was most suitable for axillary shoot proliferation, it was used in combination with other cytokinins (Kin, Zea, 2iP - 5, 10 and 20 μM each; and TDZ- 0.005, 0.01, 0.05 μM) to examine whether the combinations could further enhance shoot proliferation.

3.2.2.4.Effect of presence of cotyledons on axillary shoot proliferation from cotyledonary node: Experiments were conducted to study the effect of cotyledons on axillary shoot proliferation from cotyledonary nodes.

Cotyledonary nodes were collected from 4-5 weeks old seedlings. The cotyledonary nodes were used as follows: (i) with both cotyledons intact (ii) one cotyledon removed, and (iii) both cotyledons removed. Semi-solid MS (full strength) + BA 10 μM + sucrose 3% was used.

3.2.2.5. Elongation of shoots: Axillary shoots obtained from cotyledonary nodes and nodal explants were separated from the parent explant and transferred to elongation media (Table 3.9). Elongation of shoots was carried out on MS medium, with either BA (1 and 5 μM), or GA_3 (5 and 15 μM), or activated charcoal 0.5%. Dark incubation of separated shoots was also carried out to observe the effect on shoot elongation. After removal of shoots, the parent explant was transferred to fresh shoot induction medium (MS + BA 10 μM + sucrose 3% + agar 0.6%) for further axillary multiplication. In another approach for shoot elongation, the axillary shoots were not separated from the parent explant. Parent explant along with the proliferated shoots, was transferred to fresh shoot induction medium (MS + BA 10 μM + sucrose 3 % + agar 0.6%).

3.2.2.6. Rooting of elongated shoots: Shoots more than 2.5 cm in length were used for *in vitro* root induction. The shoots were initially cultured on semi-solid and liquid media for root induction. Medium used was MS (half-strength) + IAA (5 μM) + IBA (2.5 μM) + sucrose 1%. Phytagel™(Sigma Chemical Co., USA) was used as gelling agent. For liquid medium, shoots were inoculated on filter paper supports in tubes, each with 10 ml medium. More number of shoots rooted in liquid medium. Root induction was very poor in semi-solid medium. Therefore, half-strength MS liquid medium with sucrose 1% was selected for all rooting experiments. Auxins IAA and IBA, separately and in combinations (Table 3.10) were used to study their effect on *in vitro* rooting. For root induction, shoots were given auxin treatment in two ways:

(i) Pre-treatment with auxins: Shoots were pre-treated with auxins IAA (50, 100, 200 μM), IBA (50, 100, 200 μM), NAA (50, 100, 200 μM) individually for

varying time duration (12, 24, 48 hours). Afterwards they were cultured on auxin-free MS liquid medium.

(ii) Growth on auxin-containing media: Shoots were cultured on medium with IAA and IBA

3.2.2.7. Hardening of rooted shoots: Rooted shoots were transferred to sterilized sand:soil mixture (river sand and garden soil in the ratio 1:1 by volume) in polythene bags (4" x 2"). The mixture was initially moistened with sterile quarter-strength MS medium without sucrose. The polythene bags with the shoots were kept in greenhouse, for acclimatization at 25±2°C and 80±5 % relative humidity. In the greenhouse, each shoot was covered with another transparent polythene bag. The polybag cover was removed after 7-8 weeks. Formation of a pair of new leaves at the apex was taken as indication of survival of the shoots.

3.2.3. Effect of BA on multiple shoot induction from germinating seed and intact seedling: To induce axillary shoot production from the germinating seeds, and/or intact seedlings of cashew, mature seeds were germinated in varying concentrations of BA (10, 20, 50, 100, 250 and 500 μM). Seeds germinated in distilled water were used as control. To study whether BA could induce multiple shoots from the seeds during germination, and during further development, germinated seeds were allowed to develop into seedlings in the BA-containing medium. Percent germination was noted four weeks after inoculation.

Cotyledonary nodes and nodal explants were collected from 4-5 weeks old seedlings. Explants from each BA concentration were inoculated on (i) MS medium without cytokinin, and (ii) MS medium with 10 μM BA. Axillary shoot formation from germinating seeds, intact seedlings, and seedling explants was observed for each BA treatment. Thirty to forty explants were used for each treatment, which was repeated thrice. Cultures were incubated in the dark till two weeks after germination, and then in 16-hour photoperiod. Axillary shoot production was noted six weeks after inoculation of explants.

3.3: Results and discussion: Results obtained in different experiments are presented and discussed in this section.

3.3.1. Devising suitable method for *in vitro* germination of mature seeds: When whole seeds were inoculated on different liquid substrates (distilled water, quarter, half and full strength MS medium), and incubated for four weeks, it was observed that seed germination was between 70-80% in all substrates (Table 3.3a). Thus, the nutrient medium substrates did not have any significant effect on germination of seeds, and seed germination occurred equally well in distilled water.

Table 3.3a: Comparison of different liquid substrates used for germination of mature cashew seeds

Substrate	Percent germination after 4 weeks	Percent responding explants, 6 weeks after inoculation		Average number of shoots per explant	
		CN	NE	CN	NE
SDW	77.1 ± 0.9	70.8 ± 0.7	64.9 ± 0.6	8.2 ± 0.1	1.2 ± 0.1
MS ¼	72.3 ± 2.4	68.7 ± 0.9	62.4 ± 0.6	7.1 ± 0.1	1.1 ± 0.1
MS ½	80.0 ± 2.8	68.3 ± 1.5	63.2 ± 0.4	6.9 ± 0.2	1.2 ± 0.2
MS full	74.9 ± 1.8	69.4 ± 1.3	63.3 ± 1.1	7.2 ± 0.1	1.0 ± 0.1

{Sterilized distilled water (SDW), and MS medium with 2% sucrose were used for seed germination. Cotyledonary nodes (CN) and nodal explants (NE) were obtained from 4-5 week old seedlings. All explants were inoculated on MS + BA 10 μM + sucrose 3% + agar 0.6%., and incubated in 16-hour photoperiod. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-35 explants per treatment in each experiment. The differences between all values in each vertical column were insignificant at P≥ 0.01 and 0.05 significance levels, as analyzed by ANOVA, and Fisher's LSD test}.

Seedlings developed on each type of liquid substrate were used as source of cotyledonary and nodal explants. These explants from each substrate were inoculated on MS + BA 10 μM + sucrose 3% + agar 0.6%. Between substrates, there was no significant difference in number of explants (both, cotyledonary and

nodal) producing multiple shoots and average number of axillary shoots formed per explant (Table 3.3a). It was observed that 70.8%, 68.3%, and 69.4% of cotyledonary explants formed axillary shoots when distilled water, half, and full strength MS media respectively were used. The average number of shoots formed per cotyledonary node was 8.2 for distilled water, 6.9 for half strength MS, and 7.2 for full strength MS. For nodal explants, 64.9%, 63.2% 63.3% explants formed one axillary shoot when distilled water, half, and full strength MS media were used as germination substrates respectively (Table 3.3a).

Thus, use of nutrient medium for seed germination did not have any beneficial effect either on germination, or axillary shoot production. There was no significant difference in the number of explants forming axillary shoots, and number of shoots produced per explant, when the four substrates were compared. Distilled water worked as a satisfactory and equally effective substrate for seed germination and seedling growth, as evident from the axillary shoot formation response. Therefore, for all further experiments, seeds were always germinated, and grown into seedlings, in distilled water (Fig. 3.1 a-c).

In other woody species, seedling tissues are frequently used for *in vitro* studies, and the seeds are conveniently germinated *in vitro*. In cashew, Das and co-workers (1996) and D'Silva and D'Souza (1992) used de-coated seeds, whereas, Thimmapaiah and Samuel (1999) used whole seeds.

In the present study, it was observed that use of solid substrates (agar and cotton) for germination of seeds caused heavy leaching of phenolic exudates into the substrates (Fig. 3.1 d), necessitating transfer of seeds to fresh substrates. Exudation occurred mainly from the seedcoat. Therefore, using whole embryos (after removing seed-coat) was an alternative method to germinate seeds. However, removal of seed coat in cashew is difficult because it is very thick, hard and tough. The seeds have to be soaked in water for atleast 48 hours to soften the seed coat. Due to the tough and leathery nature of the seedcoat, it had to be cut open with some force, and chances of kernels getting mutilated and damaged during dissection increased. Additionally, the seedcoat contains corrosive cashewnut shell liquid (CNSL). It is an irritant and direct contact with

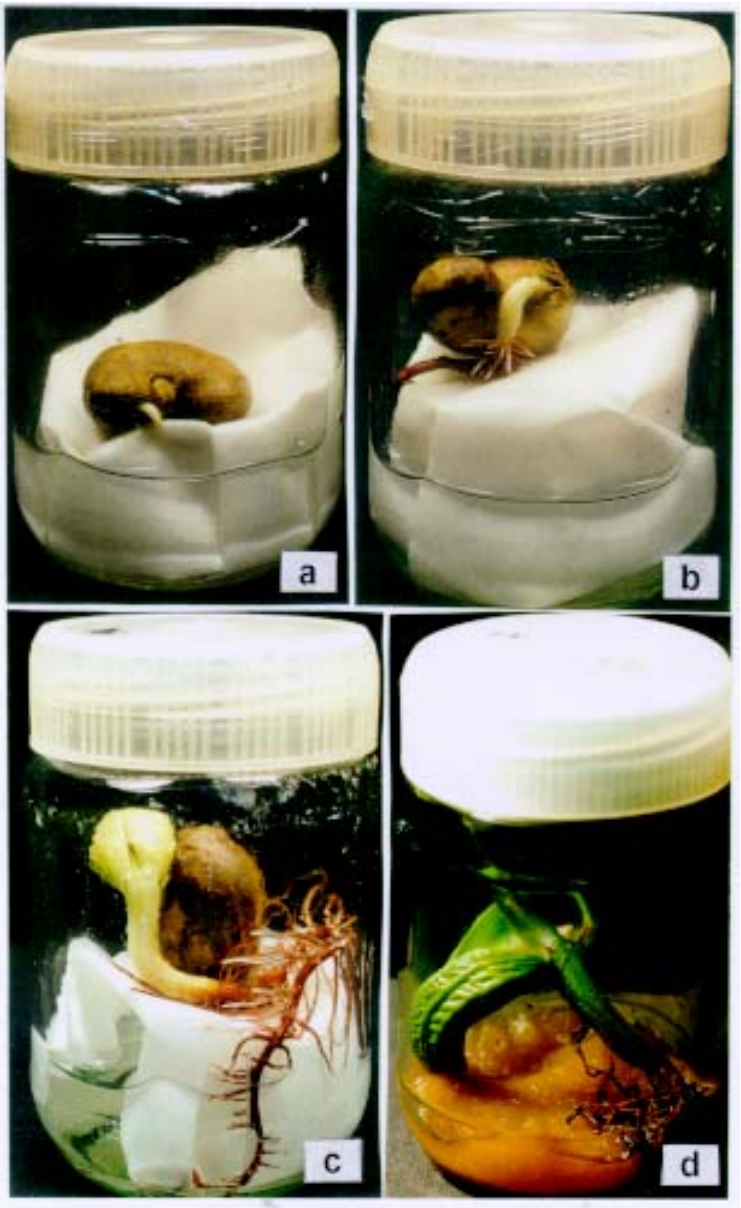


Fig. 3.1: *In vitro* germination of mature cashew seeds a, b, c: Seeds placed on filter paper supports partially immersed in distilled water; 3.1d: Leaching of phenolic exudates into cotton substrate, and hampered root growth

skin and eyes is dangerous and causes lesions. Great care has to be taken to avoid any contact with CNSL. The smooth surface and biconvex shape of the nut further make it difficult to hold the nut in a firm grip, which is essential for safe dissection.

Therefore, seeds were used along with seedcoat for all experiments. This did not involve any hazards involved in the dissection process. When these seeds were placed on filter paper supports in liquid medium, it was observed that there was no phenolic exudation from the seedcoat, as evident from the clear colourless appearance of the liquid medium throughout the incubation period of 4-5 weeks. Thus, removal of seedcoat was not required when liquid substrate was used for germination. Hence, for all experiments, seeds were used with seedcoat, and germinated using liquid substrates.

In another experiment, seeds and isolated mature zygotic embryo axes were compared for germination and seedling growth. It was observed that whole seeds always produced healthier seedlings (Table 3.3b).

Table 3.3b: Comparison between whole mature seeds and isolated mature embryo axis of cashew for germination and development of seedling *in vitro*

Explant used	Germination substrate used	Percent germination four weeks after inoculation	Percent explants (germinated seeds) forming seedling
Whole mature seeds	SDW	79.3 ± 1.8	100
	MS ¼	75.2 ± 1.9	100
	MS ½	75.8 ± 1.6	100
	MS full	76.3 ± 0.5	100
Isolated mature embryo axis	SDW	0	0
	MS ¼	0	0
	MS ½	0	0
	MS full	0	0

{Sterile distilled water (SDW), and MS medium with 2% sucrose were used for germination. Each value represents mean ±standard deviation (SD) of 3 experiments with 30-35 explants per treatment in each experiment}.

The isolated mature zygotic embryo axes could never germinate and produce seedlings, either in distilled water, or in nutrient media. Only slight elongation of hypocotyl region of the zygotic embryo axis was observed on all germination substrates. The isolated zygotic embryo axis is an explant without cotyledons. Removal of cotyledons severely affected the development of the embryo axis, and the isolated embryo axis invariably failed to develop into seedling. Thus, presence of cotyledons is indispensable for seedling formation in cashew. The cotyledons are a rich source of various complex organic nutrients and growth substances, and provide nutrition necessary for seedling formation. The cotyledons considerably increase in size, become green, thick and fleshy during germination and remain attached to the seedling till about 6 weeks after germination. The rapidly growing seedling takes all the necessary nutrition from the cotyledons. Removal of cotyledons deprives the embryo axis of these

substances, and therefore, it cannot develop further. The components of nutrient media are unable to act as substitute for the complex endogenous substances provided by the cotyledons.

There are no reports on successful culture and seedling development from isolated mature embryo axis of cashew. This observation indicates the total dependence of the mature zygotic embryo axis on the cotyledons, for further development into seedling.

Distilled water can be conveniently used for seed germination and seedling growth till 4-5 weeks after germination, and there was no need to use any nutrient medium for this purpose.

3.3.2. Designing regeneration system for variety Vengurla 1: As mentioned in part 1, mature seeds were germinated and seedlings developed *in vitro*, using distilled water. Cotyledonary node and nodal explants were obtained from these seedlings, and used for studying their response to various culture conditions (3.2.2.1. to 3.2.2.7). The responses observed for different conditions are discussed below.

3.3.2.1. Effect of explant age on axillary shoot proliferation:

To study the effect of increasing age of seedling explants on axillary shoot proliferation, cotyledonary nodes and nodal explants were collected at two weeks post-germination, through eight week post-germination stages, and inoculated on MS medium with 10 μM BA. Nodal explants were collected from four through eight- week post-germination stages. The number of explants forming axillary shoots, and shoots produced per explant were noted for each stage, six weeks after inoculation.

It was observed that age of explant affected the shoot forming capacity. The effect of age on axillary shoot production was more pronounced in cotyledonary node. For nodal explant, age of explant did not have any significant effect, and the number of shoots produced at all stages remained 1-3 per explant.

Cotyledonary node: In very young (3-4 weeks post-germination stage) (Fig. 3.2a), as well as slightly older (5 through 8 weeks post-germination stages, Fig. 3.2c) explants, fewer axillary shoots were produced per explant (5-6 and 3-4 respectively). Maximum shoots (8-9 per explant) were produced at intermediate (4-5 weeks, Fig. 3.2b) stage of seedling development (Table 3.4). After 4-5 weeks, axillary shoot production decreased with increasing age of the cotyledonary node.

With progressive development of seedling, there is progressive decrease in regeneration potential, leading to simultaneous decrease in number of shoots produced during later stages of seedling development. Therefore, the older cotyledonary nodes produced less shoots, as compared to earlier stage. However, cotyledonary nodes from very early stages (3-4 week post-germination), also failed to produce more shoots. Thus, tissues at very early stages of development also could not be induced to produce more shoots. Cotyledonary node explants need to attain a certain stage of development at which maximum response can be obtained (Fig. 3.2d), which was 4-5 weeks post-germination stage of seedling in this study.

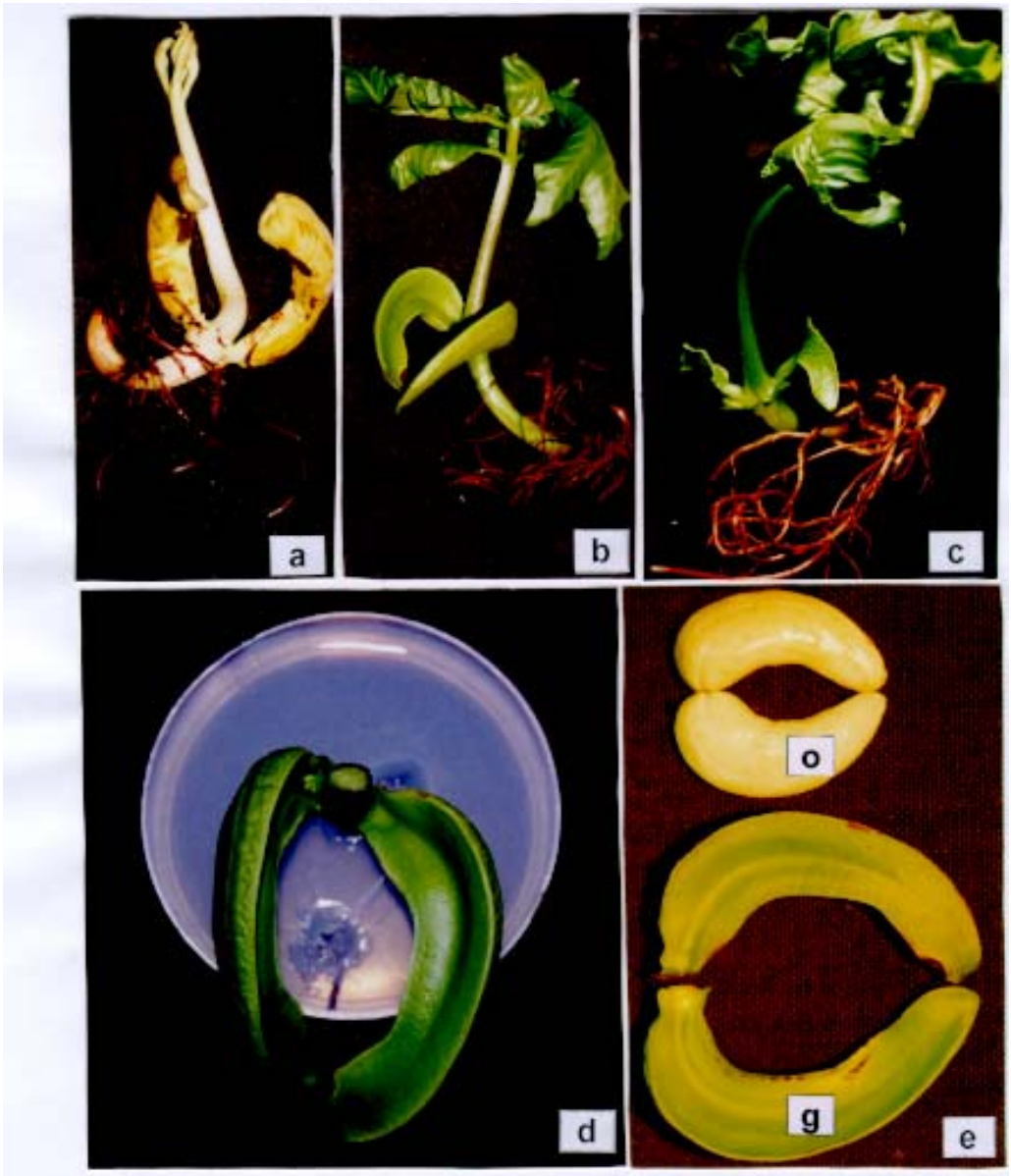


Figure 3.2: Post-germination stages (in weeks) of cashew seedlings used to obtain cotyledonary node and nodal explants

a: 3-4 weeks; b: 4-5 weeks; c: 5-7 weeks; d: Cotyledonary node from 4-5 weeks stage (optimal for shoot production); e: increased cotyledon size during germination (o: size of cotyledons before germination; g: fully expanded green fleshy cotyledons at 4-5 weeks)

Table 3.4: Effect of age of explant on axillary shoot proliferation in seedling explants {cotyledonary nodes (CN) and nodal explants (NE)} of cashew

Age of explant (weeks after germination)	Cotyledonary nodes		Nodal explants	
	Percent responding explants	Average number of shoots per explant	Percent responding explants	Average number of shoots per explant
3-4	59.6 ± 1.3	3.3 ± 0.1	-	-
4-5	70.6 ± 0.9	8.2 ±0.1	64.7 ± 1.0	1.2 ± 0.1
5-6	62.6 ± 0.8	4.4 ± 0.3	61.5 ± 2.7	1.4 ± 0.1
6-7	57.5 ± 0.5	2.8 ± 0.1	54.6 ± 0.3	1.5 ± 0.1
7-8	38.5 ± 0.9	2.2 ± 0.1	48.9 ± 0.9	1.6 ± 0.1

{Explants were obtained from 3 through 8 weeks old seedlings. All explants from each stage were inoculated on MS medium with 10 μM BA, 3% sucrose and 0.6% agar. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-35 explants per treatment in each experiment}.

Explants obtained from seedlings 3-4 weeks after germination produced an average of 3.3 axillary shoots per cotyledonary node. Cotyledonary nodes collected at 4-5 weeks after seed germination showed maximum number (average 8.2) of shoots per explant (Table 3.4). At this stage, the cotyledons were attached to the explant and were fully expanded, green and fleshy (Fig.3.2d, e).

In the stages after 4-5 weeks, the number of shoots formed per cotyledonary node decreased progressively with increasing explant age. Thus, with increasing age, there was progressive decrease in the morphogenetic potential, resulting in lesser number of shoots.

The number of shoots formed from 5-6, 6-7 and 7-8 weeks old cotyledonary nodes was 4.4, 2.8 and 2.2 shoots per explant respectively. At these stages, the cotyledons gradually shrivelled, turned yellow, and got detached from the

explant. Lowest number of shoots were produced when the cotyledons had completely detached from the cotyledonary node (7-8 weeks stage).

The number of responding explants also decreased with increasing age of cotyledonary node. At 4-5 weeks post-germination stage, 70.6% explants formed axillary shoots. This number decreased progressively from 62.6%, 57.5% to 38.5% at the 5-6 weeks, 6-7 weeks, and 7-8 weeks post-germination stages respectively.

The effect of developmental stage on axillary shoot production from cotyledonary node also appeared to be correlated with condition of cotyledons at that stage. At 4-5 weeks, cotyledons were green, fully expanded and mobilization of storage products was probably at its maximum, which had a beneficial effect on axillary shoot proliferation. As the age of explant increased, the amount of storage nutrients went on decreasing, and cotyledons shriveled and turned yellow, and finally detached from the explant. This effect of cotyledons was examined later, discussed in 3.2.4.

Thus, cotyledonary nodes obtained from 4-5 week old seedlings were the optimal stage for axillary shoot proliferation, and explants from this stage were used for further experiments. In other studies on cashew, cotyledonary node explants at the 20-day old (Das *et al*., 1996), or 3-week old stage of seedling (D'Silva and D'Souza, 1992) were used.

In other species, the cotyledonary nodes were used at varying stages of seedling development, from 7-day (*Dalbergia sissoo*, Pradhan *et al*., 1998), 10-day (*Melissa officinalis*, Tavares *et al*., 1996), 15-day (*Acacia nilotica*, Dewan *et al*., 1992), 20-day (*Sterculia urens*, Purohit and Dave, 1996) to 40-45 day-old (*Achras sapota*, Purohit and Singhvi, 1998) seedlings. The difference in age of cotyledonary node may be due to the different species used.

Nodal explants: Effect of explant age on axillary shoot formation was not prominently observed in nodal explants. Nodal explants could be obtained starting from 4-5 week-post germination stages, because at earlier stages, nodes were not yet formed on the primary shoot. In nodal explants from 4-5 weeks stage (Fig. 3.3a),

one axillary shoot (average) was formed from each explant. In nodes obtained from further stages (5-6, 6-7 and 7-8 week old stages, Fig 3.3b), the average number of shoots increased to 1.4, 1.5 and 1.6 shoots per nodal explant respectively. The increase was due to more number of explants forming two shoots per explant (Fig.3.3 c). However, when two shoots were produced, only one shoot grew and elongated. Other shoot remained stunted, and did not elongate further (Fig. 3.3 d). Removal of this shoot from the parent node did not help in enhanced growth of remaining shoot or induction of more shoots.

Thus, effectively, only one axillary shoot was formed per nodal explant at all the developmental stages. Since it was observed that a single axillary shoot showed better growth and elongation, even when two shoots emerged, for further experiments, nodal explants were also used at 4-5 weeks post-germination stage. Though the increasing age did not affect axillary shoot production very much from nodal explant, it affected the number of explants forming axillary shoots. The number of nodes forming axillary shoots decreased progressively with increasing age of explant. At 4-5 weeks post-germination stage, 64.7% of nodal explants formed axillary shoots. Lesser number of nodal explants (61.5%, 54.6% and 48.9% at 5-6, 6-7 and 7-8 weeks post-germination stages respectively) formed axillary shoots with increasing age of seedling.

3.3.2.2. Effect of basal medium on axillary shoot proliferation: When three basal media, MS, SH and WPM, were used for selecting the optimal medium for axillary shoot proliferation, 70.8% cotyledonary explants formed axillary shoots on MS medium, as compared to 47.4 % on SH and 50.9% on WPM medium (Table 3.5). Average number of shoots formed per explant was also more on MS (8.3), than SH (4.8) or WPM (5.4) media.

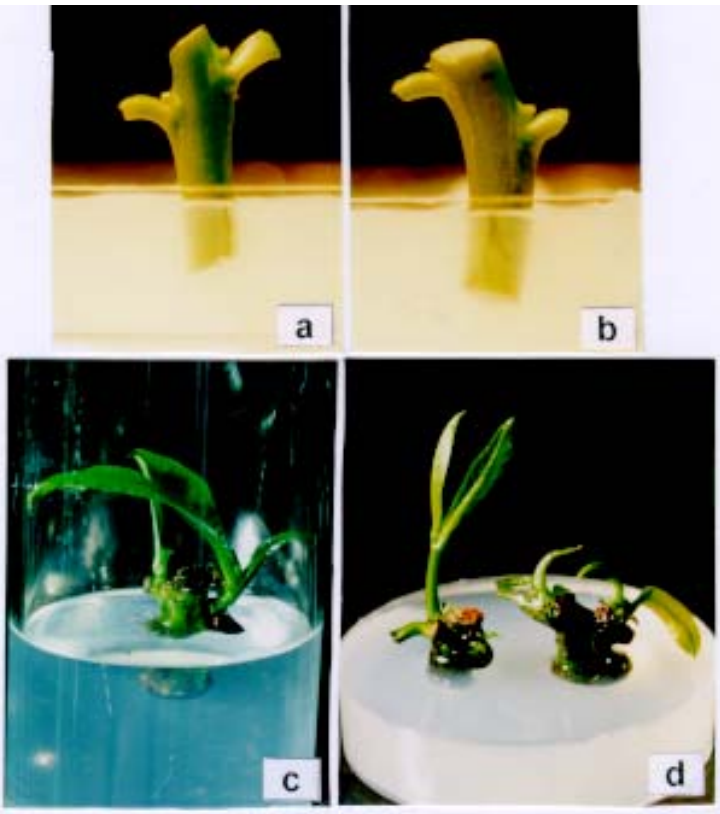


Figure 3.3: Axillary shoot formation from nodal explants of cashew seedlings

a: node from 4-5 week post-germination stage; b: node from 5-8 week old seedling; c: sprouting of axillary buds; d: elongation of one shoot

Table 3.5: Effect of different basal media on axillary shoot proliferation in seedling explants {cotyledonary nodes (CN) and nodal explants (NE)} of cashew

Basal medium	Percent explants showing axillary bud proliferation		Average number of shoots per explant	
	CN	NE	CN	NE
MS	70.8 ± 0.7	64.2 ± 0.8	8.3 ± 0.1	1.2 ± 0.1
SH	47.4 ± 1.0	43.1 ± 2.2	4.8 ± 0.2	1.1 ± 0.3
WPM	50.9 ± 2.2	41.8 ± 2.2	5.4 ± 0.3	0.9± 0.2
MS full	70.6 ± 1.1	64.6 ± 1.2	8.1 ± 0.1	1.3 ± 0.1
MS half	58.9 ± 0.9	55.1 ± 1.7	5.1 ± 0.6	0.9 ± 0.2
MS major salts half	61.3 ± 0.9	55.6 ± 0.9	5.0 ± 0.2	1.0 ± 0.2

{Explants were obtained from 4-5 week old seedlings. Medium with 10 μM BA, 3% sucrose and 0.6% agar was used. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-35 explants per treatment in each experiment}.

MS medium was also found to be suitable for the nodal explants. Though the average number of shoots produced per explant was 1-2 on all media, the number of responding explants was more (64.2%) on MS, as compared to SH (43.1%) and WPM (41.8%).

Between different strengths of MS medium, full strength MS medium supported axillary shoot formation in more number of explants (cotyledonary: 70.8%, nodal: 64.6%) and greater average number of shoots per explant (cotyledonary 8.1, nodal 1.3), than half-strength and major salts half strength media (Table 3.5). Therefore, full strength MS medium was used as basal medium for further experiments.

In other studies in cashew, MS medium with major salts at half strength (Boggetti *et al*., 1999; Thimmapaiah *et al*., 2002), MS at $3/4^{th}$ strength (Thimmapaiah *et al*., 1999) and WPM (Boggetti *et al*., 1999) have been used for proliferation of shoots from cotyledonary and nodal explants. Lievens and co-

workers (1989) used Lepoivre medium for sprouting of axillary buds. Das and co-workers (1996) have also used MS basal medium for shoot formation from cotyledonary node. They observed that addition of activated charcoal to the medium, and initial 7-day dark incubation with daily transfer to fresh medium was essential for explant survival. In the present study however, cotyledonary explants did not show any mortality when incubated in 16-hour photoperiod on medium without activated charcoal.

In *Fraxinus excelsior* however, DKW medium was found to be optimal for shoot proliferation. Cotyledonary explants failed to survive on MS medium (Hammatt and Ridout, 1992).

3.3.2.3. Effect of cytokinins on axillary shoot proliferation: Cytokinins have been often used for shoot development *in vitro*. Favorable effect of cytokinins on *in vitro* axillary and/ or adventitious shoot formation has been observed in many species (George, 1993). Various cytokinins (BA, Kin, 2iP, TDZ, Zea) were therefore used and compared, to choose the cytokinin most favorable for axillary shoot proliferation.

It was observed that one or two axillary shoots were produced from cotyledonary nodes even on medium devoid of growth regulators. The pre-existing morphogenetic potential of cotyledonary node was enhanced with the use of cytokinins. In contrast, cotyledonary explants did not develop axillary shoots in absence of growth regulators in cashew (Das *et al*., 1996), and also in *Dalbergia sissoo* (Pradhan *et al*., 1998).

It was observed that cytokinins could not elicit enhanced axillary shoot production from nodal explants. Maximum 1-3 shoots were produced per explant in presence of BA, and Kin, and only one shoot in presence of 2iP and Zea. More (4-5 per node) shoots could be produced when TDZ was used at 0.01μM, but these shoots were leafy, produced in a compact cluster, very short (0.5-1 cm long) and did not elongate. They did not survive after separation from the parent node. The different cytokinins were used separately, and the results of these experiments are presented in Table 3.6 a.

Table 3.6a: Effect of different cytokinins on axillary shoot proliferation from cotyledonary nodes and nodal explants of 4-5 week-old seedlings of cashew

Cytokinin (μM)		Cotyledonary node		Nodal explant	
		Percent responding explants	Average number of shoots per explant	Percent responding explants	Average number of shoots per explant
BA	1	70.3 ± 2.3	3.8 ± 0.6	64.4 ± 1.2	0.9 ± 0.1
	5	70.5 ± 2.5	5.8 ± 0.3	64.3 ± 1.7	1.1 ± 0.1
	10	71.1 ± 2.3	8.5 ± 0.4	65.4 ± 1.1	1.2 ± 0.1
	20	70.2 ± 1.5	14.1 ± 1	61.8 ± 1.9	1.2 ± 0.1
	50	-	-	-	-
Kin	1	65.2 ± 2.2	1.6 ± 0.5	46.6 ± 1.6	1.4 ± 0.3
	5	64.8 ± 2.4	3.2 ± 0.8	45.8 ± 1.2	1.2 ± 0.2
	10	63.7 ± 1.8	4.2 ± 0.6	48.5 ± 1.6	1.4 ± 0.2
	20	64.5 ± 0.6	3.2 ± 0.4	46.7 ± 0.7	1.2 ± 0.1
	50	-	-	-	-
2iP	5	56.9 ± 2.5	2.8 ± 0.6	39.5 ± 1.5	1.1 ± 0.1
	10	59.2 ± 1.8	2.5 ± 0.3	40.7 ± 0.9	1.3 ± 0.2
	20	59.8 ± 1.9	2.8 ± 0.2	37.4 ± 1.4	1.1 ± 0.1
	50	-	-	-	-
TDZ					
	0.005	66.7 ± 0.9	8.2 ± 0.8	52.5 ± 0.9	2.4 ± 0.2
	0.01	66.6 ± 1.1	14.2 ± 1.2	55.9 ± 0.7	3.6 ± 0.2
	0.05	62.8 ± 1.8	12.2 ± 1.1	53.6 ± 1.8	5.0 ± 0.7
	0.1	43.4 ± 4.7	2.4 ± 0.5	52.9 ± 0.4	2.2 ± 0.1
	0.5	-	-	-	-
Zea	5	49.9 ± 3.6	2.8 ± 0.4	35.3 ± 2.1	1.1 ± 0.1
	10	45.3 ± 2.9	3.2 ± 0.3	37.1 ± 1.8	1.5 ± 0.2
	20	41.7 ± 2.1	2.5 ± 0.3	35.3 ± 1.2	1.3 ± 0.1
	50	-	-	-	-

{Medium used is MS + sucrose 3% + agar 0.6%. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-40 explants per treatment in each experiment}.

