TISSUE CULTURE STUDIES IN NEEM (*Azadirachta indica* A. Juss): MICROPROPAGATION, EMBRYOGENESIS AND SECONDARY PRODUCT ANALYSIS.

A THESIS

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled " TISSUE CULTURE STUDIES IN NEEM (*Azadirachta indica*A. Juss): MICROPROPATATION, EMBRYOGENESIS AND SECONDARY PRODUCT ANALYSIS" submitted by Mrs. Medha Joshi was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

(Dr. S. R. Thengane)
Guide

Pune:

Date: 30th April 1997

KEY TO ABBREVIATIONS

$AdSO_4$	Adenine sulphate
AZ	Azadirachtin
B	Biotin
BAP	6-Benzylamino purine
B5	Gamborg's nutrient medium (1968)
CC	Column chromatography
CH	Casein hydrolysate
CM	Coconut milk
CP	Calcium pantothenate
$CaSO_4$	Calcium sulphate
$CDCl_3$	Deuterated chloroform
2,4-D	2,4 Dichloro phenoxy acetic acid
2iP	(2-iso pentenyl adenine) 6-(γ,γ-dimethylallylamino) purine
EtOH	Ethyl alcohol
FAA	Fixing agent (Formaldehye:Acetic acid:Absolute alcohol)
GA_3	Gibberellic acid
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KN	Kinetin (6-furfuryl amino purine)
LS	Linsmair and Skoog's nutrient medium (1965)
MHz	Mega hertz
$HgCl_2$	Mercuric chloride
MS	Murashige and Skoog's nutrient medium (1962)
NAA	α-Naphthalene acetic acid
HNO_3	Nitric acid
NMR	Nuclear magnetic resonance
RT	Room temperature
NaOH	Sodium hydroxide
SiO_2	Silicon dioxide
SE	Standard error
H_2SO_4	Sulphuric acid
TDZ	Thidiazuron
TLC	Thin Layer Chromatography
WB	Wood and Braun's nutrient medium (1961)

SYNOPSIS

Azadirachta indica A. Juss, commonly known as Neem, is recognized today, as potentially one of the most valuable of all arid-zone trees, because of its evergreen nature, its ability to grow in even the most arid and nutrient deficient soils, as well as its many commercially exploitable by-products and environmental beneficial attributes.

This tree is a native to the dry arid areas of India, Pakistan, Srilanka, Malaysia, Indonesia, Thailand and Myanmar, where it has been cultivated for ages. A multipurpose tree in the real sense, the tree has long been used in medicine, and also provides almost all the requirements like timber, fuelwood, fodder, oil, fertilizer or pest-repellent. It is therefore no wonder that it is recognized as "the most promising of all the plants" by National Research Council, USA. It is now been identified as " the Wonder Tree", "nature's gift to mankind", "the tree for solving global problems" or "global tree".

The present work was undertaken with the following objectives :

1. To standardize a protocol of micropropagation of neem for its mass production.
2. Investigating the optimum conditions for both the pathways of somatic embryogenesis.
3. Induction of callus and its analysis for secondary product.

This work has been divided into five chapters.

CHAPTER 1: GENERAL INTRODUCTION

This chapter brings out the role of tissue culture in general and in particular about tree/woody species in solving several global problems. It also summarizes the importance of the Neem tree in particular in the field of forestry, agriculture, medicine, etc. It includes a literature survey of the work carried out in Neem and also the present status of *in vitro* studies on it.

CHAPTER 2: MATERIALS AND METHODS (GENERAL)

This chapter describes the collection of Neem explant material, its surface sterilization, media preparation and the various culture methods used in the course of this study. Methods used for evaluating the results have been illustrated. The macro- and microelements and vitamin composition in the various basal media used for the studies have been presented in a tabular form. Procedures for transfer of plants obtained through various tissue culture methods to soil are dealt with in detail. Protocols followed for the chemical analysis of callus as well as for the histological analysis are described.

CHAPTER 3: MICROPROPAGATION AND PLANTLET FORMATION FROM *Azadirachta indica* A. Juss.

Section A: Shoot multiplication.

The optimum chemical and physical parameters for the mass production of Neem plantlets from nodal explants, were determined.

An elaborate study on the factors influencing shoot multiplication revealed that incorporation of the cytokinin 6-benzyl amino purine (BAP) into the MS medium elicited the best response, followed by Kinetin (KN) and 2- isopentenyl adenine (2iP). Combination of BAP and KN, however, was most effective. Incorporation of auxins like indole 3-acetic acid (IAA), indole 3-butyric acid (IBA) and α-naphthalene acetic acid (NAA) alone with the cytokinins failed to induce shoot multiplication. Effect of different carbon sources like, glucose, maltose, sorbitol and sucrose were also studied.

Enhancement of shoot multiplication ensued when shoots were cultured on medium containing additional vitamins like calcium pantothenate (CP) and biotin (B). Variable response of different dilutions of Murashige and Skoog's (MS) basal medium on shoot multiplication has been elucidated.

As a result of a series of experimental manipulations, a optimum medium composition for obtaining maximum shoot multiples from nodal explants was - 3/4 MS basal salts supplemented with MS vitamins, 0.05 mg/l KN, 0.1 mg/l BAP, 0.05 mg/l CP and B and 20 g/l sucrose.

The incubation temperature (25°C), light intensity (2000 lux) and photoperiod (16hr.) were found to be optimum for rapid shoot multiplication. Solid medium supported shoot multiplication while liquid medium promoted vitrification. Position of the explant on the tree and also the age of the explant influenced shoot multiplication. Number of shoots regenerated per explant went on increasing upto subculture no:7, beyond which new shoots did not form new shoots.

Section B: Root initiation from *in vitro* regenerated shoots.

Rooting could be initiated either *ex vitro* or *in vitro*. The *ex vitro* rooted plantlets however had poor survival percentage. Studies on *in vitro* root initiation revealed that transfer of shoots to plain MS medium resulted in 100% rooting as against 90% on 1/2MS supplemented with 2.0 mg/l IAA. Survival rate of both these rooted plantlets was 100%.

Section C: Transfer of plantlets to soil.

It was observed that the transfer of rooted plantlets to plain water prior to transfer to soil:sand mixture (1:1) gave better survival percentage.

CHAPTER 4: SOMATIC EMBRYOGENESIS IN *Azadirachta indica* A. Juss

Section A: Initiation

The type of auxin was found to be the deciding factor in the embryo induction pathway. When 2,4-dichloro phenoxy acetic acid (2,4-D) was used in combination with BAP or thidiazuron (TDZ), somatic embryogenesis was induced directly on the cotyledonary explants. However, when IAA was used in combination with BAP, compact nodular callus was induced followed by somatic embryogenesis. In both the media casein hydrolysate (CH) was used as an additional nitrogen source.

The embryogenic response of the callus increased by enhancing incubation period upto 8th week. However, after this there was gradual decline in embryogenic response.

Section B: Maturation and germination.

Indirect pathway: On testing various media for maturation and germination of the embryos, increased BAP concentration was seen to support maturation of embryos obtained via callus upto cotyledonary stage. 70 percent of the embryos germinated on a medium containing 2.0 mg/l BAP with complete omission of CH. The presence of auxin (IAA) was found to be essential throughout the protocol.

Direct pathway: Various media were tested for maturation and germination of the embryos induced directly on the cotyledonary surface.

(i). Embryos induced on medium supplemented with 2,4D and BAP.
The three media were tested viz.,
(a) Increase in BAP concentration with 2,4-D and keeping CH constant,
(b) Increase in BAP concentration with decrease in CH, and
(c) Total omission of 2,4-D,
all lead to either callusing or blackening of embryos.

(ii). Embryos induced on medium supplemented with 2,4-D and TDZ.

As prolonged incubation on TDZ leads to inhibition of somatic embryogenesis, these embryos were transferred to MS medium supplemented with only BAP which lead to precocious germination of the embryos. Very low concentrations of BAP promotes normal development of the embryos and also germination to plantlets.

Section C: Histology.

Indirect pathway: Meristematic pockets were observed in the inner cortical paranchymatous tissue of cotyledons which were pushed towards the periphery due to swelling and thus formed proembryogenic masses.

Direct pathway: Meristematic activity was seen in the epidermal and sub-epidermal layers of the explant which leads to the formation of embryos.

CHAPTER 5: SECONDARY METABOLITE ANALYSIS.

Preliminary experiments were carried out for the callus induction from seeds. Various parameters were studied for the induction and growth of callus, viz., basal medium, auxin (type and concentration), cytokinin (type and concentration), supplements (CH, $AdSO_4$, glycine, etc.) and carbon source.

The compact friable callus was chosen for secondary metabolite analysis. Such callus was observed on MS basal medium supplemented with MS vitamins, auxins IAA/IBA/NAA (0.5-5.0 mg/l) in combination with KN/BAP (1.0 mg/l). Ten grams calli obtained on these media were analyzed using Thin Layer Chromatography (TLC) (SiO_2 : $CaSO_4$ :: 77 : 13) with ß-sitosterol, epoxy azadiradione and de-acetyl nimbin as references.

The calli induced from cotyledonary pieces on MS basal medium supplemented with MS vitamins, IAA (0.5 mg/l) with BAP (1.0 mg/l) and NAA (0.5 mg/l) with KN (1.0 mg/l), showed significant spots on TLC. These spots showed Rf values corresponding to those of epoxy azadiradione and de-acetyl nimbin.

They were further analyzed using column chromatography, preparative TLC and ^{1}H-NMR (200,300 Hz). For column chromatography, column grade silica (60-120 mesh) was used. For preparative TLC, SiO_2 (400 mesh), with thickness of silica layer 2mm and size of the plate 20x20 cm was used. ^{1}H-NMR in $CDCl_3$ was carried out to find out the exact nature of the compounds isolated.

Further studies carried out on calli obtained on these media were:

(i) Effect of modification of MS basal media formulations on callus growth and product accumulation. These included different concentrations of phosphates, total omission of nitrates and phytohormones.

(ii) Effect of redifferentiation of callus on product accumulation.

From these studies, there was an indication of production of de-acetyl nimbin and azadiradione from dedifferentiated callus while differentiation caused only induction of monoglycerride of unsaturated fatty acid.

Conclusion

In the present work three major aspects of in vitro techniques, viz., micropropagation, somatic embryogenesis and secondary metabolite production have been studied. Even though tree/woody species are recalcitrant to in vitro culture, neem appears to be very responsive. Protocols for mass multiplication of neem has tremendous potential in rapid forestation programmes and also multiplication of selected elites. Somatic embryogenesis via callus or directly on cotyledons can be a starting point for isolation of variants and in transformation studies. Production of active principle in culture conditions can be further exploited and work can be directed solely towards in vitro production of Azadirachtin and other potent terpenoids.

CHAPTER 1

GENERAL INTRODUCTION

PART A: INTRODUCTION TO NEEM.

In the past few decades, the green revolution technologies have put forward high-input production systems that disregard the ecological integrity of land, forests, water resources and endanger the biota. These technologies have more than doubled the yield of food grains, especially in the developing countries. However, more than 20,000 pest species destroy one third of the food production annually. This amounts to losses of over one billion US dollars. Thus, the economic future of the developing countries will depend to a great extent on improving the productivity of the natural resources through the application of sustainable production methods and by improving tolerance of crops to adverse environmental conditions. In addition, control of post harvest losses caused by pests and diseases is of utmost importance.

Pest control, as practiced today in most of the developing countries relies mainly on use of imported technologies for the manufacture of synthetic pesticides like pyrethroids. However, use of synthetic pesticides is being increasingly objected by the environmentalists all over the world due to its short term and long term harmful effects on the environment as well as the public health.

As these pesticides are not biodegradable, their residues lead to contamination of terrestrial and aquatic environments, damage to beneficial insects and wild biota, accidental poisoning of humans and livestock, and the twin problems of pest resurgence and resistance. Pest resistance is increasingly becoming a serious threat to sustainability of agricultural production systems.

For these reasons, there is a need for pest control agents that are pest-specific, non-toxic to humans and beneficial organisms, biodegradable, less prone to pest resistance and resurgence and relatively less expensive.

Among the 2400 plant species studied for pesticidal properties, neem holds out the promise of providing environmental friendly natural pesticides.

For centuries, the neem tree has been revered for its range of products such as fodder, fuel, pesticides, slow-release fertilizers, medicines, cosmetics, toiletries and other services to mankind such as providing shade, shelter and pollution-free environment. Currently, it has attained a pride of place in the

international scientific research and literature. It is now being identified as **"nature's gift to mankind"**, "the **wonder tree**", "the tree for solving global problems" or "**Global tree**".

The development of natural pesticides like NEEM-based pesticides in particular is of great relevance. Considering its importance, active research and development activities are being carried out all over the world to develop the optimum process technology for the production of neem-based pesticides.

The National Research Council set up by US National Academy of Sciences has brought out a study which emphasizes the potentialities of this tree. According to this study, "All this is potentially of vital importance for the developing and underdeveloped countries, which have high rates of population growth, severe problems with agricultural pests and widespread lack of even basic medicines".

From a tree meant to solve problems of the under-developed world, neem products are now much sought after in the international commerce as well. Several isolated groups of researchers are working on varied aspects of neem research. The scientific information on neem had been consolidated and brought into focus through seminars and International Conferences. Germany played a pioneering role by organizing successively two international conferences in 1980 (Roltach-Egern) and in 1983 (Rauisccholzhaussen). The third conference was held in Nairobi, Kenya (1986). In India, Indian Agricultural Research Institute (IARI, New Delhi), hosted a National Seminar on "Neem in Agriculture" in 1983. A World Neem Conference was organized by Indian Society of Tobacco Science (Andhra Pradesh) and sponsored by IARI (New Delhi) and Indian Tobacco Company (ITC) Ltd. (Calcutta) in 1993. Recently, another International Neem Conference - "Phytopesticide for the Modern World", was held in Gatton, Australia in February 1996. This conference highlighted the universal application of neem extracts for the more environmental friendly control of insects, nematodes and fungi under both present and future conditions.

The aim of all these conferences and seminars was to bring together scientists who are working on neem so that an overview of present research can be developed and also provide a guideline for the future research.

1.1. BOTANY

Azadirachta indica A. Juss (synonym *Melia azadirachta* L.; *Melia indica* A. Juss Brand; *Antelea azadirachta* or *Melia parviflora* Moon) belongs to the family Meliaceae (Mahogany). It's centers of origin lies in southern and south-eastern Asia. It also occurs in tropical and subtropical areas of Africa, America and Australia. During the last century, it was introduced in many countries mainly for afforestation and fuelwood production in dry areas. It is now being widely planted in Fiji, Mauritius, The Carriebean, Haiti, Philippines, Puerto Rico and other countries as an avenue or shade tree and also as a producer of natural pesticides.

The neem or margosa, also called as Indian lilac, is an evergreen or deciduous, fast-growing tree which can grow upto a height of 25m. It has a spreading habit with a broad crown **(Fig. 1.1)**. In the native habitat it occurs at altitudes between 50 and 100m. It can grow on nutrient deficient soils and is tolerant to high temperatures, higher rainfall upto 400-800mm and even extended dry season (Ketkar, 1976; Radwanski and Wichens, 1981). Although the tree grows well at pH 5, its leaf litter gradually brings the surface soil pH to neutrality (Radwanski, 1969).

The leaves which may also be used for pest control, are usually medium sized, green, imparipinnately compound, alternate, exstipulate, 20-38 cm long and crowded at the ends of the branches. The asymmetric, serrate leaflets are 7-17 in number and upto 7 cm long.

Flowering in neem starts after the tree reaches 5 years of age. The flowering season starts in January and extends upto May. The inflorescence is long and slender, axillary or terminal panicle with abundant flowers **(Fig. 1.2)**. The flowers are white or pale-yellow with characteristic sweet aroma. They are small, bi-sexual, pentamerous and bracteate. They are cross-pollinated and fertilization is porogamous. The seed is ovoid or spherical, pointed above and has a thin testa. Under natural conditions the seed generally falls to ground during the rainy season and germinates in 8-15 days (Mohan Ram and Nair, 1993).

Fruits are the most important source of the ingredients of neem that affect insects in various ways. The fruits are produced in drooping panicles **(Fig. 1.3)**,

Figure 1.1: The Wonder Tree

Figure-1.1

Figure 1.2: Imparipinnately compound, alternate, exstipulate leaves and panicle inflorescence with small, bisexual, pentamerous and bracteate flowers.

Figure 1.3: Drooping panicles of neem.


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which mature usually from May to August. Each fruit is 2cm long and when ripe, has a yellow fleshy pericarp, a white hard shell and a brown oil-rich kernel. The yield ranges between 30-100 kg per tree depending upon the ecosystem, genotype and habitat.

1.2. CHEMISTRY

Upto 1940, the chemical investigations aimed at isolating active principles did not yield any well defined products mainly due to poor separation and analysis techniques prevalent at that time.

The pioneering work on the isolation and identification of the tree's constituents was initiated in India in 1942, by Siddiqui, Council of Scientific and Industrial Research (CSIR, New Delhi). He succeeded in isolating the first major bitter principle, NIMBIN, in a crystalline form. In addition, another crystalline compound Nimbinin and an amorphous bitter principle Nimbidin were also isolated. However, the structure elucidation was successful after a gap of 20 years i.e. in 1962 (Narayanan *et al*, 1962)

During the early sixties, the agricultural scientists unravelled that neem was a storehouse of biologically active materials of interest in repelling pests and in regulating release of fertilizer nitrogen (Pradhan *et al*, 1962). The bitterness of the tree is attributed to the presence of a wide array of biologically active compounds which are chemically diverse and structurally complex. In addition to the general type of constituents such as carbohydrates, fatty acids, amino acids, sugars and polysaccharides, a number of novel chemical compounds have been isolated from various parts of the tree. These compounds include protolimonoids, limonoids or tetranortriterpenoids, pentanortriterpenoids, hexanor-triterpenoids and nontriterpenoidal constituents. These have been well reviewed by Mitra(1970), Schmutterer *et al*(1981), Kraus(1983), Schmutterer and Ascher (1984) and Siddiqui *et al* (1988).