Among the cytokinins, BA and TDZ, used separately, produced more number of shoots per cotyledonary node. In presence of BA, the number of axillary shoots per explant increased progressively, with increasing BA concentration, from 1 to 20 μM (Table 3.6a). Maximum shoots (average 14.1 per

cotyledonary node) were produced at 20 μM of BA. Above 20 μM of BA, number of shoots formed per explant decreased, and shoots were fused all along their lengths, and could not be used for elongation (Fig.3.6).

Shoots formed at 20 μM of BA (average number 14.1 per explant), though more in number, were short in length (0.5-1.5 cm) and did not elongate. Only a few shoots (3-4) elongated to a length of more than 2.5 cm (Fig. 3.4c), and could be used for rooting. Thus, even though more number of shoots were formed at 20 μM BA, most of these shoots were not satisfactory for elongation and root induction. Less (average 8.5) shoots per cotyledonary node were produced at 10 μM BA, but decrease in shoot number was compensated by increased length of individual shoots (2-3.5 cm) (Fig.3.4a). Five to six of these shoots elongated further on the same shoot induction medium (Fig. 3.4b), and were found to be suitable for root induction. Therefore BA at 10 μM was decided to be the optimal concentration for axillary shoot proliferation.

More number of shoots were also produced in presence of TDZ (14.2 at 0.01 μM and 12.2 at 0.05 μM respectively) (Table 3.6a). However, the shoots were again very short in length (0.5-1.5 cm), and failed to elongate. It was observed that the shoots produced on medium with TDZ were dark green and showed leafy appearance. The shoots were produced in thick clusters, and were difficult to separate for elongation (Fig. 3.4d, e). These shoots frequently showed necrosis on separation from the parent explant, either during elongation, or rooting.

Shoots produced in presence of Zea also showed leafy appearance (Fig. 3.4 f) and reduced shoot length. Shoots produced in presence of Kin (Fig. 3.4 g), 2iP (Fig.3.4 h_1, h_2) and Zea were few (2-4) in number, as compared to number of shoots obtained with BA (8-10) and TDZ (12-14) (Table 3.6a).

Since BA at 10 μM was found to be optimal for shoot production, it was used in combination with other cytokinins (Kin, 2iP, Zea, TDZ), to see if the cytokinin combination could further enhance axillary shoot production. The results obtained using these combinations are indicated in Table 3.6 b.

Figure 3.4: Effect of cytokinins on axillary shoot production from cotyledonary nodes (basal medium MS + sucrose 3% + agar 0.6%)

a: Shoots formed on BA 10 μM; b: shoots elongated on BA 10 μM; c: shoots formed on BA 20 μM; d, e: shoots formed on 0.05 μM TDZ

Figure 3.4: Effect of cytokinins on axillary shoot production from cotyledonary nodes (basal medium MS + sucrose 3% + agar 0.6%)

f: shoots formed on Zea; g: shoots formed on Kin;
h_1, h_2: shoots formed on 2iP

Table 3.6 b: Effect of cytokinin combinations on axillary shoot proliferation in cotyledonary nodes and nodal explants of 4-5 weeks old seedlings of cashew

BA 10 μM in combination with cytokinin (μM)	Cotyledonary node		Nodal explant	
	Percent responding explants	Average number of shoots per explant	Percent responding explants	Average number of shoots per explant
Kin 5	61.9 ± 0.8	3.3 ± 0.3	44.6 ± 0.8	1.2 ± 0.2
Kin 10	63.2 ± 0.7	4.2 ± 0.3	45.9 ± 0.9	1.3 ± 0.1
Kin 20	59.5 ± 1.4	2.9 ± 0.1	45.7 ± 1.5	1.3 ± 0.1
2iP 5	55.9 ± 0.4	2.3 ± 0.1	41.7 ± 1.1	1.2 ± 0.1
2iP 10	58.5 ± 0.8	2.3 ± 0.1	41.8 ± 0.7	1.2 ± 0.2
2iP 20	58.0 ± 1.1	2.4 ± 0.1	36.6 ± 0.8	1.1 ± 0.1
TDZ 0.005	66.3 ± 1.1	7.9 ± 0.3	51.5 ± 1.5	1.7 ± 0.1
TDZ 0.01	67.0 ± 0.6	8.3 ± 0.4	52.1 ± 1.1	2.3 ± 0.1
TDZ 0.05	63.2 ± 1.3	6.8 ± 0.2	48.6 ± 1.4	2.1 ± 0.1
Zea 5	45.1 ± 0.9	1.7 ± 0.4	39.4 ± 0.6	1.2 ± 0.2
Zea 10	44.6 ± 0.5	1.1 ± 0.1	35.9 ± 1.4	1.1 ± 0.1
Zea 20	40.0 ± 0.6	1.0 ± 0.3	39.2 ± 0.4	1.0 ± 0.1

{Medium used is MS + sucrose 3% + agar 0.6%. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-40 explants per treatment in each experiment}.

Though BA and TDZ produced maximum number of shoots per explant when used individually, their combination was not effective for enhanced production of axillary shoots in case of cotyledonary node (Table 3.3b). BA at 10 μM produced an average of 8.5 shoots per cotyledonary node, and TDZ at 0.01 μM formed an average of 14.2 shoots per cotyledonary node. However, when used in combination, only 8.3 shoots were produced per cotyledonary node. As seen previously, the shoots produced from BA and TDZ combination were in clusters, leafy, very short, and were not suitable for rooting experiments. Thus in BA + TDZ combinations, morphology of shoots was influenced by TDZ.

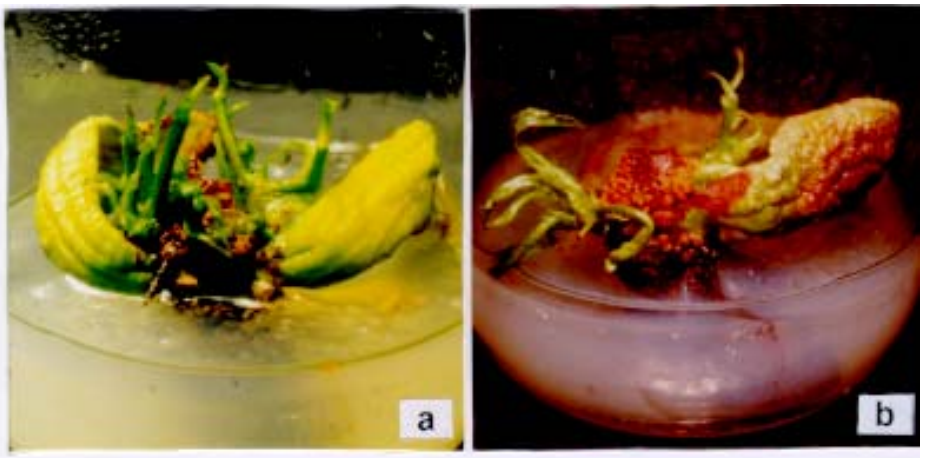


Figure 3.5 a, b: Axillary shoots with reduced shoot length

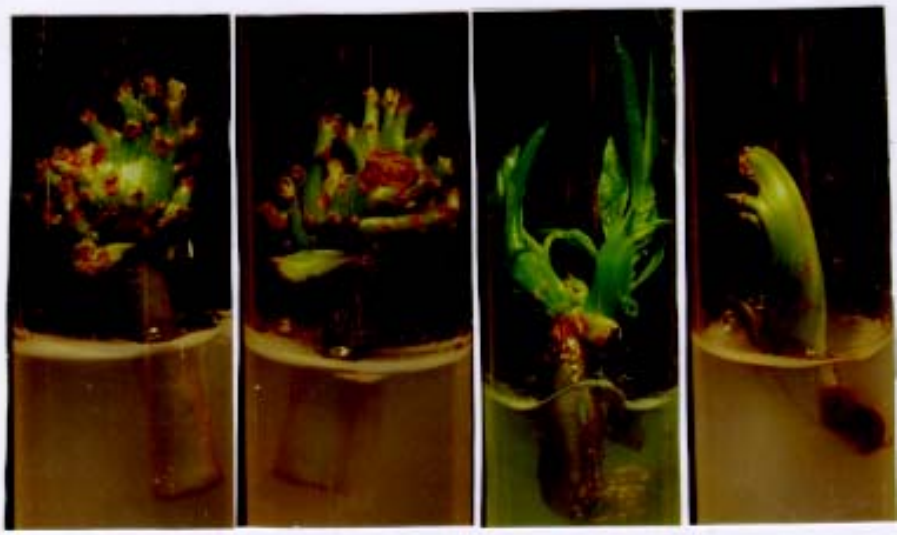


Figure 3.6: Fasciation of axillary shoots formed at high (50 μM) BA concentration

Presence of BA did not help in producing shoots of increased length and normal appearance. The number of shoots formed was lower in BA + TDZ combination, than either of them (BA and TDZ) used alone. Other cytokinins in combination with 10 μM BA were also not effective in increasing the number of shoots produced per explant, as compared to number of shoots produced by BA alone. Thus, synergistic effect of cytokinins for axillary shoot proliferation from cotyledonary node was not observed in the present study.

In case of nodal explants also, synergistic action of cytokinins was not observed, and 1-2 shoots were formed per node at all combinations used (Fig. 3.7 a). It was observed that except TDZ, no other cytokinin, used alone or in combination, had a beneficial effect on axillary shoot production from nodes. Only TDZ could form more number of shoots (about 5 per node, Fig. 3.7b). However, these shoots were leafy, clustered, short, and could not be used for root induction.

Das and co-workers (1996) found the combination of BA, Kin and Zea to be more suitable for shoot production from cotyledonary nodes of cashew. A higher BA concentration (22.2 μM) was found to be optimum for shoot production in studies carried out by D'Silva and D'Souza (1992).

It was generally observed that increase in the number of shoots per explant always led to decrease in individual shoot length and decrease in percent shoot survival. Similar observation was made in cashew (D'Silva and D'Souza, 1992) and *Melissa officinalis* (Tavares *et al*., 1996), where, increase in shoot number resulted in decrease in shoot length.

In most of the species indicated in Table 3.2, BA was found to be the most effective cytokinin for axillary shoot proliferation from cotyledonary nodes. TDZ was more effective in *Bauhinia vahlii* (Bhatt and Dhar, 2000), whereas BA and TDZ were used in combination in eastern redbud (Distabanjong and Geneve, 1997).

After removing the first flush of shoots, the parent explant was cultured in fresh shoot initiation medium for re-induction of axillary shoots. During this second flush, less number of shoots were produced (average 3-5 per explant),

and the shoots were shorter (1-2 cm) than the shoots obtained from first flush (2-3 cm in length). The decrease in number of shoots during the second flush could be due to increased age of the explant, due to which, the shoot forming potential may have been decreased. This observation was made while studying the effect of age of explant on shoot proliferation, discussed earlier (section 3.3.2.1). These shoots were found to be unsuitable for rooting. The results of these experiments are therefore, not included in the present chapter.

3.3.2.4. Effect of presence of cotyledons on axillary shoot proliferation from cotyledonary node: As discussed in section 3.3.2.1., along with explant age, cotyledons also probably affected axillary shoot proliferation from cotyledonary node. Effect of presence or absence of cotyledons on axillary shoot proliferation from cotyledonary nodes was therefore examined. Cotyledonary nodes were used as follows: (i) with both cotyledons intact, (ii) with one cotyledon, and (iii) without cotyledons. The different types of these cotyledonary explants were grown on MS+ BA 10 μM + sucrose 3% + agar 0.6%. Number of shoots produced per explant was recorded in each category. The observations are presented in Table 3.7.

Table 3.7: Effect of presence of cotyledons on axillary shoot proliferation from cotyledonary nodes of 4 –5 week old seedlings of cashew

Number of cotyledons attached to cotyledonary node	Percent responding explants	Average number of shoots per explant
2	71.1 ± 0.7	8.3 ± 0.2
1	61.9 ± 1.7	4.9 ± 0.1
0	52.9 ± 1.3	2.8 ± 0.3

{MS medium with 10 μM BA, 3% sucrose and 0.6% agar was used. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-35 explants per treatment in each experiment}.

It was observed that cotyledons affected formation of axillary shoots from cotyledonary node. Removal of cotyledons decreased the number of shoots produced per explant. More shoots (average 8.3 per explant) were formed when both cotyledons were present (Refer Fig. 3.4 a). Removal of one or both cotyledons adversely affected shoot production. Shoots formed in absence of cotyledons were few in number (average 2.8 per explant, Fig. 3.7 a,b) and they frequently showed fasciation. Such fused shoots did not develop properly, and often became necrotic on further culture.

Due to the removal of cotyledon, the explant is deprived of the complex endogenous substances, which may have resulted in decreased shoot production. The endogenous factors supplied by cotyledons may also play a role in axillary shoot production, along with exogenous cytokinins. These endogenous factors probably act synergistically with the exogenously supplied cytokinin, resulting in production of more shoots, because, in the absence of cotyledons, cytokinin alone failed to produce more shoots.

Similar beneficial effect of cotyledons was also reported in cacao, where, shoots failed to grow in culture when cotyledons were detached (Janick and Whipkey, 1985). In *Cucumis sativus*, it was suggested that the cotyledons play an important role in shoot production, probably by supplying endogenous growth regulators (Gambley and Dodd, 1991).

3.3.2.5. Elongation of shoots: Shoots obtained from the cotyledonary nodes and nodal explants were separated and transferred to different media for elongation. Shoots were inoculated on MS medium, containing (i) reduced concentration of BA (1 and 5 μM); (ii) GA_3 5 μM, (iii) Kin 5 μM. Some shoots were incubated in dark.

Separation from original explant adversely affected the further growth of shoots. Dark incubation led to browning and necrosis of shoots. Shoots grown in presence of GA_3 showed browning, and hyperhydricity. On all media used for elongation, yellowing of leaves, followed by leaf abscission was frequently observed in the separated shoots. This may be the reason for poor growth of the separated shoots. Such poorly grown shoots showed very low root induction, or

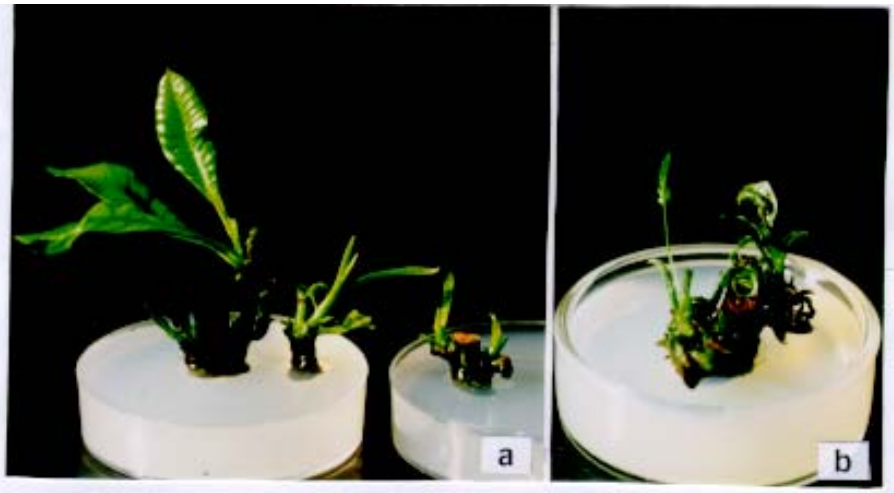


Figure 3.7: Axillary shoots formed from nodal explants (basal medium MS + sucrose 3%).
a: 1-2 shoots per node using BA, Kin, 2iP, Zea;
b: 4-6 axillary shoots per node formed in presence of TDZ

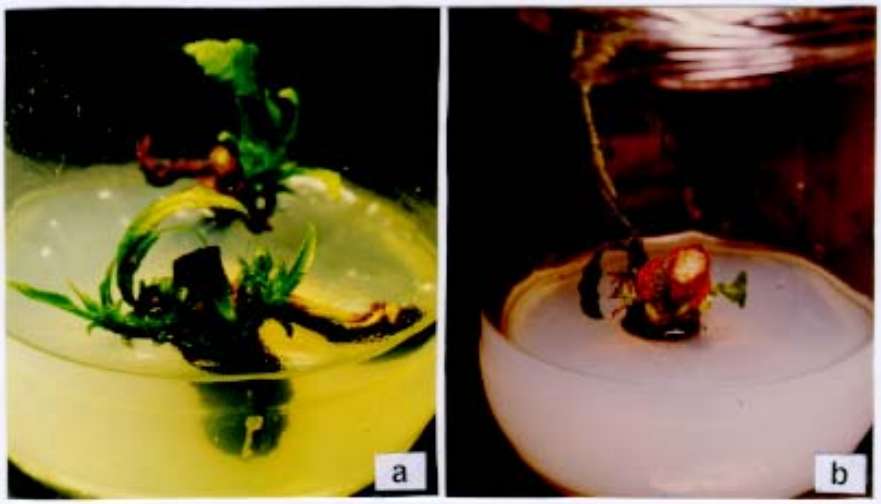


Figure 3.8: Axillary shoots formed from cotyledonary node in absence of one or both cotyledons.
a: shoots formed in presence of one cotyledon
b: shoots formed in absence of cotyledons

did not survive when transferred to rooting medium. In other studies in cashew also, shoots elongated with GA_3 were weak, hyperhydric, with decreased leaf size, and they failed to root (Boggetti *et al*., 1999).Thimmapaiah and co-workers (2002) observed that BA and GA_3 in elongation medium caused yellowing of leaves and defoliation in shoots developed from nodal explants.

To avoid damage to shoots, they were left intact on the original explant, and transferred to fresh medium with 10 μM BA, the medium found optimal for both, shoot proliferation and growth. No mortality was observed in these axillary shoots, and they showed normal growth (Refer Fig. 3.4b). Shoots longer than 2.5 cm were excised and transferred to rooting medium.

It was observed that during elongation of shoots, some cotyledonary nodes and nodal explants previously grown in presence of Kin (during shoot proliferation phase) showed spontaneous root formation. These roots were formed directly from the base of the explant (Fig. 3.9a) or base of axillary shoots (Fig.3.9b). These explants were used for hardening, since there was no need for a root induction step. This observation led to the use of low concentrations of Kin along with auxins, in the rooting medium. The addition of Kin was found to promote root formation in subsequent studies. Similar beneficial effect of cytokinin (2iP) on *in vitro* root induction in cashew shoots was also reported by Boggetti and co-workers (2002).

3.3.2.6. Rooting of shoots: When shoots more than 2.5 cm in length were given pulse treatments of auxins IAA, IBA and NAA (100, 200 and 500 μM each) at varying time intervals (12, 24, 48 hours), root induction did not occur. Therefore, the shoots were grown in medium containing auxins IAA and IBA, separately, and in combination (Table 3.9).

Table 3.9: *In vitro* rooting of shoots obtained from cotyledonary nodes and nodal explants of cashew

Growth regulator μM	Percent explants showing root induction	
	Cotyledonary node	Nodal explant
IAA 1	0	0
2. 5	0	0
5	0	0
10	5.3 ± 1.5	0
IAA 5 + Kin 0.5	13.6 ± 0.7	10.9 ± 0.5
IBA 1	0	0
2. 5	0	0
5	0	0
10	20.7 ± 1.4	14.8 ± 1.2
IBA 5 + Kin 0.5	14.4 ± 0.6	6.2 ± 0.9
IAA 2. 5 + IBA 2.5	0	0
IAA 5 + IBA 2.5	27.7 ± 0.4	24.7 ± 1.9
IAA 1 + IBA 0.5 + Kin 0.5	0	0
IAA 5 + IBA 2.5 + Kin 2.5	35.5 ± 1.2	31.7 ± 0.7
IAA 2.5 + IBA 5 + Kin 2.5	0	0
IAA 5 + IBA 5 + Kin 0.5	0	0
NAA 1	0	0
NAA 5	7.1 ± 0.1	5.1 ± 1.4
NAA 2.5 + Kin 0.5	0	0

Medium used was half strength MS + sucrose 1%, with different growth regulators as indicated in the table below. Each value represents mean ± standard deviation (SD) of 3 experiments with 25-40 explants per treatment in each experiment.

Medium containing IAA (5 μM) + IBA (2.5 μM) + Kin (2.5 μM) was the most suitable medium for root induction. Root induction occurred in 35.5 % of shoots obtained from cotyledonary node, and in 31.7% of the shoots obtained from nodal explants. Roots were formed within 3 weeks of dark incubation. Two to three roots were formed directly from the basal cut portion of the shoots, which elongated within 2-3 weeks in the rooting medium (Fig. 3.10).

Combination of IBA, NAA and activated charcoal (Das *et al*., 1996), IBA (Lievens *et al*., 1989; Boggetti *et al*., 1999), NAA (Falcone and Leva, 1990),

Figure 3.9: Spontaneous root formation from cotyledonary node in presence of Kin in shoot induction medium.

a: roots formed at base of cotyledonary node explant;
b: roots formed at base of axillary shoot

NAA alone or with IBA (Thimmapaiah and Samuel, 1999) have been reported to be effective for root induction in cashew shoots.

Figure 3.10: *In vitro* rooting of axillary shoots on rooting medium (MS ½ + IAA 5 μM + IBA 2.5 μM + Kin 2.5 μM + sucrose 1%)

3.3.2.7. Hardening of shoots: When the rooted shoots were transferred to greenhouse for hardening, the survival percentage was 43.2 after one month, and 11.3 after further 5 months in the greenhouse. It was observed that shoots kept at high humidity levels for a longer time (8-10 weeks) showed better survival (Fig. 3.11). Removal of the cover at earlier stages resulted in withering of leaves and subsequent drying of shoots. Yellowing of leaves followed by defoliation, browning, and necrosis of rooted shoots occurred during hardening period, which affected their survival rate.

The absence of a strong taproot, and the comparatively weak adventitious roots may be one of the reasons for poor establishment of the plantlets in greenhouse. The *in vitro* formed roots were brittle, and easily damaged during transfer to polythene bag. Damage to the roots had an immediate effect on shoot survival. Poor growth or defoliation of leaves during root induction may also be a reason for low shoot survival. Due to poor health of leaves, the shoots became weak and could not withstand the hardening treatment.

Rooting in woody plants is still one of the major constraints for *in vitro* propagation, and it is a serious problem with cashew also (Boggetti *et al*., 2001). In the present study, shoots less than 2 cm in length failed to survive when they were separated from the original explant. Similar observations were noted in microshoots produced from cotyledonary nodes (D'Silva and D'Souza, 1992) and nodal explants (Boggetti *et al*., 2001).

Leaf abscission and browning of shoots caused poor development of the shoots on elongation media, which must be the reason for difficulties faced during rooting of such shoots, as was also observed by Boggetti and co-workers (2001). Difficulty in rooting of shoots obtained in cotyledonary nodes and low rooting frequency were also reported by Das and co-workers (1996). Infecting the shoots with *Agrobacterium rhizogenes* increased rooting in infected shoots, but recovery of plants was very poor (Das *et al*., 1996). Very poor root induction response in shoots of cashew was also observed by Thimmapaiah and co-workers (2002). Auxin pulse treatments and *in vitro* rooting on media with auxins failed to induce rooting in shoots formed *in vitro* from nodal explants. In such shoots, yellowing of leaves was observed, resulting in defoliation. To increase shoot survival, the shoots obtained *in vitro* were micrografted on seedling root-stocks (Thimmapaiah *et al*., 2002). Rooting, and subsequent survival of the shoots during acclimatization still remain the bottlenecks for *in vitro* clonal propagation of cashew (Boggetti *et al*., 1999 and 2001; Das *et al*., 1996; Thimmapaiah *et al*., 2002).

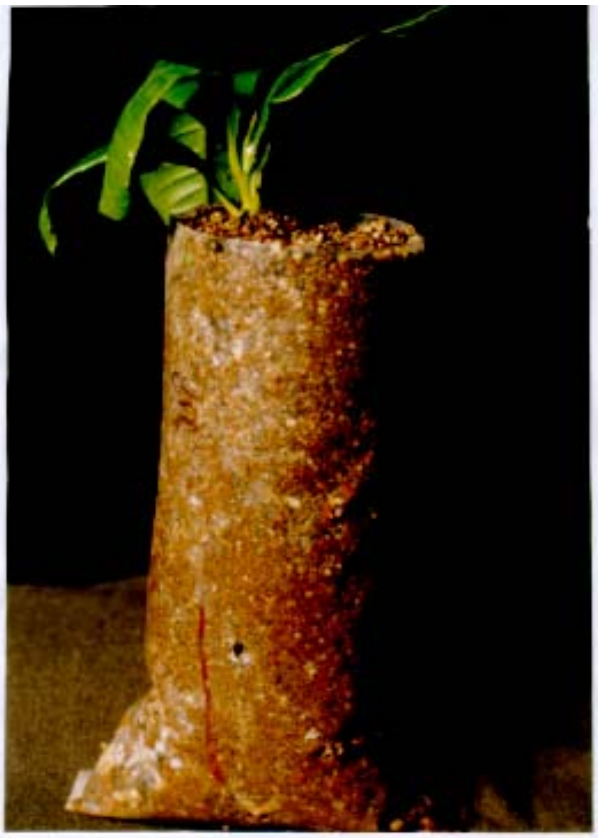


Figure 3.11a: Rooted shoot after three months of hardening in greenhouse

Fig. 3.11b: Rooted shoot after 5 months of hardening in greenhouse (shoot removed from polybag to show root growth, yellowing of leaves is seen)

3.3.3. Effect of BA on multiple shoot induction from germinating seed and intact seedling: Producing multiple shoots from seeds germinated on cytokinin containing medium has been mentioned as a method of *in vitro* shoot formation and multiplication (George, 1993). By this method, multiple shoots were obtained during seed germination in woody trees like walnut (Rodriguez, 1982), *Psidium guajava* (Mohamed-Yasseen *et al*., 1995), *Syzygium alternifolium* (Sha Valli Khan *et al*., 1997) and *Syzygium cuminii* (Yadav *et al*., 1990). Multiple shoots were formed from nodal regions of intact seedlings of *Murraya koenigii* (Bhuyan *et al*., 1997) and *Cinnamomum zeylanicum* (Rai and Jagdishchandra, 1987), when seeds were germinated and further grown into seedlings in presence of cytokinin.

For producing multiple shoots, mature cashew seeds were germinated in BA solutions of varying (10, 20, 50, 100, 250 and 500 μM) concentrations. Seeds germinated in distilled water acted as control for each treatment. Percent germination, and multiple shoot induction from germinated seeds was noted after 6 weeks. The results are given in Table 3.10.

Table 3.10: Effect of BA on germination and multiple shoot formation from germinating seeds of cashew

Concentration of BA in germination medium μM	Percent germination after 4 weeks	Percent germinating seeds showing induction of multiple shoots	Effect of BA on seedling morphology
0 (control)	79.8 ± 0.8	0	normal
10	79.2 ± 0.9	0	do
20	78.5 ± 2.1	0	do
50	78.8 ± 1.1	0	do
100	77.6 ± 1.2	0	do
250	74.3 ± 1.3	0	Stunted growth, very poor root development
500	67.6 ± 1.7	0	do

{ Seeds were germinated on filter paper supports partly immersed in distilled water, and BA solution. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-40 explants per treatment in each experiment}.

It can be seen from Table 3.10 that presence of BA in the germination medium was totally ineffective in inducing multiple shoots from the germinating seeds. BA also had no effect on the germination of seeds, as the germination percentage of BA-treated seeds was not significantly different from control (untreated) seeds, except at 500 μM. This indicates that probably the endogenous growth factors had a far stronger influence on germination than the exogenous cytokinin. It may also suggest failure of BA uptake by the roots. Germinated seeds were allowed to develop into seedlings in BA solutions. There was no formation of axillary shoots from any part of the intact seedling. The ineffectiveness of exogenous BA in inducing axillary or adventitious shoot formation from intact seedlings, again may be due to dominance of endogenous factors on seedling development.

Though BA could not induce shoots from intact seedlings, it affected the growth of seedlings at concentrations of 100, 250 and 500 μM. Seedlings formed at these concentrations showed very poor development of the root system, and only limited growth of the primary shoot (Fig. 3.12b, c). At 4-5 weeks, the seedlings from each BA treatment were used to obtain cotyledonary nodes and nodal explants, which were inoculated on (i) MS medium without growth regulators, and (ii) MS medium with BA 10 μM. Number of shoots formed per explant were noted after 6 weeks. The results are included in Table 3.11a and b.

Table 3.11a: Effect of BA pre-treatment on axillary shoot production from cotyledonary nodes of cashew seedlings

BA pre-treatment (μM)	Percent responding explants (cotyledonary node) inoculated on		Average shoots per cotyledonary node inoculated on	
	MS	MS + BA 10 μM	MS	MS + BA 10 μM
0 (control)	40.1 ± 1.8	70.3 ± 1.4	2.6 ± 0.4	8.1 ± 0.3
10	36.9 ± 0.5	52.1 ± 1.8	3.7 ± 0.6	5.7 ± 0.6
20	32.7 ± 2.5	41.3 ± 1.4	2.6 ± 0.2	3.8 ± 0.4
50	27.9 ± 1.4	30.1 ± 0.7	1.4 ± 0.3	2.3 ± 0.3
100	20.4 ± 1.3	-	1.1 ± 0.2	-
250	-	-	-	-
500	-	-	-	-

{Each value represents mean ± standard deviation (SD) of 3 experiments with 30-40 explants per treatment in each experiment}

Table 3.11b: Effect of BA pre-treatment on axillary shoot production from nodal explants of cashew seedlings

BA concentration (μM)	Percent responding explants (nodes) inoculated on		Average shoots per node inoculated on	
	MS	MS + BA 10 μM	MS	MS + BA 10 μM
0 (control)	45.6 ± 1.9	63.9 ± 1.6	1.4 ± 0.1	1.5 ± 0.2
10	38.3 ± 0.4	53.5 ± 0.7	1.2 ± 0.3	1.2 ± 0.1
20	30.9 ± 0.8	42.4 ± 1.1	1.1 ± 0.1	0.9 ± 0.1
50	22.9 ± 0.9	-	0.9 ± 0.1	-

{BA pre-treatment up to 50 μM is included, as at higher concentrations, the primary shoot remained stunted, and nodes were not obtained from seedlings. Each value represents mean ± standard deviation (SD) of 3 experiments with 30-40 explants per treatment in each experiment}

It can be seen (Table 3.11 a and b) that there was no enhanced axillary shoot production from explants treated with BA. In fact, growth of seedlings in BA solution seemed to have an adverse effect on number of responding explants and number of shoots produced per explant, as compared with untreated (control) explants. With increasing BA concentration, number of responding explants (cotyledonary nodes), and shoots produced per explant decreased (Fig. 3.12a). At higher concentrations of 250 and 500 μM of BA, none of the cotyledonary nodes formed axillary shoots, indicating a completely inhibitory effect of BA on axillary shoot formation at such high concentrations. Shoots formed at 50 μM BA were frequently fasciated (Refer Fig. 3.6). Thus, the exogenous BA had an inhibitory effect on axillary shoot formation in cotyledonary node.

Presence of BA during seedling development also adversely affected axillary shoot production from nodal explants. With increasing concentration of BA during seedling growth, there was a decrease in the number of nodes forming axillary shoots. BA treatment was not effective for increasing the number of

Figure 3.12: Effect of BA on axillary shoot production from cotyledonary node

a: decreased number of shoots from cotyledonary node obtained from seedling grown in presence of BA; b: adverse effect of BA on root development (b_1 : poorly developed root system; b_2: root system absent)

axillary shoots produced from nodes, and only a single shoot was produced in nodes from BA-treated seedlings.

Thus, use of cytokinin in the germination medium has proved useful for multiple shoot production from germinating seeds and seedlings in other species. In cashew however, such experiments indicate that exogenous cytokinin cannot overcome the effect of endogenous growth regulators. Endogenous factors play a very dominant role in seed germination, and seedling development, since both processes remain unaffected in presence of cytokinin. The seedling growth is affected only at very high (100, 250 and 500 μM) concentration of BA.

3.4: Summary

In vitro germination of mature seeds of cashew could be conveniently obtained on filter paper supports placed in distilled water. Seedlings produced by this method were used for obtaining explants, which were used for developing regeneration system.

Presence of a gradient of regeneration potential was observed along the seedling length, which decreased from base to apex. Only cotyledonary node and nodal regions were morphogenetically responsive.

Cotyledonary node was the most suitable explant for axillary shoot proliferation. Cotyledonary nodes at 4-5 weeks post-germination stage produced an average of 7-10 shoots when grown on MS medium with BA 10 μM. Shoot production was adversely affected by removal of cotyledons, which indicates the role of endogenous factors in axillary shoot production. The shoots elongated on the same shoot induction medium. Shoots were rooted using IAA 5 μM + IBA 2.5 μM + Kin 2.5 μM. Rooted plantlets were acclimatized in the greenhouse.

Germination of cashew seeds in presence of BA was ineffective in inducing multiple shoot formation from germinating seeds and developing seedlings. Endogenous factors are probably more dominant in deciding the course of developmental events during seed germination and seedling development. When seedlings were grown in presence of cytokinin, and

cotyledonary nodes and nodal explants from such seedlings were used for axillary shoot production, the shoot production capacity of these explants was found to be decreased.

3.5: Conclusions:

- Mature cashew seeds can be easily and conveniently germinated *in vitro* on filter paper supports placed in distilled water. Nutrient medium is not necessary for germination.
- Cotyledons play a very important role in seedling development. Removal of one or both cotyledons is inhibitory for seedling formation.
- In seedlings, morhogenetic potential is restricted to cotyledonary node and nodal explants. There is a gradient of morphogenetic potential in seedling, with cotyledonary node having more potential than the epicotyledonary nodes (nodal explants).
- Axillary shoots could be formed from cotyledonary node and nodal explants. Of the two, cotyledonary nodes produced more number of shoots per explant (6-10) than nodal explants (1-3 shoots per explant).
- Axillary shoot proliferation from cotyledonary node was age-dependent. Cotyledonary nodes from 4-5 week-old seedlings gave optimal response.
- BA (10 μM) was most effective cytokinin for axillary shoot formation from 4-5 weeks old cotyledonary node. Shoots elongated on fresh medium of the same composition.
- Half strength MS liquid medium with 1% sucrose, IAA (5 μM) + IBA (2.5 μM) + Kin (2.5 μM) was most suitable for *in vitro* rooting of shoots.
- Adding cytokinin to germination medium did not help in producing multiple shoots from the germinating seed.
- Exogenous cytokinin (BA) decreased the shoot forming capacity of seedling explants obtained from seedlings grown in presence of BA.

CHAPTER 4

STUDIES ON EMBRYOGENIC RESPONSE OF NUCELLAR TISSUE

CHAPTER 4

STUDIES ON EMBRYOGENIC RESPONSE OF NUCELLAR TISSUE

Somatic embryogenesis is the differentiation of bipolar embryos from somatic cells. This phenomenon was first reported in suspension cultures of carrot (Stewart *et al*., 1958). Since then, somatic embryogenesis has been reported in many plant species.

Somatic embryogenesis can occur *via* two different pathways: from Induced Embryogenic Determined Cells (IEDCs), and Pre-Embryogenic Determined Cells (PEDCs) (Sharp *et al*., 1980). In IEDCs, differentiated cells undergo redetermination and form callus. These callus cells can give rise to somatic embryos. In PEDCs, cells have pre-existing embryogenic potential. Such cells form somatic embryos, usually directly, without callus formation.

The advantages of somatic embryogenesis over other methods of *in vitro* propagation have been well established (Redenbaugh, 1993; Bajaj, 1995a). Somatic embryos being bipolar propagules, can develop into complete plantlets in a single step. Thus, the problem of root induction encountered in micropropagation or shoot regeneration systems, especially with woody species, can be avoided.

Somatic embryogenesis has been reported in about 150 woody species (Dunstan *et al*., 1995). It has been reviewed extensively in tropical and subtropical fruit crops (Litz and Jaiswal, 1991), tropical fruit trees (Akhtar *et al*., 2000; Nadgauda *et al*., 2000; RajBhansali and Singh, 2000), perennial fruit and nut crops (Litz and Gray, 1992), temperate zone fruit and nut crops (Zimmerman, 1991), specific fruit and nut and other woody species (Tulecke, 1987; Ahuja, 1993; Bajaj, 1995 a; Hammerschlag and Litz, 1992; Jain *et al*., 1995 and 2000).

Mature tissues of woody plants have very poor regeneration capacity (Benson, 2000; McCown, 2000), and juvenile tissues have to be used for *in vitro* plant regeneration. In cashew, mature tissues are extremely recalcitrant to *in vitro* culture. Therefore, seedling explants were used to develop a regeneration system, as discussed in chapter 3.

However, for clonal propagation, it would be desirable to use juvenile explants having genetic fidelity with the chosen elite mother plant.

The nucellus is a mass of tissue within an ovule, containing the embryo sac, and surrounded by integuments. Although the morphogenetic potential of nucellus has been obvious for many years, attempts to exploit this tissue for *in vitro* culture manipulations in woody species have been successful only in few species. Potential of nucellus as an explant for *in vitro* studies in woody plants has been accentuated by Rangaswamy (1982), who has mentioned that adventitious embryo development occurs from nucellus in atleast 16 plant families. A literature survey of somatic embryogenesis from nucellus in woody species, using nucellar tissue, is summarized in Table 4.1.

Nucellus being a tissue with juvenile characteristics, assumes considerable importance in a very recalcitrant species like cashew, where only juvenile tissues have responded *in vitro*. Further, as it is a maternal tissue, plants arising from nucellus would retain the desirable traits of the parent plant. Thus, this tissue can be effectively used for clonal propagation of the selected elite mother plants. Additionally, it does not have any vascular connection with the parent plant, and is therefore considered to be free of pathogens. Thus, the plants obtained from nucellus would also be disease free. Such disease-free plants have been obtained from nucellar tissue in *Citrus* (Rangan, 1984).

Experiments were conducted to study the morphogenetic response of nucellar tissue in cashew. The details of these studies are discussed in this chapter. This chapter is divided into five sections: **4.1** Experimental: collection of plant material and surface sterilization procedures; **4.2:** Culture conditions; **4.3:** Results and Discussion; **4.4:** Summary; **4.5:** Conclusions.

Table 4.1: Somatic embryogenesis from nucellus in woody species

Species	Basal medium	Growth regulators and supplements	Res-ponse	Reference
Carica pentagona	White/ MS ½	Gln + $AdSO_4$ or CH + BA + NAA; NAA + BA; BA; basal; CH + IAA; CH + $AdSO_4$ + BA	c; cp; se; con	Rojas & Kitto, 1991
Citrus variety	White + Nitsch	Cobalt chloride + CH; Kin + CH	se; con	Rangaswamy, 1958
Citrus reticulata X C. sinensis	MS	ME	se	Rangan *et al*., 1968
C. grandis, C. limon, Citrus hybrid	MS modified	ME/ $AdSO_4$ + NAA + orange juice	se	Rangan *et al*., 1969
Citrus varieties	MS modified	ME	se	Bitters *et al*., 1970
Washington Navel orange	White/ MS	Adenine + ME	se	Button & Bornman, 1971
C. sinensis, C. paradisi	MT or MS	ME or Kin + IAA GA_3	cp con	Kochba *et al*., 1972
C. sinensis	MT	ME; GA_3	se; con	Kochba *et al*., 1972
"	"	ME; Kin + IAA; GA_3	ec, se, con	Kochba & Spiegel-Roy, 1973
"	"	-	se	Button *et al*., 1974
C. reticulata, C. sinensis	MS	ME + Ad SO_4	se	Kochba et al., 1974
C. limon	MS	ME; GA_3	se; con	Starrantino & Russo, 1980
C. paradisi	MT	"	"	Vardi *et al*., 1982
C. limon, C. sinensis	MS	Galactose + lactose	"	Kochba *et al*., 1982
C. aurantium, C. jambhiri, C.paradisi	MT	glycerol	se	Ben-Hayyim & Neumann, 1983
Citrus varieties	"	ME; Kin + IAA/ ABA / Daminozide; GA_3	"	Moore, 1985
Citrus spp	MS	ME	se; con	Navarro *et al*., 1985
C. sinensis	MT	Sucrose + mannitol	se	Kobayashi *et al*., 1985

continued…..

Table 4.1 continued…..

Species	Basal medium	Growth regulators and supplements	Res-ponse	Reference
Microcitrus	MT	-	se	Vardi *et al*., 1986
C. sinensis	MS	ME/ 2,4-D + Daminozide; GA_3	"	Gmitter & Moore, 1986
C. mitis	MS	-	"	Sim *et al*., 1988
C. reticulata	MT	ME + Adenine; lactose; GA_3	"	Ling *et al*., 1990
C. sinensis	MT	lactose	se	Starrantino & Caponetto, 1990
C. unshiu	MT	Lactose; sucrose + gellan gum + GA_3	se; con	Kunitake *et al*., 1991
C. aurantium	-	-	se	Gavish *et al*., 1992
C. reticulata	MS	Kin + NAA	se, con	Gill *et al*., 1991, 1994
Citrus related genera	MT	2,4-D + BA + ME; BA; lactose; NAA	c; cm; se; con	Ling & Iwamasa, 1997
Citrus spp	MS	ME	c; se, ger	Carimi *et al*., 1998
"	MS; MS modified	ME + sucrose; lactose	c; se	Perez *et al*., 1998
C. reticulata	MS	ME/ Kin/ Kin + NAA	"	Vijayakumari & Singh, 2001
Eugenia jambos	MS*	Ascorbic acid + gln+ suc + 2,4-D; 2,4-D; BA	se; ecp; shoot dev	Litz, 1984 a
Malus domestica Borkh.	MS	-	se	Eichholtz *et al* 1979
Malus pumila Mill.	LS	-	se	James *et al*., 1984
Mangifera indica L.	MS *	ascorbic acid + gln + suc + CW + BA; CW	c & se; cp	Litz *et al*., 1982
"	"	ascorbic acid + gln + suc + 2,4-D; basal; CW + ME	c; dev & mat; ger	Litz, 1984 b
"	"	ascorbic acid + gln + suc + CW; 2,4-D; AC; BA + CH/ CW/ ME	c; c; se & mat; ger	Litz *et al*., 1984
"	MS*; B5*	ascorbic acid + gln + suc + 2,4-D; suc + 2,4-D + Kin; ; gln + suc + CH + CW; CH + CW	c; ec, cp, cm; ger	DeWald *et al*., 1989 a & b

continued…..

Table 4.1 continued….

Species	Basal medium	Growth regulators and supplements	Res-ponse	Reference
Mangifera indica L.	MS; MS**	AC + Suc + 2,4-D + GA_3; gln + 2,4-D + GA_3; AC + CH + CW + suc + ABA; BA	ec; se; mat; ger	Jana *et al*., 1994
"	B5*	Gln + suc + 2,4-D; BA + Kin	ec & se; mat	Lad *et al*., 1997
"	MS*; B5*	ascorbic acid + gln + suc + 2,4-D; basal; suc + GA_3	c,ec; se; ger	Ara *et al*., 1999
"	RO; B5-RO	AC + suc + 2,4-D + GA_3; AC + gln + 2,4-D + GA_3; AC + CH + CW + gln + suc + 2,4-D + GA_3; ABA; TDZ	ec; se; con; mat; ger	Thomas, 1999
"	MS	BA; BA + GA_3	se; ger	Laxmi *et al*., 1999
"	MS*; B5*	ascorbic acid + gln + suc + 2,4-D; gln + suc; Kin + 2,4-D/ NAA; suc + GA_3	ec; cp; mat; ger	Ara *et al*., 2000
"	Barba-Patena	suc + CW + 2,4-D; AC + CW + gln + suc	se, ger; pl	Patena *et al*., 2002
Myrciaria cauliflora	MS*	Ascorbic acid + gln+ suc + 2,4-D; BA	se; shoot dev	Litz, 1984 c
Ribes rubrum L.	Miller's Nitsch	Kin GA_3 3	se pl	Zatyko *et al*., 1975
Theobroma cacao L.	MS ½ liq MS ½ WPM	CW + ME + CH + 2,4-D + 2iP; CW + ME + 2iP; CH + suc; sorbitol + suc; fructose; 20,000 ppm CO_2	c; cp; se; mat; con; ger	Figueira & Janick, 1993
Vitis longii Microsperma	MS	2,4-D + BA; basal; BA	ec & se; cp; ger	Gray & Mortensen, 1987
Vitis vinifera and *Vitis* hybrid	Nitsch	2,4-D or NOA + BA; basal	c; se	Srinivasan & Mullins, 1980
Vitis vinifera L.	Nitsch	BA/ BA + NOA 2iP + GA_3 / White's	c,ec & se shoot dev	Mullins & Srinivasan, 1976

B5*: B5 major + MS minor salts; c: callus; cm: callus maintenance; cp: callus proliferation; con: conversion of somatic embryos; ec: embryogenic callus; ecp: embryogenic callus proliferation; ger: germination of somatic embryos; mat: maturation of somatic embryos; MS*: MS major salts half strength; MS**: MS major and minor salts half strength; pl: plantlet formation; se: formation of somatic embryos, shoot dev: shoot development;

4.1: Experimental

Collection of plant material: Immature nuts (variety Vengurla 1) were procured from the Balasaheb Sawant College of Agriculture, Dapoli, Maharashtra. Nuts were collected from 8-15 year old open cross-pollinated field grown trees. Immature nuts were collected during January – March, from second through eighth week post fertilization stages. Nuts were processed as early as possible after collection, to avoid wastage of plant material due to rapid deterioration.

Surface sterilization: Nuts were washed thoroughly under running tap water, followed by laboratory detergent wash (1% Labolene®, Qualigens, India) for 20 minutes. They were then surface sterilized with 0.1% $HgCl_2$ for 20 minutes and finally, rinsed 3-4 times with sterile distilled water in laminar airflow cabinet.

4.2: Culture conditions: The immature nuts were cut open in laminar airflow cabinet. Ovules were taken out from the nuts and bisected longitudinally into halves. Ovule halves containing the nucellar tissue were placed in disposable petriplates (85 mm x 15 mm or 55 mm x 15 mm; Laxbro, India) containing semi-solid nutrient medium supplemented with 3% sucrose, 0.5% AC (Qualigens, India) and 0.5% agar. Five to ten explants were inoculated in each petriplate. The zygotic embryos were removed from the nucellar halves and cultured separately. If zygotic embryo was not easily visible, ovular halves were inoculated along with the zygotic embryos. After 3-4 days of incubation, zygotic embryos enlarged and were easily visible, when they were removed and cultured separately. Each treatment was repeated thrice, and 40-70 explants were used per treatment every time. Initially, the nucellar explants were inoculated in dark as well as in 16-hour photoperiod. Nucellar tissue incubated in light rapidly turned dark brown, and did not form proliferating callus. Therefore, in all experiments, cultures were incubated at 25± 2ºC in dark.

Different factors affecting the *in vitro* response of nucellar tissue (developmental stage of explant, basal medium, and effect of growth regulators) were studied. Nucellar callus was maintained for growth and induction of somatic

embryos. Culture conditions for each factor studied have been mentioned separately in subsections 4.2.1 to 4.2.6. All the experiments, starting from selection of proper developmental stage, to induction of somatic embryos, were carried out during a period of five consecutive years.

4.2.1. Effect of developmental stage on proliferation of nucellar tissue: At the commencement of the present study, there were no reports on *in vitro* studies in cashew where nucellus was used as explant. Hence, it was decided to first determine the developmental stage of the nut at which the nucellar tissue would yield morphogenetic response. Accordingly, nucellar tissue was collected at different developmental stages of immature nuts, from second through eighth week post fertilization stages. The nucellar tissue from each stage was inoculated on MS medium supplemented with 2,4-D 5 μM, GA_3 5 μM, sucrose 3%, AC 0.5%, and agar 0.5%. Since maximum number of nucellar explants obtained from nuts at 3-4 weeks post-fertilization stage formed callus, explants at this stage were used for further experiments.

4.2.2. Effect of basal medium on proliferation of nucellar tissue: To carry out experiments with the nucellar tissue, it was essential to choose a suitable basal medium favorable for growth of nucellar tissue. Therefore, nucellar explants obtained from 3-4 weeks post-fertilization stage of nut development were cultured on three basal media MS, SH and WPM, and the response of nucellar explant on the three basal media was compared. MS medium, which was found to support callus formation from greater number of explants was then used at full strength, half strength, and major salts only at half strength, to determine the most optimal salt concentration. All media were supplemented with 2,4-D 5 μM, GA_3 5 μM, sucrose 3%, AC 0.5%, and agar 0.5%. Full strength MS medium was optimal for proliferation of nucellar tissue, and was then used for all experiments.

4.2.3. Effect of growth regulators and organic supplements on proliferation of nucellar tissue: The nucellar tissue was subjected to various growth regulator

treatments in initial experiments, to observe the response of nucellus to the different growth regulators. Growth regulators used were auxins 2,4-D (5 - 50 μM), 2,4,5-T (5 - 50 μM), NAA (5 - 50 μM), IAA (5 - 50 μM), IBA (5 - 50 μM), cytokinins BA (5 -50 μM), Kin (5 - 50 μM), 2iP (0.5-25 μM), TDZ (0.5-5 μM), Zea (0.5-25 μM), and GA_3 (0.5- 50 μM). Organic supplements CH (50-2000 mg/l), glutamine (25-2000 mg/l) and CW (2-20% v/v) were also used.

Morphogenetic response of nucellus was governed by the growth regulators in the medium. Separate growth regulator combinations were required for the different stages of callus formation, formation of embryogenic mass, and expression of somatic embryos respectively. The nucellar tissue proliferated rapidly on five types of media, based on growth regulators used. Three media from each type were selected for initial inoculation of nucellar tissue.

4.2.4. Maintenance of nucellar callus and formation of granular embryogenic mass: During the course of experiments it was realized that nucellar callus showed continued growth on few media containing combinations of 2,4-D and GA_3. Growing and multiplying the nucellar callus was necessary to continue the experiments. Therefore, the initial calli, obtained on each of the five media, were transferred to and maintained on media having combinations of 2,4-D (5 and 10 μM) and GA_3 (5, 10, 15, 20 and 30 μM). These media were supplemented with 0.05% CH, 10% CW (v/v), 4% sucrose, 0.5% AC, and 0.5% agar. It was seen that white granular masses were formed on some maintenance media, which appeared embryogenic.

4.2.5. Induction of somatic embryos, and observation of somatic embryos under microscope: The granular embryogenic mass formed during maintenance of nucellar callus was transferred to a few media for further growth of embryogenic callus, and differentiation of somatic embryos. These media were supplemented with combinations of 2,4-D (5 μM) and GA_3 (5, 10, 15, 20 and 30 μM). Somatic embryos developed on one of these media.

The nucellar tissue had to be cultured sequentially on three different media for initial callus formation, formation of embryogenic mass, and differentiation of somatic embryos respectively. These three media have been used to successfully obtain somatic embryos for three consecutive years. Medium on which the nucellar tissue had initially formed callus was labeled as (callus) Initiation Medium. The medium on which the callus formed embryogenic mass was called Maintenance Medium. Medium on which somatic embryos differentiated from embryogenic mass was called Expression Medium.

Formation of embryogenic callus and development of somatic embryos were observed with the help of stereomicroscope (Zeiss, Germany). Section 4.3.5 describes the observations made after transfer of embryogenic mass on different media, formation of somatic embryos, and microscopy studies on development of somatic embryos.

4.2.6. Maturation and conversion: Somatic embryos formed on embryo Expression Medium were transferred to different maturation media containing combinations of ABA (1-30 μM), PEG (2, 4, 10%), maltose (6, 8, 10%), sucrose (6, 8, 10%) and gelrite (0.2, 0.5, 0.8, 1%). Coconut water and CH were used at 5% and 0.025% respectively. MS medium without growth regulators was also used for maturation. It was used with and without AC (0.5%). For all maturation and conversion media, gelrite (0.2%) was used as gelling agent. The different maturation media used are indicated in Table 4.9. MS medium without growth regulators was most suitable for development of somatic embryos.

4.2.7. Histology: The embryogenic masses, containing developing somatic embryos were fixed in FAA (formaldehyde: acetic acid: alcohol), and further dehydrated in the tertiary butyl alcohol series. They were embedded in paraffin wax (melting point 58-60°C, Qualigens, India) and sectioned at 10 μm thickness using a rotary microtome. The sections were stained with haematoxylin and observed under light microscope (Zeiss).

4.2.8: Long-term maintenance of nucellar callus: The embryogenic nucellar callus obtained in the above experiments was maintained in liquid and semi-solid medium. Embryogenic callus has still retained the embryogenic potential, and can be used as source of somatic embryos. The medium used for maintenance is MS with 5 μM 2,4-D, 3% sucrose, and 0.05% CH, with and without (0.5%) agar. The callus on semi-solid medium was transferred to fresh medium after every 5-6 weeks. For callus maintained in liquid medium on rotary shaker in dark, spent medium is removed and fresh medium added after every 8 days. Suspension cultures are transferred to fresh flasks for continued multiplication.

4.3: Results and discussion

The results of different experiments described in Section 4.2 are discussed below, in subsections 4.3.1 through 4.3.9.

4.3.1. Effect of developmental stage on proliferation of nucellar tissue: To elicit maximum response from the nucellar tissue, it was necessary to select the explant at the correct stage of nut development. To achieve this, immature nuts were collected at different times, from 2nd week through 8th week post-fertilization stages (Fig. 4.1). Nucellar explants obtained from each stage were inoculated on MS medium with 2,4-D 5 μM + GA_3 5 μM + sucrose 3% + AC 0.5% + agar 0.5%. Response of nucellus from each developmental stage was observed (Table 4.2).

Fig. 4.1: Immature nuts of cashew used at different developmental stages (weeks after fertilization)

a: nuts at 1-3 weeks; b: nut at 3-4 weeks; c: nut at 6-8 weeks; d, e, f: immature nuts dissected to show size of fertilized ovule; g: ovule at 3-4 weeks stage, chosen for further studies; h: ovule at 3-4 weeks stage bisected for use in experiments, nucellus is exposed to view.

Table 4.2: Effect of developmental stage of immature nuts on proliferation of nucellar tissue in cashew

Developmental stage of explant (weeks after fertilization)	Percent explants (nucellus) showing callus proliferation 3 weeks after inoculation
2-3	4.6 ± 0.5
3-4	50.6 ± 1.3
4-5	18.4 ± 0.6
5-6	4.2 ± 1.1
6-7	0
7-8	0

(Medium used was MS + 2,4-D 5 μM + GA_3 5 μM + sucrose 3% + AC 0.5% + agar 0.5%. Values are mean ± standard deviation (SD) of three experiments, with 40-70 explants used per treatment in each experiment. Cultures were incubated at 25 ± 2ºC in the dark).

It can be seen from Table 4.2, that maximum number of explants (50.6%) showing callus proliferation (Fig. 4.2b) were observed at 3-4 weeks post-fertilization stage.

At 2-3 weeks post-fertilization stage, only 4.6% of the nucellar explants proliferated to form callus. The callus showed rapid browning within 3-4 days, and did not grow further (Fig. 4.2a). Similarly, response of explants declined progressively from 4-5 week post-fertilization stage. At 4-5 weeks post-fertilization stage, only 18.4% of the nucellar explants showed callus proliferation. At the 5-6 weeks post-fertilization stage, the number of responding explants dropped further, to only 4.2%. The callus formed from explants at 4-5 week, and 5-6 week post-fertilization stages showed very poor growth (Fig. 4.2c) and did not grow on further maintenance. None of the explants from the 6-7 week, and 7-8 week post fertilization stages formed callus.

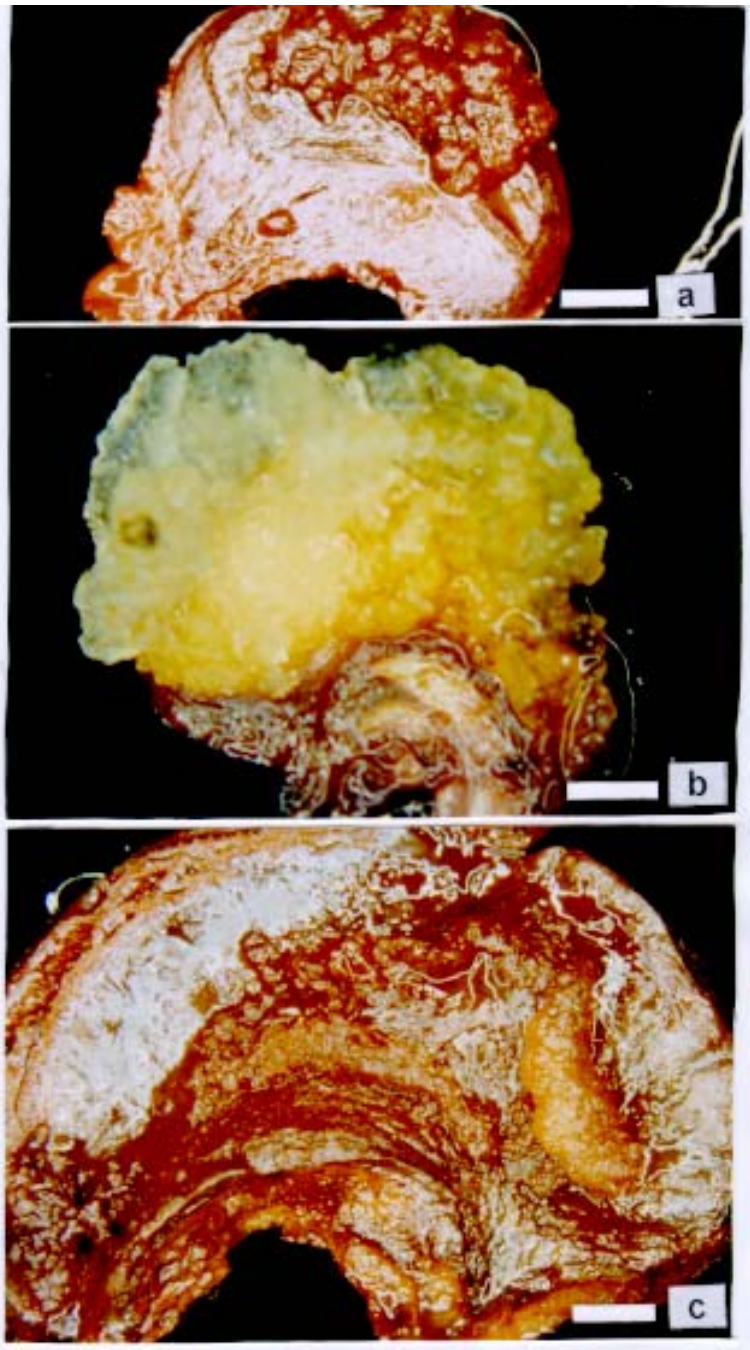


Fig. 4.2: Response of nucellar tissue at different developmental stages

a: nucellar callus formed from 2-3 weeks post-fertilization stage; b: yellowish proliferating callus formed from nucellus obtained from immature fruits at 3-4 weeks; c: nucellar callus formed at 4-7 weeks post-fertilization stages; (a-c: bar= 6 mm)

Callus formed from nucellus explants obtained at 2-3 weeks, and from 4th through 8th week post fertilization stages (Fig.4.2 a,c) did not grow even after maintaining the callus for a prolonged period (10-15 weeks), and could not be used for experiments. Explants from these stages were, therefore, unsuitable for further studies.

The nucellar tissue at 3-4 weeks post-fertilization stage of development proliferated rapidly within 3 weeks after inoculation. The nucellar tissue formed a proliferating yellowish or creamish callus. The callus filled the cavity of the ovular halves in 2-3 weeks (Fig. 4.2b), and proliferated further onto the medium surface. Maximum number of explants (50.6%) showed proliferating callus at this stage (Table 4.2). This callus showed continued growth on further incubation, and therefore, could be used for further experimentation. Hence, nucellar explants at 3-4 weeks post-fertilization stages were chosen for all the further experiments.

Ananthakrishnan and co-workers (1999) observed that nucellus from 2-3 weeks post-fertilization stage of cashew was embryogenic, whereas Cardoza and D'Souza (2002) found 2-week post fertilization stage suitable for embryogenic response. This variation in responsive stage of nut development may be due to the different varieties of cashew used in these studies. Such difference in embryogenic response between varieties, with respect to stage of fruit development has also been observed in mango (Thomas, 1999) and citrus (Bitters et al., 1970).

The importance of appropriate developmental stage of the fruit for induction of embryogenesis from nucellus has been noted in other species also. In mango, a particular stage of fruit development was more suitable for embryogenic response from nucellus (Litz, 1984b; Litz *et al*., 1995). In citrus, the nucellus had to be excised at an appropriate stage of development (70-120 days post-pollination) to initiate nucellar embryogenesis *in vitro* (Rangan, 1984). In a study on *Luffa* and *Trichosanthes*, nucellus excised before pollination, and 6 weeks post pollination, failed to proliferate, showing browning within 2 weeks of culture (Rangaswamy and Shivanna, 1975). Thus, morphogenetic potential of nucellus of cashew depends on developmental stage of the nut.

4.3.2. Effect of basal medium on proliferation of nucellar tissue: When nucellar explants at 3-4 weeks post-fertilization stage were inoculated on three different basal media MS, SH and WPM, it was observed that the number of explants forming proliferating callus was significantly more (50.1%) on MS medium, as compared to SH (38.7%) and WPM (32.5%) basal media (Table 4.3). MS medium was further used at different strengths (half, major salts half, full). Full strength MS medium was found to be most suitable, as more number of explants (51.3%) formed proliferating callus in full strength medium, than on half strength (34.4%) or only major salts at half strength (38.3%) media (Table 4.3). Hence, full strength MS medium was used for all further experiments.

Table 4.3: Effect of basal medium on proliferation of nucellar tissue of cashew

Basal medium used	Percent nucellar explants showing callus proliferation 3 weeks after inoculation
MS	50.1 ± 3.0
SH	38.7 ± 2.0
WPM	32.5 ± 1.6
MS full strength	51.3 ± 2.2
MS major salts half strength	38.3 ± 1.3
MS half strength	34.4 ± 2.9

{Nucellar explants were obtained from immature nuts at 3-4 weeks post-fertilization stage. Medium used was MS + 2,4-D 5 μM + GA_3 5 μM + sucrose 3% + AC 0.5% + agar 0.5%. Values are mean ± standard deviation (SD) of three experiments, with 40-70 explants used per treatment in each experiment. Cultures were incubated at 25 ± 2ºC in the dark}.

MS basal medium has been used also in other studies in cashew (Ananthakrishnan *et al.*, 1999; Cardoza and D'Souza, 2002), and in mango (Jana *et al*., 1994). In

other studies in mango, several basal media, like MS with half strength major salts (Litz *et al.*, 1984; DeWald *et al.*, 1989 a and b; Ara *et al.*, 1999 and 2000), Rugini Olive (RO) (Thomas, 1999), Barba-Patena (BP) (Patena *et al.*, 2002), B5 major + MS minor salts and vitamins (Lad *et al.*, 1997) have been used for nucellar culture of mango. MS medium has been used at half strength for culture of nucellus in cacao (Figueira and Janick, 1993). However, for citrus (Kochba *et al.*, 1972; Moore, 1985, to mention a few), Murashige and Tucker basal medium has been routinely used for culture of nucellar tissue.

4.3.3. Effect of growth regulators and organic supplements on proliferation of nucellar tissue: Screening for suitable growth regulators was conducted over a period of two years, and media supporting *in vitro* growth of nucellus were identified. These media were used for culture of nucellar tissue in the subsequent studies. Embryogenic callus and somatic embryos were obtained during further studies carried out over a period of two years. Therefore, efforts were concentrated on development of somatic embryos, and establishing a regeneration protocol. Due to low embryogenic response, and availability of plant material only for a short period once every year, one particular sequence of media was followed for obtaining somatic embryogenesis. As a result, some media combinations remain to be investigated for possible induction of somatic embryogenesis. Observations on experiments to select the initial media for callus formation are discussed below.

Nucellar tissue did not grow, and did not form somatic embryos on medium without growth regulators (Table 4.4b), even when such medium was enriched with CH, CW, and glutamine. This implied the embryogenically undetermined state of the nucellar cells (IEDCs), requiring external stimulus for induction of somatic embryogenesis. In contrast, the nucellar tissues of polyembryonic varieties of mango (Litz *et al*., 1982 and 1984) and citrus (Bitters *et al*., 1970, Gavish *et al*., 1992) readily formed somatic embryos on media lacking growth regulators, due to presence of embryogenically pre-determined cells (PEDCs) in the nucellus.

In presence of appropriate growth regulators, the nucellar tissue formed rapidly proliferating yellowish callus. Auxin 2,4-D used alone and in combination was effective for callus formation. This callus had to be grown on a sequence of suitable media to make it embryogenic. Continuous supply of exogenous auxin, with suitable change in auxin and gibberellin concentration was necessary for the stages of callus formation, obtaining embryogenic callus, and differentiation of somatic embryos. Callus formed at low auxin concentration became embryogenic on exposure to increased auxin concentration for a period of 12-14 weeks. The embryogenic callus differentiated into somatic embryos after transfer to medium with lower auxin, and higher gibberellin concentration.

Auxins IAA, IBA, 2,4,5-T, and cytokinins (BA, Kin, 2iP, TDZ, Zea), used separately and in combinations, did not result in callus formation (Fig. 4.3a, b). Nucellar tissue responded very poorly on media with these growth regulators. Only 1-5% of nucellar explants formed callus on few of these media. This callus became black, and did not grow on further incubation (Fig. 4.3c, d). Therefore these media were not used for further experimentation. The results of these media are also not included in the present study. Media used for inoculation of nucellar tissue, but found unfavorable for callus formation are indicated in Table 4.4 a.

Table 4.4 b includes the media on which the nucellar tissue proliferated rapidly within three weeks after inoculation, to form yellowish callus. The appearance and consistency of the callus was more or less similar on all these media. The nucellar tissue formed a rapidly growing callus on 48 media. These 48 media are divided into five groups on the basis of growth regulators used. The five groups are: Group 1: media with only 2,4-D; Group 2: media with only GA_3; Group 3: media with combinations of 2,4-D and GA_3; Group 4: media with combinations of 2,4-D, GA_3 and BA; Group 5: media with combinations of 2,4-D, GA_3 and NAA.

Though BA alone was unable to form nucellar callus, it supported callus formation when used along with 2,4-D and GA_3. Similarly, NAA used alone was ineffective for proliferation of nucellus, but supported it when used with 2,4-D and

GA_3. The highest number of nucellar explants (57.57%) formed proliferating callus on medium with 2,4-D 5 μM + GA_3 20 μM.