Among the tetranortriterpenoids, three compounds obtained from the seed are quite active as insect feeding deterrents, toxicants, and (or) disruptants of growth, against a large variety of insect species and nematodes (Warthern Jr., 1979). These specific compounds are melantriol, salanin and azadirachtin first isolated by Lavie *et al* (1967) Henderson *et al* (1964) and Butterworth and Morgan

(1968), respectively.

Out of the three, azadirachtin (AZ) is the most active principle in neem seeds. The percentage of AZ in the seed varies from 0.3% (India) to 0.6% (Africa). AZ works at very low concentrations, viz., 1-10 parts per million, but is sensitive to UV light and the action of acids. The direct effect of AZ is on the neuroendocrine system and it also disturbs the homeostatic situation in an insect in a irreversible way (Rembold, 1993).

The structure of AZ was established after a gap of almost a decade in 1986. Three international teams viz., Nakanishi *et al* (Turner *et al*, 1987), Morgan *et al* (Bilton *et al*, 1987) and Kraus *et al* (1987), arrived at the correct structure of AZ based on detailed NMR and x-ray crystallographic studies.

AZ(A) was the first member of the group of meliacins, isolated by Butterworth and Morgan. So far, 13 analogues/ derivatives have been reported from neem, namely Azadirachtin A, B, D, E, F, G, H, I, and K, 3-desacetyl-3-cinnamoyl azadirachtin, vepaol, isovepaol, 1,3-diacetyl-11,19-deoxa-11-oxo-meliacarpin and 11-methoxy-azadirachtinin (Rembold, 1990).

The synthesis of AZ has offered a challenge to synthetic organic chemists because of its complexity. Scientists at the Imperial College of London, led by Dr. Steven Ley, have achieved an important breakthrough in synthesizing the furan and decalin fragments of AZ (Ley *et al*, 1993). Dr. Ley in collaboration with entomologist Monique Simmonds of Royal Botanical Gardens, London, have shown that the two parts have different insecticidal properties. Dr. Ley has further improved AZ resistance to acids by alkylating the hydroxyl group. They have made the molecule more resistant to UV light by hydrogenating its double bonds, thus increasing the shelf life of AZ.

Today, more than 100 triterpenoids have been reported from neem. A large series of tetranortriterpenoids and pentanortriterpenoids were isolated from seed oil by Kraus and Cramer (1978) and Kraus *et al*(1981), Lucke *et al* (1980), Garg and Bhakuni (1984) and Kubo *et al* (1984). Other components isolated are ß-sitosterol (Quasim and Dutta, 1970), fatty acids (Skellon *et al*, 1962), amino acids (Mitra and Misra, 1967) and flavonoids (Subramaniam and Nair, 1972).

Table 1.1: Some of the important constituents and their location in the plant.

Plant part	Chemical constituents
Bark	Nimbin,Nimbinin,Nimbidin, Nimbindiol,tannins
Flowers	Flavonoids, Nimbosterol, Quercetin
Heart-wood	Nimbatone
Leaves	Quercetin, Nimbosterol, gedunin
Seed	Azadirachtin,de-acetly nimbin,meliacin, melantriol, salanin

A project on the utilization of neem as pest control agent was initiated by Nagasampagi and his group at National Chemical Laboratory (NCL, Pune), India, in 1979. A simple process was developed for obtaining two pest control fractions, namely Neemrich I and Neemrich II from neem seed/cake/oil for commercial use. Both the fractions contain different compounds. Neemrich I contains mostly fatty acids, little amount of nimbin and azadiradiones and was found to show oviposition deterrent activity. Neemrich II contains 4% AZ with 24 derivatives related to AZ skeleton and around 95 to 96 other tetranortriterpenoids. This fraction exhibits insect-antifeedant and growth-inhibitory activity.

Their long term research has led to a number of publications in prestigious journals (Nagasampagi, 1993; Rojatkar and Nagasampagi, 1994, 1995), three Indian patents (Nagasampagi *et al*, 1991) and one US patent (Nagasampagi *et al*, 1995). They have also transferred the technology for production of these fractions to four Indian industries viz., Indofil India Ltd., Mumbai, Maharashtra; Voltas India Ltd., Hyderabad,Andhra Pradesh; Monofix India Ltd., Hubli, Karnataka and Bioscense Ltd., Mumbai, Maharashtra. **Table 1.9**, highlights the landmarks in the chemical investigations of neem.

1.3. USES

The commercial value of neem was known from the Vedic period in India over 4000 years ago and the domestic uses have been mentioned by Kautilya in his 'Arthashastra', as early as 4th Century B.C. Neem has multifarious properties and uses in forestry, agriculture, as an insecticide and in the pharmaceutical and

cosmetic industries (Koul *et al*, 1990).

1.3.1. Biomass production and utilization:

Full grown neem trees yield between 10 to 100 tons of dried biomass per hectare, depending on rainfall, site conditions, spacing, ecotype or genotype. Proper management of neem can yield harvests of about 12.5 cubic meter (40 tons) of high quality solid wood per hectare.

The wood is aromatic and moderately heavy (sp.gr. = 0.56 - 0.85; mean = 0.68, National Academy of Sciences, USA, 1980). The wood resembles teak in its strength and properties.

Table 1.2: Properties of neem timber in comparison to teak.

Properties	Quality Index	
	Teak	Neem
Weight	100	124
Strength (as beam)	100	087
Stiffness (as beam)	100	081
Suitability (as post)	100	082
Shock resistance	100	105
Shape retention	100	077
Shear	100	129
Surface hardiness	100	131
Nail holding ability	100	144

Source: CSIR (1985)

The timber seasons well, except for end splitting. Being resistant to fungi and insect attack and immune to termite attack, it is used in making fence posts, poles for house construction, furniture, ship and boat furniture, agricultural implements, etc.

Pole wood is especially important in developing countries. The tree's ability

to coppice is highly suitable for pole production (Vietmeyer, 1992). Neem grows fast and is a good source of fuel wood and fuels; the charcoal has a high calorific value.

1.3.2. Scope in reforestation and agroforestry:

Neem is a valuable forestry species in India and Africa and is becoming popular in tropical America. Being a hardy tree, it is ideal for reforestation programmes and for rehabilitating degraded semi-arid and arid lands, and coastal areas.

With growing concern to prevent the spread of desert and ameliorate the desert environment, neem has become an important plantation species. According to Radwanski (1969), the tree improves soil fertility and water holding capacity as it has a unique property of calcium mining, which changes the acidic soils to neutral.

From the agroforestry point of view, neem has the advantage of supplying much needed fodder and fuel to desert inhabitants. In countries from Somalia to Mauritania, neem has been used for halting the spread of the Sahara Desert.

Neem is useful as windbreaks in areas of low rainfall and high windspeed. It can protect crops from desiccation. In the Majjia valley in Niger, over 500 km of windbreaks comprising of double rows of neem trees have been planted to protect millet crops which resulted in 20% increase in grain yield (Benge, 1989).

Being hardly leafless, it is widely planted for shade and ornamental purposes in drier parts. It is therefore popular as an avenue tree. A decade ago, 50,000 neem trees were planted in the Plains of Arafat near Mecca in Saudi Arabia to provide relief from the blazing summer sun to pilgrims (Ahmed, 1989).

1.3.3. As a pesticide:

Due to the high cost of petroleum based synthetic pesticides, ecological hazards and many other undesirable problems, botanical pesticides are gaining importance.

Of the more than 2400 plant species studied for insecticidal and insect

repellent properties, only neem holds the promise of being highly effective, non-toxic and environmentally harmless means of controlling and eliminating pests.

In the past decade, attention has been focused on the pest control value of neem as its products are highly competitive in price and quality. Neem does not kill but incapacitates pests. It shows diverse biological effects, which include the repellent, anti-feedant, oviposition deterrent, growth regulatory and sterilant activities. It also impairs egg hatchability.

The neem products have been found to be highly effective in controlling more than 300 species of insect pests affecting different crops (Schmutterer, 1990).

Various neem products have been used as pest control agents viz., neem oil (NO), neem seed kernel water extract (NSKE), neem cake extract (NCE) and neem seed bitter (NSB) (Singh, 1993). The bioefficacy of the neem derivatives has attracted attention of the pesticide industry in India and worldwide. Nearly two dozen products are being marketed in India **(Table 1.3)**. Most of the products are either oil-based or based on various extractives.

Table 1.3. Some of the important products currently available in the market.

Product	Manufacturer
Achook	M/S Godrej Agrovet Ltd., Mumbai, Maharashtra
Neemasol	EID Parry India Ltd., Madras, Tamil Nadu
Neemgold	M/SSouthern Petro Chemical Industries Corporation Ltd., Madras, Tamil Nadu
Agrisef	Meghavyl Industries, Gundlav, Gujarat
RD-9 Repelin	M/S ITC-ILTD Ltd., Rajahmundry, Andhra Pradesh
Fortune Aza	Fortune Biotech Ltd., Secunderabad, Andhra Pradesh
Suneem	Sunida Exports, Mumbai, Maharashtra

1.3.4. As a fertilizer:

Long before the introduction of chemical fertilizers, the oilseed cakes of particularly those of groundnut, castor and mahua were used as a source of plant nutrients, specially by the growers of cash crops like cotton and sugarcane. However, field experiments conducted during 1896-1931 near Pune (Ketkar, 1976), revealed that the neem cake has high nitrification rate (55.6%) as compared to castor (52%) and groundnut (48.6%). Thus, with the current thrust on sustainable agriculture and organic farming, the use of natural resources like neem has a greater practical significance especially in augmenting efficient use of nitrogen.

The neem seed cake, a residue of the oil extract, has a greater concentration of N, P, Ca, Mg than farmyard manure and has a high sulphur content relative to other oil cakes (Sinha, 1960).

Bains *et al* (1971), were the first to show under field conditions that treatment of urea with an acetone extract of neem kernel reduces ammonia volatilization caused by nitrifying bacteria. Urea coated with neem oil is more effective than urea blended with neem cake in reducing ammonia volatilization (Singh and Singh, 1984).

A systematic investigation of the neem seeds through a protocol involving extraction, screening of different extractives for nitrification, isolation of pure major constituents and their detailed screening for nitrification retardation was carried out by Devkumar (1986). Out of the three fractions obtained from fresh seeds *viz., pure lipids devoid of bitter (NL), lipid associated meliacin fraction (LM) and* hexane-insoluble polar meliacins (PM), the meliacin fractions were found to be the seats of nitrification retardation activity.

When alcoholic extract of the cake was mixed with urea and ammonium sulphate fertilizers, high nitrate levels could be achieved. Inhibition of nitrification by neem cake was reported (Chhonkar and Misra, 1978), to last for a much longer period. After 160 days of incubation, ammonium nitrogen content in soil

was 17.8 ppm with urea blended with neem cake as compared to 5.6 ppm with prilled urea.

Neem thus serves as an excellent manure, due to its anti-microbial activity and slow decomposition action leading to release of nutrients. These attributes have led to production of many neem-based commercial products as shown in Table 1.4 .

Table 1.4. Some of the commercially available neem-based fertilizers.

Product	Manufacturer
Nimin	M/S Godrej Agrovet Ltd., Mumbai, Maharashtra
Jeevan soil conditioner	M/S MCDA Agro Pvt. Ltd., Mumbai, Maharashtra
Neem manure	M/S Swastik Chemicals Works, Mumbai, Maharashtra
Humi-gold	M/S Fertiplant Engg. Company, Mumbai, Maharashtra
Wellgro	M/S ITC-ILTD Ltd., Rajahmundry, Andhra Pradesh
Organic and Neem cake mixed NPK	M/S Jaisingpur Mills, Kolhapur, Maharashtra

1.3.5. As a therapeutic agent:

There are several references to use of 'Nimba' as a handy remedy from the Vedic times. The words Nimba, Nimban and Nimbapatra along with their compounds occur in the Vedas and Atharvaveda parisista (1500 B.C.). Nimba was known to posses a reputation in the society because of its health promotive and curative properties. It is called the 'Kalpavrksa' of the earth and a domestic doctor in India due to its common availability and wide efficacy.

All the parts of neem viz., roots, stem, leaves, flowers, fruits, seeds, bark and gum have therapeutic uses. Neem trees remove toxicity from unhealthy and

noxious air that may otherwise cause health problems.

The pharmacological properties of neem includes anti-inflammatory, anti-leprosy, antiseptic, anti-arthritic, anti-pyretic, anti-microbial and anti-helminthic actions.

The leaves as crude drug are used in leprosy, cholera, jaundice and skin diseases. The leaf extract is either taken orally or the juice is applied on the skin. Neem seed oil has been used for antimalarial, anti-helminthic, vermifuge, febrifuge, antiseptic and anti-microbial purposes. It is also used to control bronchitis and as a healing agent against various skin disorders (Chopra *et al*, 1952; Radwanski, 1977; Sinniah and Bhaskaran, 1981).

Certain organosulphur compounds of *A. indica*, such as sulphone dapsone, have been found to be anti-leprotic. These sulphones (Gupta and Joshi, 1983), along with aqueous leaf extracts also act as anti-malarial agents. The analgesic and anti-pyretic effects of the components are encouraging. These have been shown to reduce eosinophil count by 75% in eosinophilia patients (Gopinath, 1983).

Another widely studied effect of neem has been its antifertility properties. Riar *et al* (1988), reported that neem oil is absorbed through the vaginal mucosa into its circulation and exerts antifertility effects. Its oral administration to female albino rats has prevented pregnancy (Khare *et al*, 1984; Lal *et al*, 1987), with normal restoration of fertility after 30 days. Dr. Talwar and coworkers (Indian Council of Medical Research, ICMR, New Delhi), have formulated a neem based oral contraceptive, 'Praneem', for post-coital application. Praneem contains the bitter fraction of neem oil (Talwar *et al*, 1993). This polyherbal cream was tested in rabbits, monkeys and also human beings. Moreover, the cream has dual benefits of contraceptive protection and anti-microbial effect.

Table 1.5. Biological properties of neem preparations.

Preparation	Biological activity		
	As a pesticide	Therapeutic use	Others
Oil	antifeedant, growth regulatory	antifertility, anti-microbial	soap making
Seed kernel extract	oviposition deterrent, antifeedant	anti-inflammatory	-
Leaf extract	-	blood purifier, antileprotic	-
Deoiled seed cake	-	-	organic manure
Root extract	-	-	Preperation of toddy
Bark/Stem extract	-	antipyretic, antiseptic	-

1.3.6.1. Livestock health:

The use of 'Nimba' in veterinary medicine dates back to times of Mahabharata (5561 B.C.). Nimba was known to posses a reputation in society because of its health-promoting and curative properties. Even today, a large number of indigenous herbs and plants are used in veterinary practice along with neem. Different formulations are used for the treatment of various diseases in cattle e.g. rinderpest, malignant sore throat, tuberculosis, leprosy, parasitic infestations, rheumatism, milk fever, etc.

Almost every part of the tree finds application in indigenous veterinary medicine. The stem and root bark and young fruits are reported to posses astringent, toxic and anti-periodic properties. The leaves are effective in helminthiasis. They posses antiviral, antiseptic properties. The hot infusion of

leaves is used as anodyne for fomenting swollen glands, bruises and sprains. The fruit is used as a tonic, purgative, emollient and as an antihelminthic. The oil is mainly used against skin disorders. It is found to be antipyretic and non-irritant. Neem oil has shown antihyperglycaemic effect in dogs (Bhargava *et al*, 1985).

The neem leaves and twigs have appreciable quantity of total digestible nutrients, specially digestible crude protein. They are therefore used in small quantities in animal and poultry feed. Neem leaves have low fiber content, but they help in alleviating the copper deficiency when feeding with straw and dry fodder. In cattle and goats, if fed immediately after parturition, it helps to increase secretion of milk. However, its use in poultry feed has to be restricted as it has deleterious effects on the birds.

1.3.6.2. Livestock products:

The alcoholic extract of fresh stem bark is useful in improving animal product technology. The stem bark contains 12-16% of tannins and bitter principles nimbin (0.04%), nimbinin (0.02%) and nimbidin(0.4%). When the alcoholic extract was rubbed on the goat skin, the goat skin was found to be superior to avaram-tanned goat skins in lightness of colour, feel and tannin strength. The Central Leather Research Institute, Madras, has developed a method of curing hides and skins with a mixture of organic compounds obtained from margosa oil. The leathers prepared by this method do not deteriorate in quality during storage (Singh, 1993).

1.3.7. Toiletries and Cosmetics :

Neem is reputed to have many curative properties and is widely accepted as herbal medicine. In general, leaf and bark extracts, neem oil and isolated compounds of neem are now being used by industries in their products.

The largest outlet of neem oil in India is the soap industry, which markets about 10 different brands of bathing and laundry soaps **(Table 1.5)**. Neem oil is also used in the manufacture of dog soaps and shampoo that controls ticks and fleas. In Germany, it is used in making herbal hair oil, hair tonic and nail oil.

Neem twigs are used daily by millions in India and Pakistan as disposable tooth brushes. Today, extracts of neem bark 'Silvose T' and 'Silvose TRS' are

used in tooth pastes and mouth wash. Neem is also being used in cosmetics due to its skin-care properties **(Table 1.6)**.

Table 1.5: Some Neem based toiletries marketed in India.

Product	Manufacturer
Soaps and Shampoos	
Margo	M/S Calcutta Chemical Co. Ltd., Calcutta
Feu Drop	M/S Nand Kishore Khanna & Sons, Mumbai
Kutir Neem Sandal Soap	M/S Thanjavur Sarvodaya Sangh, Tamil Nadu
Nirmala Neem Dog Soap	M/S Nirmal Enterprises, Mumbai
Dentrifice and Mouth Wash	
ORA	M/S Nand Kishore Khanna & Sons, Mumbai
Nimodent	M/S Hamdard Co., Karachi
Dr. Grandel's Neem tooth-paste	Keimdiat Gmbh, W. Germany

Table 1.6. Some Neem based cosmetics marketed in India.

Product	Use	Manufacturer
Neemtulsi	ayurvedic prickly heat powder	M/S Puma Ayurvedic Herbal Pvt. Ltd. Nagpur
Neemal	face-pack	——"——
Licika	anti-lice oil	——"——
Clean N Care	skin care	M/S Dabur India Ltd., Delhi
Curoline	antisceptic cream	M/S Chemicure Laboratories Pvt. Ltd., Udaipur
Kailas Jeevan	skin care cream	M/S Ayurvedic Sumsodhanalaya, Pune
Lotion Capillaire Oile Capillaire	hair and scalp tonic	Dr. Hauschha Wald-Heilmittel, Gmbh, W. Germany

PART B: ROLE OF BIOTECHNOLOGY IN NEEM

Biotechnology is defined as 'The integrated use of biochemistry, microbiology and chemical engineering in order to achieve the technological applications of the capacities of microbes and cultured cells'. It holds enormous promise to fulfill some of humanity's most fundamental needs - from increasing food and energy supplies to improving health care.

Since the last two and a half decades and more, plant cell and tissue culture has emerged as a strong branch of plant biology with possibilities for complementing the conventional methods in finding solutions to some of the practical problems in agriculture and forestry. Plant cell and tissue culture in simple terms, is defined as a technique by which single plant cells, unorganized groups of cells (callus) or organized cell masses (tissue) can be induced to multiply or regenerate into plants by manipulation of the chemical and physical environment of the culture.

The pre-requisites of successful plant cell and tissue culture are tissue specificity (genotype, source), media composition (minerals, phytohormones, other organics, supporting agents), culture conditions (light, temperature, gases, size of vessel), culture period and interactions between all the above mentioned factors. The precise requirement of all these factors are species specific.

The different pathways for plant production by tissue culture are:-

a. axillary shoot elongation
b. organogenesis
c. somatic embryogenesis

For the commercial propagation of plants, axillary shoot elongation is preferred. It is the easiest method and maintains genetic stability better than propagation by organogenesis (McCown and McCown, 1987). The merit of using this technique as a means of regeneration is that incipient shoot has already been differentiated *in vivo*. Thus, to establish a complete plant, only elongation and root differentiation are required (Hu and Wang, 1983). This system is nowadays described as conservative because of its ability to produce true-to-

type plants without genetic change.

In organogenesis, adventituous shoots are induced from sites other than bud meristems. Rarely are roots induced before shoots and often there is a passage through a callus stage. Organogenesis has great potential for multiplication. However, it faces several problems; first of all the plantlets regenerated are not genetically true-to-type. Secondly, there is variation in growth behaviour of the plantlets which leads to heterogeneous growth under field conditions. This is because in *in vitro* conditions, some clones show plagiotropic growth and poor vigour, while other clones show orthotropic growth and vigour typical of young seedlings.

A more promising propagation technique for the economical production of large number of plantlets is somatic embryogenesis. Somatic embryogenesis is the *in vitro* production of embryo-like structures from somatic tissue which are potential miniature plants. For mass multiplication, somatic embryogenesis is preferred over organogenesis. In organogenesis *in vitro* induced shoot and root are two separate steps. However, somatic embryos are a bipolar entity and hence, in embryogenesis one difficult step of root induction is bypassed, thus decreasing labor costs.

Among the different species of plants which are responsive in culture, the woody species were the most neglected ones as they were assumed to be intractable in culture. However, the vegetative propagation of these species using tissue culture has progressed significantly over the years and the list of species which are propagated by *in vitro* methods have been reviewed from time to time by several workers viz., Bajaj (1986), Bonga and Durzan (1987), Hanover and Keathley (1988), Ahuja (1993), Jain *et al* (1995). These include timber yielding (rosewood, sandalwood, teak, eucalyptus), fast growing, nitrogen fixing trees (*Acacia*, *Alibizzia*, *Leuceana* sp.), palms (coconut, date, oil), horticultural crops (mulberry, apple, citrus), medicinals (*Taxus*, *Mappia*) and also gymnosperms (pine, larch, spruce, *Podophyllum*).

In addition to solving problems related with mass multiplication, plant cell and tissue culture has tremendous potential for the production of active principles from plants which are important in pharmaceuticals. The role of plant cell and

tissue culture in the production of important compounds directly in culture has been proved over the years. The most successful story to date is that of shikonin from *Lithospermum erythrorhizon* (Mizukami *et al*, 1977). Other commercially important active principles isolated from tissues grown in culture are atropine (Khanna *et al*, 1977), rosmarinic acid (Razzaque and Ellis, 1977), camptothecin (Misawa *et al*, 1985), etc.

As compared to conventional propagation plant cell and tissue culture system has certain advantages for the production of secondary compounds. The system can provide a weather, disease independent and continuous homogenous supply of plant material in a uniform physiological state. Such material can also be used to generate undiscovered novel compounds in addition to potentially known compounds. Moreover, higher levels of secondary compounds than the parent plant can be produced.

The three approaches for production of secondary metabolites are:

a. *De novo* synthesis: In this the desired product accumulates spontaneously in the production medium. For this, generally high yielding lines are chosen (Zenk *et al*, 1977).

b. Precursor feeding: This includes exogenous supply of a cheap biosynthetic precursor to the culture to increase the yield of the final product.

c. Biotransformation: This includes the production of valuable products by bioconversion of cheap precursors using cell cultures.

Although the suspension cultures are the most preferred culture systems for the production of secondary metabolites, an alternate approach is to use immobilized plant cells. In the immobilization technique, the cells are entrapped in different polymeric matrices e.g. alginate, carragenum, agar, agarose, etc. The cells are thus protected from liquid shear and they can also be used in a bioreactor for the continuous conversion of substrate into product. It also allows the easy separation of the product and the metabolic inhibitors accumulated can

be easily dialyzed away. Moreover, the high cell density results in increased productivity (Thengane and Mascarenhas, 1987).

1.4. Scope of Biotechnology in Neem Research:

Even though neem is an indigenous, multi-purpose tree with wide adaptability and valuable in forestry, agroforestry and pharmacology, certain basic aspects like information on the tree genetics, propagation, agronomy, scientific collection and processing and storage of the raw materials has been lacking. The product development, standardization, stabilization, shelf-life, etc., are also inadequately attended. Thus, there are several areas in every aspect of neem research and use where gaps in knowledge exist.

The world neem conference pointed out some areas where future research should aim:

1. Research on genetic divergence in neem and their phenotypic stability.

2. Research on maximising the viability, vigour and storability of neem seed.

3. Selection of varieties with higher oil content and high yielding capacity.

4. Research on of quality control and general biological properties.

5. Methods to check the photodegradability of neem formulations.

6. Efficacy of insect repellents and antifeedant property of neem can be enhanced by improved ecotypes, better timing and application methods.

7. Use of tissue culture and biotechnology approaches to obtain large number of neem trees with high yield in shorter time.

8. Making neem cultivation mandatory in all social forestry and other gardening programmes.

9. Future research on aspects of manufacturing ready-to-use and effective neem formulations.

1.5. LITERATURE SURVEY

1.5.1 Tissue culture studies :

There are numerous potential applications of in vitro techniques towards improvement of neem. The interest lies in mass propagation on a commercial scale, in developing high yielding lines with improved seed quality with high oil content and production of active principles from callus tissue.

Several workers have reported successful regeneration of plantlets in *A. indica* by using a variety of explants like mature and immature embryos (Rangaswamy and Promila, 1972; Thiagarajan and Murali, 1996), stem (Sanyal *et al* 1981; Jaiswal and Narayan, 1984), axillary buds (Jaiswal and Narayan, 1983; Drew, 1996; Joshi and Thengane, 1996), leaves (Schulz, 1984; Narayan and Jaiswal, 1985; Ramesh and Padhya, 1987), bark (Sanyal and Datta, 1986), cotyledons (Muralidharan and Mascarenhas, 1989; Shrikhande *et al*, 1993; Nirmala Kumari *et al*, 1996), anther (Gautam *et al*, 1994) and inflorescences (Nirmala Kumari *et al*, 1996). **Table 1.7** enumerates the tissue culture studies carried out on neem.

In most cases, proliferation of shoot and root meristems was either direct or via callus. The first report of morphogenesis was from Delhi University by Rangaswamy and Promila (1972), where development of normal seedling or differentiation of 10-20 shoot buds from plumular callus was observed when mature embryos were cultured on minimal medium containing Hoagland's solution in distilled water. However, shoot bud morphogenesis was not accompanied with root formation.

In most of the reports, plant regeneration has been achieved on Murashige and Skoog's (MS, 1962) basal medium supplemented with different growth hormones like 2, 4-dichloro phenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), 6-benzyl amino purine (BAP), kinetin (KN) and 2-isopentenyl adenine (2iP), individually or in

various combinations and concentrations (0.05 - 2.0 mg/l). In all the reports, differentiation of either shoot or root meristems was via callus.

Sanyal *et al* (1981, 1983, 1986, 1988) conducted a series of experiments to study the effect of hormonal regime on regeneration and histochemical differentiation in neem. They used stem bark as their explant. MS medium and a specially formulated SD (Sanyal and Datta, unpublished data) medium which contained calcium nitrate, sodium sulphate, potassium chloride, sodium nitrate, ammonium sulphate and sodium dihydrogen phosphate, vitamins used by Chaturvedi and Mitra (1974) as well as two vitamins biotin and riboflavin were used as basal media. Among the different auxins (IAA, IBA, NAA, 2,4-D) and cytokinins (BAP, KN) tried singly in different concentrations (0.1-1.0 mg/l); NAA and IAA at high concentrations induced roots. IBA and 2,4-D failed to induce either shoots or roots. Whereas BAP at 0.5 mg/l produced shoot buds earlier and in higher number than KN. Addition of adenine sulphate (10 mg/l) gave best results with development of leafy shoots. Sucrose (30 or 50 g/l) was used as carbon source in all the experiments.

Jaiswal and Narayan (1984), reported production of callus as well as green protuberances from the cut ends of stem segments cultured on MS medium supplemented with 0.5 mg/l BAP or KN. However, further development of these shoot buds was not favorable on the same medium. Addition of auxins IAA or NAA (0.05 - 0.1 mg/l) to the same medium promoted shoot bud growth. Vigorous elongation of the shoot buds was observed within 60 days. BAP (0.5 mg/l) along with NAA (0.1 mg/l) induced both shoot as well as root.

Stem segments cultured on MS medium supplemented with BAP (0.5 mg/l) and 2,4-D (0.1 mg/l) induced yellowish white callus. This callus when subcultured onto MS medium supplemented with IAA or NAA (0.05-1.0 mg/l) produced roots after 20-30 days in culture. However, if this callus was subcultured onto medium containing BAP/KN (0.05 - 1.0 mg/l), shoot bud formation took place after 30-35 days. Regenerated shoots when transferred to medium containing IAA (1.0 mg/l) in combination with KN (0.05 mg/l) developed roots within 4 weeks of transfer.

Another popular explant used for morphogenesis has been leaf discs. Leaflet explants cultured on MS medium containing 0.5 mg/l 2,4-D in combination with BAP (0.05 mg/l) produced callus (Narayan and Jaiswal, 1985). Shoot buds were

induced from this callus when subcultured onto medium supplemented with BAP (0.05 - 1.0 mg/l) alone. Addition of NAA (0.05 mg/l) increased the frequency of shoot formation (62.5 ± 8.5). Rooting (3-5 roots) was maximum (50%) on MS liquid medium supplemented with IAA (1.0 mg/l).

Ramesh and Padhya (1987), however, used Wood and Braun (WB,1961) medium and studied the effect of cytokinins alone and in combination with IAA or NAA. Leaf discs subjected to cytokinins alone, showed formation of buds (4-5) directly from the margins. However, cytokinins in combination with IAA induced rhizogenesis, whereas with NAA induced callogenesis. They have also reported (Ramesh and Padhya, 1990), that combination of KN and BAP (4μM) each and adenine sulphate (16μM) was optimal for the production of 18-20 shoot buds from excised leaf discs. WB medium supplemented with IAA (6μM) was used for rooting.

Induction of bipolar structures was observed by Muralidharan and Mascarenhas (1989), when cotyledonary explants were cultured on Gamborg's (B5, 1968) medium containing cytokinins BAP/KN/2iP (0.5 - 5.0 mg/l). On hormone free media, these bipolar structures produce leafy appendages along with a single tap root. No studies were conducted to confirm the nature of these bipolar structures.

The first conclusive evidence of induction of somatic embryogenesis in *Azadirachta indica* was demonstrated by Shrikhande *et al* (1993).

Islam *et al* (1996), obtained induction of somatic embryogenesis from nucellar tissue of immature seeds and from mature and immature cotyledons. The cultures were initiated an MS and Linsmaier and Skoog's (LS, 1965) medium supplemented with different concentrations of cytokinins and auxins, either singly or in combinations. Nucellus, 40-45 days after fertilization was found to be most responsive. Basal medium with 2.0 mg/l BAP and 0.2 mg/l NAA induce callus. According to them callus induction was genotype dependent. They also claimed that the basal medium was the deciding factor i.e., LS was found to be effective for indirect somatic embryogenesis via callus stage, and MS for direct somatic embryogenesis pathway.

Direct somatic embryogenesis in *Azadirachta indica* was reported by Joshi and Thengane (1996a). According to them, type of auxin was found to be the deciding factor in embryo induction pathway. When 2,4-D was used in combination with BAP, somatic embryos were induced directly on the cotyledonary explant. However, when IAA was used, callus induction was followed by somatic embryogenesis (Shrikhande *et al*, 1993).

Recently induction of adventituous shoots has been observed from immature embryos (Thiagarajan and Murali, 1993), shoot tips and axillary buds (Gurumurthi and Stanley, 1992; Drew, 1993; Joarder *et al*, 1996; Joshi and Thengane, 1996a & b; Nirmala Kumari *et al*, 1996). About 5-10 shoots per explant were regenerated using MS basal medium supplemented with cytokinins.

Thiagarajan and Murali (1996), optimized the conditions to establish *in vitro* plantlets of neem by culturing seed embryos, collected from immature fruits (30 days from flowering). High frequency regeneration of plantlets from intact embryos were obtained on medium containing 3 g/l sucrose, NAA (0.1 mg/l) and BAP (0.3 mg/l).

1.5.2 Studies on secondary metabolites of Neem:

Sanyal *et al* (1981, 1983, 1986, 1988) carried out a series of experiments to stimulate production of active principles from callus obtained from stem bark or cotyledons. They studied the *de novo* synthesis of nimbin and ß-sitosterol. The callus raised from the young stem bark was completely devoid of nimbin. However, they observed that nimbin and ß-sitosterol were present in callus tissue induced from cotyledons. The concentration of nimbin decreased with the age of callus. Nimbin synthesis increased markedly only with organogenesis. Root formation alone, accompanied the least quantity of nimbin; shoot formation alone accompanied more nimbin whereas redifferentiation into both root and shoot accompanied the highest nimbin synthesis. They also observed an increased biosynthesis of nimbin and decrease of ß-sitosterol by adding glycine in the culture media. Glycine probably triggered the biosynthesis of nimbin, blocking the ß-sitosterol pathway (Sanyal *et al*, 1988).

Ramesh and Padhya (1996), have reported selection of stable cell lines producing higher levels of compounds, particularly the flavonoids. Holowach-

Keller *et al* of Rohm and Hass Company (1994) have reported a method for producing Azadirachtin. Azadirachtin production was also reported by Wewetzer (1996), from callus initiated from bark tissue. He studied the variation of azadirachtin content from trees of different geographical origins (Nigeria, Nicaragua and Cuba).

Enhanced secretion of azadirachtin was reported by Kuruvilla *et al*, 1997, by permeabilising neem cells using DMSO, Triton X-100, Chitosan as permeabilizing agents. Triton X-100 has proved to increase several fold increase in the secretion of azadirachtin.

Raj Kumar *et al*, 1997, also detected azadirachtin in callus cultures initiated from young leaves of *A. indica*, after 20 weeks of incubation on MS medium supplemented with NAA 1.0 mg/l), BAP (0.5 mg/l) and glycine (0.3 mg/l).

Other phytochemicals reported from cell cultures are gedunin (Rajagopal and Ramaswamy, 1996), two new tetranortriterpenoids, Neeflone and Neeflonolide, which resemble close to azadirone in structures (Srinivas *et al*, 1996). **Table 1.8** enumerates the *in vitro* studies on secondary metabolites carried in neem.