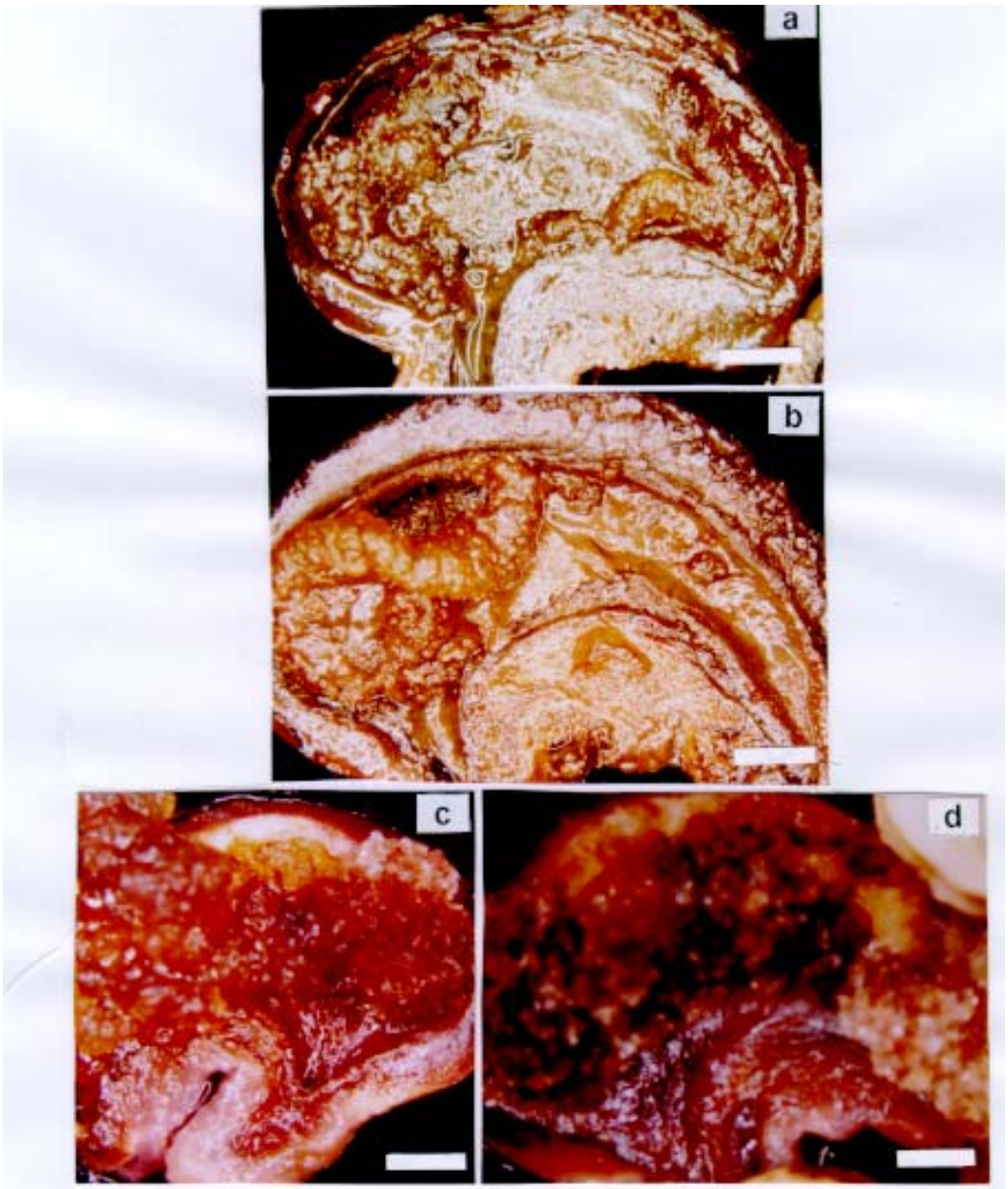


Fig. 4.3: Response of nucellus in presence of IAA, IBA, 2,4,5-T, and cytokinins (BA, Kin, 2iP. TDZ, Zea) (basal medium MS + sucrose 3% + AC 0.5% + agar 0.5%)

a, b: no response of nucellar tissue; c, d: only 1-5% explants formed callus in presence of above growth regulators, callus showed rapid browning, and did not proliferate (a-d: bar= 6 mm)

Table 4.4a: Different media and media constituents tested for initiation of callus from nucellar tissue of cashew

(Basal medium: MS + sucrose 3% + AC 0.5% + agar 0.5%, concentrations of growth regulators in μM)

Media No.	1	2	3	4	5	6	7	8	9
CH (g/l)	-	-	0.5	0.5	0.5	-	2	2	-
L-glutamine (g/l)	-	-	0.4	1	-	0.5	0.2	-	2
CW (% v/v)	-	10	5	-	5	10	-	-	-

Media No.	10	11	12	13	14	15	16	17	18	19	20	21
2,4-D	-	-	-	-	-	-	-	2.5	5	-	5	-
GA_3	5	20	50	2.5	15	30	5	-	-	-	15	10
BA	5	-	-	1	5	5	5	5	2.5	-	10	5
NAA	-	5	2.5	5	2	-	5	-	-	2.5	2	-

Media No.	22	23	24	25	26	27	28	29	30	31	32	33
2,4-D	10	5	-	-	-	-	-	-	5	5	-	5
GA_3	-	-	-	-	-	15	30	-	-	-	15	15
BA	2.5	-	5	-	-	-	-	5	-	10	5	-
Kin	5	5	5	10	5	5	5	5	10	5	5	5
NAA	2.5	10	-	5	5	-	-	5	-	-	-	-

Media No.	34	35	36	37	38	39	40	41	42	43	44	45	49
IAA	5	-	5	-	5	5	-	-	10	10	-	-	-
IBA	-	5	5	-	-	5	10	-	-	-	10	-	-
BA	-	-	-	5	-	10	-	5	2	-	5	-	2
Kin	-	-	-	-	10	-	5	5	-	5	-	-	-
TDZ	-	-	-	0.1	0.05	-	-	0.1	0.1	-	-	-	-
2,4,5-T	-	-	-	-	-	-	-	-	-	-	-	5	5

Media No.	50	51	52	53	54	55	56	57	58	59
2iP	5	-	-	2	-	-	5	5	2	-
Zea	-	0.1	-	-	0.1	0.1	0.1	-	-	0.1
NAA	-	-	5	-	5	-	-	5	-	2
2,4-D	-	-	5	10	-	5	5	-	-	-
2,4,5-T	-	-	-	-	-	-	-	-	5	5

Table 4.4 b: Proliferation of nucellar tissue of cashew on different nutrient media

Growth Regulator μM	Percent explants showing nucellar proliferation	Growth of nucellar callus, weeks after inoculation		
		3 weeks	6 weeks	12 weeks
No growth regulator	0	-	-	-
Group 1: only 2,4-D				
2,4-D 5	43.9 ± 0.7	p	b	c
10	44.6 ± 1.2	"	"	s
15	46.6 ± 0.3	"	i	-
20	48.6 ± 0.9	"	i	-
30	37.3 ± 0.1	"	i	-
50	32.4 ± 1.4	"	-	-
Group 2: only GA_3				
GA_3 5	50.2 ± 2.6	p	b	s
10	51.3 ± 0.5	"	"	"
15	51.2 ± 1.0	"	"	"
20	53.7 ± 2.8	"	"	"
30	41.9 ± 0.7	"	i	-
50	41.6 ± 0.7	"	-	-
Group 3: 2,4-D + GA_3				
2,4-D 5 + GA_3 5	51.5 ± 1.3	p	br	c
" + GA_3 10	55.1 ± 0.8	"	b	c
" + GA_3 15	56.2 ± 1.1	"	"	c
" + GA_3 20	57.6 ± 1.9	"	"	c
" + GA_3 30	46.7 ± 0.5	"	"	s
" + GA_3 50	37.2 ± 1.1	"	i	-
2,4-D 10 + GA_3 5	43.8 ± 0.7	p	br	c
" + GA_3 10	45.4 ± 0.7	"	"	c
" + GA_3 15	47.8 ± 1.1	"	"	c
" + GA_3 20	48.9 ± 0.4	"	"	c
" + GA_3 30	54.9 ± 0.7	"	"	s
" + GA_3 50	35.4 ± 0.9	"	i	-
2,4-D 15 + GA_3 5	35.6 ± 0.6	p	b	-
" + GA_3 10	41.8 ± 0.7	"	"	-
" + GA_3 15	46.1 ± 1.3	"	"	-
" + GA_3 20	47.3 ± 1.1	"	i	-
" + GA_3 30	42.5 ± 0.8	"	i	-
" + GA_3 50	34.6 ± 1.1	br	-	-

Table 4.4b continued…….

Growth Regulator μM	Percent explants showing nucellar proliferation	Growth of nucellar callus, weeks after inoculation		
		3 weeks	6 weeks	12 weeks
2,4-D 20 + GA_3 5	54.8 ± 2.1	p	i	-
" + GA_3 10	49.4 ± 1.9	p	i	-
" + GA_3 15	49.7 ± 0.9	p	i	-
" + GA_3 20	55.9 ± 0.8	br	i	-
" + GA_3 30	40.5 ± 0.2	br	i	-
" + GA_3 50	39.2 ± 1.4	br	i	-
Group 4: 2,4-D + GA_3 + BA				
2,4-D 5 + GA_3 5 + BA 5	35.7 ± 0.7	p	br	s
" + " 15 + "	46.2 ± 1.9	"	"	s
" + " 30 + "	36.2 ± 0.5	"	"	s
2,4-D10 + $GA_3$5 + "	34.3 ± 0.4	p	i	-
" + " 15 + "	36.4 ± 1.2	"	"	-
" + " 30 + "	48.6 ± 2.9	"	"	-
Group 5: 2,4-D + GA_3 + NAA				
2,4-D 5 + GA_3 5 + NAA 5	34.5 ± 0.7	p	br	s
" + " 15 + "	42.9 ± 1.2	"	"	s
" + " 30 + "	36.1 ± 0.6	"	"	s
" 10 + GA_3 5 + "	36.4 ± 0.7	p	i	-
" + " 15 + "	37.2 ± 1.8	"	i	-
" + " 30 + "	49.5 ± 2.6	"	i	-

{Nucellar explants were obtained from immature nuts at 3-4 weeks post-fertilization stage. Basal medium used was MS + 3% sucrose + 0.5% AC + 0.5% agar. Values represent mean ± standard deviation (SD) of three experiments, with 40-70 explants used per treatment in each experiment. Cultures were incubated at 25± 2ºC in dark.
b- blackening of callus; br- browning of callus; c- continued growth of callus; i- intense blackening of callus; s- very slow growth of callus; - no further growth of callus}.

The callus obtained on each medium in Table 4.4b, was observed further for a period of 12 weeks. The callus was transferred to fresh medium after every 4 weeks during this period.

The callus showed a similar pattern of growth on all the 48 media till six weeks after inoculation. During 0-3 weeks after inoculation, nucellar tissue proliferated very rapidly to form a creamish or yellowish callus (Fig. 4.4b) on all 48 media (Table 4.4b). During 3-6 weeks after inoculation, the rapid growth of the nucellar callus slowed considerably on all 48 media. Slow growth was accompanied by gradual browning (Fig. 4.4c), and later complete blackening (Fig. 4.4d) of callus. This was not the effect of media, but the inherent growth pattern of the tissue. Frequent transfer of explants to fresh medium, and addition of AC did not prevent browning. The nucellar callus of mango was also slow growing and turned dark brown after a few days in culture (Litz, 1984b). Extensive browning of ovular explants was also noticed in cacao (Figueira and Janick, 1993).

During further incubation period of 6-12 weeks, blackened callus of some nucellar explants on one medium from Group 1 and few media from Group 3 formed a proliferating yellowish/ creamish/ white granular mass after 10-12 weeks. The calli formed on media from Groups 2, 4 and 5 were very slow growing, or did not show any further growth. Slow-growing callus remained black/ brown throughout the 12-week observation period, and also during further maintenance. The callus which did not grow further, was dark brown or black, friable and necrotic. Callus growth observed on the five groups of media, between 6-12 weeks is as follows:

Group 1: Media with only 2,4-D: Out of six media in this group, callus formed at 5 μM of 2,4-D gradually became yellowish, with granular appearance (Fig. 4.5a). This granular yellowish callus continued to proliferate throughout the observation period. Callus formed on medium with 10 μM 2,4-D was slow-growing. Callus formed in the remaining four media (with 15, 20, 30 and 50 μM 2,4-D) became black, friable (Fig. 4.5b) and stopped growing after 6 weeks. Thus, out of six media in this group, only one medium, containing 5 μM 2,4-D formed callus showing continuous growth. Higher 2,4-D concentrations were inhibitory for callus growth.

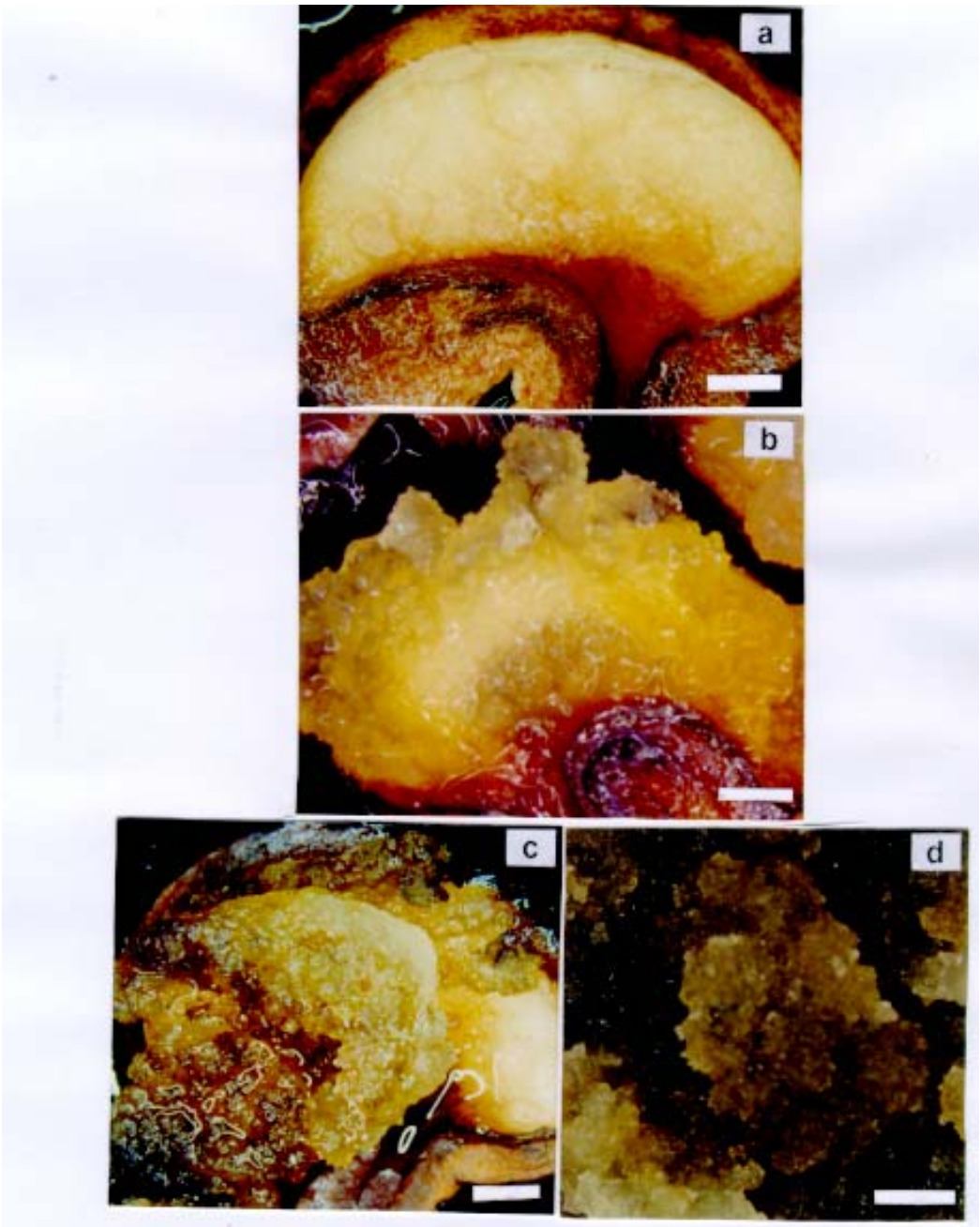


Fig. 4.4: Callus formation from nucellar tissue
a: nucellus at the time of inoculation; b: yellowish proliferating callus formed after 3 weeks; c: gradual browning of callus begins; d: browning/ blackening of callus after 6 weeks (a-c: bar= 6 mm; d: bar= 3 mm)

Group 2: Media with only GA_3: Proliferating callus was not formed on any medium from this group. Out of six media, slow-growing callus was formed on four media (with GA_3 concentrations of 5,10, 15 and 20 μM). This callus remained black, and did not form any proliferating mass in 12 weeks. Callus formed on media with GA_3 30 and 50 μM became necrotic and stopped growing after six weeks (Fig. 4.5c).

Group 3: Media with combinations of 2,4-D and GA_3: This was the largest group, with 24 media. These media contained 2,4-D at concentrations of 5, 10, 15 and 20 μM. Each concentration of 2,4-D was used in combination with a series of GA_3 concentrations (5, 10, 15, 20, 30 and 50 μM). Out of the 24 media, callus formed in eight media (with 2,4-D 5 and 10 μM) showed continuous growth (Table 4.4b), and continued to proliferate (Fig. 4.5d). The callus formed on all media with concentration of 2,4-D at 15 and 20 μM stopped growing after 6-8 weeks. In this group also, only lower concentrations (5 and 10 μM) of 2,4-D were suitable for continued callus growth.

Group 4: Media with 2,4-D, GA_3 and BA: Out of six media in this group, 2,4-D was used at 5 μM in three media, and at 10 μM in the remaining three. Callus formed in the three media with 5 μM of 2,4-D showed slow growth (Fig. 4.5e), but callus formed in media with 10 μM 2,4-D blackened and stopped growing.

Group 5: Media with 2,4-D, GA_3 and NAA: In this group also, there were six media, three with 2,4-D at 5 μM and three with 2,4-D at 10 μM. Slow-growing callus was formed at 5 μM of 2,4-D (Fig. 4.5f). Raising the 2,4-D concentration to 10 μM resulted in inhibition of callus growth.

In all the five groups, concentration of 2,4-D in the medium was a crucial factor affecting the growth of callus. Generally, callus formed at lower (5 and 10 μM) concentrations of 2,4-D showed further growth. Higher (15 μM and

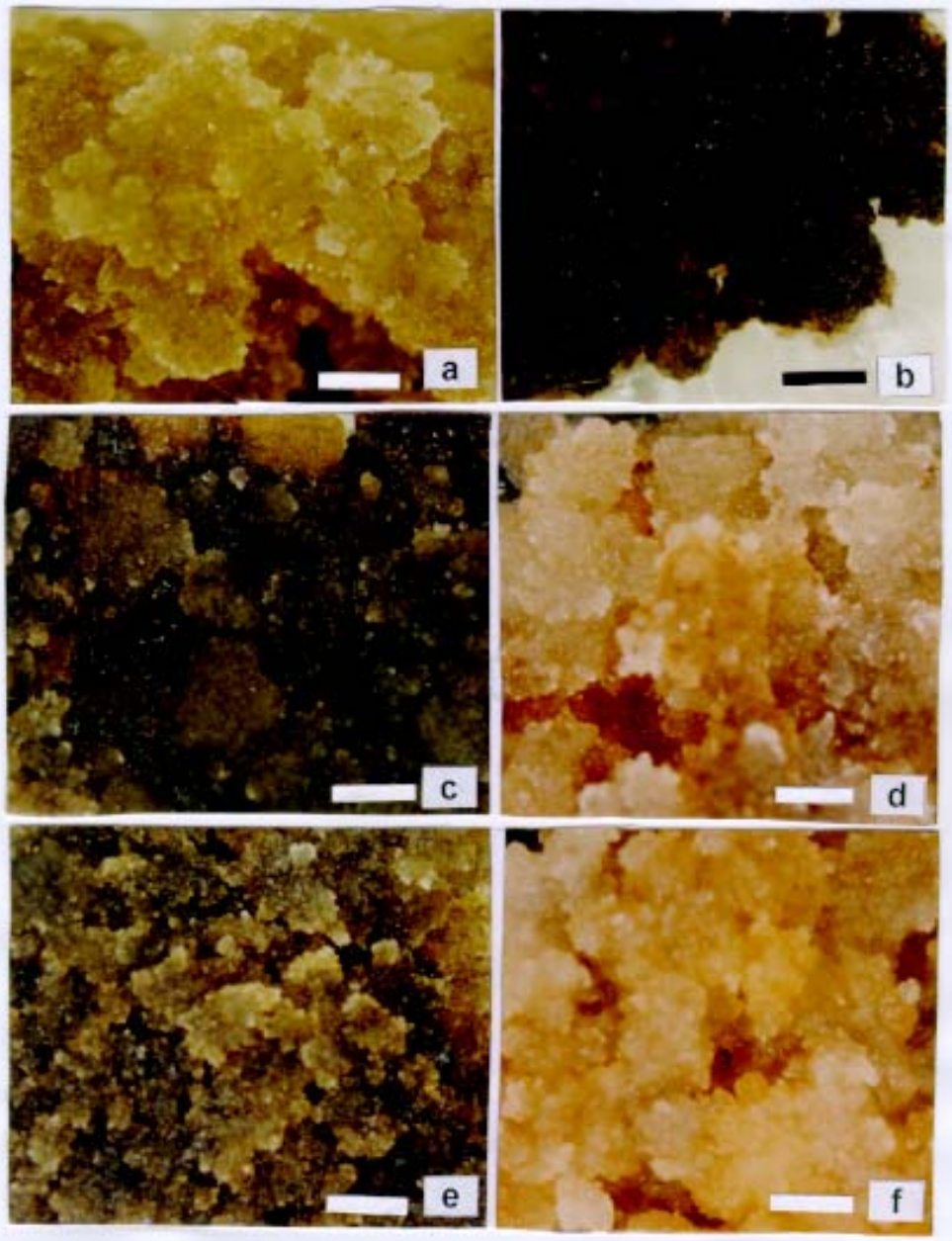


Fig. 4.5: Appearance of nucellar callus initially formed in presence of different growth regulators after 12 weeks of incubation (basal medium MS + sucrose 3% + AC 0.5% + agar 0.5%) (a-f: bar= 3 mm)

a: callus on 2,4-D 5 μM; b: callus on 2,4-D 50 μM; c: callus on GA_3 30 & 50 μM; d: callus on 2,4-D (5 or 10 μM) + GA_3 (5, 10, 20, 30 μM) combinations; e: callus on 2,4-D 5 μM + BA 5μM + GA_3 (5, 15, 30) media; f: 2,4-D 5 μM + NAA 5μM + GA_3 (5, 15, 30) media; (d, e, f: representative callus, appearance was more or less similar on these media)

above) concentrations of 2,4-D were inhibitory for callus growth. In media where BA or NAA was used along with 2,4-D and GA_3, slow-growing callus formed at 2,4-D concentration of 5 μM only, and callus formed at 10 μM 2,4-D did not grow further. High concentration of GA_3 (50 μM) also always inhibited callus growth.

Thus, out of 48 media, callus showed continuous growth in eight media, and slow growth in ten media. On the remaining media, callus became dark brown or black, and did not grow further. From these studies, two types of calluses were obtained for further experiments – slow growing (Fig. 4.6a), and continuous growing (Fig. 4.6b). The continuous growing callus continued to proliferate following transfer to fresh medium. This callus was then subjected to various treatments to induce somatic embryogenesis. The callus was transferred to following media: (results in parentheses)

(i) medium with 2,4-D 1 and 5 μM – (callus transferred to 2,4-D 1μM showed very slow growth, callus transferred to 2,4-D 5 μM continued to proliferate as long as it was maintained in culture); (ii) medium with 2,4-D 5 μM + GA_3 5 μM – (callus continued to proliferate); (iii) medium without growth regulators (iv) medium without growth regulators, but enriched with organic supplements CH, CW, glutamine (v) medium with BA 1 and 5 μM, (vi) medium with 2,4-D 5 μM + BA 5 μM; (vii) medium with 2,4-D 5 μM + Kin 5 μM, (viii) medium with BA 5 μM + GA_3 5 μM – {callus transferred to media (iii)- (viii) became brown, and stopped growing}.

It must be mentioned here, that nucellar callus obtained during the experiments carried out for determination of (i) the correct developmental stage and (ii) optimal basal medium was also multiplied and used for the above experiments.

Callus formed initially on media with combinations of 2,4-D and GA_3 continued proliferating when transferred to medium with 5 μM 2,4-D, but never differentiated to form somatic embryos, throughout the experimental period (Fig. 4.6c). Callus initially formed on medium with 5 μM 2,4-D and maintained on the

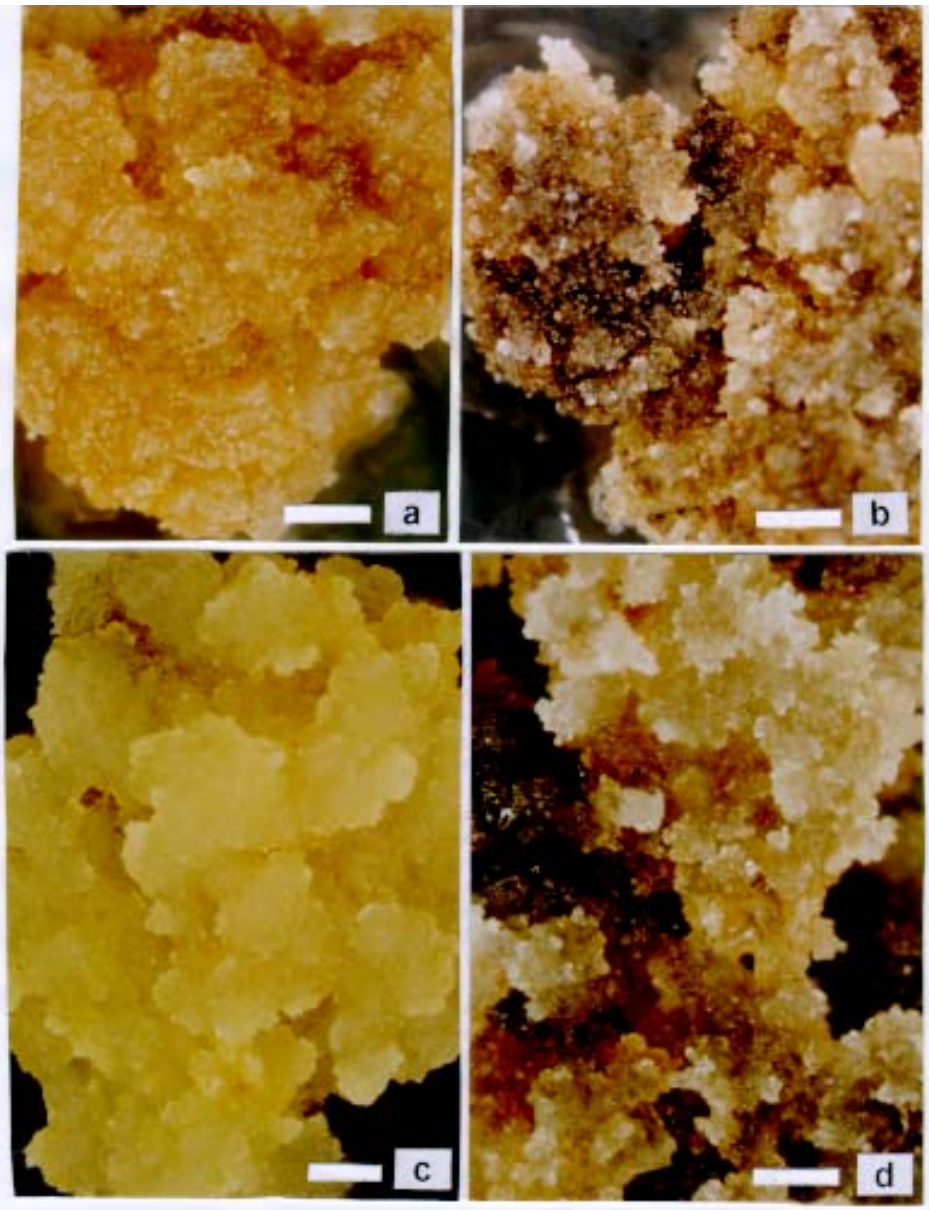


Fig. 4.6: Appearance of nucellar callus obtained on further maintenance (basal medium MS + sucrose 3% + AC 0.5% + agar 0.5%) (a-d: bar= 3mm)

a: slow-growing callus; b: continuously proliferating callus; c: callus initially formed on media with 2,4-D + GA_3 and maintained on medium with 2,4-D 5 μM; d: callus initially formed, and further maintained on medium with 2,4-D 5 μM

same medium appeared granular, but did not differentiate into somatic embryos. Apparently, auxin alone was not sufficient for differentiation of somatic embryos, and additional components were required along with 2,4-D for development of somatic embryos from callus.

Slow growing callus obtained in the initial experiments was also transferred to similar media as above. However, this callus remained black, and did not form, proliferating mass. This callus therefore was not useful for continued experiments.

Thus, in the initial studies, proliferating granular callus was obtained on a few media, but attempts to induce somatic embryogenesis from this callus were not successful. These experiments indicated that combination of 2,4-D and GA_3 was favorable for initial callus formation, and continued callus growth, but not sufficient for differentiation of somatic embryos. Some important observations made during the above experiments helped in deciding the course of further experiments. These observations were:

1. Continued incubation of the initial callus on the same initial medium was not suitable for obtaining a proliferating callus. Therefore, in subsequent studies, transferring the nucellar callus at the early stage of initial rapid growth could be attempted for obtaining a granular proliferating mass.

2. Some media from Group 3 (2,4-D + GA_3 combinations) were suitable for initial callus formation and further callus growth, but not for somatic embryo formation. Media from other groups were suitable only for initial callus formation. In further experiments, callus could be initially formed on media of other groups. This callus could be transferred at the proliferation stage to media of Group 3, in attempts to obtain continuous callus growth, and possibly, subsequent embryogenic cultures.

3. Only lower concentrations of 2,4-D (5 and 10 μM) were effective in forming continuous proliferating callus. Callus formed at higher 2,4-D concentrations (30 and 50 μM) invariably became necrotic and stopped growing. Therefore, in further experiments, only lower (5 and 10 μM) 2,4-D concentrations were used in media.

From the above observations, it was decided that the media from Groups 1, 2, 4 and 5 would be used for initial callus formation. Media from Group 3 were not selected for callus initiation, because it was already seen in the initial experiments that the callus initially formed on these media proliferated, but could not differentiate to form somatic embryos.

From Group 1 (media with only 2,4-D), only two media were chosen for initial callus formation, since, higher concentrations of 2,4-D were inhibitory for further callus growth. Similarly, from Group 2 (media with only GA_3), three media were chosen, since callus formed at GA_3 concentrations of 30 and 50 μM became, friable, necrotic, and did not grow further.

From six media each from Groups 4 and 5, three media were chosen from each group for initial callus formation.

In subsequent studies, eleven media (two from Group 1, and three media each from Groups 2, 4 and 5) were chosen for initial callus formation. It was decided that the remaining media would be used after studying the response of callus obtained on the 11 selected media. The 11 media selected for initial inoculation of nucellar tissue are listed below. The basal medium for all the eleven media was MS + sucrose 3 % + AC 0.5% + agar 0.5%. They were numbered as 'I 1' through 'I 11'(I: initiation). The media contained: (concentration in μM):

1. 2,4-D 5 (I 1)
2. 2,4-D 10 (I 2)
3. GA_3 10 (I 3)
4. GA_3 15 (I 4)
5. GA_3 20 (I 5)
6. 2,4-D 5 + GA_3 15 + BA 5 (I 6)
7. 2,4-D 10 + GA_3 15 + BA 5 (I 7)
8. 2,4-D 10 + GA_3 30 + BA 5 (I 8)
9. 2,4-D 5 + GA_3 15 + NAA 5 (I 9)
10. 2,4-D 10 + GA_3 15 + NAA 5 (I 10)
11. 2,4-D 10 + GA_3 30 + NAA 5 (I 11)

The nucellar explants were inoculated on these eleven media. The nucellar tissue proliferated on these media to form a yellowish callus in 3 weeks. It was previously observed that continued growth of this callus on the same medium formed slow growing callus. Therefore, it was necessary to transfer this callus to other media, which would support further callus growth.

It was also observed earlier that some media of Group 3 (with combinations of 2,4-D and GA_3) were suitable for continued callus growth. Therefore, these media from Group 3 were used for further growth of callus. The media chosen for further callus maintenance are listed below. They were numbered as 'C 1' through 'C 9'. The basal medium for these was MS + CH 0.05% + CW 10% v/v + sucrose 4% + AC 0.5% + agar 0.5%.

The media contained (concentration in μM):

(i) 2,4-D 5 + GA_3 5 (C 1)
(ii) 2,4-D 5 + GA_3 10 (C 2)
(iii) 2,4-D 5 + GA_3 15 (C 3)
(iv) 2,4-D 5 + GA_3 20 (C 4)
(v) 2,4-D 5 + GA_3 30 (C 5)
(vi) 2,4-D 10 + GA_3 5 (C 6)
(vii) 2,4-D 10 + GA_3 10 (C 7)
(viii) 2,4-D 10 + GA_3 15 (C 8)
(ix) 2,4-D 10 + GA_3 20 (C 9)

The rapidly growing 3-week old yellowish callus formed on each of the eleven media was transferred to media with 2,4-D and GA_3 combinations, mentioned above. Growth of callus from each initial medium was observed. The discussion regarding growth of callus from each of the 11 media, and the conclusions drawn are included in the following section 4.3.4.

4.3.4. Maintenance of nucellar callus: As mentioned earlier, nucellar tissue was inoculated on 11 media (I 1 through I 11). Three weeks after inoculation, the explants showing proliferating callus on each of the 11 media were distributed on the 9 media selected for further callus growth (C 1 through C 9). These media were supplemented with CW 10% + CH 0.05% + 4 % sucrose + 0.5% AC + 0.5% agar. The media were enriched with organic supplements to ensure better growth of the nucellar callus. Further growth of the 3-week old yellowish callus was observed on these different media. The main observations recorded during maintenance of nucellar callus were:

1. Irrespective of the composition of initial medium and medium used for further growth, callus formed on all initial media blackened completely within three weeks of transfer to callus maintenance medium.

2. Growth of blackened callus was very slow, as compared to the initial rapid proliferation noticed in the three weeks following inoculation. Slow growth of callus occurred on most of the callus maintenance media.

3. On some callus maintenance media, completely blackened callus formed proliferating mass, which appeared embryogenic.

The media on which the nucellar callus formed granular embryogenic proliferating mass are indicated in Table 4.5a.

Table 4.5a: Formation of embryogenic mass from nucellar callus of cashew on different media

Medium for initial callus formation	Medium used for further callus maintenance and growth								
	C 1	C 2	C 3	C 4	C 5	C 6	C 7	C 8	C 9
I 1	c	s	**em**	s	s	s	s	c	s
I 2	s	s	s	s	s	c	c	c	s
I 3	s	s	s	s	s	c	c	s	s
I 4	c	s	s	s	s	s	s	s	s
I 5	s	s	s	s	s	s	s	s	s
I 6	s	s	s	s	s	s	s	**em**	c
I 7	s	s	s	s	s	s	s	s	s
I 8	s	s	s	s	s	s	s	s	s
I 9	s	s	s	**em**	s	s	s	c	c
I 10	s	c	s	s	s	s	s	s	s
I 11	s	s	c	c	s	s	s	s	c

{Basal medium for callus initiation (I 1- I 11) is MS + 3 % sucrose + 0.5% AC + 0.5% agar. Basal medium for callus maintenance (C 1 – C 9) is MS + CH 0.05% + CW 10% + 4 % sucrose + 0.5% AC + 0.5% agar. Nucellar explants were obtained from nuts at 3-4 weeks post-fertilization stage. 800-900 explants were inoculated on each initial medium. Out of these, 400-500 explants formed proliferating callus. Explants with proliferating callus were distributed (40-50 explants per medium) on the nine maintenance media for further growth. I- initiation medium; C- callus maintenance medium; c - continued growth of callus; **em**- granular embryogenic callus; s-blackened, slow growing callus}

When the rapidly growing 3-week old callus formed on three initiation media (I 1, I 6 and I 9), was transferred to callus maintenance media, it blackened gradually. Blackened primary callus then formed white granular embryogenic mass. The embryogenic mass was formed on three different maintenance media. Callus initiated on I 1 formed embryogenic mass on C 3. Similarly, callus initiated on I 6 and I 9, formed embryogenic masses on C 8 and C 4 respectively (Table 4.5 a).

Only some surface portions of the primary blackened callus formed the proliferating embryogenic mass. A similar observation was made in grape (Mullins and Srinivasan, 1976), where embryogenic callus appeared in some sectors of the proliferated nucellar tissue, and rest of the callus was nonembryogenic. The granular embryogenic mass proliferated on the surface of the blackened primary callus. It continued proliferating even after separation from the blackened callus. The callus formed on I 1, I 6 and I 9 formed embryogenic mass on C 3, C 8 and C 4, after 12, 9, and 20 weeks respectively (Fig. 4.7a, b, c). On the rest of the callus maintenance media, the callus remained black and slow-growing, or, it became yellowish and showed continued growth. The formation of embryogenic mass on different media is summarized in Table 4.5b.

Legend to Figure 4.7:

Granular proliferating masses formed from nucellar callus (basal medium for callus initiation: MS + suc 3% + AC 0.5% + agar 0.5%; for callus maintenance MS + CH 0.05% + CW 10% v/v + suc 4% + AC 0.5% + agar 0.5%;
a callus initially formed on 2,4-D 5 μM (I 1) transferred to 2,4-D 5 μM + $GA_{3\ 15}$ μM (C 3)
b: callus initially formed on 2,4-D 5 μM + BA 5 μM + GA3 15 μM (I 6), transferred to 2,4-D 10 μM + GA_3 15 μM
c: callus initially formed on 2,4-D 5 μM + NAA 5 μM + GA_3 15 μM (I 9), transferred to 2,4-D 5 μM + GA_3 20 μM (C 4); (a-c: bar= 1.5mm)

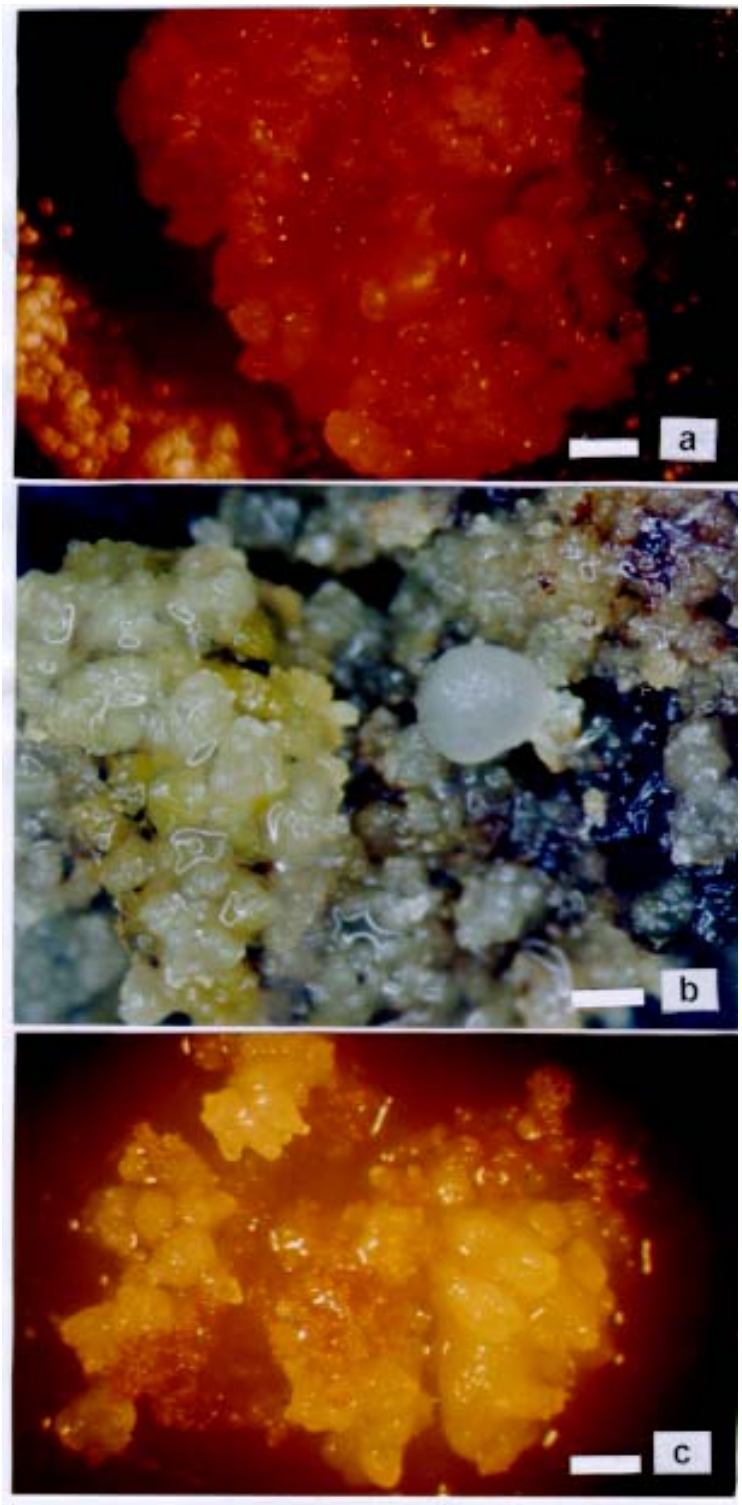


Figure 4.7

Table 4.5b: Media used for initial callus formation and callus maintenance, resulting in embryogenic mass formation from nucellar callus of cashew

Initial medium (values in μM)	Callus maintenance medium (values in μM)	Percent explants forming embryogenic mass	appearance of embryogenic mass, weeks after transfer to medium for maintenance
I 1 (2,4-D 5)	C 3 (2,4-D 5 + GA_3 15)	12.8	12 weeks
I 6 (2,4-D 5 + GA_3 15 + BA 5)	C 8 (2,4-D 10 + GA_3 15)	6.1	9 weeks
I 9 (2,4-D 5 + GA_3 15 + NAA 5)	C 4 (2,4-D 5 + GA_3 20)	6.9	20 weeks

{Basal medium for callus initiation (I 1, I 6, I 9) is MS + 3 % sucrose + 0.5% AC + 0.5% agar. Basal medium for callus maintenance (C 3, C 4, C 8) is MS + CH 0.05% + CW 10% + 4 % sucrose + 0.5% AC + 0.5% agar. Explants were obtained from nuts at 3-4 weeks post-fertilization stage. 800-900 explants were inoculated on each initiation medium, I 1, I 6 and I 9, from which, about 400-450 explants formed proliferating callus. On distribution, 40-50 explants with proliferating callus were transferred to C 3, C 8 and C 4 respectively. Out of these, 3-6 explants formed embryogenic callus}.

The granular masses formed on the three media were allowed to proliferate further on the same medium, medium with reduced 2,4-D content (5 and 1 μM), and medium without growth regulators. It was however, seen that the granular embryogenic callus could not differentiate into somatic embryos beyond the globular stage. The growth of granular callus was very slow on (i) medium without growth regulators, and (ii) medium with 1 μM 2,4-D. Very low concentration (1 μM) of 2,4-D, and absence of growth regulators did not help in differentiation of somatic embryos. These media were also unsuitable for continued proliferation of the embryogenic mass. Thus, a minimum concentration of 5 μM 2,4-D was necessary for maintaining the embryogenic mass.

During the next season, nucellar explants were inoculated only on the three initial media, namely I 1, I 6 and I 9, since callus formed on these media had formed embryogenic masses. The rapidly growing callus formed on I 1, I 6, and I 9 was transferred to callus maintenance media – C 3, C 8 and C 4 respectively. As

observed earlier, the primary callus formed embryogenic mass on the callus maintenance media. Explants forming embryogenic mass were distributed to media with 2,4-D (5 μM) and GA_3 (5-30 μM), along with CH 0.05%, CW 10%, sucrose 4%, AC 0.5% and agar 0.5%. Response of embryogenic mass was observed on these media.

It was seen that the callus initially formed on I 6 (medium with 2,4-D 5 μM + GA_3 15 μM + BA 5 μM), and maintained on C 8 (medium with 2,4-D 10 μM + GA_3 15 μM + CH 0.05% + CW 10%), differentiated into somatic embryos on medium with 2,4-D 5 μM + GA_3 30 μM + CH 0.05% + CW 10%. The observations are given in Table 4.6.

Table 4.6: Growth of granular proliferating mass formed from nucellar tissue of cashew

Initial medium (conc. in μM)	Medium of granular mass formation (conc. in μM)	Medium used for further growth of granular mass (conc. in μM)	Formation of somatic embryos **(se)** (Number of weeks after transfer, percent responding explants)
I 1 2,4-D 5	C 3 2,4-D 5 + GA_3 20	2,4-D 5 + GA_3 5 " + GA_3 10 " + GA_3 15 " + GA_3 20 " + GA_3 30	Embryos remained in globular stage
I 6 2,4-D 5 + GA_3 15 + BA 5	C 8 2,4-D 10 + GA_3 15	" + GA_3 5 " + GA_3 10 " + GA_3 15 " + GA_3 20	Callus remained granular, did not differentiate into somatic embryos
		" + GA_3 30	**se^1 (8 weeks, 0.3)**
I 9 2,4-D 5 + GA_3 15 + NAA 5	C 4 2,4-D 5 + GA_3 20	2,4-D 5 + GA_3 5 " + GA_3 10 " + GA_3 15 " + GA_3 20 " + GA_3 30	Callus remained granular, did not differentiate into somatic embryos

{se^1 : Somatic embryos appeared 8 weeks after transfer to medium with 2,4-D 5 μM + GA_3 30 μM, and showed normal development till cotyledonary stage. Basal medium is MS + CH 0.05% + CW 10% v/v + sucrose 4% + AC 0.5% + agar 0.5%. 900-1000 explants were inoculated on each initiation medium (I 1, I 6 and I 9). Out of these, about 400-500 explants formed proliferating callus, which were transferred to C 3, C 8 and C 4 respectively. Out of these, 25-40 explants formed embryogenic granular callus. Explants with embryogenic callus were distributed on the five media indicated in Table (5-8 explants per medium)}.

Granular mass formed on medium with 2,4-D 10 μM + GA_3 15 μM, CH 0.05% + CW 10% v/v + sucrose 4% + AC 0.5% + agar 0.5%, formed somatic embryos on further maintenance. Somatic embryos formed on medium with 2,4-D 5 μM + GA_3 30 μM, CH 0.05% + CW 10% v/v + sucrose 4% + AC 0.5% + agar

0.5% (Table 4.6). The somatic embryos showed normal development, and were used for further studies.

The granular mass formed on medium with 2,4-D 5 μM retained the granular appearance. However, the globular somatic embryos could not develop through the further stages of embryogenesis. The granular mass formed on C 4 medium also did not give rise to somatic embryos. The inability of this granular callus formed on C 4 medium to develop into somatic embryos may be due to initial exposure of nucellar callus to two auxins, 2,4-D and NAA, and further prolonged growth of callus in presence of auxin (2,4-D).

Thus, it was observed that callus formed on MS + 2,4-D 5 μM + GA_3 15 μM + BA 5 μM + sucrose 4%, AC 0.5%, and agar 0.5%, formed granular embryogenic mass when maintained on medium with 2,4-D 10 μM + GA_3 15 μM, CH 0.05%, CW 10% v/v, sucrose 4%, AC 0.5%, and agar 0.5%. The former medium was therefore chosen as the Callus Initiation Medium, and the latter as the Callus Maintenance Medium. The granular mass formed on Maintenance Medium gave rise to somatic embryos on medium with 2,4-D 5 μM, GA_3 30 μM, CH 0.05%, CW 10% v/v, sucrose 4%, AC 0.5%, and agar 0.5%. This medium was therefore selected as the embryo Expression Medium for further studies. The formation of somatic embryos from the embryogenic mass, and development of somatic embryos is discussed in the following section 4.3.5.

4.3.5. Formation of somatic embryos and observation of development of somatic embryos under microscope: The white granular proliferating mass formed on Maintenance Medium appeared embryogenic. For further growth, and differentiation of somatic embryos, this callus was transferred to different media with 2,4-D and GA_3, which are previously indicated in Table 4.6. Composition of these media are given in Table 4.7, along with response of embryogenic mass on each medium.

Table 4.7: Different media used for proliferation of white granular mass formed from nucellar callus of cashew

Growth regulators μM	Response of nucellar callus (after 7-8 weeks of transfer from Maintenance Medium) (percent explants)
2,4-D 5 + GA_3 5	proliferation
2,4-D 5 + GA_3 10	"
2,4-D 5 + GA_3 15	"
2,4-D 5 + GA_3 30	somatic embryos (0.3)

{Basal medium is MS + CH 0.05% + CW 10% + 4 % sucrose + 0.5% AC + 0.5% agar. 5-8 explants with granular embryogenic callus were used per medium. Percent explants showing somatic embryo formation calculated for explants initially inoculated (900-1000) on I 6}

The granular mass continued proliferating on the media with 2,4-D 5 μM + GA_3 (5, 10, 15, 30 μM). This proliferating mass did not show exudation of phenolics, or blackening, as was seen earlier during the initiation and maintenance steps. The white granular mass, when transferred to medium with 2,4-D 5 μM + GA_3 30 μM + CH 0.05% + CW 10% v/v, formed somatic embryos after 6-8 weeks. On other media, the granular mass did not differentiate to form somatic embryos.

From the initial callus formation stage till formation of somatic embryos, the concentration of 2,4-D in the medium played an important role in deciding the fate of the nucellar explant. At the stage of initial callus formation from nucellar tissue, it was observed that though more number of explants formed proliferating callus at higher (15 μM and above) concentrations of 2,4-D, such callus did not grow further, and could not be used for experiments.

Callus formed at low (5 μM) concentration of 2,4-D showed continued growth through 6-10 weeks of further maintenance, but did not become embryogenic.

Combination of 2,4-D and GA_3 was favorable for proliferation of nucellar tissue, and also for further growth, but the callus formed on this medium lost the granular appearance at later stages.

Addition of BA at low concentration (5 μM) to the initial medium with 2,4-D (5 μM) and GA_3 (15 μM) was necessary for triggering embryogenic development in the nucellar tissue. Probably BA affects the endogenous hormone balance in some way, and is required to 'switch on' the embryogenic pathway in cells of the nucellus. Removal of BA from the medium was essential for further growth of callus, as continued presence of BA resulted in very slow growth of callus (Table 4.4b). Omitting BA, and increasing the concentration of 2,4-D from 5 μM in Initiation Medium, to 10 μM in Maintenance Medium, was necessary for further growth and proliferation of nucellar callus. Increased concentration of 2,4-D was also necessary for production of granular embryogenic mass.

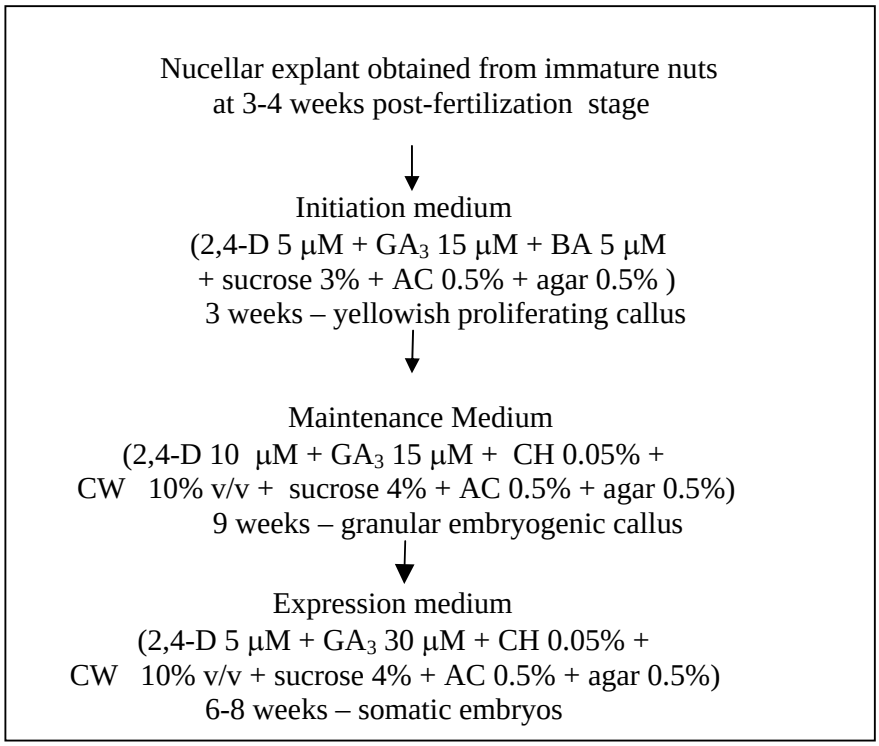
Continued growth of embryogenic callus in presence of 10 μM 2,4-D did not favor somatic embryo formation. For the granular embryogenic callus to form somatic embryos, it was again essential to reduce the auxin concentration, and increase the gibberellic acid concentration in the nutrient medium. The embryogenic callus formed in presence of 10 μM of 2,4-D and 15 μM of GA_3 gave rise to somatic embryos when it was transferred to medium with a lower concentration of 2,4-D (5 μM) and a higher concentration of GA_3 (30 μM).

A 3-week initial exposure to BA (5 μM) in Initiation Medium was necessary to trigger the embryogenic pathway in the nucellar tissue. Higher concentration of 2,4-D (10 μM) in Maintenance Medium was necessary for formation of embryogenic callus. Decrease in 2,4-D concentration (from 10, to 5 μM) and simultaneous increase in GA_3 (from 15, to 30 μM) concentration in Induction Medium was essential for the expression of somatic embryos from the embryogenic callus.

Thus, the balance of 2,4-D, GA_3 and BA in the nutrient medium affected the embryogenic response from the nucellar tissue. Any shift in the balance (with respect to concentration, and exposure period to the growth regulator) had adverse

effect on induction of embryogenesis from nucellar tissue. Induction of somatic embryogenesis from the nucellar tissue of cashew is summarized in the flowchart below.

Sequence of media used for induction of somatic embryogenesis from nucellar tissue of cashew



The details of the initiation medium, maintenance medium and induction medium used for obtaining somatic embryos are given in Table 4.8.

Table 4.8: Induction of somatic embryogenesis in nucellar callus of cashew: composition of Initiation, Maintenance and Induction media

Supplements	Initiation	Maintenance	Induction
BA (μM)	5	-	-
2,4-D (μM)	5	10	5
GA_3 (μM)	15	15	30
CH %	-	0.05	0.05
CW % (v/v)	-	10	10
Sucrose %	3	4	4
Response of nucellar tissue	Yellowish proliferating callus after 3 weeks	Formation of white granular embryogenic mass after 9 weeks	Appearance of somatic embryos after 7-8 weeks
Number of explants used / Number of explants showing response	1187/ 538	538/ 26	26/17

(Basal medium used was MS + AC 0.5% + agar 0.5%.)

Presence of auxin was found to be necessary for induction of embryogenic callus. Callus formed in absence of auxin (on medium with only GA_3) was never observed to be embryogenic even after long periods of subculture and maintenance. It has been reported that auxins, particularly 2,4-D, commonly act as inducers of embryogenesis (Tisserat *et al*., 1979; Ammirato, 1983).

Dependence on auxin, particularly 2,4-D, for embryo induction has also been observed in other systems using nucellar tissue. In mango, 2,4-D was used alone (Litz, 1984b; DeWald *et al*., 1989 a and b; Ara *et al*., 1999 and 2000), or with GA_3 (Jana *et al*., 1994). In grape, 2,4-D was used with BA (Gray and Mortensen, 1987), and in cacao, with 2iP (Figueira and Janick, 1993), for embryo induction.

In citrus, however, auxin was not at all necessary for embryogenesis from nucellar tissue. Auxins IAA, NAA and 2,4-D suppressed embryogenesis, whereas

inhibitors of auxin synthesis, and anti-auxin compounds like aza-indole favoured the formation of embryogenic callus (Kochba *et al*., 1977; Moore, 1985).

In the present study, somatic embryos were formed on medium with 2,4-D + GA_3, as is also reported in mango (Jana *et al*., 1994). However, in citrus, Daminozide, an inhibitor of GA_3 synthesis, is reported to have produced the largest number of somatic embryos (Gmitter and Moore, 1986; Moore, 1985). Cytokinins used alone and in combination with auxin never formed proliferating callus in this study. In citrus, however, BA alone, or with IAA, could stimulate embryoid formation from nucellar tissue (Rangan *et al*., 1968).

Somatic embryogenesis and the subsequent stages of development of the somatic embryos often require a particular sequence of different growth media formulations and culture conditions (Ammirato, 1983). In the present study, three different media were used sequentially to obtain somatic embryos from nucellar tissue.

To obtain somatic embryos from the nucellar tissue, separate media have been used serially at each stage (initiation, proliferation and embryo induction) also in mango (DeWald *et al*., 1989 a and b; Jana *et al*., 1994), cacao (Figueira and Janick, 1993), grape (Mullins and Srinivasan, 1976; Gray and Mortensen, 1987) and citrus (Perez *et al*., 1998; Carimi *et al*., 1998, to mention a few).

Development of somatic embryos

The development of the somatic embryos was studied with the help of stereomicroscope (Zeiss). The globular stage somatic embryos (Fig. 4.8b) soon developed into heart shaped embryos (Fig. 4.8c) on the same embryo induction medium. However, if the somatic embryos were retained on the embryo induction medium after heart stage of development, they reverted to callus.

Hence, globular stage somatic embryos were transferred to hormone-free MS basal medium for their further development. On media devoid of any growth regulators, somatic embryos showed normal development from globular to heart, torpedo (Fig. 4.8d, e), and early cotyledonary stages (Fig.4.9).

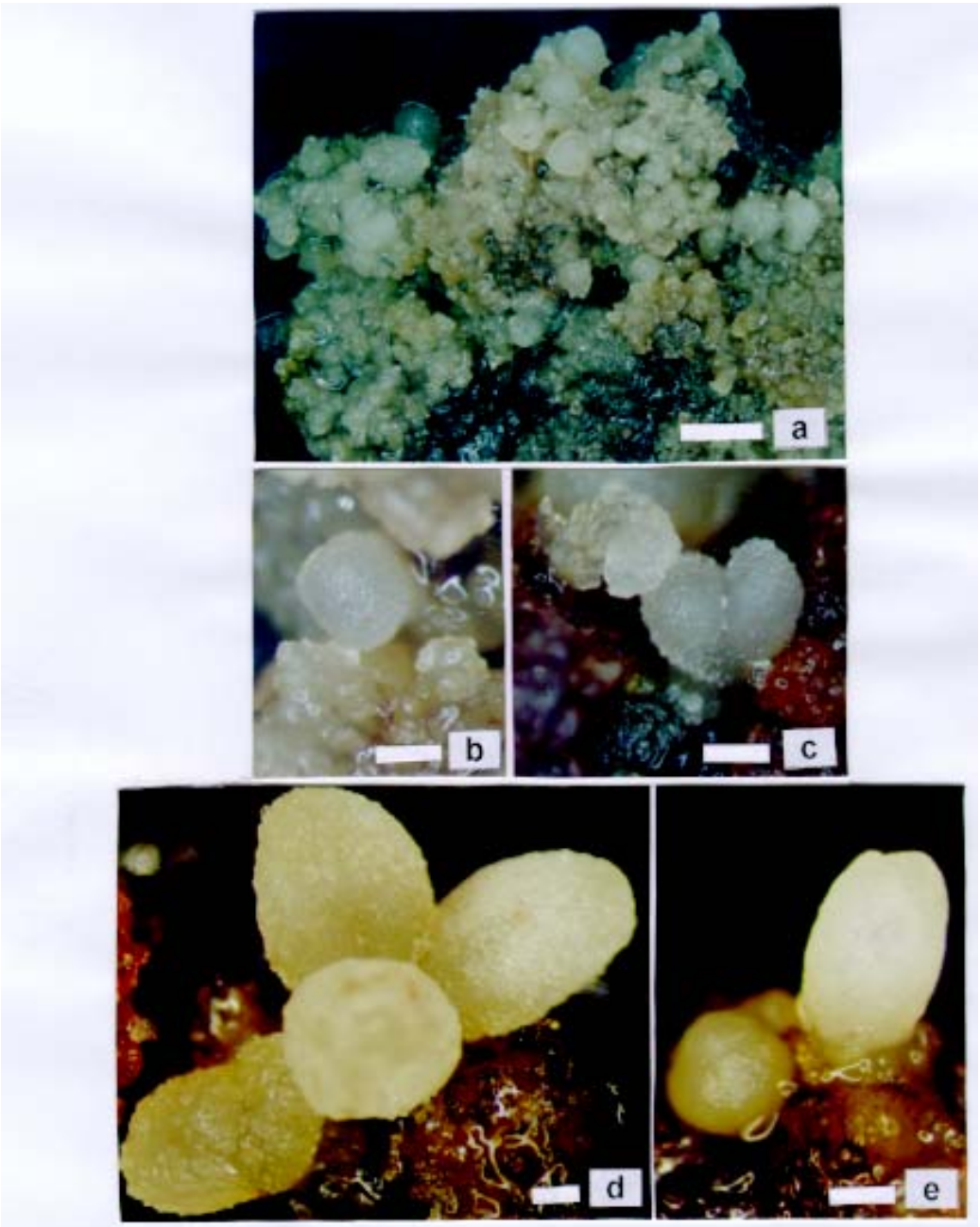


Fig. 4.8: Differentiation and development of somatic embryos from embryogenic callus

a: granular embryogenic mass formed on 2,4-D 10 + GA_3 15 μM, transferred to 2,4-D 5 + GA_3 30 μM, differentiated into somatic embryos; b: globular stage somatic embryo; c: heart stage somatic embryo; d, e: torpedo stage somatic embryo (a: bar= 6 mm; b-e: bar= 1 mm)

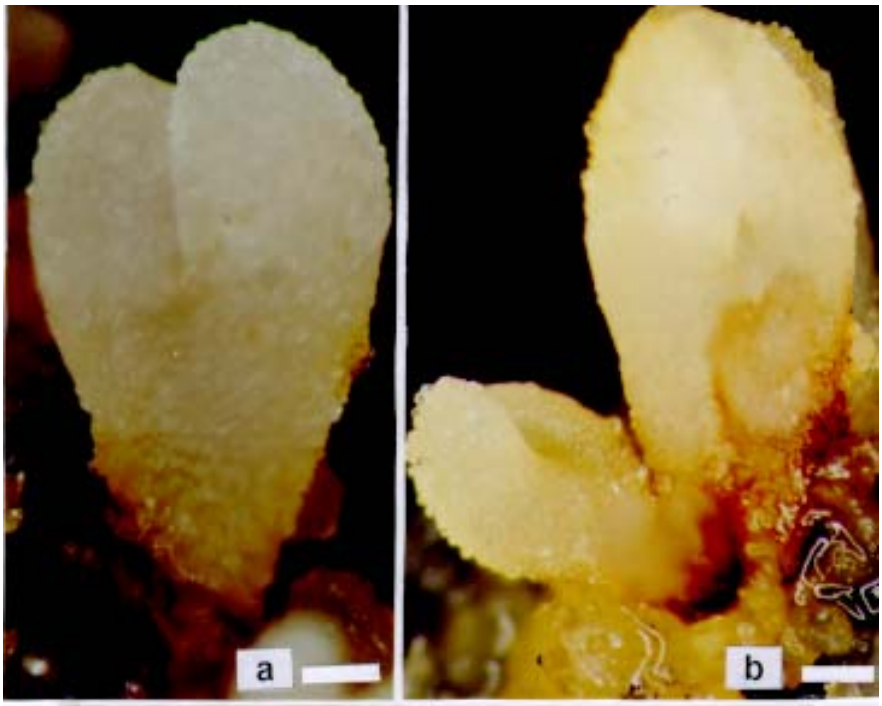


Fig. 4.9: a, b, c, d: Cotyledonary stage somatic embryos (a-d: bar= 2 mm)

Development of somatic embryos was not synchronous (Fig. 4.10a, b). Embryos in different developmental stages could be observed simultaneously in the same culture. Few somatic embryos from the embryogenic cluster grew more rapidly and probably suppressed the growth of the other embryos. In the

cotyledonary stage somatic embryos, the cotyledons were much reduced in size (3-7 mm), as compared to cotyledons in the zygotic embryo (more than 1.5 cm in length at the late cotyledonary stage).

In some somatic embryos, secondary somatic embryos were seen to be arising from the tip of the radicle of the primary somatic embryo (Fig. 4.10c, d). These secondary embryos were much smaller in size than the primary embryo. Some abnormalities were observed during development of somatic embryos. Hypertrophy of globular somatic embryos, fasciation, more than two cotyledons, asymmetric cotyledon development, were some of the abnormalities (Fig. 4.11a, b, c). In some embryos, there was total absence of the apical dome though the root pole and cotyledons appeared normal. Some somatic embryos became hyperhydric in culture. These hyperhydric embryos did not develop further, and often became necrotic. Similar abnormalities in the morphology of somatic embryos have been observed in mango (Litz *et al*., 1995; Jana *et al*., 1994), citrus (Rangan, 1984), grape (Mullins and Srinivasan, 1976), and many other woody species.

4.3.6. Maturation and conversion studies of somatic embryos: Medium devoid of growth regulators was suitable for the normal development of the embryos. To observe the effect of different maturation agents (ABA, maltose, PEG, gelrite at 0.4%), the somatic embryos were also transferred to different maturation media, as indicated in Table 4.9.