In spite of the extensive research work carried out through the years, there are still some short comings in neem research programme, which cannot be overlooked. As neem is an open pollinated tree, it exhibits a wide variation in bioactive principles. Moreover, its propagation is only by seeds and this is limited by seed viability.

There is also a need to develop superior planting material. Moreover, development of a suitable cell culture system for the *in vitro* production of the active principles on a large scale is equally important.

The present work was undertaken with the following objectives:

1. To standardize a protocol of micropropagation for its mass production.

2. To investigate the optimum conditions for both the pathways of somatic

embryogenesis.

3. To induce callus and analyse it for secondary product and to study the effect of differentiation on chemical expression *in vitro*.

The work has been divided into five chapters, namely:

Chapter 1: General Introduction

Chapter 2: Materials and Methods (General)

Chapter 3: Micropropagation and plantlet formation of *Azadirachta indica* A. Juss.

Chapter 4: Somatic embryogenesis in *Azadirachta indica* A. Juss.

Chapter 5: Secondary metabolite analysis.

Table 1.7: Tissue culture studies on *Azadirachta indica*.

Explant	Response	Results	References
Mature embryo	callus	organogenesis	Rangaswamy and Promila, 1972
Stem bark	callus	-	Sanyal *et al*, 1981
Stem, leaf axillary buds	callus	organogenesis	Jaiswal and Narayan, 1983
Cotyledon	callus	organogenesis	Sanyal *et al* , 1983
Leaf	callus	organogenesis	Schulz, 1984
Leaf	callus	organogenesis	Narayan and Jaiswal, 1985
Stem bark	callus	organogenesis	Sanyal and Datta, 1986
Leaf discs	callus	morphogenesis	Ramesh and Padhya, 1987
Cotyledon	callus	-	Sanyal *et al*, 1988
Cotyledon	bipolar structures	morphogenesis	Muralidharan and Mascarenhas, 1989
Leaf discs	morpho-genesis	plantlet formation	Ramesh and Padhya, 1990
Anther	-	androgenesis	Gautam *et al*, 1991
Cotyledon	callus	embryogenesis	Shrikhande *et al* , 1993
Anther	callus	morphogenesis	Gautam *et al*, 1994
Immature embryo	germination	plantlets	Thiagarajan and Murali, 1996
Cotyledons induction	shoot	morphogenesis	Nirmala Kumari *et al* , 1996
Nucellar tissue and cotyledons	callus	embryogenesis	Islam *et al*, 1996
Cotyledon	-	embryogenesis	Joshi and Thengane, 1996a
Axillary buds	shoot induction	plantlets	Joshi and Thengane, 1996b
Axillary buds	shoot induction	plantlets	Drew, 1996
Axillary buds	shoot induction	plantlet formation	Venkateswarlu *et al*, 1997
Shoot tips	shoot buds, embryos, meristematic nodules	-	Sharma *et al*, 1997

Table 1.8: *In vitro* secondary metabolite studies

Source	Study undertaken	Reference
Young stem bark callus	Relation of chemo-differentiation with morpho-differentiation	Sanyal *et al*, 1981
Cotyledon callus	Relation of production of nimbin and ß-sitosterol to redifferentiation	Sanyal *et al*, 1983
Young stem bark callus	Effect of nutritional environment on redifferentiation	Sanyal and Datta, 1986
Cotyledon callus	Effect of glycine on *in vitro* biosynthesis of nimbin and ß-sitosterol	Sanyal *et al*, 1988
Stem, leaf, flower callus	Accumulation of flavonoids Kaempferol and quercetin	Ramesh and Padhya, 1996
Leaf, bark callus	Variation of AZ production with geographical origin	Wewetzer, 1996
Leaf callus	Correlation of AZ content *in vivo* and *in vitro*	Mordue *et al*, 1996
Callus	Permeabilization of cells for higher AZ yield	Kuruvilla *et al*, 1997
Leaf callus	Production of AZ	Rajkumar *et al*, 1997.

Table 1.9: Landmarks in chemical investigations of Neem

Study Undertaken	Reference
Identification of contents of neem oil	Warden, 1888
Refining of neem oil and isolation of margosopicrin	*Warson et al*, 1923
Identification of two amorphous bitters	Seshadri *et al*, 1940
Isolation of Nimbin, Nimbinin and Nimbidin	Siddiqui, 1942
Isolation of fatty acids from neem oil	Dasa Rao and Seshadri, 1942
Isolation of Nimbidinic acid and Nimbic acid	Siddiqui and Mitra, 1945
Carbohydrate content of neem gum	Mukherjee and Srivastava, 1955
Molecular weight of nimbin	Narashimhan, 1959
Isolation of nimbiol 4 and ß-sitosterol	Sengupta *et al*, 1960
Structure elucidation of nimbin	Narayanan *et al*, 1962
Identification of other fatty acids	Skellon *et al*, 1962
Isolation and structure elucidation of salanin	Henderson *et al*, 1964
Isolation and structure elucidation of melantriol	Lavie *et al*, 1967
Identification of amino acid content of neem seed meal	Mitra and Misra, 1967
Isolation of Azadirachtin (AZ)	Butterworth and Morgan, 1968
Isolation of Azadirone and its analogues	Lavie *et al*, 1971
Isolation of flavonoids	Subramaniam and Nair, 1972
Identification of tetranortriterpenoids in neem seed oil	Kraus and Cramer, 1978 Kraus *et al*, 1981
Identification of organo-sulphur compounds in seed oil	Gupta and Joshi, 1983
Identification of pentannortri-terpenoids	Garg and Bhakuni, 1984 Kubo *et al*, 1984
Structure elucidation of AZ by NMR and X-ray crystallography	Turner *et al*, 1987; Bilton *et al*, 1987; Kraus *et al*, 1987
Identification of isomeric AZs	Rembold, 1990
Identification of other protomeliacins	Siddiqui *et al*, 1991
Synthesis of two fractions of AZ	Ley *et al*, 1993

CHAPTER 2

MATERIALS AND METHODS (GENERAL)

2.1. GLASSWARE PREPARATION

2.1.1.Cleaning

Borosil glass tubes (150mm x 25 mm), Erlenmeyer flasks (250 ml and 100 ml) and Laxbro disposable petriplates (55 mm size) were used in most of the experiments. The newly purchased glassware was acid-bathed in 30% nitric acid before washing with tap water and finally rinsed with distilled water. Used tubes or flasks containing old agar were first autoclaved to remove contaminants and the liquefied agar drained. The glassware was then soaked in boiling sodium carbonate solution (washing soda 10%) for one hour, acid-bathed for 30 minutes, followed by washing under running tap water. They were then rinsed in distilled water and dried at room temperature before use.

2.1.2. Sterilization

The Laxbro petriplates being sterile and disposable were used as such. The tubes and flasks were plugged with absorbent cotton and autoclaved at about 120°C and 15 pounds pressure per square inch (lb. psi) for one hour.

The other accessories required for medium, explant preparation and explant dissection were pipettes, glass petriplates, filter paper pads, forceps, scalpels, arrowheads, etc. The mouth end of graduated pipettes (1, 2, 5 and 10 ml) were plugged with cotton and wrapped individually in wrapping paper or aluminium foil. The glass petriplates were wrapped with wrapping paper or aluminium foil. The dissection instruments such as forceps, scalpels, arrowheads, etc., were placed in test-tubes or autoclavable plastic bags. Ordinary filter papers were cut into 20 x 14 cm size and placed into autoclavable plastic bags. All the glassware and instruments were autoclaved at 120°C, 15 lb. psi for one hour.

2.2. CHEMICALS

Inorganic salts used were of Analar grade (BDH, E. Merck India Ltd., Bombay), vitamins, hormones and other organic additives were obtained from Sigma Chemicals Co., USA, Difco Laboratories, USA or LOba Chemie, Mumbai, India. Gelling agent used was agar-agar from Qualigens, India.

Coconut milk was collected from tender coconuts and filtered through cotton. This was then autoclaved at 120°C and 15 lb. psi for 20 minutes. The coconut milk was again filtered through Whatman filter paper No.1 when hot. After thorough cooling it was dispensed into plastic bottles and stored at -20°C.

2.3. PREPARATION OF MEDIA

Murashige and Skoog's (MS) nutrient medium was used throughout the work. Its formulation is presented in **Table 2.1 A&B.** In addition, media were supplemented with growth regulators, other additional vitamins, organic supplements and carbon sources.

Auxins: Indole 3-acetic acid (IAA), Indole 3-butyric acid (IBA), α-Napthalene acetic acid (NAA) and 2,4-dichloro phenoxy acetic acid (2,4-D) were the auxins used. NAA and 2,4-D being thermostable were added to the medium prior to autoclaving while IAA being thermolabile had to be filter sterilized.

Cytokinins: 6-benzyl amino purine (BAP), Kinetin (KN) and, 6-γ,γ-(dimethyl allyl amino) purine (2iP) and thidiazuron (TDZ) were used.

Other additional vitamins: Calcium pantothenate (CP) and Biotin (B) were used.

Organic supplements: Casein hydrolysate (CH) and Coconut milk (CM) were used.

Carbon sources: Varying concentrations of sucrose, glucose, maltose and sorbitol were used as carbon source.

The preparation of stock solutions of the various growth regulators and vitamins, their storage and sterilization is described in **Table 2.2.**

For the preparation of all the culture media, glass distilled water was used. All the major and minor salts and vitamins were added before adjusting the pH. After adding the growth regulators, additional organic sources and the carbon source, the pH was adjusted using 1N HCL or 1N NaOH. Agar-agar was then

added to the medium. The medium was then melted and dispensed into test-tubes (20 ml) or flasks (30 ml) . Liquid medium was dispensed into the tubes with supports of Whatman filter paper No. 1 to avoid submerging of cultures.

All culture media were autoclaved at 120°C and 15 lb. psi for 20 min. As and when required, autoclaved agar media were dispensed (approx. 10 ml per petriplate) into sterile petriplates before solidification. Heat labile additives, if any, were filter sterilized through Millpore membrane (0.22 µm) and added to the poured medium before it solidifies.

2.4. CULTURE CONDITIONS

All the operations like sterilization and inoculations were carried out in a laminar flow cabinet. The forceps, scalpels, needles used for dissection were flamed in between after dipping in 70% ethanol. Dissections required for the preparation of the explants were done on sterile filter papers.

Cultures were incubated at 25±2°C in a culture room either in the dark or under cool fluorescent illumination at an intensity of ~ 35 µE/m²/s under 16/8 hour photoperiod. Petriplates were sealed with parafilm (Klin wrap, Flexo film wraps , Mumbai) before incubation.

2.5. MEASUREMENT OF GROWTH

Growth of callus tissue was measured on the basis of the increase in fresh weight. The tissue was weighed in a previously weighed petriplate before inoculation and after any particular treatment at an interval of 20 days. Tissues were first spread on a piece of filter paper to blot out any moisture that might have accumulated on the tissue, before weights were taken.

Embryo counts were taken under the inverted microscope (Carl Zeiss make). In the indirect somatic embryogenesis pathway, the embryos of various stages were counted together as the growth was non- synchronous. The embryos over the entire explant were counted. The explants were chosen randomly. Twenty explants were taken for counting per replicate and the number of replicates were three.

For the direct pathway, only globular stage embryos were observed at one end of the cut cotyledonary piece, thus were counted as such. Callusing was also observed at the cut ends of the cotyledonary explant. Thus, the number of cultures showing either embryogenesis (E) or callusing (C) or both (E+C) , have been counted. The embroys induce directly from the explant did not show step-wise development and hence the other stages like heart and torpedo, could not be counted. These embryos showed precocious germination.

For regeneration induction experiments, percent sprouting and the number of shoots per explant were counted after 20 days. For multiplication experiments, the shoot length and number of shoots were counted after every 20 days. The cultures were removed on filter paper and the number of shoots were counted before reinoculation. The shoot length was measured on the autoclaved graph paper. For each experiment, the number of cultures counted were ten and the number of replicates were three.

2.6. STATISTICAL ANALYSIS

For the statistical analysis of the various data, standard deviation and standard error was calculated according to the standard formulae (Snedecor and Cochran, 1967). The Annova test (2 way) was done using Lotus release 3.1 (Lotus Corporation Inc., USA), to analyse the significant treatments of media and subculture in regeneration experiments, at The Agharkar Research Institute, Pune.

2.7. HISTOLOGICAL STUDIES

Histological studies were carried out according to standard procedure (Johansen, 1940). Absolutely dirt free material was used for histological studies to avoid difficulties in sectioning. The tissue was cut into pieces of 5mm x 3mm size and placed immediately in the fixative - either FAA (40% Formaldehyde : glacial Acetic acid: Ethyl alcohol 5:5:90) or acetic alcohol (glacial acetic acid: ethyl alcohol 25:75) solution for 24 hour. Tissues were then washed thoroughly under running tap water and stored in 70% ethyl alcohol in refrigerator. The tissues were then passed through alcohol and xylol series for dehydration and clearing respectively.

For cold infiltration, xylol was saturated with fine chips of paraffin wax (m.p. 59-60 °C). The material was further kept in oven at temperature slightly higher than the melting point of paraffin. Under these conditions, paraffin melts and displaces the evaporating xylol. Two changes of molten paraffin were made to ensure that no trace of xylol was left behind.

The tissue was then embedded in solid paraffin blocks. This was accomplished by simply pouring the molten paraffin into a mold, arranging the tissue in it and cooling it. Serial sections 8 - 10 µm thick were cut with rotary microtome (Reichert-Jung), floated in water and mounted on the glass slides.

Sections were then passed through the xylol : ethyl alcohol series. For double staining, sections were stained either with Hematoxylene (1% aqueous) - eosin (1% in absolute alcohol) or saffranin (1% aqueous) - light green (1% in absolute alcohol). The sections were finally mounted with DPX- 4- 189- (2-chloro-N- (4- methoxy-6-methyl-1,3,5-triazin-2-yl amino carboxyl) benzene sulfonamide) (BDH), using (22 x 50 mm) cover glasses. The histological observations and photomicrographs were taken under Docuval (Carl Zeiss) microscope.

2.8. PREPARATION OF EXPLANTS

2.8.1. Cotyledonary explants

Cotyledonary pieces as explants were obtained from three types of neem seeds, unripe green fruits, ripe yellow fruits and seeds stored for one year.

1. The unripe green fruits **(Fig 2.1)**, were plucked from the 20-30 year old mature trees growing in the National Chemical Laboratory campus in Pune. After surface sterilization, the fruits were then cut horizontally into two halves to facilitate the removal of cotyledon pieces from the pericarp **(Fig. 2.2)**. Explants consisted of pieces of cotyledons with the embryo axis dissected out **(Fig. 2.3)**.

2. Fresh ripe yellow fruits were collected, the fleshy mesocarp removed and seeds along with the hard endocarp were dried at room temperature. These seeds were either used immediately or stored in a cloth bag at 4°C were treated as follows.

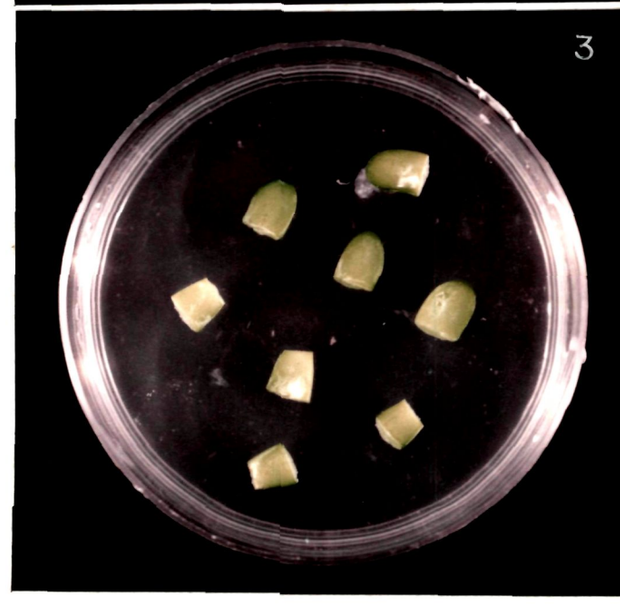
Figure 2.1: Immature fruits of neem.

Figure 2.2: Immature fruits cut horizontally to facilitate removal of the cotyledons.

Figure 2.3: Cotyledons inoculated abaxially onto the medium.


1


2


3

The outer hard endocarp was broken and seeds along with the testa were surface sterilized and then left to imbibe in sterile water for 42 hours. The swollen seeds were then taken out and seed coat removed using a needle and a pair of forceps. The decoated seeds consisting of embryo along with intact cotyledons were thus obtained. The embryo was excised and the cotyledons cut into 2 pieces horizontally were used as explants.

2.8.2. Terminal/Axillary buds

In general, neem trees flower after 5 years of age. Therefore the trees growing in the NCL campus, of the age of 2-5 years (girth = 20-40 cms) and 20-30 years (girth = 110-150 cms) were chosen to collect the bud-wood material. The axillary and terminal buds were collected in the sprouting season i.e. from January to April. Axillary buds from neem trees at different developmental stages viz., mature flowering (MF); mature non-flowering (MN); coppiced branches of mature flowering (CF) and coppiced non-flowering (CN) branches , were collected. The leaves were excised and the branches cut into sections comprising of one single node (size 1.0 -1.5 cm). Terminal buds along with 2 apical leaves were also inoculated.