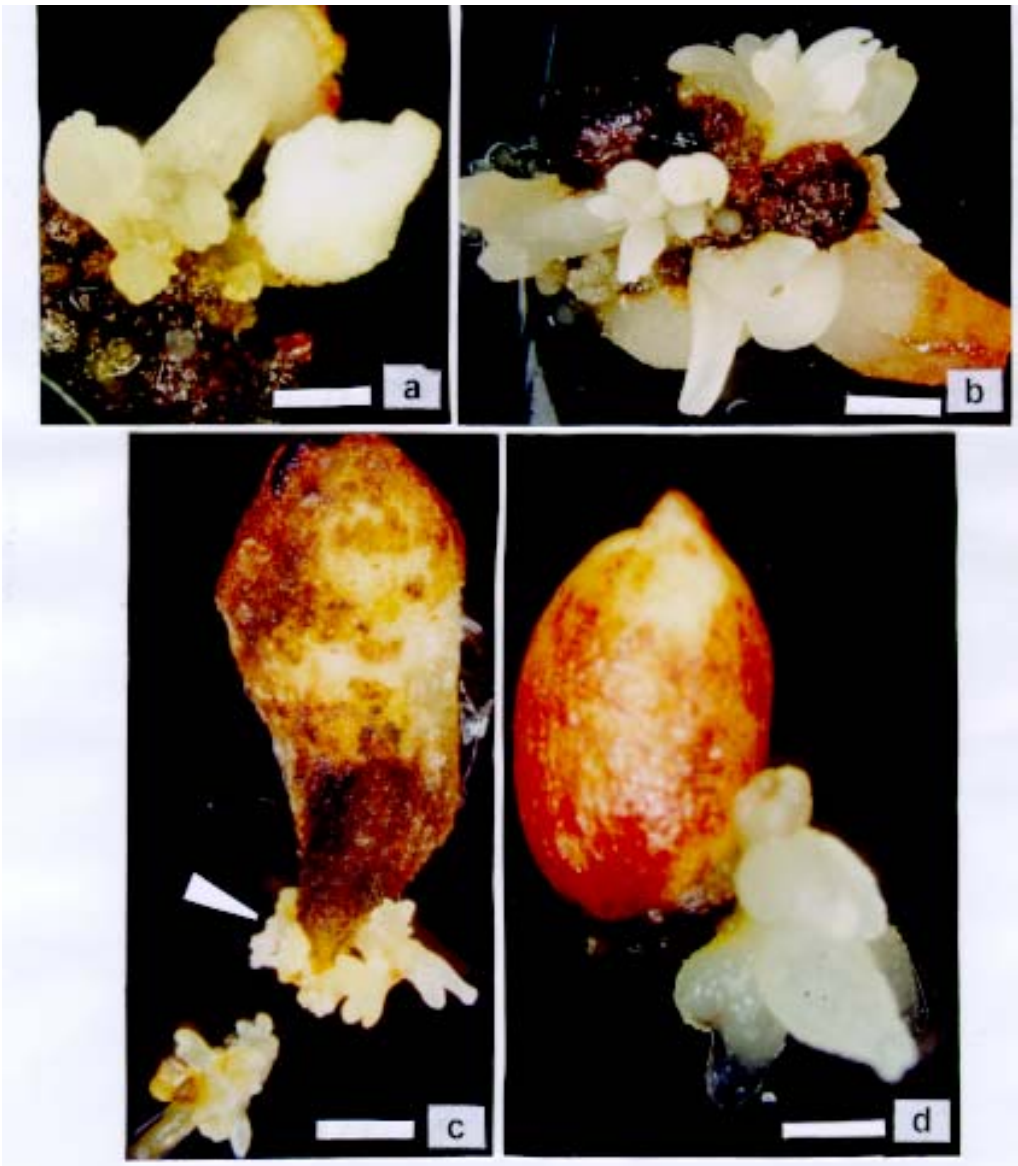


Fig. 4.10: a, b: asynchronous development of somatic embryos; c, d: secondary somatic embryos (arrowhead) arising from radicle tip of primary somatic embryos (a-d: bar= 3 mm)

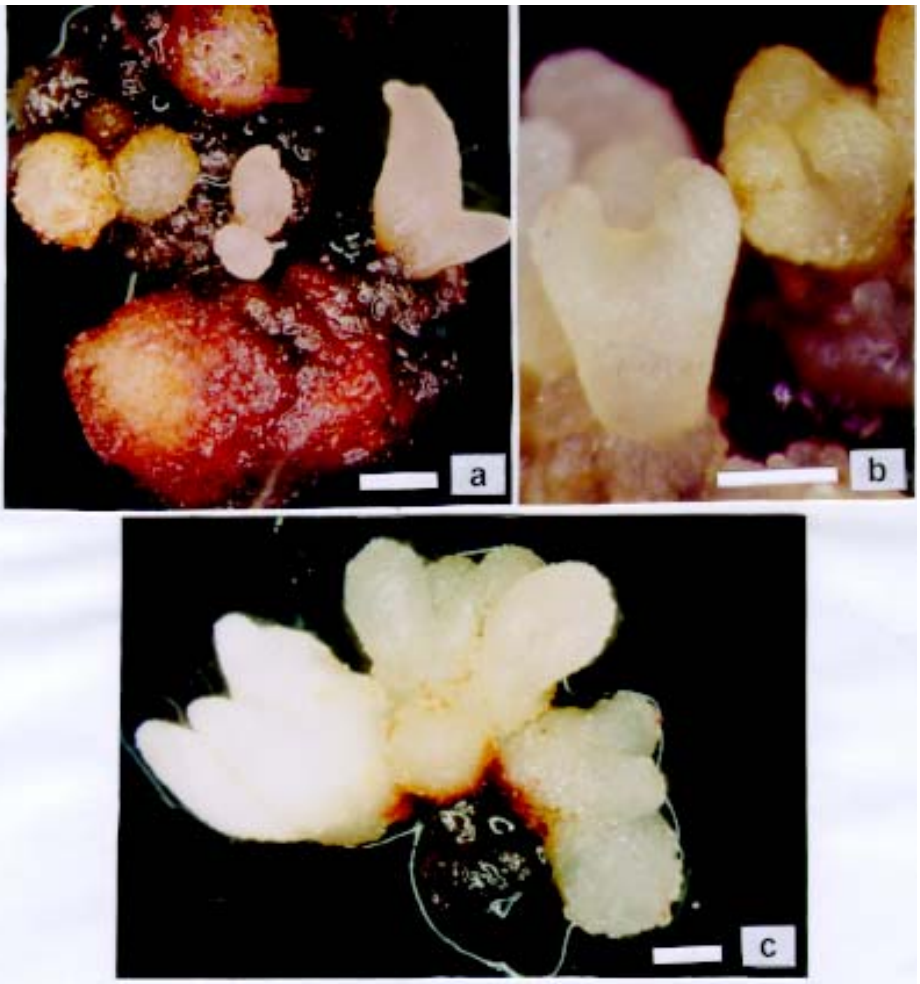


Fig. 4.11: Abnormalities observed in somatic embryos

a: hypertrophied globular stage embryos; b: absence of apical dome, more than two cotyledons; c: fused somatic embryos, with more than two cotyledons each (a-c: bar= 2 mm)

Table 4.9: Response of somatic embryos obtained from nucellar callus of cashew to different maturation agents in the media

Maturation agents used in the medium	Response of somatic embryos
ABA 2, 5, 10, 20 and 30 μM	Callusing of entire embryos, blackening and necrosis on further incubation
PEG 5 and 10%	Enlargement and blackening of embryos
Maltose 4, 6, 8%	Root development at 6 and 8%
BA 5 μM or Kin 5 μM	No enhancement in shoot axis development
ABA (1 and 2 μM) + PEG (5 and 10%)	Blackening and necrosis
ABA (1 and 2 μM) + maltose (6 and 8%)	Root development, only at ABA 1 μM + maltose 8%
Maltose (6 and 8%) + PEG (2 and 4%)	Enlargement of embryos, browning on further culture
AC 0.5%	Normal development to cotyledonary stage
AC 0.5% + gelrite 0.4%	"
AC 0.25% + gelrite 0.4%	"
Sucrose 6,8, and 10%	Development to cotyledonary stage at 6%, browning of embryos at 8 and 10% on further maintenance
CH 0.025% and CW 5% v/v (separately and in combination)	Normal development of embryos till cotyledonary stage, no enhancement of growth

(Basal medium used is MS + sucrose 3% + gelrite 0.2%.)

On media containing ABA 1 μM + maltose 8%, root development was observed in somatic embryos (Fig. 4.12b). But the shoot poles did not develop. In media containing only ABA, enlargement and callusing of the somatic embryos

was observed at all ABA concentrations (Fig. 4.12a). Somatic embryos showed gradual blackening and necrosis on all media containing ABA as maturation agent. Ananthakrishnan and co-workers (1999) also observed that ABA led to callusing of somatic embryos of cashew. Cardoza and D'Souza (2002) have used 3 μM ABA for somatic embryo maturation. However, successful conversion of these embryos has not been reported.

Polyethylene glycol (PEG) also proved to be unsuitable as a maturation agent (Table 4.7). In presence of PEG, either alone, with ABA, or with maltose, somatic embryos enlarged without showing any further development. On further exposure to PEG, the embryos started blackening.

In attempts to improve the growth of somatic embryos, CW (5%) and CH (0.025%) were added to the medium not containing growth regulators. These supplements did not have any beneficial effect on the development of somatic embryos.

When somatic embryos were grown on medium not containing growth regulators, but containing AC, development of root and shoot poles was observed. The somatic embryos developed normally, without showing hypertrophy, callusing, blackening and necrosis, as observed in other maturation media (Table 4.7). Thus, medium devoid of growth regulators, and containing AC was favorable for normal growth and development of somatic embryos in the present study. Increased (0.4%) concentration of gelrite was superior to ABA, PEG, sucrose, and maltose for normal embryo development. However, development of the cotyledons remained rudimentary on this medium (Fig.4.12c, d). Development of cotyledons of the somatic embryos seems to be crucial for better maturation of somatic embryos. Growth of somatic embryos on these media was similar to that obtained on media with AC.

In mature zygotic embryo of cashew, the embryo axis is very much reduced in size (0.4-0.6 cm length) as compared to the cotyledons (2-2.5 cm in length) (Refer Fig. 3.2e), which form the major bulk of the seed. It is evident, that during zygotic embryogenesis in cashew, accumulation of storage products, associated with the development of cotyledons, is the most important and prolonged phase.

As mentioned in Chapter 3, mature zygotic embryo axis can develop into plantlet only in presence of cotyledons. When the cotyledons were detached, the embryo axis could not germinate, and failed to develop into a plantlet. Thus, cotyledons play a key role in the development of the embryo axis during germination. In the absence of cotyledons, germination did not occur, even in mature embryo axes. There was complete absence of development of the plumule in the mature zygotic embryo, and plantlet could not be obtained. This indicates that the cotyledons are essential for the embryo axis to develop into plantlet (conversion).

In large seeded species, sequential events during maturation of zygotic embryo are very complex, and not completely understood. This poses difficulties for bringing about normal development of somatic embryos *in vitro* in such species (Wang and Janick, 1984). In the present system, poor development of cotyledons may be an important factor affecting the subsequent maturation and conversion of somatic embryos.

Considering the importance and indispensability of cotyledons for germination and initial growth of mature zygotic embryos, proper growth and complete development of the cotyledons of the somatic embryos would certainly help in better maturation and conversion rates.

In both mango and citrus, the nucellus is an explant with a strong embryogenic potential, evident from the numerous reports on embryogenesis using this tissue in these species (Table 4.1). In polyembryonic varities of mango and citrus, simply the removal of nucellus from the influence of zygotic embryos initiates the embryogenic pathway, without need for any additional media supplements. In these cases, the nucellus is already programmed for embryogeny, therefore, growth and development of nucellar embryos occur rapidly.

In comparison, nucellar tissue of cashew has proved to be a recalcitrant explant. The nucellar cells appear to be embryogenically non-determined, in contrast to nucellar tissue of mango and citrus.

In cashew, so far there are only three reports of somatic embryogenesis from nucellus explant (Anathakrishnan *et al*., 1999; Gogte and Nadgauda, 2000;

Cardoza and D'Silva, 2002). In all the three studies, there have been difficulties in normal development of somatic embryos. Plantlet recovery has not been reported in any of the studies (Anathakrishnan *et al*., 1999; Cardoza and D'Silva, 2002). This may be because of recalcitrancy even of the juvenile tissue like nucellus, in this species.

Though progress achieved in cashew may seem slow as compared to other woody species, it is significant. The increased number of studies in cashew since the past decade (Table 1.6 a, b and c), indicate that the tissue is not completely recalcitrant. The advent of modern technology and discovery of novel chemicals would open new possibilities of experimenting. More concerted efforts, use of better chemicals and growth regulators, and increased understanding of the tissue physiology would certainly be of much use in overcoming the difficulties faced at present.

A complete and reliable regeneration system would prove immensely valuable for variety improvement programs for a woody nut tree like cashew, where long generation cycles, lengthy juvenile phase and early fruit drop are some of the major hurdles in conventional methods of improvement. Such a regeneration system would also be useful for cryopreservation of the long-term embryogenic callus, which is invaluable for germplasm conservation and maintaining the diversity within the species.

4.3.7. Histological studies: Histological sections of embryogenic callus and somatic embryos were observed under light microscope (Zeiss). Somatic embryos were formed in the peripheral or superficial layer of the embryogenic callus (Fig. 4.13b). The embryogenic cells in the callus showed prominent nuclei and densely stained cytoplasm. Globular embryos developed singly or in clusters (Fig. 4.13b) from the superficial cells of the callus. Torpedo (Fig. 4.13c) and cotyledonary stages (Fig. 4.13d) of development were also observed in the sections. In sections of cotyledonary stage somatic embryos, formation of vascular structures could not be seen in the apical region. This observation corroborated with the morphological appearance of

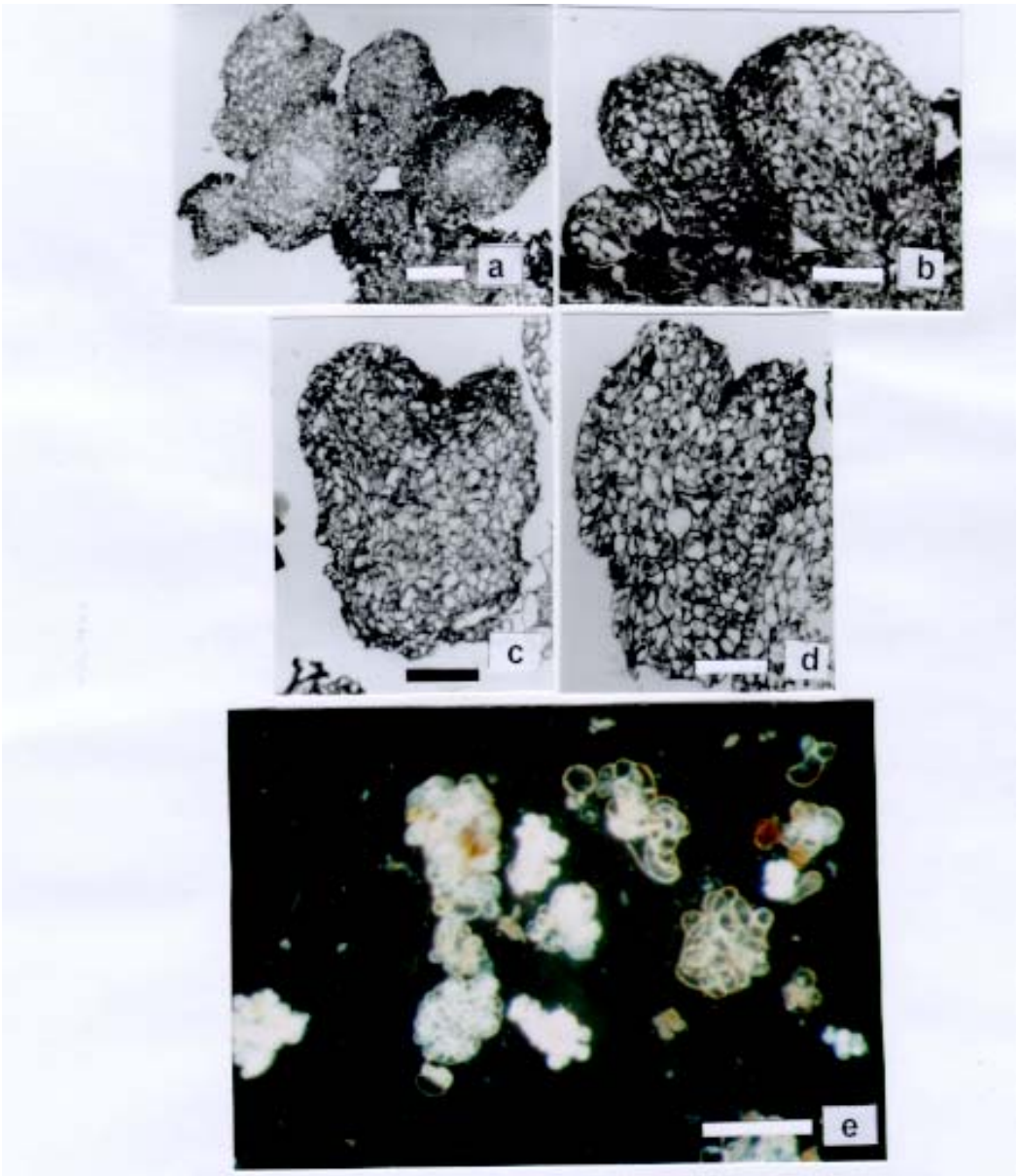


Fig. 4.13: Histological studies of somatic embryos formed from nucellar callus

a: mass of somatic embryos; b: globular somatic embryos; c: torpedo stage somatic embryos; d: early cotyledonary stage somatic embryo; e: suspension culture of embryogenic callus; (a-d: bar= 0.1 mm; e: bar= 3 mm)

somatic embryos, in which apical dome could not be seen on examination under microscope.

4.3.8. Establishment of cell suspension culture: The nucellar callus on semi-solid maintenance medium was also transferred to liquid medium for maintenance and proliferation. Experiments during initiation and maintenance of callus had indicated that low (5 μM) concentrations of 2,4-D were suitable for proliferation of this tissue. Therefore, liquid medium containing 2,4-D (5 μM) as the only phytohormone, was used for proliferation. Coconut water and CH did not have any promoting effect on growth of cell suspension.

Proliferating callus on maintenance medium grew very rapidly in the liquid medium (Fig.4.13e), after an initial lag phase of about three weeks. However, differentiation of somatic embryos did not occur in liquid medium. Semi-solid medium was better for growth and development of somatic embryos.

The embryogenic callus is being maintained on semi-solid medium containing 2.5 - 5 μM 2,4-D using gelrite as the gelling agent. The embryogenic callus initiated in 1997 and 1998 is also being maintained and it has been observed that this callus has retained its embryogenic potential, as evident from appearance of globular somatic embryos on callus surface. Auxin at minimum concentration seems to be necessary for continuous division and growth of the callus cells. Embryogenic callus system, on semi-solid medium, and as suspension, has implications for protoplast formation and regeneration systems, which can be extrapolated for genetic transformation experiments. The ability of the nucellar callus to maintain its embryogenic potential for such long periods has implications for cryopreservation, and for long-term conservation of germplasm. The technique would be very useful after an efficient regeneration system via somatic embryogenesis is established. In citrus, the nucellar callus has been used for protoplast generation and plantlet formation (Kobayashi *et al*., 1985) and also for cryopreservation (Perez *et al*., 1999).

4.4: Summary

The nucellar tissue of cashew has embryogenic potential. Nucellar tissue obtained from 3-4 weeks post-fertilization stage of nut development formed a proliferating yellowish callus on callus Initiation medium (MS + 2,4-D 5 μM + GA_3 15 μM + BA 5 μM + sucrose 3% + AC 0.5% + agar 0.5%) in 3 weeks. This callus turned completely black on Maintenance Medium (MS + 2,4-D 10 μM + GA_3 15 μM + CH 0.05% + CW 10 % + sucrose 4% + AC 0.5% + agar 0.5%). The blackened callus formed a white granular proliferating mass after 9 weeks. The granular mass was transferred to embryo Induction Medium (MS + 2,4-D 5 μM + GA_3 30 μM + CH 0.05% + CW 10 % + sucrose 4% + AC 0.5% + agar 0.5%). On this medium, somatic embryos were formed after 7-8 weeks. The somatic embryos developed till cotyledonary stage on MS medium with AC 0.5%. The nucellar callus is being maintained on semi-solid and liquid MS medium with 2,4-D 5 μM + CH 0.05% + sucrose 3% with/ without agar 0.5 %.

4.5: Conclusions

- Nucellar tissue of cashew has embryogenic potential
- Nucellar explants from 3-4 week post fertilization stage of immature nuts were most suited for induction of somatic embryogenesis
- Induction of embryogenesis was dependent on the balance of growth regulators in the medium
- BA was essential during callus initiation. Increased concentration of 2,4-D was necessary for formation of embryogenic callus. Lower concentration of 2,4-D and higher concentration of GA_3 was favorable for somatic embryo formatio.
- Three different media (Initiation, Maintenance and Induction Media), were used sequentially for induction of embryogenic callus. Minimum time required from initiation of callus to formation of globular somatic embryos was 18 weeks
- Embryogenic callus has been amenable to long-term maintenance and proliferation in suspension culture and on semi-solid medium

Part of work in chapter 4 has been published as follows:

Gogte S and Nadgauda R (2000) Induction of somatic embryogenesis in cashewnut (*Anacardium occidentale* L.). *In Vitro Cell Dev. Biol.* 36: 41-46.

Nadgauda R, Mathur G, Gogte S (2000) Somatic embryogenesis research on fruit trees in India. In: SM Jain, PK Gupta, RJ Newton (Eds) *Somatic Embryogenesis in Woody Plants Vol 6*, Kluwer Academic, The Netherlands, pp 193-213.

Nadgauda RS and Gogate SS (2003) *In vitro* regeneration and genetic transformation of cashewnut (*Anacardium occidentale* L.). In: PK Jaiwal and RP Singh (Eds*) Plant Genetic Engineering Vol 6: Improvement of Fruit Crops*, Sci-Tech Publishers, U.S. A., pp 41-64.

CHAPTER 5

DIRECT SOMATIC EMBRYOGENESIS FROM IMMATURE ZYGOTIC EMBRYO

CHAPTER 5
DIRECT SOMATIC EMBRYOGENESIS FROM IMMATURE ZYGOTIC EMBRYO

In woody tree species, obtaining regeneration from mature explants is difficult due to recalcitrancy of these tissues. Merkle and co-workers (1998) have stated that as woody trees mature their tissues become increasingly refractory and do not respond to treatments designed for induction of morphogenetic response. In such woody species, it becomes necessary to utilize juvenile tissues, like zygotic embryos, for developing a regeneration system. The embryogenic potential of zygotic embryos has been exploited to a great extent in various woody angiosperms. Regeneration procedures developed through somatic embryogenesis, using immature zygotic embryos as explant are summarized in Table 5.1.

Variety improvement in cashew is achieved mainly through conventional breeding practices (Table 1.4, Chapter 1). Prolonged juvenile phase, lesser number of hermaphrodite flowers, low fruit set, and premature fruit fall (Pillai and Pillai 1975, Nawale *et al*., 1984; Pattnaik *et al*., 1985), hinder the success of breeding programs in cashew. Efforts to propagate the high-yielding improved varieties by vegetative propagation are inadequate in meeting the increasing demand for elite planting material.

In view of these considerations, the zygotic embryo was selected for experiments. A regeneration system using zygotic embryo as explant would be valuable for multiplying superior hybrid varieties, and would give a boost to the ongoing strategies of variety improvement. Additionally, such a system would be an invaluable asset for germplasm conservation. Further, observations on *in vitro* growth response of the zygotic embryos would be helpful in designing suitable medium for maturation and conversion of somatic embryos of nucellar origin.

Table 5.1: Somatic embryogenesis in woody and fruit tree species, using immature zygotic embryo as explant

Species	Basal medium	Growth regulators used for induction and proliferation	Growth regulators used for germination	Reference
Acacia arabica	MS	2,4-D + BA	BA + ABA	Nanda & Rout, 2003
Acacia catechu	WPM	Kin + NAA	-	Rout *et al*., 1995
Acacia mangium	MS	TDZ + IAA; GA_3	-	Xie & Hong, 2001
Acer palmatum	MS	2,4-D + BA; BA + NAA	-	Vlasinova & Havel, 1999
Aesculus hippocastanum	MS	2,4-D + Kin; 2,4-D	GA_3+ IAA	Radojevic, 1988
	MS	BA + NAA	-	Kiss *et al*., 1992
Albizzia julibrissin	MS	2,4-D	-	Burns & Wetzstein, 1998
Carya illinoensis	WPM	2,4-D + BA + CH	-	Merkle *et al*., 1987
	do	do	IBA	Wetzstein *et al*., 1989
	DS	2,4-D + Ad; BA + Pic	-	Corte-Olivares, 1990
	TM	BA + Kin + IBA/ IBA; basal	4ºC dark 2 months, $AgNO_3$ + BA	Yates & Reilly, 1990
	WPM	BA+NAA; basal		Mathews & Wetzstein, 1993
Cercis canadensis	SH	2,4-D	-	Trigiano *et al*., 1988
	WPM	do	-	Geneve & Kester, 1990
Coryllus avellana	MS	2,4-D + Kin; BA + IBA	BA + Kin + IBA	Berros *et al*., 1995
Dalbergia sissoo	MS	2,4-D + Kin	ABA	Das *et al*., 1997
Elm	MS	BA	Not reported	Corredoira *et al*., 2002
Feijoa sellowiana	LP	Gln	BA	Dal Vesco & Guerra, 2001
	MS	2,4-D + Kin	GA_3+ Kin	Cruz *et al*., 1990
Hardwickia binata	MS	2,4-D	ABA/ BA + NAA	Chand & Singh, 2001

continued……

Table 5.1 continued…..

Species	Basal medium	Growth regulators used for induction and proliferation	Growth regulators used for germination	Reference
Juglans cinerea	DKW	BA + Kin + IBA; BA/2,4-D	-	Pijut, 1993
Juglans regia	DKW (mod)	BA + Kin + IBA; basal	2-4ºC 8-10 weeks	Tulecke & McGranahan, 1985
Juglans (hybrid)	TM	-	-	Cornu, 1988
Juglans (intergeneric hybrid)	TM	-	-	McGranahan *et al*., 1989
Liquidambar	BBMG	2,4-D + BA + CH; 2,4-D; AC	-	Merkle *et al*., 1998
Liquidambar (hybrid)	do	2,4-D + CH; WPM + 2,4- D + CH	-	Vendrame *et al*., 2001
Liriodendron tulipifera	BBMG	2,4-D + BA + CH; basal	-	Merkle & Sommer, 1986
Liriodendron (hybrid)	MS	2,4-D + BA + CH; 2,4-D + BA	-	Merkle *et al*., 1993
Magnolia species	BBMG	2,4-D + BA + CH; 2,4-D	-	Merkle & Weicko, 1990
Myrtus communis	MS	2,4-D/ 2,4-D + BA/TDZ	-	Parra & Amo-Marco, 1998
Olea europaea	OM	BA	-	Rugini, 1988
Persea americana	MS	Pic	-	Mooney & van Staden, 1987
	MS	do	-	Pliego-Alfaro & Murashige, 1988
	B5 MS	do	BA; IBA	Witjaksono & Litz, 1999a & b
Prunus avium	MS + Morel Vit	2,4-D + Kin	IAA; Zea	DeMarch *et al*., 1993
	do	BA + Kin + NAA + CH + Gln	ABA + Mal; WPM, 4ºC (2 months)	Reidiboym *et al*., 1998
Prunus species	MS	2,4-D + BA + Kin + CH; basal	BA	RajBhansali *et al*., 1990

continued……

Table 5.1 continued……

Species	Basal medium	Growth regulators used for induction and proliferation	Growth regulators used for germination	Reference
Quercus acutissima	MS MS	BA + IBA/ IBA; basal BA+ IBA; basal	BA BA	Kim *et al*., 1994 Kim *et al*., 1997
Quercus robur	WPM	BA/ BA+ GA_3/ IBA	BA	Chalupa, 1990
Quercus suber	MS	2,4-D + BA/ basal	-	Gingas & Lineberger, 1989 Bueno *et al*., 1992
Robinia pseudoacacia	FMG	2,4-D+BA; 2,4-D	-	Arrillaga *et al*., 1994
Simmondsia chinensis	MS MS	2,4-D + BA + NAA; 2,4-D	- -	Lee & Thomas, 1985 Wang & Janick, 1986
Theobroma cacao	MS	NAA + CW	-	Pence *et al*., 1979
Tilia cordata	MS	2,4-D	IBA	Chalupa, 1990
Vitis vinifera *Vitis rotundifolia*	ER liq N6	Basal; WPM + BA + AC NOA + BA; MS + AC	- MS + BA	Emershad & Ramming, 1994 Gray, 1992

(BBMG: modified Blayde's major + Brown's minor salts + MS iron + Gresshof & Doy vitamins; DKW: Driver Kuniyuki Walnut; DS: Dunstan & Short; ER: Emershad-Ramming; FMG: Finer & Nagasawa macro + MS micro salts + Gresshof & Doy vitamins; Gln: glutamine; liq: liquid; Mal: maltose; N6: Nitsch; NOA: naphthoxy acetic acid; OM: Olive Medium; Pic: picloram; TM: Tulecke McGranahan)

The present chapter describes studies conducted on *in vitro* response of immature and mature zygotic embryos of cashew.

In initial pilot experiments, the immature and mature zygotic embryos were used as intact explants (embryo axis with both cotyledons attached). Intact mature and immature zygotic embryos were inoculated on MS medium containing auxin 2,4-D 5 μM, and sucrose 3% (auxin was used since it has been frequently used for induction of somatic embryogenesis in many species) (Tisserat, 1980).

It was observed that the intact mature zygotic embryo showed only germination in auxin containing medium. No other response was observed, even

after prolonged culture period. Due to absence of any morphogenetic response from whole mature zygotic embryos, it was decided to use this explant as isolated embryo axes. The isolated mature embryo axis showed callus formation in presence of 2,4-D. Therefore, for all the experiments, isolated mature embryo axes were used as explants, instead of whole mature zygotic embryos. The immature zygotic embryos were used intact, and as excised parts.

Results of experiments proved that immature zygotic embryos of cashew have embryogenic potential. Further, the embryogenic response is dependent on the developmental stage of the immature zygotic embryos, and growth regulators present in the nutrient medium.

Immature zygotic embryos at 3 to 5 weeks post-fertilization stage formed somatic embryos, when inoculated on MS medium with 2,4-D (5 μM) + BA (5 μM) + GA_3 (3 μM) + sucrose 3% + AC 0.3% + agar 0.5%. Somatic embryos were formed directly from the tip of the radicle of immature zygotic embryos. This system can be used for propagation of hybrid varieties, and rootstocks. Further, as the embryogenesis is via direct pathway, the system also has implications for genetic manipulations.

Hereafter, throughout this chapter, the terms ‘immature zygotic embryos’ and ‘mature zygotic embryos’ are indicated by the abbreviations IZE/ IZEs and MZE/ MZEs respectively. The experiments and the results obtained are discussed below in different sections, as follows:

5.1. Experimental: collection of plant material, surface sterilization; **5.2. Culture conditions:** Different parameters were considered for study (Developmental stage of explant, growth regulators, photoperiod. Embryogenic potential of excised parts of IZEs was also examined. Maturation and conversion studies for somatic embryos obtained from IZEs were conducted. Culture conditions for each parameter are mentioned separately; **5.3: Results and discussion**: The results obtained for each parameter are discussed separately; **5.4:** Summary; **5.5:** Conclusions

5.1: Experimental

Collection of plant material: The immature and mature nuts of cashew (variety Vengurla 1) were obtained from the Balasaheb Sawant College of Agriculture, Dapoli, Maharashtra. The nuts were collected from 8-15 year old open pollinated field grown trees.

Collection of IZEs: Immature nuts were collected every week through January-March from second through eighth week post fertilization. The immature nuts were brought to the laboratory. They were processed for *in vitro* culture as soon as possible, to minimize wastage of plant material due to deterioration.

Collection of MZEAs: Mature nuts were dusted with the fungicide Bavistin®. They were stored in polythene bags at 4-8°C and used as required.

Surface sterilization of:

IZEs: The immature nuts were washed thoroughly under running tap water, followed by wash with laboratory detergent (1% Labolene®, Qualigens, India) for 20 minutes. They were surface sterilized with 0.1% $HgCl_2$ for 20 minutes, and rinsed 3-4 times with sterile distilled water in laminar airflow cabinet.

MZEAs: The mature nuts were surface sterilized as described previously in Chapter 3, Section 3.1. These nuts were soaked in sterile distilled water for 72 hours. Then they were dissected (description in Chapter 3, Section 3.2.1) to obtain entire decoated seeds.

5.2: Culture conditions

IZEs: The immature nuts were cut open in laminar airflow cabinet. The ovules were taken out, and seedcoat was discarded. Ovules were bisected longitudinally. IZEs were gently separated from the ovular halves. If the IZEs were not easily visible during bisectioning, IZEs were cultured along with ovular halves. After

incubation period of 3-4 days, IZEs enlarged, and were easily visible. They were then picked out and cultured separately.

MZEAs: The cotyledons were excised from the entire seeds to obtain isolated mature embryo axes (MZEAs). The isolated mature embryo axes were used for experiments.

The IZEs and MZEAs were placed in disposable petridishes (55 mm x 15 mm or 85 mm x 15 mm, Laxbro, India) on semi-solid full-strength MS basal medium containing sucrose 3%, with and without activated charcoal 0.5%, supplemented with different growth regulators. It has been discussed in Chapters 3 and 4, that MS medium was most suitable for *in vitro* culture of seedling and nucellar explants, respectively. During these experiments, mature zygotic embryos (used for germination study), and the immature zygotic embryos, (obtained during dissection for nucellar tissue), were also cultured on MS medium. It was observed that MS medium was suitable for growth of mature and immature zygotic embryos. Therefore, MS basal medium was used for all experiments using these explants, and separate standardization of basal medium was not carried out.

Medium was gelled using 0.5% agar (Qualigens, India). All growth regulators were added to nutrient media before autoclaving, except ABA, which was added after autoclaving as a filter sterilized solution. The pH of all media was adjusted to 5.8 with 0.1 N NaOH/ HCl. The media was sterilized by autoclaving at 121°C and 1.1 kg/cm^2 pressure for 20 minutes. All cultures were incubated at 25±2°C in the dark. For observing the effect of photoperiod, the cultures were incubated at 25±2°C in 16-hour photoperiod.

For all experiments with IZEs, 40 to 70 explants, and for experiments with MZEAs, 25 to 30 explants were used per treatment. Each treatment was repeated thrice. In case of MZEAs, because of difficulties involved in dissecting the explants from the mature nut (Chapter 3, section 3.1), low number of explants were used.

Parameters studied for induction of embryogenic response were developmental stage of explant (only for IZE), growth regulators and

photoperiod. Embryogenic potential of excised portions of IZEs was examined. Maturation and conversion studies of somatic embryos obtained from IZEs were carried out. The culture conditions for each of these factors are mentioned under separate headings.

5.2.1. Effect of developmental stage of IZE on embryogenic response: In most of the studies where somatic embryos have originated from IZEs, it has been observed that the developmental stage of IZE was a crucial factor for induction of embryogenic response. Therefore, in the present study, effect of developmental stage of IZE on embryogenic response was investigated. IZEs were obtained at different developmental stages (from 2 through 8 weeks post-fertilization). Intact whole IZEs were inoculated on MS medium + sucrose 3% + AC 0.3% + agar 0.5%. The medium was supplemented with auxin 2,4-D (5 μM), which has been widely used for inducing somatic embryogenesis. The response of IZEs at the different developmental stages was observed. As only IZEs from 3 to 5 weeks post-fertilization stage gave rise to somatic embryos directly, after 5-7 weeks of incubation, IZEs at this stage were used to study the other parameters.

5.2.2. Effect of growth regulators and organic supplements on embryogenic response from IZEs and MZEAs: To study how growth regulators affect induction of embryogenesis, IZEs at 3-5 weeks post-fertilization stage were used. The IZEs and MZEAs were exposed to different growth regulators added to nutrient medium. The growth regulators used were 2,4-D (5 - 50 μM), 2,4,5-T (5 - 50 μM), NAA (5 - 50 μM), IAA (5 - 50 μM), IBA (5 - 50 μM), cytokinins BA (5 - 50 μM), Kin (5 - 50 μM), Zea (0.5 - 25 μM), 2iP (0.5 – 25 μM), TDZ (0.5 - 5 μM), and GA_3 (0.5 - 50 μM). Organic supplements CH (50 - 2000 mg/l), glutamine (25 - 2000 mg/l) and CW (2 - 20% v/v) were also used in some media. The media are given in Table 5.3a, and media in which embryogenic response was obtained from IZEs are given in Table 5.3b. The process of induction of embryogenesis and development of somatic embryos is described in the

discussion part of this parameter (Section 5.3.3) under the heading 'Induction of somatic embryogenesis in IZEs and development of somatic embryos'.

5.2.4. Photoperiod: Various light incubation conditions have been adopted for induction of somatic embryos from IZEs in different species. The effect of photoperiod on embryogenic response from IZEs of cashew was therefore examined. IZEs at 3-5 post-fertilization stage were inoculated on MS + 2,4-D (5 μM) + BA (5 μM) + GA_3 (3 μM) + sucrose 3% + AC 0.3% + agar 0.5%, in two sets. One set was incubated in dark, and the other set was incubated in 16-hour photoperiod. Dark incubation of IZEs was more favorable for induction of embryogenesis, hence, in all experiments, IZEs were incubated in dark.

5.2.5. Embryogenic potential of excised portions of IZE: In some woody species, apart from intact IZEs, excised parts of IZEs have also given rise to somatic embryos. Therefore, in the present study, the embryogenic potential of excised parts of IZEs was studied. For experiments, the IZEs at 3 to 5 weeks post-fertilization stage were used as (i) whole intact explants, and (ii) excised parts. The excised parts used were: isolated embryo axis, embryo axis in transverse and longitudinal sections, whole cotyledons, cotyledons in transverse and longitudinal sections. All the explants were inoculated on MS + 2,4-D 5 μM + BA 5 μM + GA_3 3 μM + sucrose 3% + AC 0.3% + agar 0.5%.

5.2.6. Maturation and conversion of somatic embryos: Somatic embryos obtained from IZEs were transferred to different maturation media, which included (i) MS medium without growth regulators, without AC, (ii) MS medium with 0.5% AC, without growth regulators (iii) MS medium with different maturation agents like polyethylene glycol (PEG) (5, 10%), ABA (5, 10, 20, 30 μM), sucrose (6%, 8%, 10%), maltose (4%, 6%, 8%) and gelrite (0.4%, 0.6%, 1%) in varying combinations (Table 5.6). Somatic embryos were incubated for 5-6 weeks on maturation media and the response on each medium was observed. As MS + ABA 20 μM + maltose 3% was most suitable for maturation, this

medium was used as maturation medium. Embryos were transferred from maturation medium to germination medium (PGR-free MS medium). Somatic embryos germinated on the germination medium. All the maturation and germination media were gelled with gelrite at 0.2% and cultures were incubated in dark.

5.2.8. Histology: For histological studies, terminal portions of the radicle of IZEs (with the developing somatic embryos attached) were excised and fixed in FAA (formaldehyde: acetic acid: alcohol), and further dehydrated in the tertiary butyl alcohol series. They were embedded in paraffin wax (melting point 58-60°C, Qualigens, India) and sectioned (8-10 μm thickness) using a rotary microtome (Leica, Germany). The sections were stained with haematoxylin and observed under light microscope (Zeiss, Germany).

5.3: Results and discussion

In many woody species, embryogenesis has been reported using juvenile and immature tissues as explants, particularly zygotic embryos, either mature or immature. This type of embryogenesis, especially induced in zygotic embryos, is considered to occur from Pre Embryogenic Determined Cells (PEDCs), where the cells of the explant are physiologically closer to the embryonic phase. It is believed that in such explants, the cells of the explant can be comparatively easily induced to follow the embryogenic pathway (Sharp *et al*., 1980; Williams and Maheshwaran, 1986). In woody plants, though mature explants are unresponsive, the zygotic embryos frequently show potential for somatic embryogenesis (Table 5.1). In the present study in cashew also, success was achieved in inducing embryogenic response from IZEs. The mature zygotic embryo however, did not show embryogenic response.

5.3.1. Effect of developmental stage of IZEs on embryogenic response: The developmental stage of IZEs strongly affects the embryogenic response. When IZEs were collected at different stages of development (from 2-3 weeks through 7-8 weeks post-fertilization stages, Refer Fig. 4.1a, b, c), and inoculated on medium with 5 μM 2,4-D, IZEs from the 3-4 week and 4-5 week post-fertilization stages formed somatic embryos. Before discussing these results in detail, the response of IZEs at 2-3 weeks post fertilization, and from 5 through 8 weeks post-fertilization stages is briefly described in the following two paragraphs.

Response of IZEs at 2-3 week post fertilization stage:

The IZEs at 2-3 weeks post-fertilization stage (Fig. 5.1a) enlarged within 2-3 days of inoculation. They frequently became hyperhydric. After 2-3 weeks of incubation on the 2,4-D containing medium, hypertrophy and callusing of entire embryos was observed (Fig. 5.1b). The IZEs at this stage did not show any embryogenic response. These IZEs frequently became necrotic, and did not show further growth in culture.

Response of IZEs at 5-8 weeks post fertilization stages:

When IZEs from 5–8 weeks post-fertilization stages (Fig. 4.1f) were inoculated on medium with 2,4-D, the IZEs enlarged within 3-4 days of inoculation. The cotyledons enlarged and radicle elongated and developed rapidly, and the IZEs germinated precociously within 1-2 weeks after inoculation (Fig. 5.1g). Presence of 2,4-D in the medium did not have any effect on growth of IZEs. These IZEs did not show callus formation, and did not become hyperhydric. All IZEs germinated to form plantlets. No other response, apart from germination, was observed in IZEs at these stages of development.

Seedlings formed from these IZEs were however, very weak and much reduced in size than the seedlings obtained from mature seeds (Refer Fig. 3.2b) As previously mentioned in Chapter 3 (Section 3.3.1.), the cotyledons play a

vital role in development of zygotic embryo into a healthy seedling. At 5-8 weeks post-fertilization stages, the development of cotyledons and process of accumulation of storage products is incomplete. Though the IZEs germinated (due to intact cotyledons), the seedling produced was not robust, probably due to incomplete development of cotyledons. The immature cotyledons may be unable to supply all the complex substances necessary for the healthy development of seedling. It is clear from the above discussion that IZEs from 2–3 week, and from 5 to 8 weeks post-fertilization stages did not show embryogenic response in the present study.

Response of IZEs at 3-4 weeks, and 4-5 weeks post fertilization stages (Embryogenic response): IZEs at 3-4 weeks (Fig. 5.1c) and 4-5 weeks post-fertilization stages (Fig. 5.1d) started swelling soon after inoculation. During 4-5 weeks after inoculation, gradual changes were observed in the IZEs as they started germinating precociously. Elongation of hypocotyls, enlargement and elongation of the cotyledons, and slight elongation of plumule was observed in precociously germinating IZEs. Five to seven weeks after inoculation, 8.2% of IZEs at 3-4 week post-fertilization stage, and 5.8% of IZEs at 4-5 week post-fertilization stage, formed somatic embryos (Table 5.3). Somatic embryos were formed directly from the tip of the developing radicle of the IZEs (Fig 5.1d).

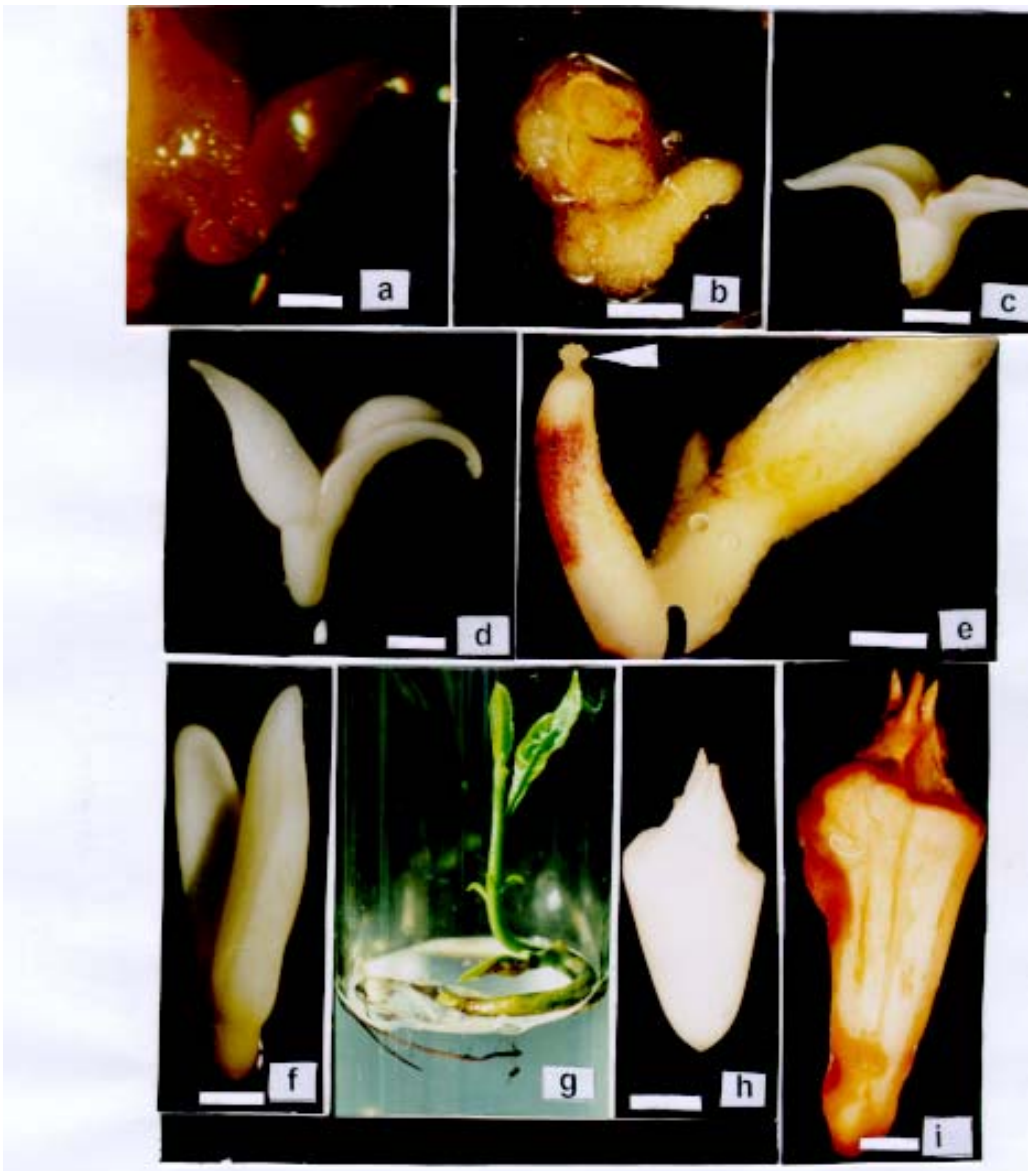


Fig. 5.1: Response of immature zygotic embryo (IZE) at different post-fertilization developmental stages

a: IZE at 2-3 weeks stage, and b: callusing of entire embryo; c: IZE at 3-4 weeks stage; d: IZE at 4-5 weeks stage, and e: Initiation of direct somatic embryogenesis at the radicle tip (arrowhead) of IZE; f: IZE at 5-7 weeks stage, and g: precocious germination of IZE; h: MZEA; i: unresponding MZEA; (a,c,d,f: bar= 10mm; b,e,h,i: bar= 6mm)

Table 5.2: Effect of developmental stage of IZEs (immature zygotic embryos) of cashew on embryogenic response

Developmental stage of IZE (weeks after fertilization)	Percent embryogenic explants (6 weeks after inoculation)
2-3	0
3-4	8.23 ± 2.5
4-5	5.84 ± 0.5
5-6	0
6-7	0
7-8	0

(Medium used was MS + 2,4-D (5 μM) + sucrose 3% + agar 0.5% + AC 0.3%. Each value is the mean ± standard deviation (SD) of three experiments with 40-70 explants per treatment in each experiment).

During initiation of embryogenic response, a knoblike structure was observed at the radicle tip of the precociously germinating IZEs (Fig. 5.2a), which formed a single, or, cluster of globular somatic embryos (Fig 5.2b-e). The somatic embryos were formed before the radicle developed into the primary tap root.

From the above observations, it was concluded that the developmental stage of the IZEs was a decisive factor for induction of somatic embryogenesis. However, Cardoza and D'Souza (2000) have reported embryogenic response from IZEs at 2 weeks post-fertilization stage. This difference in the developmental stage could be due to the different varieties used.

The above observations lead to the conclusion that there exists a "physiological window" for induction of embryogenesis from developing IZEs of cashew. Similar observations about the existence of a window period, with only a particular stage of zygotic embryo development being able to give rise to somatic embryos, have been made in other species.

In eastern redbud, initiation of somatic embryogenesis was strongly influenced by the developmental stage of IZE (Trigiano *et al*., 1995). In olive, embryogenesis was induced in IZEs from 75 days post fertilization stage only

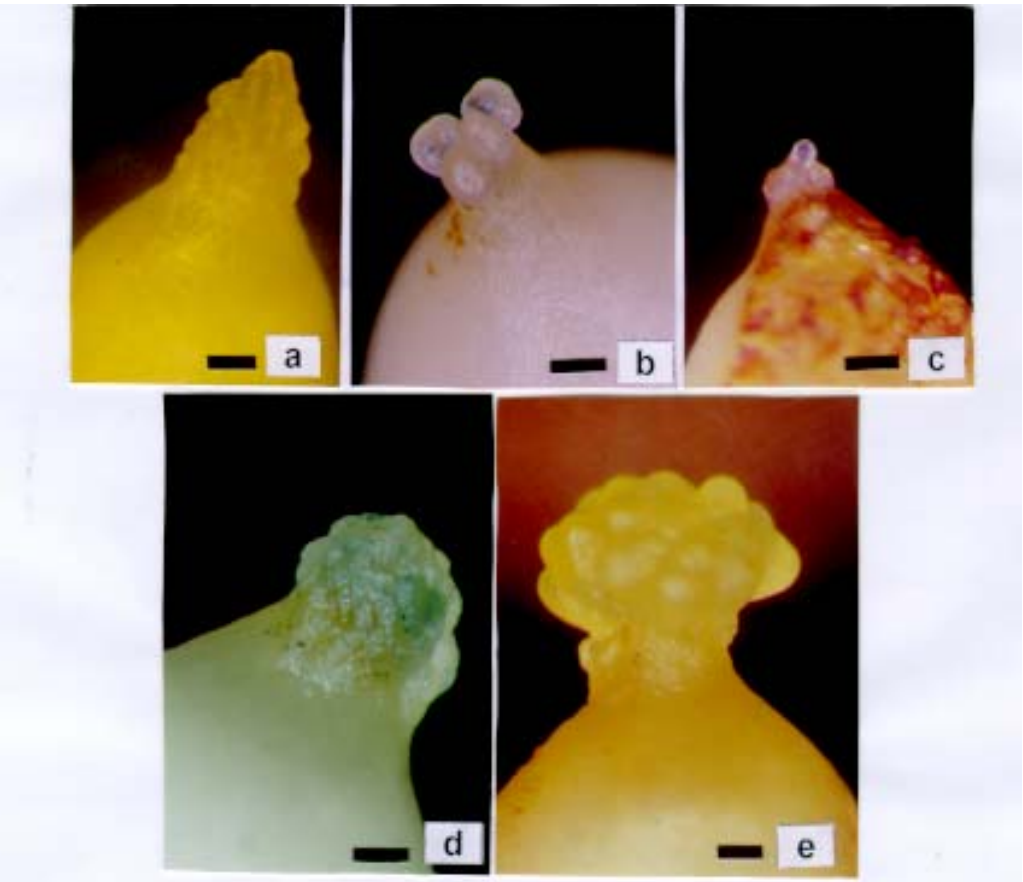


Fig. 5.2: Initiation of direct somatic embryogenesis from radicle tip of IZE (a-e: bar= 0.5 mm)

a: knob-like structure formed at radicle tip of IZE; b-e: development of globular somatic embryos directly, without callus formation

(Rugini, 1988, 1995). In yellow poplar, IZEs at 7-9 weeks post pollination stage (Sotack *et al*., 1991), and in sweetbay magnolia, IZEs at 3 weeks post anthesis (Merkle and Wiecko, 1990) had the highest embryogenic potential.

In most of the species mentioned in Table 5.1, existence of " window period" has been observed, and embryogenesis has been reported to occur only at specific developmental stage of the IZE. However, in horse chestnut, developmental stage of IZE did not affect induction of somatic embryogenesis (Radojevic, 1988).

5.3.2. Effect of growth regulators on embryogenic response from zygotic embryos: The results obtained from IZEs are discussed first. The effect of growth regulators on MZEAs is discussed later.

(a) Effect of growth regulators on embryogenic response from IZEs: Embryogenic response was observed in IZEs on few media, which are shown in Table 5.3 b (following Table 5.3a) below.

Table 5.3a: Different media and media constituents tested for induction of somatic embryogenesis in immature and mature zygotic embryos of cashew

(Basal medium: MS full strength + sucrose 3% + AC 0.5% + agar 0.5%; concentrations of growth regulators in μM)

Media No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GA_3	10	-	-	-	-	-	-	15	-	-	-	-	10	-
BA	-	5	5	-	5	-	5	-	-	5	-	-	-	-
IAA	-	-	-	-	-	5	5	-	-	-	-	-	-	-
IBA	-	-	-	-	-	5	-	5	-	-	-	-	-	-
2,4,5-T	5	5	-	-	5	-	-	-	-	-	-	-	-	-
Kin	-	-	-	-	-	-	-	-	5	5	5	-	-	-
2iP	-	-	-	-	-	-	-	-	-	-	-	0.05	0.05	-
Zea	-	-	-	0.01	-	-	-	-	-	-	-	-	-	-
TDZ	-	-	0.01	-	-	-	-	-	-	-	0.05	-	-	-

Media no.	15	16	17	18	19	20	21	22	23	24	25	26
2,4-D	5	5	-	-	-	-	5	5	-	-	-	5
GA_3	30	15	-	15	15	-	30	-	15	-	30	15
BA	-	-	10	5	-	-	10	10	5	10	-	-
Kin	-	5	-	-	-	10	-	-	5	2	-	-
NAA	-	-	5	-	5	5	-	5	-	5	5	2

Media no.	27	28	29	30	31
IAA	5	-	10	10	-
IBA	-	10	5	-	10
BA	-	5	5	-	-
Kin	-	-	-	5	5

Table 5.3 b: Direct somatic embryogenesis from immature zygotic embryo of cashew: Effect of different growth regulators on induction, and further development of somatic embryos

Growth regulators (μM)	% embryogenic explants ± S.D.		Morphological appearance of somatic embryos six weeks after induction
	3-4 week post-fertilization stage	4-5 weeks post-fertilization stage	
No growth regulators	0	0	-
2,4-D (5)	9.3 ± 1.9 [a]	4.12 ± 0.4 [a]	Callusing of entire embryo
2,4-D (10)	9.7 ± 1.2 [a]	3.98 ± 1.0 [a]	do
GA_3 (15)	7.6 ± 0.8 [a]	4.34 ± 0.3 [a]	Vitrification of embryo
2,4-D (5) + Kin (5)	9.5 ± 0.8 [a]	4.97 ± 0.6 [a]	N
2,4-D (5) + BAP (5)	6.9 ± 0.9 [a]	3.44 ± 0.6 [a]	N
2,4-D (5) + BAP (5) + GA_3 (3)	10.3 ± 1.2 [a]	5.62 ± 1.3 [a]	N
2,4-D (5) + BAP (5) + GA_3 (15)	8.2 ± 2.2 [a]	4.83 ± 0.2 [a]	N

{Basal medium is MS + sucrose (3%) + agar (0.5%) + AC (0.5%). IZEs were used at 3 to 5 week post-fertilization stages. Cultures were incubated in dark. N: normal development of somatic embryo till cotyledonary stage. Each value represents the mean ± standard deviation (S.D.) of three experiments with 40-70 explants per treatment in each experiment. Difference between values followed by the same letter is insignificant at P 0.05 and P 0.01 as analysed by Fisher's LSD test}.

From Table 5.3 b, it can be seen that growth regulators were essential for induction of embryogenesis from IZEs. No embryogenic response was observed when IZEs were grown on medium devoid of growth regulators.

However in longan (Lai *et al*., 2000), and walnut (Cornu, 1988), growth regulators were not necessary, and IZEs formed somatic embryos on basal medium without growth regulators.

The growth regulators which were effective in inducing embryogenic response from IZEs of cashew were: 2,4-D, GA_3, BA and Kin (Table 5.3b).
Of these four growth regulators, 2,4-D when used alone, and GA_3 when used alone, could induce embryogenesis. The cytokinins BA and Kin, could not induce embryogenesis when used as the only growth regulator in the medium.

It was observed that somatic embryos formed on medium with 2,4-D alone showed callusing during further incubation (Table 5.3b). The callused somatic embryos did not show further development, and their growth was arrested. Detrimental effects of 2,4-D have also been observed in pecan somatic embryos, where 2,4-D caused high degree of developmental abnormalities, and poor development of shoot apex (Wetzstein *et al*., 2000).

Somatic embryos formed in presence of GA_3 as the sole growth regulator, became hyperhydric, during their development through torpedo or cotyledonary stages (Table 5.3b). These embryos became necrotic during continued incubation. Thus, 2,4-D and GA_3 could induce embryogenic response from IZEs when used alone, but they were not suitable for normal development of somatic embryos.

Though BA and Kin were unable to induce embryogenic response when used alone, they were effective when used along with 2,4-D in inducing embryogenic response from IZEs. Between BA and Kin, Kin induced embryogenesis in more number of IZEs (9.5% at 3-4 weeks, and 4.97 % at 4-5 weeks stages respectively) as compared to BA, which induced embryogenesis in fewer (6.9% at 3-4 weeks, and 3.44% at 4-5 week stages respectively) explants (Table 5.3b).

In media with 2,4-D 5 μM + BA 5 μM, and 2,4-D 5 μM + Kin 5 μM, the somatic embryos developed normally till late cotyledonary stage, without showing any abnormalities. Callusing of embryos did not occur even if the medium contained 2,4-D.

A combination of 2,4-D (5 μM), GA_3 (3 μM) and BA (5 μM) was observed to be most suitable for embryogenic response. Maximum number of IZEs (10.3% at 3-4 weeks and 5.62% at 4-5 week stages respectively) produced somatic embryos on this medium (Table 5.3b). Normal development of somatic embryos occurred on this medium also, as was observed on media with 2,4-D + BA, and 2,4-D + Kin. Callusing and necrosis of somatic embryos was not observed. Continued incubation of somatic embryos on this medium for more

than 6 weeks did not have any adverse effect on growth of somatic embryos, in spite of presence of 2,4-D and GA_3.

The cytokinins present in the above media may play a role in suppressing the callusing effect of 2,4-D. They probably also suppressed the hyperhydricity caused by GA_3.

In the present study, it was observed that

(i) media containing only auxin (2,4-D) was favorable for somatic embryo induction, but not for normal development of somatic embryos.

(ii) media having only cytokinin were ineffective in inducing embryogenic response.

(iii) media having both, auxin (2,4-D) and cytokinin (BA or Kin) could induce embryogenesis in IZEs and were also suitable for normal development of somatic embryos.

Therefore it was concluded that auxin-cytokinin combinations were favorable for embryogenic response from IZEs. The auxin-cytokinin balance in nutrient medium was necessary for induction of embryogenesis, as well as proper growth and development of somatic embryos. In such combinations, the auxin component is responsible for commencing of embryogenic pathway in explant, and the cytokinin component probably has an influence on further development of somatic embryos.

From the discussion in Section 5.3.1 (Effect of developmental stage of IZE) and the present section (5.3.3, Effect of growth regulators) it can be concluded that:

(i) Induction of embryogenesis was restricted to a particular developmental stage of IZE.

(ii) Exogenous growth regulators are necessary to trigger the embryogenic pathway in IZEs.

The developmental stage is a measure of the intrinsic physiology of IZE. Plant growth regulators in the nutrient medium are exogenous factors affecting the response of IZEs. Therefore, the embryogenic response from IZEs depends on two factors:

(i) intrinsic physiological condition of the IZE, and

(ii) interaction between exogenous and endogenous growth regulators.

Both factors play an equally important role in induction of somatic embryogenesis. Embryogenic response was induced in only those IZEs where both these factors were in equilibrium with each other.

In present studies, auxins IAA, IBA, NAA, 2,4,5-T, cytokinins 2ip, TDZ, Zea, and combinations of 2,4-D and GA_3 were completely ineffective in inducing embryogenic response from IZEs.

However, NAA was effective in inducing embryogenesis from IZEs of cacao (Pence, 1995). BA alone induced embryogenesis in IZEs of olive (Rugini, 1988) and elm (Corredoira *et al*., 2002). Indole butyric acid (IBA) was used with cytokinin for embryo induction from IZEs of *Acacia mangium* (Xie and Hong, 2001) and *Quercus acutissima* (Kim *et al*., 1994).

Among the different auxins used, only 2,4-D could induce embryogenic response from the IZEs. In *Aesculus hippocastanum* (Radojevic, 1988) and longan (Lai *et al*., 2000), 2,4-D was said to be indispensable for induction of somatic embryogenesis from IZEs. However, 2,4-D inhibited somatic embryogenesis from IZEs in olive (Rugini, 1988, 1995), and was completely ineffective in inducing embryogenesis in IZEs of oak (Chalupa, 1990).

Auxin-cytokinin combinations were effective for somatic embryo induction in other species also. Combination of 2,4-D and BA has been used for embryogenic induction from IZEs of black locust (Arrillaga *et al*., 1994) and red oak (Gingas and Lineberger, 1989), whereas, combination of 2,4-D and Kin was effective for inducing embryogenesis in IZEs of horse chestnut (Radojevic, 1988).

It was observed that CW, glutamine, and CH had no effect on the embryogenic response of the IZEs. Induction of embryogenesis occurred in media with and without these supplements. They did not have any beneficial effect on embryogenic induction, or further development of the somatic embryos in cashew.

However, CH was used to induce embryogenesis from IZEs of pecan (Merkle *et al*., 1987; Wetzstein *et al*., 1989). It was used along with glutamine for inducing embryogenesis from IZEs of cherry (DeMarch *et al*., 1993), and with CW for embryo formation from IZEs of cacao (Pence *et al*., 1979 and 1980). On the other hand, CH inhibited germination of somatic embryos in yellow poplar (Merkle *et al*., 1990). In *Vitis*, medium lacking glycine was found to be optimal for growth of embryogenic callus and germination of somatic embryos (Gray, 1992).

Induction of somatic embryogenesis and development of somatic embryos: On media with 2,4-D + BA, 2,4-D + Kin, and 2,4-D + BA + GA_3, somatic embryos developed normally through the globular, heart, torpedo, early and late cotyledonary stages of development, as described below.

During the initiation of embryogenesis, tip of the radicle of precociously germinating IZEs formed a knob-like structure (mentioned previously in the concluding part of section 5.3.1), instead of developing the primary root. Observations of this structure under stereo-microscope revealed that the knob formed sigle, or cluster of globular stage somatic embryos.

It was observed that the somatic embryos arose from the tip of the radicle, or region very close to the radicle tip. Whether arising in clusters, or singly, somatic embryos developed through the normal heart (Fig 5.3a, b), torpedo and cotyledonary (Fig 5.3c-f) stages, while still attached to the parent zygotic embryo. Absence of synchrony in the development of somatic embryos (Fig. 5.4a) in a cluster was frequently observed. Few somatic embryos in the cluster developed more rapidly than the others. Somatic embryos could be easily separated from the parent IZE at the torpedo or cotyledonary stage. The detached embryos continued their further development even after separation from the parent explant.

Structural abnormalities like fasciation (Fig. 5.4b), multiple cotyledons (Fig. 5.3e, f), fused or asymmetrically developed cotyledons (Fig. 5.4c), and absence of apical dome were observed in few somatic embryos.

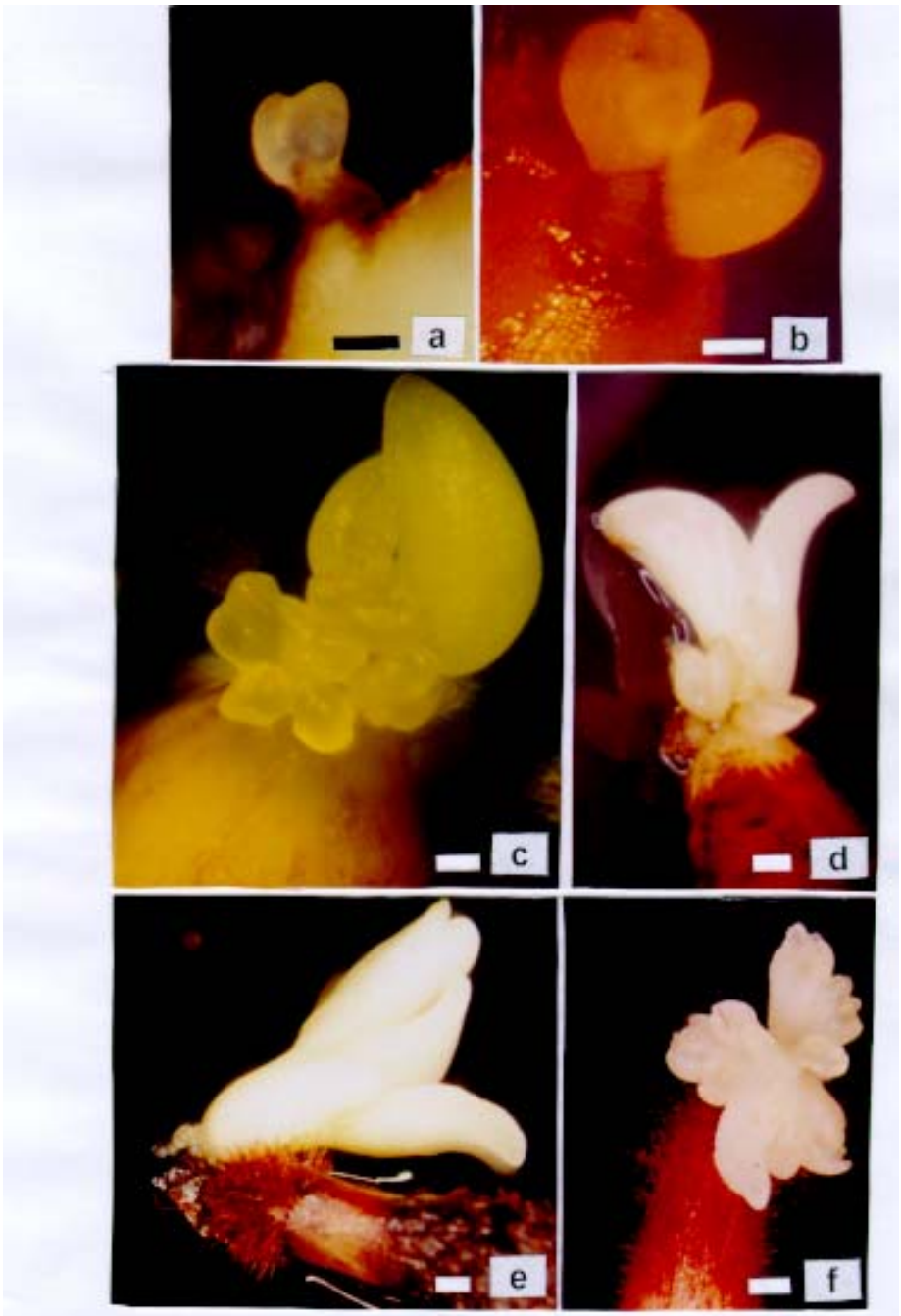


Fig. 5.3: Development of somatic embryos formed from IZEs a, b: heart stage somatic embryos; c-f: cotyledonary stage somatic embryos (a-f: bar= 0.5 mm)

During the development of the somatic embryos, the parent IZEs showed elongation and enlargement of cotyledons, and normal development and elongation of the plumule. However, development of the radicle appeared to be arrested.

Few other species in which the radicle, or radicle-hypocotyl zone of IZEs has directly given rise to somatic embryos are *Castanea* (Merkle *et al*., 1992), black locust (Arrillaga *et al*., 1994), and *Vitis* (Emershad and Ramming, 1994). Secondary somatic embryos formed directly from radicular region of primary somatic embryos of walnut (Tulecke and McGranahan, 1985) and pecan (Yates and Reilly, 1990).