2.8.3. Surface sterilization

The explants were first washed under running tap water, followed by treatment with 10% savlon for 2 minutes and washed thoroughly under tap water. These were then transferred to a sterile flask and surface sterilized in a laminar flow unit. They were treated with 70% ethanol for 2 minutes followed by 0.1% $HgCl_2$ for 10 minutes and washed thoroughly with sterile distilled water.

2.9. CHEMICAL ANALYSIS

Callus (10gm) induced on various media was used for chemical analysis. The medium adhering to the calli was removed and the calli were surface dried, and then extracted with acetone in a Soxhlet unit for three hours. Acetone extract was further completely dried and redissolved with fresh acetone to form an acetone soluble fraction. About 3 µl of this fraction was spotted on a Thin Layer Chromatography (TLC) plate (SiO_2 : $CaSO_4$ 77:13, activated at 100°C for 1 hour), along with β-sitosterol, de-acetyl nimbin and epoxy azadiradione as reference.

The TLC plates were run using 20:80 acetone:pet ether (60-80 °C)as solvent. The plates were then sprayed with H_2SO_4:HNO_3 (1:1) and spots were developed by heating the plates at 150°C. ^{1}H-NMR (200 MHz) in $CDCl_3$ was carried out to find out the exact nature of the compound.

TABLE 2.1.A. Chemical Composition of MS Basal Medium.

Chemical	Formula	Weight in mg/l
Major Salts		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Magnesium sulphate	$MgSO_4.7H_2O$	370
Potassium ortho phosphate	KH_2PO_4	170
Calcium chloride	$CaCl_2.2H_2O$	440
Sodium salt of ethylene diamine tetra acetic acid	$Na_2EDTA.2H_2O$	37.3
Ferric sulphate	$FeSO_4.7H_2O$	27.8
Minor Salts		
Boric acid	H_3BO_3	6.2
Cobalt chloride	$CoCl_2.6H_2O$	0.0025
Cupric sulphate	$CuCl_2.5H_2O$	0.025
Sodium molybdate	$NaMoO_4.2H_2O$	0.25
Potassium iodide	KI	0.83
Zinc sulphate	$ZnSO_4.7H_2O$	8.6
Manganese sulphate	$MnSO_4.4H_2O$	22.3

Table 2.1 B : *Organic composition of MS Basal Medium.*

Name	Weight in mg/l
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.5
Glycine	2.0

Table 2.2 : Preperation of stock solutions, their storage and sterilization.

PGR/Vitamins	Mol. wt.	Solubility	Sterlisation	Storage
Auxins				
IAA	175.2	1N NaOH or EtOH	co-autoclave preferably filter sterilize	0°C
IBA	203.2	—"—	co-autoclave	0-5°C
2,4-D	221.0	50% EtOH	—"—	RT
NAA	186.2	1N NaOH	—"—	RT
Cytokinins				
$AdSO_4$	368.3	water	--"---	0°C
BAP	225.3	1N NaOH or 1N HCl	—"—	RT
KN	215.2	—"—	—"—	0°C
2iP	203.2	—"—	—"—	0°C
TDZ	220.2	—"—	—"—	0°C
GA_3	346.4	50% EtOH	co-autoclave/ filter sterilize	RT
Vitamins				
Biotin	244.3	water	co-autoclave	0°C
Calcium pantothenate	238.3	—"—	filter sterilize	0-5°C
Thia-HCl	337.3	—"—	co-autoclave	RT
Pyr-HCl	205.6	—"—	—"—	RT
Niacin	123.1	—"—	—"—	RT

RT = *Room temperature.*

CHAPTER 3

MICROPROPAGATION AND PLANTLET FORMATION OF *Azadirachta indica* A. Juss

3.1 INTRODUCTION

The conservation and management of natural resources is one of the major problems facing mankind. The population explosion, increased industrial activity and recurrent use of synthetic insecticides is adding up to the problems of maintaining the quality of the environment (Higgins *et al*, 1985). Some of these problems could be circumvented either by utilization of degraded land/forests for increasing productivity or by rapid replanting of superior quality planting material. In addition to conventional techniques like genetics and plant breeding, biotechnology is playing an important role to solve some of these problems via improved agriculture, waste-treatment procedure, energy conservation and health care. Since the last two decades and more, plant cell and tissue culture has emerged as a strong branch of plant biology. Besides its use for mass multiplication of superior pheno/genotypes, it also provides a means for regenerating novel plants.

The application of micropropagation techniques to semi-tropical and tropical trees is not common and success in obtaining viable plantlets through clonal propagation, organogenesis or embryogenesis in these tree species is limited (Refer **Table 3.1**).

Most of the tissue culture studies in semi-tropical and tropical trees, is restricted to fruit trees due to their increasing demand in international market (Litz and Jaiswal, 1991). Among the fruit crops whose production is very important, conventional propagation is either inefficient (*Calocapum sapota*) or virtually impossible (*Garcina mangostana*). However, rapid and unpremediated deforestation in the developing countries have focused the attention towards multipurpose tree species of different genera, viz., *Eucalyptus, Populus, Acacia, Dalbergia, Tectona, Casuarina*, etc. (Brown and Sommer, 1982).

Table: 3.1. Tissue culture studies in semi-tropical and tropical tree species

Botanical name	Common name	Reference
Carica papaya	Papaya	Litz, 1985; Litz and Conover, 1978; Yie and Liaw, 1977; Rajeevan and Pandey, 1986
Ficus carica	Fig	Muruthi et al, 1982;
Mangifera indica	Mango	Litz et al, 1982;
Citrus spp.	Orange, lime, etc.	Spiegel-Roy and Vardi, 1984; Barlasse and Skene, 1986
Malus domestica	Apple	Korban and Chen, 1992.
Annona spp	Custard apple etc.	Nair et al, 1984; Jordan, 1988
Phoenix dactylifera	Date palm	Reynolds and Murashige, 1979
Euphoria spp	-	Litz, 1988
Santalum album	Sandalwood	Lakshmi Sita, 1986
Dalbergia latifolia	Rosewood	Lakshmi Sita et al, 1986
Tectona grandis	Teak	Mascarenhas et al, 1993
Eucalyptus spp	Eucalyptus	Lakshmi Sita, 1993
Alnus, Acacia Elaegnus etc.	Nitrogen fixing trees	Dhawan, 1993

Azadirachta indica A. Juss is an indigenous tree having multifarious properties and wide adaptability to various ecological environments. It is particularly valuable in forestry, land reclamation and conservation, revegetation of wastelands and for biomass production (Gupta, 1994). It is also of great commercial importance due to its nitrogen fixing, pesticidal, medicinal and pharmacological properties (Koul *et al*, 1990). In spite of the wide adaptability of the tree, its propagation is limited due to low seed viability. Moreover, its vegetative propagation by root suckers, shoot cuttings, air layering and grafting is difficult (Dogra and Thapiyal, 1993). *In vitro* techniques especially the method of axillary bud proliferation can provide an alternative means of propagation. The main advantages of this method are high multiplication ratesand the plantlets formed are genetically stable. Thus, this method would be useful for propagation of superior neem trees showing faster growth and higher yield of active compounds.

Most of the earlier work in tissue culture of neem, comprises studies on organogenesis from callus cultures raised from anthers, cotyledons, embryo/ seedlings and stem tissue. Organogenesis from callus was obtained either for the *de novo* production of secondary metabolites, as chemo-differentiation was seen to be related to morpho-differentiation (Sanyal *et al*, 1981, 1983, 1986) or for regeneration studies (Jaiswal and Narayan, 1983, 1984; Ramesh and Padhya, 1990).

Joarder *et al* (1996), reported the formation of 1-3 shoots per culture from axillary buds of woody trunks of 5-40 year old trees. They observed an inverse relation between age of the tree and number of shoots per explant. Older trees with greater diameter of trunk gave good response than trees with smaller diameter. Average number of shoots per culture was 1.03 for 5 year old tree and 1.39 for 40 year old tree on MS basal medium supplemented with BAP (0.2 mg/ l), NAA (0.01 mg/l) and sucrose (30 g/l). Transfer of two weeks old culture of nodal explants,on growth-regulator free MS medium, was found to be essential for successful sprouting of the axillary buds. They further observed that the shoot multiplication rate increased with increasing number of subcultures and declined after 5-6 subcultures. Shoots 5, 8 and 10cm in length were tested for rooting capacity. 82% rooting was observed when actively growing shoots were inoculated on MS half strength medium supplemented with 0.5 mg/l IBA. The *in*

vitro regenerated plantlets were successfully transferred to soil and acclimatized to outdoor conditions. However, further transfer to field and their evaluation data is not reported.

Axillary buds from young non flowering trees (Joshi and Thengane, 1996b) or from coppiced shoots (Gurumurthi and Stanley, 1992), were found to be more responsive. 3-5 multiple shoots were obtained from axillary buds of coppiced shoots of 18-20 year old tree, when cultured on MS basal medium supplemented with KN (0.9 µM) and BAP (1.3 µM) by Gurumurthi and Stanley (1992). These shoots produce abundant roots even without the supply of auxins.

Nirmala Kumari *et al* (1996) have reported the production of 12-15 shoots from shoot tips of neem on MS basal medium supplemented with 1.5 mg/l BAP and CH. However, the age of the tree from which shoot tips were collected was not specified. They report range of efficiency of shootlet development (12.5% to 92%) on MS basal medium supplemented with 1.1 mg/l BAP and CH (200 mg/l). Rooting percentage was found to be from 67% to 93% on MS 1/2 supplemented with IBA (0.5 to 2.0 mg/l).

Recently, Venkateswarlu *et al* (1997), have reported that tissue cultured neem plants showed better collar diameter and greater uniformity in height than saplings raised from seeds. The best performance recorded was a height of 8 feet and girth of 4.5 inches in seven months from the clone no Hy-1.

In the present investigation, a detailed work was undertaken to standardize a protocol for the large-scale production of neem plantlets through shoot proliferation, a part of which has been earlier reported by the author (Joshi and Thengane, 1996b), where from a single bud about 50 plants can be produced within four months.

3.2 MATERIALS AND METHODS

3.2.1 *Collection of explant material and its surface sterilization*

Axillary buds were collected during sprouting and non-sprouting season from the neem trees at different developmental stages:

1. Mature flowering trees (MF).
2. Mature non-flowering trees (MN).
3. Coppiced branches of mature flowering trees (CF).
4. Coppiced branches of mature non-flowering trees (CN).

The age of the trees growing in NCL campus could not be specified as their plantation dates were not known. However, the maturity of the neem trees was determined from the girth of the tree. Thirty five to forty centimeters (35-40 cm) long branches were collected from these trees. The leaves were excised and the branches cut into segments of 1.5 cm comprising of one single node. These were then surface sterilized as mentioned in Chapter 2, section 2.8 and inoculated onto the induction medium.

3.2.2 Induction experiments

The effect of season, age of the explant and the effect of various cytokinins on sprouting was studied simultaneously in the preliminary studies. For these studies, the buds from the different seasons as well as from different trees were inoculated on MS basal medium supplemented with MS vitamins, cytokinins like BAP, KN or 2iP (1.0 mg/l) alone or in combination of two with 1.0 mg/l of each, sucrose (20 g/l) and gelled with agar (4 g/l).

In the preliminary experiments, the coppiced branches of the mature non-flowering trees responded best in the MS basal medium supplemented with BAP and KN. Therefore, for all further studies coppiced branches of 2-5 year old non-flowering trees were used as explant. To optimize the concentrations of cytokinins, BAP and KN, for sprouting, the buds were inoculated on MS basal medium supplemented with cytokinins BAP or KN (0.1 to 3.0 mg/l) alone or in combination and sucrose (20 g/l).

In addition, auxins like IAA or NAA (0.05-0.1 mg/l) and other carbohydrate sources like glucose (20 g/l) or maltose (20 g/l) or sorbitol (10 g/l) alone were tested for their effects on the number of sprouts per explant. These auxins and carbohydrates were added to MS basal medium supplemented with BAP (0.5 mg/l), KN (1.0 mg/l) and sucrose (20 g/l). Increase in sucrose to 30 g/l or 40 g/l was also tested.

3.2.3 Multiplication experiments

After 20 days, the explants showing response were subcultured onto shoot proliferation medium. Number of experiments were carried out to maximize the production of shoots from one axillary bud. These include:

1. Use of low concentrations of cytokinins, (BAP and KN; 0.05 - 0.5 mg/l) as compared to induction medium.
2. Use of additional vitamins CP and B at 0.05 and 0.1 mg/l concentrations.
3. Use of dilutions of MS macro and microelements keeping MS vitamins constant i.e. full strength.

Finally, the effect of subculture was studied using the following media:

Medium A - MS full + BAP and KN (0.2 mg/l)
A1 - MS 1/2 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l)
A2 - MS 3/4 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l)
Medium B - MS full strength + BAP (0.1 mg/l) + KN (0.05 mg/l)
B1 - MS 1/2 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l)
B2 - MS 3/4 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l)

The measurement of growth, i.e., data on percentage of explants showing response, number of shoots per explant and shoot length was done according to the methods described in Chapter 2, Section 2.5.

3.2.4 Rooting experiments

Ex vitro as well as *in vitro* rooting experiments were carried out using shoots of length more than 2.5 cm in length.

1. *Ex vitro* experiments: The individual shoots were dipped into rooting solution (IAA 100 ppm) for 1/2 hour and then directly transferred to sand:soil (1:1) mixture.
2. *In vitro* experiments: In this again two types of experiments were conducted:
   a. Transfer of shoots to hormone free MS medium gelled with agar.
   b. Transfer of shoots to MS 1/2 strength liquid medium supplemented with IAA (0.5, 1.0 and 2.0 mg/l).

The shoots were incubated in dark for 48 hours and then transferred to hormone free MS 1/2 strength in light conditions with 16/8 hour photoperiod. The rooted plants were transferred to sand:soil mixture (1:1) in polybags, acclimatized to green house conditions and then shifted to field conditions after 3 months.

3.2.5 Field evaluation of tissue culture raised plants

The morphogenetic characters of the tissue culture raised plants were compared with seed raised plants after one year (**Table 3.7**).

3.3 RESULTS

3.3.1. Initiation

3.3.1.1. Effect of age of explant

The effect of season, age of the explant and the effect of various cytokinins on induction of sprouting was studied simultaneously.

Table: 3.2: Effect of season, age of the explant on sprouting induction.

Cytokinins (1.0 mg/l)	Response							
	Sprouting				Non Sprouting			
	MF	MN	CF	CN	MF	MN	CF	CN
BAP	C	S	C	S	NR	C	C	C
KN	C	S	C	S	NR	C	NR	C
2iP	NR	C	C	NR	NR	NR	NR	NR
BAP+KN	C	S	C	S	NR	C	C	S
KN+2iP	NR	C	NR	S	NR	NR	NR	C
BAP+2iP	NR	C	NR	S	NR	NR	NR	C

Medium: MS + 20g/l sucrose

S = sprouting; C = callusing; NR = no response.

The sterilized nodal explants were inoculated on MS medium supplemented with MS vitamins and cytokinins BAP, KN, 2iP (1.0 mg/l) alone or in combination of two **(Fig. 3.1)**.

Explants from coppiced branches of non-flowering trees showed 85% sprouting within 7 days. The part of the explant which was in contact with the medium swelled as an indication of meristematic activity. This was accompanied by abscission of subtending leaf. The portion of the explant above the medium did not show any callusing **(Fig 3.2)**.

Bud growth from nodal explants of the other trees viz., mature flowering, mature non-flowering and coppiced branches of mature flowering trees was negligible; most of the explants turned brown and died. However, 9% of the nodal explants from mature non-flowering tree showed initiation of a single sprout after 20 days of incubation on initiation medium. Continued incubation did not improve the number of sprouts. However, the explants became thick and the entire explant formed callus **(Fig. 3.3)**.

The newly sprouted and the coppiced branches from non-flowering trees were more responsive than those from mature, flowering trees. The newly sprouted branches of mature trees were mainly reproductive ones and these in culture turned brown and died.

3.3.1.2. Effect of position of explant

The buds of the newly sprouted branches, which were nearer to the apical bud were more responsive. After about tenth axillary bud, bark formation was observed and such buds showed no response in culture **(Fig. 3.4)**.

3.3.1.3. Effect of cytokinins

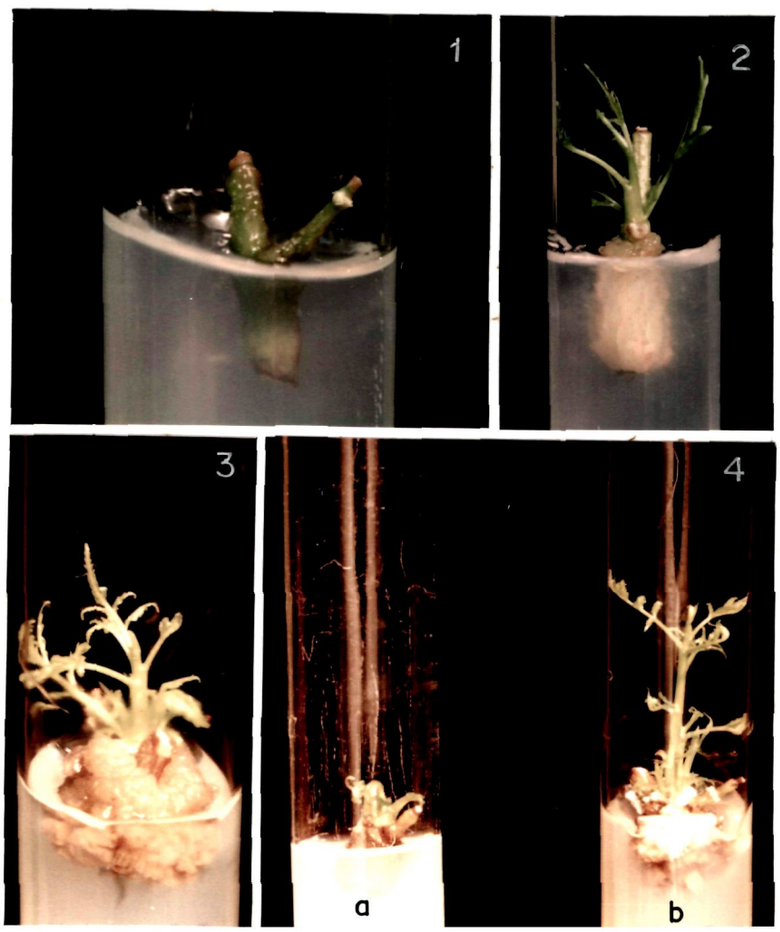
In the preliminary experiments, effect of cytokinins BAP, KN and 2iP (1.0 mg/l) alone or in combination were tested. Of the cytokinins tested individually, BAP alone showed maximum number (85%) of cultures with multiple shoots followed by KN (58%). 2iP alone had absolutely no effect on sprouting **(Table 3.3; Fig. 3.5)**. However, combination of BAP and KN was found to be the best with induction of 2-4 shoots per explant with about 64% frequency **(Fig. 3.6)**.

Figure3.1: Nodal explant from young, non flowering coppiced branch.

Figure 3.2: Explant showing initiation within 7 days of incubation.

Figure3.3: Callusing of the entire explant from mature non-flowering tree.

Figure 3.4: Comparison of sprouting response.
a = explant from mature flowering tree;
b = explant from young, non-flowering coppiced branch.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Combination of 2iP with either BAP and KN had negligible effect with 3-5% sprouting.

Table 3.3: Effect of different cytokinins on multiple shoot induction

Cytokinin (mg/l)	Percent cultures with multiple shoots±S.E.	Number of shoot per culture
BAP	85±4.5	2-3
KN	58±5.3	2-3
2iP	NIL	NIL
BAP+KN	63.6±8.5	4-5
BAP+2iP	5.2±1.1	1-2
KN+2iP	3.3±0.7	1-2

Medium : MS + 2% sucrose + cytokinin (1.0 mg/l); Number of cultures studied = 10/replicate; Number of replicates = 3; Results taken after 20 days of incubation.

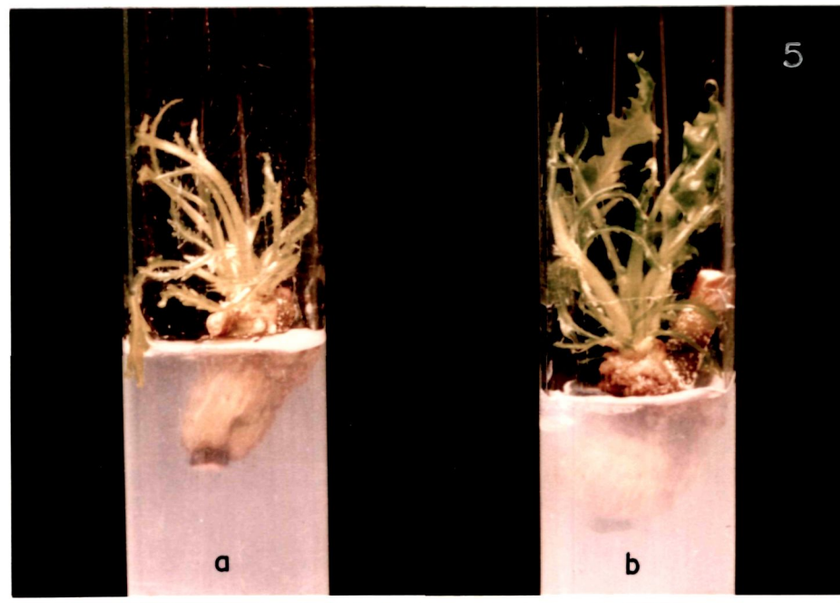
Since the preliminary experiments indicated the synergistic effect of BAP and KN on sprouting, further experiments were conducted to evaluate the optimum concentration of both the cytokinins. **Table 3.4** shows the effect of cytokinins on sprouting after 20 days of incubation. The explants were incubated on MS basal medium supplemented with MS vitamins and BAP/KN (0.1 - 3.0 mg/l) alone or in combination.

The combination of BAP (0.5 mg/l) and KN (1.0 mg/l) produced best results in percent sprouting (82%) and number of sprouts per explant (2-3) **(Fig. 3.6)**. Higher concentration of cytokinins (1.5, 2.0 and 3.0 mg/l) favoured only callusing of buds from the explant base **(Fig. 3.7)**.

Figure 3.5: Sprouting on individual cytokinin supplemented medium
a= medium with KN (1.0 mg/l);
b=medium with BAP (0.5 mg/l).

Figure3.6: 2-3 sprouts produced on medium supplemented with combination of KN and BAP.

Figure 3.7: Higher concentrations of cytokinin favour callusing.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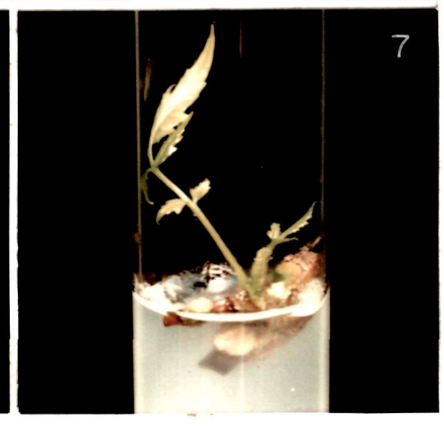
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Table 3.4: Effect of BAP and KN alone or in combination on sprouting

Cytokinin (mg/l)		Percent cultures showing sprouting	Mean sprouts per explant ±S.E.
BAP	KN		
0.1	—	16±8.2	one
0.2	—	39±5.3	one
0.3	—	41±7.1	one
0.5	—	67±8.5	one
1.0	—	43±6.2	one
1.5	—	29±6.2	one
2.0	—	16±2.1	one
3.0	—	4±2.1	one
—	0.1	20±2.4	one
—	0.2	28±3.1	one
—	0.3	28±4.0	one
—	0.5	30±3.1	one
—	1.0	68±5.3	one
—	1.5	30±3.5	one
—	2.0	15±2.8	one
—	3.0	9±2.1	one
0.5	0.5	49±4.2	two
0.5	1.0	82±2.8	three
1.0	0.5	40±5.3	one
1.0	1.0	20±3.5	one

Medium : MS + 2% sucrose.

3.3.1.4. Effect of auxin

Addition of auxins like IAA and NAA (0.05 - 0.1 mg/l) to the induction medium did not favour induction of multiple shoots. Only single stunted sprout was observed with either of the auxins with callusing of the explant **(Fig. 3.8)**.

3.3.1.5. Effect of additional carbohydrate

Supplementation of additional carbohydrate source, like glucose, maltose and sorbitol, to the induction medium did not help in increasing the number o

multiples per explant **(Fig. 3.9)**.

Glucose (20 g/l), maltose (20 g/l) and sorbitol (10 g/l) were supplemented to the induction medium in addition to sucrose (20 g/l). Addition of maltose (20 g/l) induced fasciated shoots, while the addition of sorbitol (10 g/l) induced stunted growth.

Addition of glucose (20 g/l), however induced 3 sprouts per explant. However, on further incubation these shoots did not elongate. Callusing was observed around the axil of such shoots after 15 days of incubation **(Fig. 3.10)**. Increase in sucrose concentration (30-40 g/l) also had deleterious effect on shoots, where even though the number of shoots increased they appeared vitrified.

3.3.1.6. Effect of incubation

About 85% of the buds from coppiced branches of young, non-flowering trees showed sprouting within 7 days. Further incubation on initiation medium upto 15 days increased this percentage to 97 %. However, incubation on the same medium for more than 21 days, did not increase the number of sprouts nor was there any significant shoot elongation.

The non-sprouted nodal explants were transferred tofresh induction medium and incubated for 30 days. After 15 days, out of the remaining nodal explants, only 53% showed sprouting. However, the sprouts were stunted. The remaining 47% of the nodal explants did not show sprouting even after 30 days of incubation.

3.3.2. Multiplication

In order to optimize a suitable medium for the mass multiplication of shoots from a single nodal explant, the effect of various media on number of shoots and their size was assessed.

3.3.2.1 Effect of hormone concentration

In an attempt to increase the number of shoots per explant, initially the sprouted buds were transferred to lower concentrations of BAP and KN (0.05 - 0.5 mg/l), in various combinations. Low cytokinin concentration was not effective

Figure3.8: Effect of addition of auxin to initiation medium, with callusing of explant base.
40 = medium with IAA; 41 = medium with NAA.

Figure 3.9: Effect of addition of additional carbohydrate, sorbitol, maltose and glucose respectively.

Figure3.10: Stunted vitrified shoot developed on glucose supplemented medium, callusing at axillary portion.


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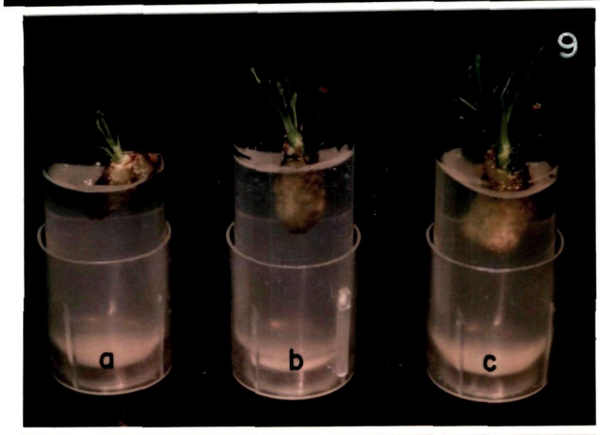
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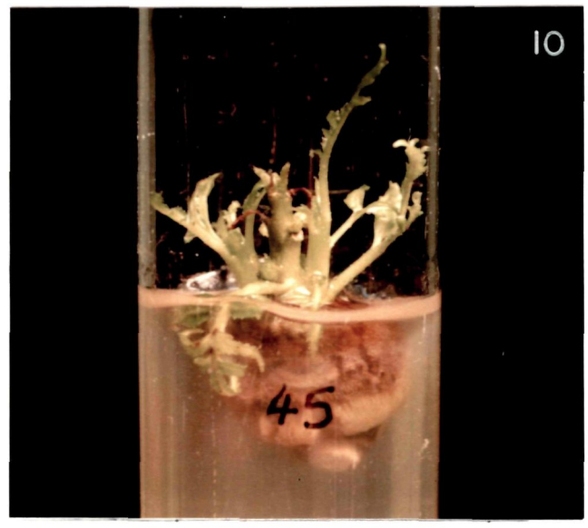
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in increasing the number of shoots per explant. However, rapid elongation was observed with lower cytokinin concentration **(Table 3.5; Fig. 3.11)**.

MS basal medium supplemented with 0.1 mg/l BAP and 0.05 mg/l KN (medium B), produced 2 shoots per explant with shoot length 3.9 cm. While MS basal medium supplemented with BAP and KN (0.2 mg/l each) (medium A), produced 3 shoots per explant with reduced shoot length (3.0 cm)**(Fig. 3.12)**.

Table 3.5: Effect of cytokinin concentration on shoot length

Cytokinin		Average length
BAP (mg/l)	KN	(cm)
0.05	0.05	0.8
0.05	0.1	1.6
*0.1	0.05	3.9
0.1	0.1	2.2
**0.2	0.2	3.0
0.2	0.5	1.2
0.5	0.5	0.8

Medium: MS + 2% sucrose. * = Medium B; ** = Medium A

3.3.2.2. Effect of additional vitamins

For further improvement in proliferation, shoots were separated from the explants, cut in section of 1-2 nodes and inoculated on media A and B supplemented with additional vitamins CP and B at the concentration 0.05 or 0.1 mg/l each.

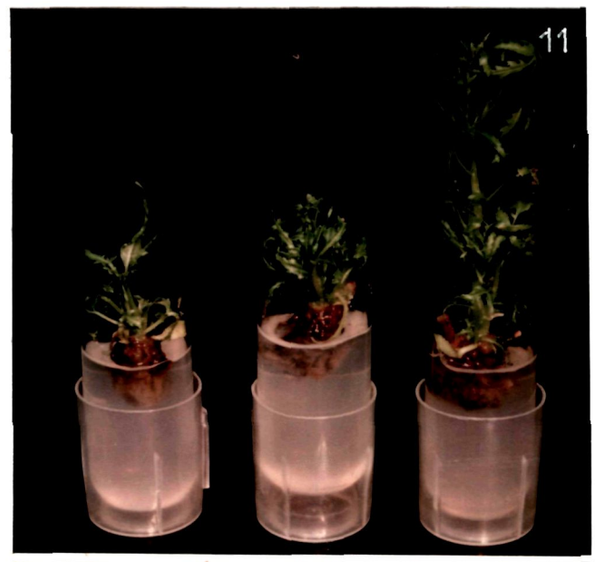
CP and B at 0.05 mg/l concentration gave better response than at 0.1 mg/l concentration. **Figure 3.13** shows the comparison of shoot length, on the four media tested.

On addition of CP and B (0.1 mg/l each) to medium A, there was no elongation and only 40% of the cultures showed increase in shoot number upto 5. However, addition of CP and B (0.1 mg/l) to medium B showed an increase in shoot number to 6 and shoot length to 4.2 cm **(Fig. 3.13)**.

Figure 3.11: Effect of lowered concentration of cytokinins; rapid elongation observed on medium B.

Figure3.12: Effect of lowered concentration of cytokinins; culture on medium A.

Figure 3.13: Effect of addition of CP and B to the medium;
a = medium A + CP and B (0.1 mg/l);
b = medium A + CP and B (0.05 mg/l);
c = medium B + CP and B (0.1 mg/l);
d = medium B + CPand B (0.05 mg/l).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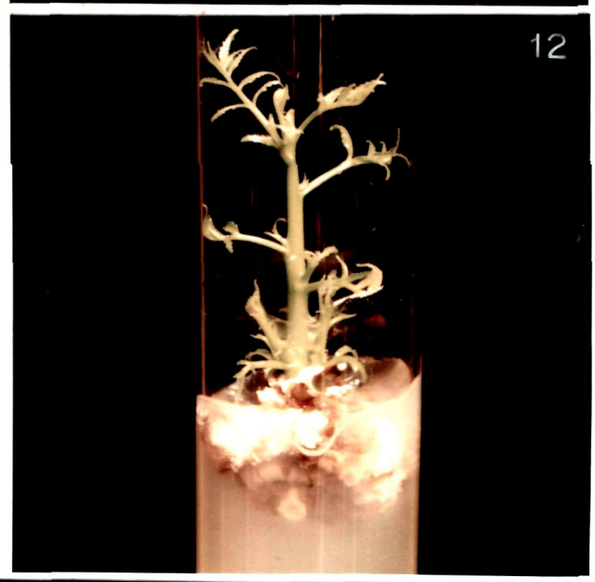
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Lowering of CP and B to 0.05 mg/l concentration, improved elongation and number of shoots. About 80% of the cultures on medium A showed 2-6 shoots/ culture. The best results were obtained on medium B i.e., MS basal medium supplemented with 0.05 mg/l CP and B, where shoot number increased to 5-8 and shoot length to 3.5-5.0 cm. On this medium shoots were more greener and healthy.

Thus a combination of low cytokinins (BAP; 0.1 mg/l and KN; 0.05 mg/l) and additional vitamins (CP; 0.05 mg/l and B; 0.05 mg/l) proved best for elongation as well as proliferation **(Fig. 3.14)**.

3.3.2.3. Effect of concentration of macronutrients

The effect of macronutrients along with compositions of media A and B on shoot number and length is shown in **Table 3.6**.

All combinations of B showed good results i.e. 5-8 shoots per explant, with shoot length of 2.5-4.2 cm. Whereas only 3/4 dilution of medium A showed 5 shoots per explant with average length 3.5 cm **(Fig. 3.15)**.

Table 3.6: Effect of dilution of macronutrients

Medium	Macronutrients	Average Number	Average length (cm)
A	Full	**2**	**3.0**
A2	3/4	**5**	**3.5**
A1	1/2	**1**	**1.5**
B	Full	**5**	**3.5**
B2	3/4	**8**	**4.2**
B1	1/2	**6**	**2.5**

Medium A: MS + BAP(0.2 mg/l) + KN(0.2 mg/l) + 2% sucrose
Medium B: MS + BAP(0.1 mg/l) + KN(0.05 mg/l) + 2% sucrose

Figure 3.14: Shoots on medium B.

Figure 3.15: Comparison in terms of shoot length of effect of dilution of MS macronutrients;
a = 1/2 dilution of medium A;
b= 3/4dilution of medium A;
c = 1/2 dilution of medium B;
d = 3/4 dilution of medium B.