In this study, only the radicle tip of IZEs formed somatic embryos. In other species however, different parts of IZEs have formed somatic embryos directly. Epidermal layers of cotyledons in *Cercis* (Trigiano *et al*., 1988; Geneve and Kester, 1990); cotyledons of whole IZEs in red oak (Gingas and Lineberger, 1989) and pecan (Yates and Reilly, 1990); and glandular hair-like structures in cacao (Pence *et al*., 1980; Pence, 1995) are the different parts of IZEs which formed somatic embryos directly.

IZEs of cashew never formed embryogenic callus on any medium used. At higher concentrations of 2,4-D (above 10 μM), callus was formed from IZEs (Table 5.3b), but this callus was non-embryogenic and non-proliferative. However, IZEs of jojoba (Wang and Janick, 1986), *Acacia mangium* (Xie and Hong, 2001), and sweetgum (Vendrame *et al*., 2001) formed somatic embryos through an intermediate callus phase. Whereas in olive (Rugini, 1988 and 1995), pecan (Corte-Olivares, 1990), and Japanese maple (Vlasinova and Havel, 1999), IZEs gave rise to somatic embryos directly, as well as through embryogenic callus phase.

(b) Effect of growth regulators on embryogenic response from MZEAs: When MZEAs were grown in presence of different growth regulators, it was observed that cytokinins did not have any effect on the MZEAs. None of the cytokinins used (BA, Kin, 2iP, TDZ and Zea) could induce any response from the MZEAs, and the embryo axes remained totally unresponsive even after 7-8 months in culture (Fig. 5.4d).

In presence of 2,4-D and 2,4,5-T, swelling of the entire embryo axes occurred, and the lateral edges of enlarged MZEAs showed slight callus formation. However, no further response could be obtained from these embryo axes. The enlarged and callused MZEAs never gave rise to somatic embryos directly, or through embryogenic callus on any medium used. Almost always the hypocotyl region of the MZEA elongated slightly, after which, further growth and development of the MZEA was arrested. The rudimentary embryonic shoot never elongated and developed to form shoot (Fig. 5.4e).

Thus, the fully mature embryo axis failed to give embryogenic response in this study. It was also observed earlier, in section 5.2.1., that with increasing age of the zygotic embryo, the embryogenic potential decreased.

5.3.4. Effect of photoperiod on embryogenic response from IZEs: When IZEs from 3 to 5 weeks post-fertilization stage were cultured on MS + 2,4-D 5 μM + BA 5 μM + GA_3 3 μM + sucrose 3% + AC 0.3% + agar 0.6%, and one set was incubated in dark, and the other in 16-hour photoperiod, dark incubation was found favorable for induction of embryogenesis. The number of IZEs showing embryogenic response was more (8.53% from 3-4 week and 5.72% from 4-5 weeks stages respectively) when they were incubated in dark. Very few IZEs (2.17% from 3-4 weeks, and 1.86% from 4-5 week stages respectively) could form somatic embryos when they were incubated in 16-hour photoperiod (Table 5.4).

Table 5.4: Effect of photoperiod on embryogenic response from IZEs (immature zygotic embryos) of cashew

Light conditions	Percent embryogenic response from IZEs, 6 weeks after inoculation	
	3-4 week post-fertilization stage	4 –5 week post-fertilization stage
Dark	8.53 ± 1.04	5.72 ± 0.66
16-hour photoperiod	2.17 ± 0.58	1.86 ± 0.5

{Medium used was MS + 2,4-D (5 μM) + BA (5 μM) + GA_3 (3 μM) + sucrose 3% + agar 0.5% + AC 0.3%. Each value is the mean ± standard deviation (SD) of three experiments with 40-70 explants per treatment in each experiment}.

The IZEs incubated in dark remained white or creamish in color. In these IZEs, etiolation of plumule occurred. Germination was not affected by dark-incubation, and all the dark-incubated IZEs germinated within 3-4 weeks of inoculation. In IZEs incubated in 16-hour photoperiod, IZEs started turning green, and germinated precociously. The plumule elongated and opening of apical leaves was observed in these IZEs. Very few IZEs could form somatic embryos when incubated in 16-hour photoperiod in this study. Presence of light seemed to inhibit embryogenic induction and favored precocious germination of IZEs.

Similarly, light strongly inhibited embryogenic induction from IZEs of olive (Rugini, 1988, 1995) and longan (Lai *et al*., 2000). In other species, embryogenesis from IZEs has been reported in varying photoperiod conditions: 12-hours for *Vitis* (Emershad and Ramming, 1994); 14-hours for *Myrtus communis* (Parra and Amo-Marco, 1998); 16-hours for cacao, Pence *et al*., 1979) and continuous light for jojoba (Lee and Thomas, 1985). However in hazelnut, photoperiod conditions did not affect embryogenesis, and embryogenic response could be induced from IZEs incubated in 16-hour photoperiod as well as in dark (Berros *et al*., 1995).

5.3.5.Embryogenic potential of excised portions of IZE: Excised parts of IZEs (isolated embryo axis, transversely and longitudinally bisected embryo axis, whole cotyledons, transverse and longitudinal sections of cotyledons) never formed somatic embryos on any medium.

The isolated embryo axis remained unresponsive even after 3-4 months of culture, and only slight enlargement of the hypocotyl regions occurred (Fig. 5.5a) After 2-3 months, browning of the embryo axis was observed. Isolated immature zygotic embryo axes failed to germinate and develop into plantlet. Similar observation was made in Chapter 3 (Section 3.3.1), where isolated mature zygotic embryo axes had also failed to develop into seedling. These observations validate presence of cotyledons as necessary for development of zygotic embryo into a normal plantlet.

The separated cotyledons also did not show any embryogenic or organogenic response. Some cotyledons became hyperhydric, and showed callusing (Fig 5.5b).

In the present study, only whole intact IZEs could form somatic embryos. The physical integrity of IZEs was essential for the formation of somatic embryos. As discussed previously, explant physiology and endogenous growth regulators are an important factor for expression of somatic embryogenesis from IZEs. These endogenous factors were left unaffected in intact undamaged IZEs, which was the condition required for formation of somatic embryos. In excised portions of IZE, internal balance of the endogenous factors was disturbed and their transport possibly interrupted due to excision, due to which, the excised parts could never form somatic embryos.

However, longitudinally bisected IZEs of *Magnolia* (Merkle and Wiecko, 1990), transversely bisected IZEs of *Quercus,* (Kim *et al*., 1997), slices of embryonal axis and cotyledons of peach and nectarine (RajBhansali *et al*., 1990) formed somatic embryos directly. Isolated embryo axes of *Albizzia julibrissin* (Burns and Wetzstein, 1998) and hazelnut (Berros *et al*., 1995) were also observed to be embryogenic.

Fig. 5.4: a: Asynchronous development of somatic embryos at the tip of radicle of IZE; b: fused somatic embryos; c: asymmetric cotyledon development; d: unresponding MZEA; e: enlargement and callusing of entire MZEA, without affecting shoot pole (arrowhead) (a-e: bar= 10 mm)

Immature cotyledons never formed somatic embryos in this study, as was also observed in eastern redbud (Geneve *et al*., 1990) and pecan (Yates and Reilly, 1990), where, immature cotyledons failed to produce somatic embryos. Embryogenic potential of the immature cotyledons is, however, evident in other nut crops like eastern black walnut (Neuman *et al*., 1993), English walnut (Tulecke and McGranahan, 1985) and pistachio (Onay *et al*., 1995).

5.3.6. Maturation and germination of somatic embryos: From experiments on maturation and conversion of somatic embryos obtained from IZEs, it was observed that **s**omatic embryos developed normally on the embryo induction medium till early cotyledonary stage. For further development, they were transferred to MS medium with or without AC (0.5%), and not containing any growth regulators.

The embryos developed further on MS medium till late cotyledonary stage. At this stage, the embryos showed precocious germination, where only elongation of root pole was seen. It was realized that precocious germination of somatic embryos had to be prevented for their successful conversion. Maturation medium seemed to be necessary for the maturation of the somatic embryos, and for their proper development and successful conversion. Therefore, somatic embryos at cotyledonary stage were transferred to different media (Table 5.5) for further maturation.

Table 5.5: R**esponse of somatic embryos to different maturation agents used in media**

Media components (μM)	**Response of somatic embryos**
ABA (10)	Precocious germination
ABA (10) + maltose 3%	-do-
ABA (10) + PEG 10%	Necrosis
ABA (20) + maltose 3%	Quiescence, normal appearance
ABA (20) + maltose 8%	Enlargement
ABA (20) + maltose 3% + PEG 5%	Blackening
ABA (30)+ maltose 6%	Callusing
ABA (30) + maltose 3% + PEG 5%	Callusing, browning
PEG 10%	Necrosis
maltose 8% +PEG 5%	Browning and further necrosis
Gelrite 0.9% + AC 0.3%	Precocious germination
Gelrite 0.5%	-do-
Sucrose 8%, 10%	-do-

(Basal medium: MS + gelrite 0.2% + sucrose 3%)

It was observed that MS medium with combination of ABA (20 μM) and maltose (3%) was optimal for maturation of somatic embryos. The somatic embryos were cultured for 5-6 weeks on this maturation medium. However, growth of the embryos was slow on this medium. The main axis of somatic embryo showed elongation (but not germination). There was slight growth, and divergence of the cotyledons, and the apical dome could be observed. Growth of cotyledons did not continue further. Though cotyledons showed only partial development on this medium, the embryos did not revert to callus phase, or show browning and necrosis, as observed on maturation media with PEG or maltose (Table 5.5).

ABA was effective for maturation at 20 μM. At lower concentrations of ABA, somatic embryos germinated precociously. And at higher concentrations they formed callus (Table 5.5). Sucrose, maltose and PEG were found to be unsuitable as maturation agents, as the somatic embryos showed precocious germination, browning and necrosis respectively, in media with these supplements (Table 5.5). Gelrite supported normal growth of somatic embryos, but did not prevent their precocious germination.

After 5-6 weeks on maturation medium, somatic embryos were transferred to the germination media, which were (i) MS + sucrose 3% + agar 0.6%, and (ii) MS + sucrose 3% + AC 0.5% + agar 0.6%. Somatic embryos germinated within 1-3 weeks of transfer to germination media. Somatic embryos elongated at the radicular end, with subsequent root development (Fig 5.5c,d). Root formation was accompanied by opening of the cotyledons, to expose the apical dome. Slight elongation of the plumule was observed. The cotyledons of somatic embryos were smaller (2-4 mm) than those of zygotic embryos (30-40 mm or more, Refer Fig. 3.2e).

Mature zygotic embryos never developed into plantlets in the absence of cotyledons (Chapter 3, Section 3.3.1). Fully developed cotyledons are indispensable for development into plantlet. In case of somatic embryos, it is observed that the cotyledons do not develop fully, which affects their development into plantlet (conversion). Proper development of the cotyledons might be important for normal development of the root system and primary shoot, leading to better conversion rates of the somatic embryos (Gogte and Nadgauda, 2000).

High sucrose concentrations have been reported to be beneficial for the *in vitro* synthesis of storage lipids in cacao (Pence *et al*., 1981, Janick *et al*., 1982) and jojoba (Wang and Janick, 1986). Cotyledons of cashew also contain lipid compounds as storage products. In attempts to obtain better growth of cotyledons (by inducing synthesis of storage lipids) of somatic embryos, media with high (8, 10 and 15%) sucrose concentrations were used for maturation (Table 5.5). However, sucrose was ineffective in improving the development of cotyledons.

Accumulation of storage substances is a key step in zygotic embryogenesis. In cashew, this step is prolonged and occupies major part of the developmental phase. During this step, compounds are stored, for later use by the germinating zygotic embryo, until it attains autotrophy. Lack of storage substances like triglycerides, adversely affect the later stages of development, and subsequent conversion of somatic embryos into plantlets (Feirer *et al*., 1989). Difficulties in the conversion of somatic embryos obtained from IZEs of cashew have also been reported in earlier studies (Jha, 1988; Cardoza and D'Souza, 2000), and plantlets could not be obtained from the embryoids and somatic embryos respectively.

In other species, basal medium devoid of growth regulators has been used for further development and maturation of somatic embryos of IZE origin, as in peach and nectarine (RajBhansali *et al*., 1990) and Quercus (Kim *et al*., 1997). Basal medium devoid of growth regulators has been used for germination of somatic embryos of *Myrtus communis* (Parra and Amo-Marco, 1998), and sweetgum (Merkle *et al*., 1998). Different growth regulators have been used for conversion/ germination of somatic embryos, like BA in *Quercus acutissima* (Kim *et al*., 1994 and 1997); IBA in linden (Chalupa, 1990) and GA_3 in *Acacia mangium* (Xie and Hong, 2001).

Additional studies on maturation treatments would be helpful for successful conversion and plantlet production. The maturation medium should be effective in preventing the precocious germination of the somatic embryos and the embryos should undergo a period of quiescence, which has been reported to be beneficial for the maturation of grape somatic embryos (Gray, 1987). As development of healthy plantlet from embryo strongly depends on the presence of cotyledons, medium supporting development of the cotyledons would certainly be more effective as maturation medium for somatic embryos of cashew. Better development of cotyledons would ensure better conversion rates in somatic embryos of this species.

Poor conversion rates of somatic embryos are commonly observed in other woody species also. In oak, low frequency of somatic embryo conversion is a serious problem. Development of oak somatic embryos is frequently blocked after cotyledon formation (Chalupa, 1995). In elm (Corredoira *et al*., 2002), conversion of somatic embryos has not been reported. To overcome the problem of poor conversion rate in avocado, somatic embryos were induced to form shoots, which were then rooted separately in IBA-containing medium (Witjaksono and Litz, 1999b). Poor conversion frequency has also been reported in many woody species, some of which are walnut (Tulecke and McGranahan, 1985), red oak (Gingas and Lineberger, 1989), chestnut (Vieitez, 1995), and hazelnut (Berros *et al*.,1995). In fact, conversion of somatic embryos into plantlets seems to be the major hurdle in the success of somatic embryogenesis in woody tree species (Merkle,1995).

It is evident that low conversion rates of somatic embryos is a problem faced in many woody species, even in those species, where embryogenic systems have been developed and studied for considerable period. In a highly recalcitrant species like cashew, difficulties faced during somatic embryo conversion come as no surprise. However, with the availability of novel growth regulators and chemicals, and certainly with more experimentation, the conversion rates can be improved in this species. The poor conversion rates reported earlier, were gradually and subsequently improved with continued experimentation over a period of time, in oak (Chalupa, 1995) and walnut (Preece *et al*., 1995).

5.3.8. Histology: In histological studies, it was confirmed that somatic embryos arose from the outermost cell layer of the tip portion of the radicle. They developed from the tip, or region immediately near the tip of the radicle. The initial development of globular somatic embryos at or near the tip of the radicle was identified by group of densely stained cells at the peripheral layer. There was no intervening callus tissue, and globular embryos arose directly from the peripheral cell layer (Fig. 5.6a). The sections showed the torpedo stage (Fig.

5.6b) of embryo development. There was no vascular connection between the parent explant and the developing embryos.

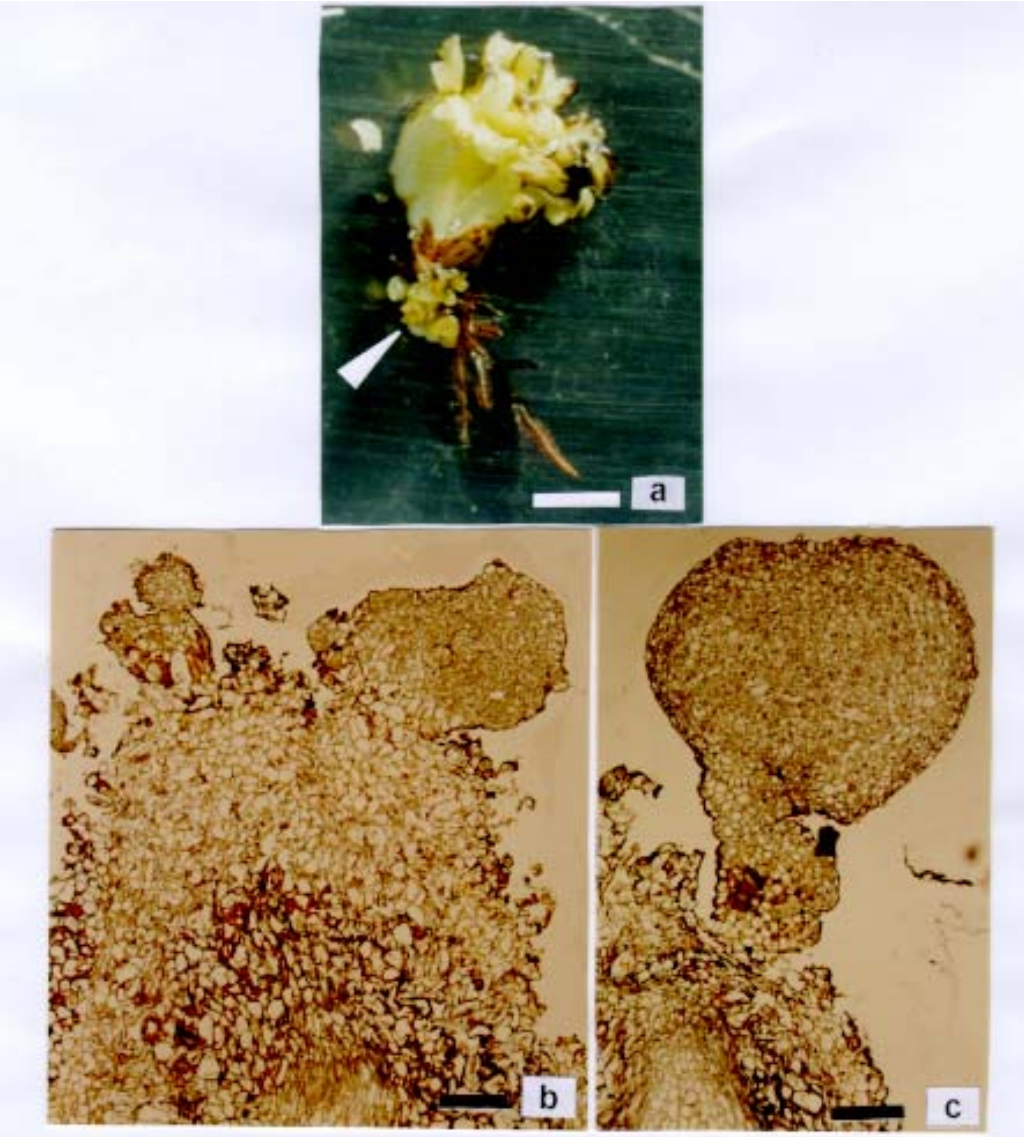


Fig. 5.6: a: Germination of fasciated somatic embryo, secondary somatic embryos (arrowhead) arising from radicle tip of primary somatic embryo (bar= 10 mm); b: Section through globular stage somatic embryos; c: section through torpedo stage somatic embryo; (b, c: bar = 0.1 mm)

5.4: Summary

The IZEs of cashew have embryogenic potential. Induction of somatic embryogenesis from this explant depends on:

(i) The developmental stage of the IZEs: This factor has strong influence on induction of somatic embryogenesis. IZEs from 3 to 5 weeks post-fertilization stage only formed somatic embryos.

(ii) Physical integrity of IZEs: Only intact undamaged IZEs showed embryogenic response. Embryos were formed directly from tip of the radicle of the IZEs.

(iii) Exogenous growth regulators are necessary for induction of embryogenesis. Auxin-cytokinin combinations were favorable for somatic embryogenesis, and MS + 2,4-D 5 μM + BA 5 μM + GA_3 3 μM + sucrose 3% + AC 0.3% + agar 0.5% was most suitable medium for formation, and normal development of somatic embryos.

Dark incubation was favorable for somatic embryo formation. The embryogenic response was very poor from IZEs incubated in presence of light. Somatic embryos matured on MS + ABA 20 μM + maltose 3%. They germinated on MS medium without growth regulators. After improvement in the conversion rates of somatic embryos, the system can be used effectively for propagation of rootstocks and hybrid varieties. Used in combination with an appropriately designed breeding program, the present embryogenic system could contribute to propagation and improvement of cashew.

As IZEs form somatic embryos directly, they can be used for genetic transformation procedures, and subsequent multiplication of transformants. The system would also be useful for a better understanding of the zygotic embryogenesis in cashew. It would certainly prove valuable in gaining an insight into the different physiological changes associated with the development and maturation of the zygotic embryo. These studies would, in turn, help in designing appropriate medium for successful maturation and conversion of somatic embryos originating from IZEs, as well as nucellus.

5.5: Conclusions

- The IZE (immature zygotic embryo) of cashewnut has embryogenic potential
- The embryogenic response is strongly dependent on developmental stage of the IZE. IZEs only at 3 to 5 weeks post-fertilization stage could form somatic embryos
- The embryogenesis occurred through direct pathway, without intervening callus phase
- Low concentration of auxin (2,4-D, 5 μM) was necessary to induce embryogenic response from the IZE
- Embryogenesis occurred from IZEs incubated in dark, on MS medium with 2,4-D 5 μM + BA 5 μM + GA_3 3 μM +sucrose 3% + AC 0.3% + agar 0.5%
- Somatic embryos formed from the IZEs developed normally till late cotyledonary stage
- ABA (20 μM) and maltose (3%) were found to be suitable for maturation of somatic embryos. Embryos germinated on MS medium without growth regulators
- Better cotyledon development in somatic embryos would improve the conversion of somatic embryos

A comparative account of somatic embryogenesis from nucellar tissue and immature zygotic embryo in cashew

In the present study, somatic embryogenesis could be induced from nucellar tissue and the immature zygotic embryo. During the course of studies, some similarities, and some differences were observed in these two embryogenic systems.

Similarities observed in induction of somatic embryogenesis were as follows:

Similarities	Nucellus	IZE
Developmental stage showing embryogenic response	3-4 week post fertilization stage	3-5 weeks post fertilization stage
Presence of plant growth regulators	Essential	
Requirement for low concentration (5 μM) of auxin 2,4-D	Used with GA_3 and BA for callus initiation; with GA_3 for somatic embryo formation	Used with GA_3 , & BA/ Kin for somatic embryo formation
Requirement for low concentration (5 μM) of cytokinin BA	Needed for formation of embryogenic mass	Needed for normal development of somatic embryos
Difficulties in maturation and conversion of somatic embryos	shoot apex development very poor or absent. Size of cotyledons very much reduced. Somatic embryos germinated precociously	

The differences observed in these embryogenic systems were:

Differences	Nucellus	IZE
Pathway of somatic embryogenesis	Indirect	Direct
Time required for appearance of somatic embryos	18-20 weeks	5-7 weeks
Number of media required for expression of somatic embryos	three different media, used sequentially	Only one medium sufficient
Effect of ABA on somatic embryos during maturation	Hypertrophy and callus formation at all concentrations	No adverse effect at 20 μM
Proliferation of embryogenic cultures	Embryogenic callus amenable to long-term maintenance	Repetitive embryogenesis not observed, embryogenic cultures have to be established afresh

Part of the work in chapter 5 has been published as follows:

Gogate SS and Nadgauda RS (2003) Direct induction of somatic embryogenesis from immature zygotic embryo of cashew (*Anacardium occidentale* L.). *Sci. Hort*. 97: 75-82.

Part of the work in chapter 5 is included in :
Nadgauda RS and Gogate SS (communicated) Somatic embryogenesis in Cashew (*Anacardium occidentale* L.) to be published as part of book on Somatic Embryogenesis in Woody Plants, SM Jain (Ed).

SUMMARY AND CONCLUSIONS

Cashew is an important tropical evergreen tree. Cashewnuts are the chief product of commercial importance having worldwide popularity. With increasing realization of the export potential of this crop, there is an urgent need to develop and propagate superior and higher yielding varieties, which can cater to international market.

The conventional methods of vegetative propagation are unable to meet the increasing demand for quality planting material. Developing an effective method of generating planting material on a large scale would be beneficial. Developing a system of plant regeneration using *in vitro* techniques would be an effective way of plant multiplication. Such a system can also be used for improving existing varieties through genetic manipulations.

The present work was therefore initiated with the objective of developing an *in vitro* method of plant multiplication. When the present studies were initiated, there were few reports on *in vitro* propagation of cashew, and all were on experiments using seedling tissues, or seed-derived explants. Initial experiments were conducted with mature axillary buds. However, no morphogenetic response was obtained from this explant. Among the different explants used, morphogenetic response *in vitro* was obtained only from juvenile explants, like cotyledonary node of seedlings, nucellus, and immature zygotic embryos. Important findings of the present study are summarized in the following paragraphs:

Axillary shoot proliferation from cotyledonary node of seedling:

To undertake studies with seedling tissues, it was necessary to develop a convenient method of *in vitro* seed germination to obtain axenic seedlings. In other studies, seeds were dissected *in vitro*, and placed on nutrient medium to obtain seedlings. Due to the presence of CNSL, removal of seedcoat in cashew is hazardous, and seeds got mutilated during dissection. Hence, a suitable and

simple method was developed, where mature seeds were germinated on filter paper supports placed in distilled water. Healthy seedlings could be obtained by this method. Nutrient medium was not needed for germination, and dissection of seeds was also not necessary. This method of seed germination may also be useful in studies where axenic seedlings are required. Minimal requirements of this method also make it an inexpensive process of obtaining seedlings and seedling-derived explants.

Seeds were germinated by the above method, and seedlings were used for obtaining cotyledonary node and nodal explants required for experiments. Effect of different cytokinins on axillary shoot proliferation was studied. It was found that BA was most effective when used at 10 μM, and could induce 7-10 axillary shoots per explant. In earlier report, a higher concentration of BA was found optimal, and though more shoots were formed, they were unsuitable for rooting.

Age of explant affected axillary shoot production from cotyledonary nodes. Cotyledonary nodes at 4-5 weeks post-germination stage were most suitable for axillary shoot production, whereas, explants collected from 3-week old seedlings were used in previous reports. The difference in explant age may be due to different varieties used for experiments. Previous study had not indicated the effect of cotyledons on axillary shoot production. Therefore, the effect of presence or absence of cotyledons on axillary shoot formation was studied. It was found that cotyledonary nodes inoculated along with cotyledons produced more shoots, indicating the beneficial effect of cotyledons on shoot production.

It was found that shoots left intact on the parent explant elongated on the medium with 10 μM BA (on shoot production medium). Separate medium for shoot elongation was not required. In fact, transferring shoots to a separate medium hampered shoot growth. Half strength MS liquid medium with IAA 5 μM + IBA 2.5 μM + Kin 2.5 μM + 1% sucrose was most suitable for *in vitro* root induction. Whereas, only auxins were found suitable for root induction in earlier studies. Survival of shoots during acclimatization step was low, probably due to absence of a strong tap root.

There were no reports on use of cytokinins for induction of axillary/ adventitious shoots from seeds during germination. Hence, studies were carried out, where BA was added to germination medium. However, shoots were not formed from seeds during germination, and during seedling development. Presence of cytokinin adversely affected axillary shoot formation in cotyledonary nodes taken from seedlings grown in BA-containing medium. These studies may help in better understanding of seedling development, and physiology.

Induction of somatic embryogenesis in nucellar tissue:

When present study was commenced, there were no reports on somatic embryogenesis from nucellar tissue of cashew. Efforts were therefore focussed on developing such a regeneration system. Developmental stage of the nut played an important role in induction of embryogenic response. Nucellar tissue obtained from 3-4 weeks post-fertilization stage of nut development formed a proliferating yellowish callus. This callus further formed embryogenic mass, which later differentiated into somatic embryos.

Induction of embryogenesis was dependent on the balance of growth regulators in the medium. BA was essential during callus initiation stage. Removal of BA, and increased concentration of 2,4-D was necessary for formation of embryogenic callus. Lower concentration of 2,4-D and higher concentration of GA_3 was favorable for differentiation of somatic embryos. Three different media (Initiation, Maintenance and Expression Media), were used sequentially for initiation of callus, formation of embryogenic mass, and differentiation of somatic embryos respectively.

The nucellus formed a rapidly proliferating callus on Callus Initiation medium (MS + 2,4-D 5 μM + GA_3 15 μM + BA 5 μM + sucrose 3% + AC 0.5% + agar 0.5%) in 3 weeks. This callus showed rapid growth only during the three weeks immediately following inoculation. The 3-week old callus was transferred to callus Maintenance Medium (MS + 2,4-D 10 μM + GA_3 15 μM + CH 0.05% + CW 0.1 % + sucrose 4% + AC 0.5% + agar 0.5%). The callus started

blackening after transfer to Maintenance Medium. The completely blackened primary callus formed a white granular embryogenic mass on Maintenance Medium after 9 weeks. The embryogenic mass differentiated into somatic embryos when it was transferred to embryo Expression Medium (MS + 2,4-D 5 μM + GA_3 30 μM + CH 0.05% + CW 0.1 % + sucrose 4% + AC 0.5% + agar 0.5%). On Expression Medium, somatic embryos appeared after 7-8 weeks. The somatic embryos were transferred to MS + AC 0.5%, for further development. On this medium, somatic embryos developed till cotyledonary stage and few somatic embryos in cotyledonary stage germinated precociously. The nucellar callus is being maintained on semi-solid and liquid medium.

The somatic embryos of nucellar origin could serve as means of clonal propagation of the chosen elite varieties.

In the course of this work, only two more reports were published regarding induction of somatic embryogenesis from nucellus. In one report published simultaneously with the present study (Ananthakrishnan *et al*., 1999), development of somatic embryos up to torpedo stage only was obtained. Whereas in the present study, somatic embryos developed to cotyledonary stage (Gogte and Nadgauda, 2000). In a subsequent report (Cardoza and D'Souza, 2002), germination of somatic embryos was reported, but conversion to plantlet did not occur. In the present study also, germination of somatic embryos has been observed, but it is precocious, and proper maturation of somatic embryos, and complete development of cotyledons seems necessary for conversion of somatic embryos.

Induction of somatic embryogenesis in immature zygotic embryo (IZE):

During initiation of these studies, there was one preliminary report regarding formation of embryo-like structures from zygotic embryo. As IZE was another juvenile explant obtained during studies conducted with nucellus, it was also used for possible induction of somatic embryogenesis. Somatic embryogenesis was also induced from immature zygotic embryos (IZEs).

Developmental stage of zygotic embryos was crucial for expression of embryogenic response. IZEs from 3 to 5 weeks post-fertilization stage only formed somatic embryos. It was observed that physical integrity of IZEs was necessary for somatic embryo production. Only intact undamaged IZEs formed somatic embryos.

Embryos were formed directly from tip of the radicle of the IZEs, in 5-7 weeks after inoculation. Auxin-cytokinin combination in the medium was favorable for somatic embryogenesis. More number of explants formed somatic embryos on MS + 2,4-D 5 μM + BA 5 μM + GA_3 3 μM + sucrose 3% + AC 0.5%. The somatic embryos showed normal development on the same medium. Somatic embryos matured on MS + ABA 20 μM + maltose 3%. They germinated on MS + AC 0.5%. More experiments are needed for improvement in conversion rates of somatic embryos.

In the time period of these experiments, another study of somatic embryogenesis from IZE was reported. In this study, IZEs were embryogenically responsive at very young stage of development. They formed somatic embryos only when they were in contact with the nucellar tissue, in presence of picloram in the medium. In the present study however, IZEs at very young stage (1-3 weeks post-fertilization) never formed somatic embryos. This difference in developmental stage showing embryogenic response again may be due to the different varieties used. In this study, IZEs could form somatic embryos when inoculated free of the ovular tissue. Auxin 2,4-D was necessary for induction of embryogenic response. In both studies, germination of somatic embryos has been obtained, however, conversion to plantlet remains to be achieved.

Once a suitable medium is formulated for somatic embryo conversion, the somatic embryo of IZE origin can be used effectively for propagation of selected rootstocks and hybrid varieties. Used in combination with an appropriately designed breeding program, the embryogenic system using IZEs could contribute to propagation and improvement of cashew. As IZEs form somatic embryos

directly, they can be used for genetic transformation procedures, and subsequent multiplication of transformants.

From the above results, it can be concluded that cashew is a recalcitrant species, and cultures have been established from juvenile explants only. In all experiments, developmental stage of the explant was important to obtain morphogenic response. Of the different explants used, cotyledonary nodes of seedlings and immature zygotic embryo are untested genotypes, and therefore may not be the explants of choice for clonal propagation of the chosen variety. However, these systems can be used for multiplying hybrid varieties. Such systems may be valuable for cashew, where variety improvement is carried out by breeding, and long generation cycles, low fruit set, and early fruit drop impede the success of breeding programs.

Nucellus being a maternal tissue, obtaining a regeneration system using this explant will be valuable for vegetative propagation of the selected superior elite mother plant.

More experiments need to be done for improving the survival rate of the axillary shoots during the hardening stage. Similarly, studies are necessary to devise an effective medium for conversion of somatic embryos obtained from nucellus and IZEs, in order to utilize the somatic embryos as propagules. Persistent efforts, and availability of novel chemicals will certainly help in achieving the desired success in this species.

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1. Nadgauda RS and Gogate SS (communicated) Somatic embryogenesis in cashew (*Anacardium occidentale* L.), to be published as part of book on Somatic Embryogenesis in Woody Plants, SM Jain (Ed)

2. Gogate SS and Nadgauda RS (2003) Direct induction of somatic embryogenesis from immature zygotic embryo of cashew (*Anacardium occidentale* L.). *Sci. Hort*. **97**: 75-82.

3. Nadgauda RS and Gogate SS (2003) In vitro regeneration and genetic transformation of cashewnut (*Anacardium occidentale* L.). *In*: PK Jaiwal and RP Singh (Eds) *Plant Genetic Engineering* Vol. 6 (*Improvement of Fruit Crops*), Sci-Tech Publishers, U.S.A., pp 41-64.

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