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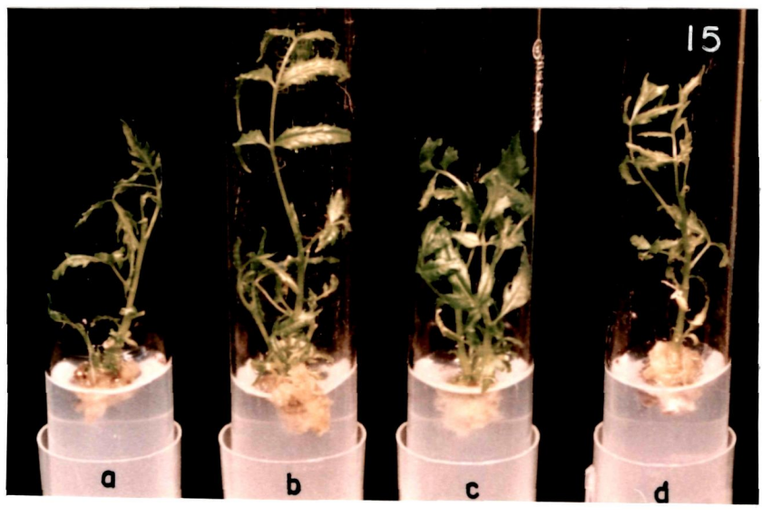
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Dilutions of MS to 3/4 concentration was more effective than that of 1/2 concentration, irrespective of the cytokinin concentration used. With 1/2 MS, the increase in shoot number and length was slow with successive subcultures, while with 3/4th strength, the elongation was rapid. **Figure. 3.15** compares the effect of various MA dilutions with respect to shoot length.

3.3.2.4. Effect of subculture

Shoots of various sizes (0.5 - 5.0 cm) was observed on media A, A1, A2, B, B1, B2. The shoots of sizes more than 1.5 cm were isolated, cut into sections of 1-2 nodes and used as inoculum for further multiplication. The shoots of size less than 1.5 cm were inoculated in bunches of 3-4 shoots. All these shoots were inoculated on media with different MA dilutions for subsequent subculture.

Table 3.7 a&b shows the effect of subculture and media on shoot proliferation. From the Annova test **(Table 3.7a)**, it was clear that media A2 and B2 were superior to A1 and B1. Out of the two media, A2 and B2, B2 had proved best for mass production of neem plantlets as it showed rapid increase in shoot number (17 at S3 to 50 at S6) and shoot length (2.6 cm at S3 to 4.4 cm at S6) at every subculture **(Table 3.7b; Fig. 3.16)**. The subculture upto S6 was significant, after which gradual decrease in shoot number as well as shoot length was observed.

Table 3.7a: Annova test

Source	Shoot Number		Shoot Length	
	DF	MS	DF	MS
MEDIA	3	4015.367	3	26.81094
SUBCULTURE	5	1102.937	5	5.49586
INTER	15	350.9167	15	1.557978
ERROR	216	11.66296	216	0.884722
TOTAL	239		239	

S.E. (m) = 1.079952
S.E. (d) = 1.527052

S.E. (m) = 0.1372852
S.E. (d) = 0.1941213

Table 3.7b: Effect of subculture and media compositions on shoot proliferation.

	Average number of shoots						Average shoot length (cm)					
Medium	**S3**	**S4**	**S5**	**S6***	**S7**	**S8**	**S3**	**S4**	**S5**	**S6***	**S7**	**S8**
A1	12.9	12.0	15.3	14.3	11.5	10.7	1.9	2.4	1.8	1.7	1.7	1.4
A2*	15.7	23.1	31.2	25.4	18.5	10.4	3.6	3.2	3.5	3.8	2.8	2.4
B1	15.5	18.9	10.8	13.4	9.6	7.0	2.9	2.2	3.2	2.8	2.4	1.8
B2*	17.2	27.6	49.5	47.1	29.6	18.0	3.6	4.1	4.7	4.4	3.2	2.5

A1 = MS 1/2 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l)
A2 = MS 3/4 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l)
B1 = MS 1/2 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l)
B2 = MS 3/4 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l)
Period of subculture = 20 days ; S3 - S8 = subculture numbers.
C.D. (for shoot number) = 2.993022
C.D. (for shoot length) = 0.380477 * = Significant media or subculture.

Figure 3.16: Effect of subculture.

Figure 3.17: 3-4 roots observed on hormone free medium.

Figure3.18: 1-2 sturdy roots observed on MS 1/2 liquid medium supplemented with IAA (2.0 mg/l).


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3.3.3. Rooting

Experiments were carried out for the induction of ex-vitro as well as *in vitro* rooting. Shoots of length 2.5 cm or more were used for rooting experiments.

3.3.3.1. *Ex vitro* rooting experiments

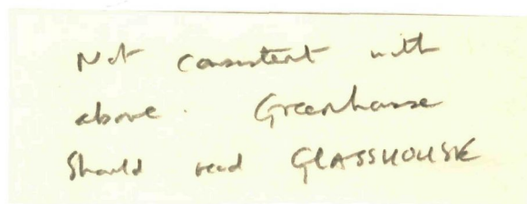
The individual shoots were directly transferred to polybags containing sand:soil (1:1) mixture. The plantlets were grown in greenhouse under 70% humidity, where 90 percent survival was observed after two weeks. However, these shoots started wilting after one month and the survival rate drastically reduced to 25%.

3.3.3.2. *In vitro* rooting experiments

For *in vitro* induction of roots two types of media were used:

i. Hormone free solid medium.
ii. MS 1/2 strength liquid + IAA (0.5, 1.0 and 2.0 mg/l).

In the first experiment 85 percent rooting was observed after 15 days where 2-6 thin long roots developed **(Fig. 3.17)**. These plants when transferred to soil showed 90% survival under green house conditions.



In the second experiment, with MS 1/2 strength medium supplemented with IAA, rooting was observed within 5 days of incubation on medium supplemented with 2.0 mg/l IAA. 80 percent rooting was observed **(Fig. 3.18)**, with one or two sturdy roots. With 1.0 mg/l IAA only 40 percent rooting was noted, while 0.5 mg/l did not show any response.

3.3.4. Acclimatization

The rooted plantlets that were transferred directly to soil, wilted during the first week of exposure to green house conditions. Therefore, plantlets were gently washed to remove agar/medium sticking to them and kept in tap water for one week, under room conditions. These were then transplanted to polybags containing sand and soil (1:1) mixture and later shifted to green house where they were maintained under a very high humidity (90-100%) for 10-15 days, where survival was 100 percent **(Fig. 3.19)**. Plants acclimatized under these conditions were shifted to field conditions after three months **(Fig. 3.20)**. **Table 3.8** shows the field evaluation data of such plants, taken after 1 year.

Table 3.8. Field evaluation of in vitro plantlets

Character	Control	Regenerants
Height (cm)	30.9±7.5	83.6±10.2
Number of branches	3.9±1.4	6.0± 1.5
Height(cm) of first branch from ground	5.6±1.6	16.0± 1.7
Number of leaves	46.3±5.1	81.6± 8.3
Size(cm) of leaves	1.35 x 3.6 ±0.15 ±0.4	1.63 x 5.17 ±0.18 ±0.5

Mean of 10 plants.

From the table it is clear that the growth of the *in vitro* derived plantlets was faster than that of the control plants. The emergence of the first branch was at the height of 16 cm as compared to that of 5.6cm in control plants. Thus, all the desirable characters viz., rapid growth, canopy development and large number of branches and leaves, and the bigger size of the leaves were noted in the *in*

Figure 3.19: Rooted plants to polybags.

Figure 3.20: One year old plant in field.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vitro derived plants.

3.4 DISCUSSION

Over the years tissue explants from a number of tree species were grown and made to generate plantlets *in vitro*. However, only in a limited number of tree species have the *in vitro* techniques advanced far enough for mass production of plantlets. Organ cultures, meristem cultures and propagation by axillary shoot elongation are more stable and involve minimum genetic risk in clonal propagation. However, axillary shoot elongation is preferred for the commercial propagation of hardwood species because it is the easiest method available which maintains genetic stability better than propagation by organogenesis and makes available the culture material abundantly (McCown and McCown, 1987).

Establishment of explants in culture and successful micropropagation depends on applying the correct combination of physical and chemical conditions (Debergh and Read, 1991).

In neem, cultures initiated from explants from juvenile material like non-woody branches of young non-flowering (2-5 year old) trees or from coppiced branches were easy to stabilize than those derived from explants from older material (Joshi and Thengane, 1996).

In general juvenile explants such as embryos, cotyledons, hypocotyls or bud explants from seedlings are more responsive *in vitro* (Ahuja, 1993). Nozeran (1984), had attributed this to the fact that, in juvenile state, the cells are constantly dividing and thus the gene expression can be transmitted to daughter cells and continues when shoots are propagated, thus maintaining genetic integrity.

The most frequent explant used is short single-node stem section (Zimmerman, 1984). Culture initiation, is the most critical stage of micropropagation because the potential for multiple shoot generation lies within the single bud. The problems imposed at this stage are microorganism contamination (Debergh and Maene, 1984), phenolic oxidation (George and Sherrington, 1984) and tissue maturity (Bonga, 1982). The physiological state of the parent plant at the time of excision has a definite influence on the response of the buds. Explants from actively growing shoots at the beginning of the growing

season generally give best results (Seabrook *et al*, 1976). A common approach has been the use of apical bud from a vigorous shoot, often growing at the top of the plant. Likewise, use of apical or axillary buds from branches near the top of the plant has been advised (Marks and Myers, 1992). Similar results were obtained in neem, where it was observed that axillary buds from newly sprouted branches of young trees which are nearer to the apical bud were responsive. The ability of juvenile explants from woody species to multiply in culture suggests that it should be possible to *in vitro* propagate their elite trees if they can be induced to develop juvenile shoots by suckering, coppicing or hormone treatment (Garland and Stoltz, 1981).

Adventituous shoot formation generally requires treatment with auxin and cytokinin. The exogenous requirement for the hormones depends on their endogenous level in the plant which is variable with the tissues, plant type and phase of plant growth (Bonga and von Aderkas, 1988; Bornman, 1983). In neem, cytokinin alone was found to be suitable for sprouting as well as multiplication. Cytokinins are more effective when applied in higher concentrations. BAP is the most widely used cytokinin as it is most effective than other cytokinins (Flinn *et al*, 1986; Lu *et al*, 1991). Combinations of several cytokinins are sometimes preferred over BAP alone (Martinez Pulido *et al* 1990). Lu *et al* (1991), observed that in cultures of *Picea rubens* the combination of BAP and KN initiated shoots at the same rate as BAP alone. In neem also, of the cytokinins tested individually, BAP alone showed maximum number of cultures with multiple shoots,followed by KN and 2iP **(Table 3.3)**. However, the combination of BAP and KN was found to be best with respect to number of shoots which was 4-5 per explant. The shoots initiated on BAP-KN combination were of better quality.

Consequently, the presence of an auxin in the medium was not found to be obligatory in neem cultures. Jaiswal and Narayan (1984) have reported that use of IAA and NAA (0.05-0.1 mg/l) favoured development of shoot buds from callus induced on KN and BAP supplemented medium. Narayan and Jaiswal (1985), reported that percentage of shoot formation on BAP medium combined with lower concentrations of NAA (0.05 mg/l) was higher as compared to that on medium containing BAP alone. However, in the present studies, addition of the IAA and NAA did not induce multiple shoot formation from the axillary buds. Only a single sprout which showed stunted growth was observed when either of the

auxins were added to the induction medium **(Fig. 3.8)**.

Normally, plant tissues produce most of the vitamins required in their metabolism. However, under *in vitro* conditions endogenous production of some of the vitamins is insufficient for optimal growth. MS vitamin combination is optimal for tissue cultures. A few vitamins, such as niacin, thiamine are routinely added in the medium. Other vitamins like pantothenic acid, folic acid, riboflavin, biotin are sometimes added in the medium (Bhojwani and Razdan, 1983). Gupta *et al* (1983), have reported the use of additional vitamins like calcium pantothenate and biotin at very low concentrations (0.05 - 0.1 mg/l) to promote formation of healthier shoots in Eucalyptus with sturdy stems and also to curb the callusing of shoots at the cut ends. In the present investigation also, supplementation of CP and B improved multiplication of neem.

The most popular medium for hardwood tree species is MS medium, or modifications thereof, at various levels of dilution. Recently, WPM (Wood and Braun, 1961) medium is also being used more commonly for woody species. The factors that most often determine whether a medium is suitable for a particular species or a particular type of development are ionic strength, total nitrogen, ammonium/nitrate ratio, calcium deficiency and chloride sensitivity of the tissues (McCown and Sellmer, 1987).

In most of the cultures of woody species, full strength MS medium is found to be inhibitory for optimum growth. This can be overcome by reducing the amount of ammonium or total nitrogen (Vieitez *et al*, 1983). Woody species have frequently been found to grow best on dilute solutions of MS (George and Sherrington, 1984). Increased survival as well as increased rate of multiplication has been reported on MS salts when used at 3/4 strength (Harris and Stevenson, 1979), as well as on 1/2 or 1/4 strength (Mekers, 1977). Present investigations confirm that MA dilutions at 3/4 concentrations were more effective, where rapid elongation was observed. With 1/2 concentration, the increase in shoot number and length was slow.

In vitro cultures require a continuous supply of carbohydrates from the medium, since the photosynthetic activity of *in vitro* tissues is reduced because of low light intensity, limited gas exchange and high relative humidity (Kozai,

1991). Carbohydrates are also necessary in tissue culture as osmotic agents (Thorpe, 1982).

Sucrose has been the carbohydrate of choice in most of the reports on micropropagation of woody species (Thompson and Thorpe, 1987). In most of the species sucrose at a final concentration between 2-4% is used. Despite its widespread use, sucrose is not always the most effective carbohydrate for shoot induction. Fructose and glucose were found to be best source for mulberry bud cultures (Oka and Ohyama, 1982). Glucose has proved to be effective carbon source for tissue culture of *Alnus* species (Tremblay and Laconde, 1984; Welander *et al*, 1989), *Potentilla fruticosa* and *Ficus lyrata* (Wain Wright and Scarce, 1989). However, Romano *et al* (1995) have observed that even though 2% glucose provides high proliferation rate, the shoots produced were compact and difficult to handle during transfers. The role of fructose in aseptic cultures has been found to be controversial (Oka and Ohyama, 1986; Chavin and Salesses, 1988).

Sorbitol has been demonstrated to be effective for apple and related species (Pua and Chong, 1985; Marino *et al*, 1993). It was found that sorbitol was essential for the initiation stage and supported growth only upto first subculture. This is because sorbitol is the main photosynthate and translocation carbohydrate in many of the species (Coffin *et al*, 1976). Sorbitol does not stimulate shoot proliferation (Romano *et al*, 1995), but it is provided in the medium to regulate osmotic potential in plants where it is not taken up or metabolized.

In the present study, the most effective carbohydrate, for shoot proliferation was sucrose (20 g/l). Shoot length as well as number increased with increase in sucrose concentration upto 40 g/l. However, these shoots showed vitrience. Combination of fructose, glucose or sorbitol with sucrose does not improve shoot length as well as shoot number.

Shoots *in vitro* often undergo episodic flush/quiescent cycles initially. The rate of multiplication by axillary branching can be substantially enhanced by using suitable conditions. There is limit to which shoot multiplication can be achieved in a single passage, after which further axillary branching stops. At this stage, the miniature shoots can be planted on a fresh medium of the same composition and multiplication can be repeated. After a number of subcultures shoot growth

stabilizes and shoot initiation and growth becomes continuous.

In neem, B2 medium, viz., MS 3/4 supplemented with BAP (0.1 mg/l), KN (0.05 mg/l), CP and B (0.05 mg/l) and sucrose (20 g/l) was found to be optimum in terms of shoots per explant as well as shoot length. It was observed that shoot growth is limited upto 5th or 6th subculture, after which the number of shoots decreases **(Table 3.7)**.

In some species, excessive callusing of shoots occurs. This can be overcome by reducing the cytokinin level in medium to levels below the optimal for shoot initiation (McCown and McCown, 1987). Finally, the successful transfer of tissue culture plants and their acclimatization determines the economic viability of the protocol (Conner and Thomas, 1982). The transplants must undergo a period of acclimatization i.e. a period of transitional development in which anatomical and physiological characteristics undergo changes to escape the influence of *in vitro* culture conditions (Donnelly and Vidaver, 1984).

Two methods were undertaken for the acclimatization of *in vitro* grown neem shoots:

1. *Ex vitro* rooting
2. *In vitro* rooting and then transfer to soil.

The main advantage of using *ex vitro* rooting conditions is that rooting and acclimatization can be carried out simultaneously, thereby omitting one of the steps and eventually reducing production cost. In the present studies, a drastic reduction in survival rate (from 90% to 25%) after 2 weeks of transfer to green house conditions, was observed in the case of neem. This can be mainly attributed to the fact that in unrooted shoots, the evaporation rates are more due to reduced stomatal control, and the shoots are photosynthetically non-competent (Donnelly and Tisdall, 1993). Moreover, the roots may be poorly developed and do not *function* normally.

Narayan and Jaiswal (1985) have reported the production of roots on MS basal medium supplemented with 1.0 mg/l IAA within 8-10 days. However, in the present investigation maximum rooting was observed with 2.0 mg/l IAA, followed by 1.0 mg/l and 0.5 mg/l. These rooted plantlets when kept in tap water under

room conditions for one week before transplanting to soil, showed better acclimatization. Under field conditions, these plants showed all the desirable traits viz., rapid growth, canopy development and large number of branches and leaves and the bigger size of leaves **(Table 3.8)**.

3.5 CONCLUSION

The results presented reveal that an adult *Azadirachta indica* tree can be propagated through *in vitro* culture of nodal explants. A combination of the cytokinins BAP and KN was essential for the successful sprouting of the axillary buds. When the sprouted buds were maintained on the same medium, elongation did not occur. However, when the sprouted buds were transferred to low concentrations of the cytokinins, rapid elongation of shoots was observed. The addition of vitamins like calcium pantothenate and biotin and the use of 3/4 strength of MS macronutrients promoted faster multiplication as well as elongation.

By using the method described above, a considerable number of plants can be propagated from a single nodal segment by continuous subculturing of shoot propagules. This method can be used for the successful propagation of superior neem trees showing faster growth and higher yield of active compounds.

CHAPTER 4

SOMATIC EMBRYOGENESIS IN *Azadirachta indica* A. Juss

4.1.INTRODUCTION

In the area of forest biotechnology, somatic embryogenesis is regarded as the *in vitro* system of choice for mass propagation of superior and genetically engineered forest tree ecotypes (Gupta *et al*, 1991). Somatic embryogenesis has a number of advantages over other micropropagation techniques like:

i. A tap root is always a rule in plantlets derived from somatic embryos (except in monocots). This is significant in the survival and health of the tree.

ii. Somatic embryoids are produced in much higher numbers than the adventitious shoots in other systems (organogenesis and axillary shoot proliferation).

iii. When induced in cell suspensions, somatic embryos are amenable to automatic handling thereby saving labor costs.

iv. By suitable encapsulation techniques, somatic embryos can be handled like natural seeds for storage and mechanized sowing.

v. Somatic embryogenesis by its nature is more suitable for procedures like genetic engineering and somaclonal variation technology (Merkle *et al*, 1990).

Thus clonal propagules obtained via somatic embryogenesis will have significantly lower costs than those produced using other micropropagation systems.

Somatic embryogenesis has been clearly defined by Haccius (1978), as a non-sexual developmental process which produces a bipolar embryo from somatic tissue. Two pathways of *in vitro* embryogenesis have been described:

1. Direct pathway: Embryos originate directly from tissues in the absence of callus proliferation.

2. Indirect pathway: Callus proliferation is pre-requisite to embryo development (Sharp *et al*, 1982).

Various factors such as genotype, explant, pre-conditioning of the explant, media requirements and origin of somatic embryos have been studied extensively (Thorpe *et al*, 1991; Litz and Gray, 1992). Studies at the structural, metabolic and molecular levels on the complex sequence of events associated with embryogenesis have also been carried out (Thorpe and Kumar, 1993). Furthermore, embryogenic cultures of forest trees have also been shown to make excellent target material for gene transfer via both the methods viz., *Agrobacterium* Ti-plasmid mediated and ballistic transformations (McGranahan *et al*, 1989; Wilde *et al*, 1992; Ellis *et al*, 1993).

Despite the difficulties in regeneration of woody species via somatic embryogenesis, several forest and fruit tree species have been regenerated and the list is ever increasing. Reviews on forest trees (Tulecke, 1987; Thorpe *et al*, 1991), tropical and sub-tropical fruit crops (Litz and Jaiswal, 1991), perennial fruit and nut crops (Litz an Gray, 1992), temperate zone fruits and nut crops (Zimmerman, 1991), cover the status of somatic embryogenesis and other modes of micropropagation for these crops.

The present status indicates that woody plants can no longer be considered as recalcitrant. A number of plant species which could not be propagated via meristem or shoot tip culture have been found to be amenable to somatic embryogenesis.

The most important factor contributing to the success is perhaps the right choice of explant. Immature embryo and nucellar tissue have proven to be the most responsive source of explant because of the presence of predetermined cells for production of somatic embryogenesis (Sharp *et al*, 1980). Some of the tree species like citrus, coffee and sandalwood have exhibited higher morphogenetic potential. This has been attained by using the right choice of the explant, auxin and cytokinin combinations, high levels (5-6%) of sucrose and additives like malt extract, L-glutamine in the media (Litz *et al*, 1985). Moreover, citrus and coffee have been used as model systems to study the process of somatic embryogenesis. Recent reports on a new cytokinin like compound thidiazuron (TDZ) shows the potential for regeneration of recalcitrant tree species (Huetteman and Preece, 1993).

Thus, it is widely believed that embryogenic cultures will eventually be employed for commercial-scale production of clonal propagules of forest trees. However, a survey of literature reviewing progress in forest tree somatic embryogenesis, reveals that, there remain significant barriers to application of somatic embryogenesis for mass propagation of commercially important forest trees.

By taking advantages of features such as repetitive embryogenesis and pro-embryogenic mass (PEM) production, somatic embryogenesis production can be scaled-up for some hardwoods (Srivastava, 1991). The bottleneck in the whole process is their conversion to plantlets. Treatments such as cold stratification and desiccation, which mimic pre-germination conditions in the seed, can have a significant impact with recalcitrant somatic embryos. Moreover, efforts are also being made to solve the problems of embryo synchronization, proliferation, maturation and germination at the production level.

There are numerous potential applications of *in vitro* culture techniques towards improvement of *Azadirachta indica*. Its mass propagation on a commercial scale and development of high yielding lines with improved seed quality with high oil content via tissue culture methods, are some of the areas where tissue culture will have its impact.

The first conclusive evidence of induction of somatic embryogenesis in *Azadirachta indica* was demonstrated by Shrikhande *et al*(1993).

Earlier, Muralidharan and Mascarenhas (1989), have reported the induction of bipolar structures from cotyledon induced callus on Gamborg's B5 basal medium supplemented with BAP and KN or 2iP. However, no studies were conducted to confirm the nature of these structures.

Islam *et al*(1996), obtained plant regeneration via somatic embryogenesis from nucellar tissue of immature seeds and from mature and immature cotyledons of *Azadirachta indica*. The cultures were initiated on Murashige and Skoog's (MS, 1962) and Linsmaier and Skoog's (LS, 1965) medium supplemented with different concentrations of cytokinins and auxins, either singly or in combination.

They reported that the choice of the basal medium was the deciding factor for the embryogenic pathway.

The present work was undertaken to optimize the conditions for the *in vitro* regeneration of neem from immature cotyledonary tissue via direct as well as indirect somatic embryogenesis. In neem, since the seed being the main source of its active compounds, it was the choice of explant. The cotyledons of the immature seeds were more responsive in culture regarding both the pathways of somatic embryogenesis. A step wise evaluation in optimizing the conditions for the induction of somatic embryogenesis, their maturation and germination, acclimatization to field conditions and their evaluation is reported. Part of the work has been published by the author earlier (Shrikhande *et al*, 1993; Joshi and Thengane, 1996a).

4.2.MATERIALS AND METHODS

Unripe mature fruits were surface sterilized, the cotyledons removed aseptically and the embryo axis excised. The remaining cotyledon was cut horizontally into two pieces (0.5 cm each) and inoculated abaxially onto the medium.

MS basal media were supplemented with MS vitamins, casein hydrolysate (CH, 1000 mg/l), IAA/2,4-D (0.5 - 5.0 mg/l), BAP/KN/TDZ (0.1 - 3.0 mg/l) and sucrose (50 g/l). The medium was solidified with 0.7% agar-agar after pH adjustment to 5.7. The auxins and cytokinins were incorporated into the medium prior to autoclaving. The media were autoclaved at 120°C and 15 lb. psi for 20 minutes. Cultures were incubated at 25±2°C in dark.

The type of auxin used was the deciding factor to determine the pathway of embryogenesis. MS basal medium supplemented with MS vitamins, CH (1000 mg/l), IAA (0.5 mg/l), BAP (1.0 mg/l) and sucrose (50 g/l) gave rise to embryogenic callus which later on yielded embryos. On MS basal medium supplemented with MS vitamins, CH (1000 mg/l), 2,4-D (0.5 mg/l), BAP (1.0 mg/l) or TDZ (0.5 mg/l) and sucrose (50 g/l) direct somatic embryogenesis was observed.

The cultures were incubated in dark at 25±2°C, till the emergence of the

embryos from the callus or from the cotyledonary surface. After the formation of the embryos the cultures were transferred to white fluorescent illumination, under 16/8 hour photoperiod, at 25±2°C. The subculture period was 21 days.

4.2.1.Indirect pathway

To optimize the conditions of embryo induction, various experiments were carried out.

In the preliminary experiments, the cotyledonary pieces were inoculated on MS basal medium supplemented with MS vitamins, IAA (0.5 - 5.0 mg/l), alone or in combination with either BAP or KN (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l), to test the effect of different concentrations of auxin, alone or in combination with cytokinins, on callus induction.

To optimize the concentration of each supplement used, different experiments were conducted. To optimize the cytokinin type and concentration, required for the induction of embryogenic callus, different concentrations of BAP or KN (0.2 - 3.0 mg/l) were tested with MS basal medium supplemented with MS vitamins, IAA (0.5 mg/l), CH (1000 mg/l) and sucrose (50 g/l).

To optimize the type and concentration of carbohydrate required for the induction of embryogenic callus, different concentrations of glucose and sucrose (30, 50, 70 and 100 g/l) were tested with MS medium supplemented with MS vitamins, IAA(0.5 mg/l), BAP(1.0 mg/l) and CH (1000 mg/l).

For maturation and germination of embryos, following media combinations were tested, viz.,

a. Lowered concentration of CH upto 200 mg/l or total omission of CH.
b. Omission of IAA with or without lowered concentration of CH to 200 mg/l.
c. Increased BAP concentration upto 2.0 mg/l with lowered concentration of CH or omission of CH.

To increase the germination percentage of the somatic embryos, the following steps were followed:

1. The embryogenic mass was transferred to MS liquid medium, supplemented

with CM (5%), IAA (0.1 mg/l), GA_3(0.01 mg/l), BAP (2.0 mg/l) and sucrose (20 g/l).

2. The germinated embryos were transferred to MS half strength plain liquid medium supplemented with sucrose (20 g/l) only.

When the plantlets grow to a height of more than 2.5 cm, they were then transferred to sterile sand:soil mixture (1:1) in polybags, acclimatized under green house conditions and then shifted to field conditions after 3 months. The field evaluation of these plants for comparison of their morphogenetic characters to those of seed raised plants was done over the year.

4.2.2.Direct pathway

Cotyledons were inoculated on MS medium supplemented with MS vitamins, 2,4-D (0.5 - 5.0 mg/l) alone or in combination with BAP or KN (1.0 mg/l) or TDZ (0.5 mg/l), CH (1000 mg/l) and sucrose (50 g/l) to test the effect of growth regulators alone or in combination on embryo induction.

To optimize the concentration of BAP required for the induction of somatic embryos, the cotyledons were inoculated on MS basal medium supplemented with 2,4-D (0.5 mg/l), BAP (0.2-3.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l).

To evaluate the number of days required to make explant embryogenically determined, the explants were exposed to optimum concentration of auxin, and then transferred to MS plain medium with or without activated charcoal (0.25 g/l) after incubation on induction medium for 7, 11, 15 and 21 days.

For embryo maturation and germination, various media with total omission or reduction of CH (0, 200, 500 mg/l); lowering of 2,4-D (0.2-0.5 mg/l) and increase of BAP to 2.0 mg/l were tested.

The data on percentage of explants showing embryogenic response, percentage of embryos germinating and converting to plantlets, etc., was selected and evaluated according to the methods described in Chapter 2, Section 2.5.

To procure evidence for the type of somatic embryogenesis pathway, the 20 and 40 day old embryogenic masses were fixed for histological studies. In case of direct pathway, 11 and 21 day old cultures were fixed. The procedure of fixing of the material and its processing is described in Chapter 2, Section 2.7.

4.3.RESULTS

The type of auxin used was observed to influence the type of embryogenic pathway. MS basal medium supplemented with 2,4-D induced somatic embryos directly on the cotyledonary surface **(Fig. 4.1a)**, whereas MS basal medium supplemented with IAA induced somatic embryos via callus phase **(Fig. 4.1b)**.

4.3.1.Indirect Pathway:

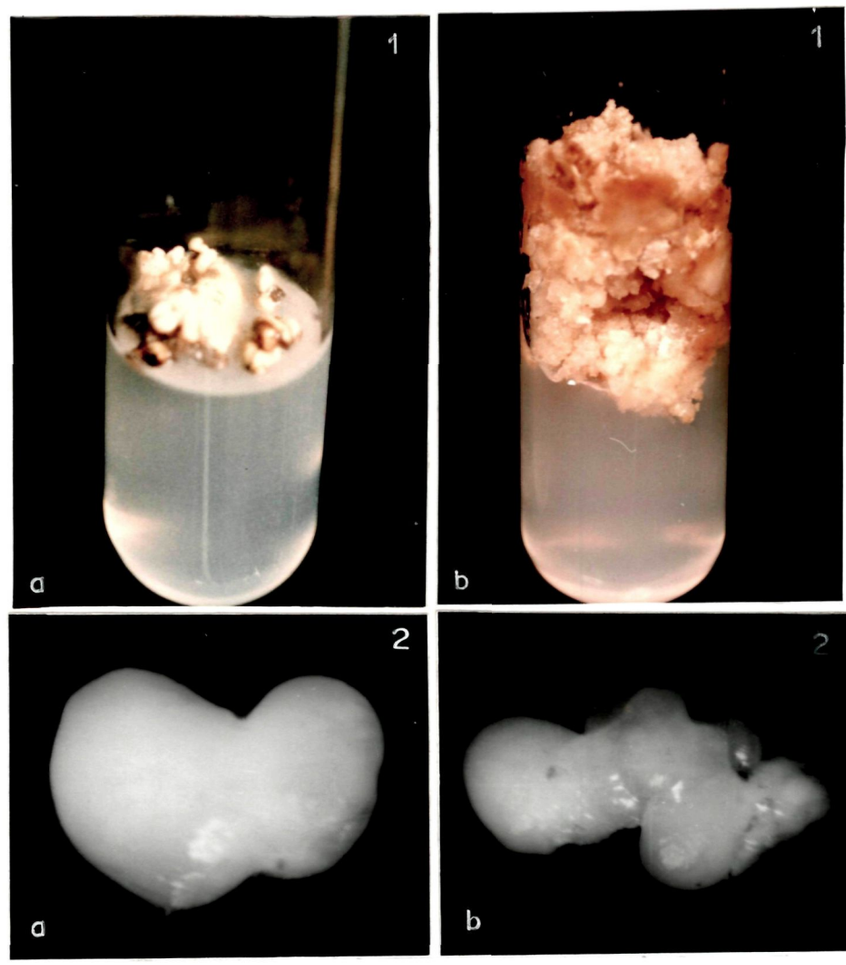
Preliminary experiments showed that IAA (0.5-5.0 mg/l) alone and in combination with KN (1.0 mg/l) induced callusing and rhizogenesis. Combination of IAA with BAP (1.0 mg/l) also showed callusing. However, along with callusing somatic embryogenesis was observed on MS basal medium supplemented with IAA (0.5 mg/l) in combination with BAP (1.0 mg/l).

Within 1-2 weeks of incubation in dark, swelling and callusing of the cotyledonary tissue at the cut surfaces was observed. The callus was compact and nodular. The callus was recultured after 21 day interval. Initiation of shiny globular masses was evident after two subcultures (i.e. after 40-45 days) on combination of IAA (0.5 mg/l) and BAP (1.0 mg/l). These, preliminary experiments suggest that the somatic embryos could be induced from callus on MS basal medium supplemented with MS vitamins, IAA (0.5 mg/l), BAP (1.0 mg/l), CH(1000 mg/l) and sucrose (50 g/l). To confirm the requirement of particular cytokinin and its optimal concentration, BAP and KN concentrations were varied from 0.2-3.0 mg/l **(Table 4.1)**. The response in the presence of KN was not significant and only 10% cultures showed embryo induction with 0.2 and 0.5 mg/l KN. However, on prolonged incubation on the respective KN media for more than 21 days, these somatic embryos reverted back to callus formation.

All concentrations of BAP induced embryogenesis. At 1.0 mg/l BAP concentration, 60% cultures showed induction of embryos. Further increase in

Figure 4.1: Effect of type of auxin on embryogenic pathway.
a= MS basal supplemented with 2,4-D;
b = MS basal supplemented with IAA.

Figure 4.2: Induction of abnormal embryos (20x):
a = fusion of two embryos
b = fusion of 2-4 embryos


1
a
1
b
2
a
2
b

concentration of BAP (3.0 mg/l) did not improve the frequency of embryogenesis.

Table 4.1: Effectof different concentrations of cytokinins oninduction of somatic embryogenesis.

Cytokinin		Percent response
BAP(mg/l)	KN (mg/l)	
0.2	—	40
0.5	—	45
1.0	—	60
3.0	—	40
—	0.2	10
—	0.5	10
—	1.0	—
—	3.0	—

Medium: MS + IAA (0.5 mg/l) + CH (1000 mg/l + sucrose (50 g/l)

The effect of different concentrations and combinations of sucrose and glucose on embryogenic efficiency **(Table 4.2)** was tested as a part of the study. With increase in sucrose concentration from 50 g/l to 100 g/l, 70% embryogenesis was observed. However, the number of abnormal embryos increased especially at 70 g/l and 100 g/l sucrose concentration **(Fig. 4.2 a&b)**. All combinations of glucose with sucrose, at the concentrations tested, had an adverse effect on embryo differentiation which resulted in increased callusing. Thus, 50 g/l sucrose appears to be optimal for induction of normal embryos.

Thus, it was confirmed that for induction of embryos via callus, optimum medium required was MS basal medium supplemented with IAA (0.5 mg/l), BAP (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l) **(Initiation medium)**.

Figure 4.3: Effect of incubation period on embryogenic efficiency of callus.

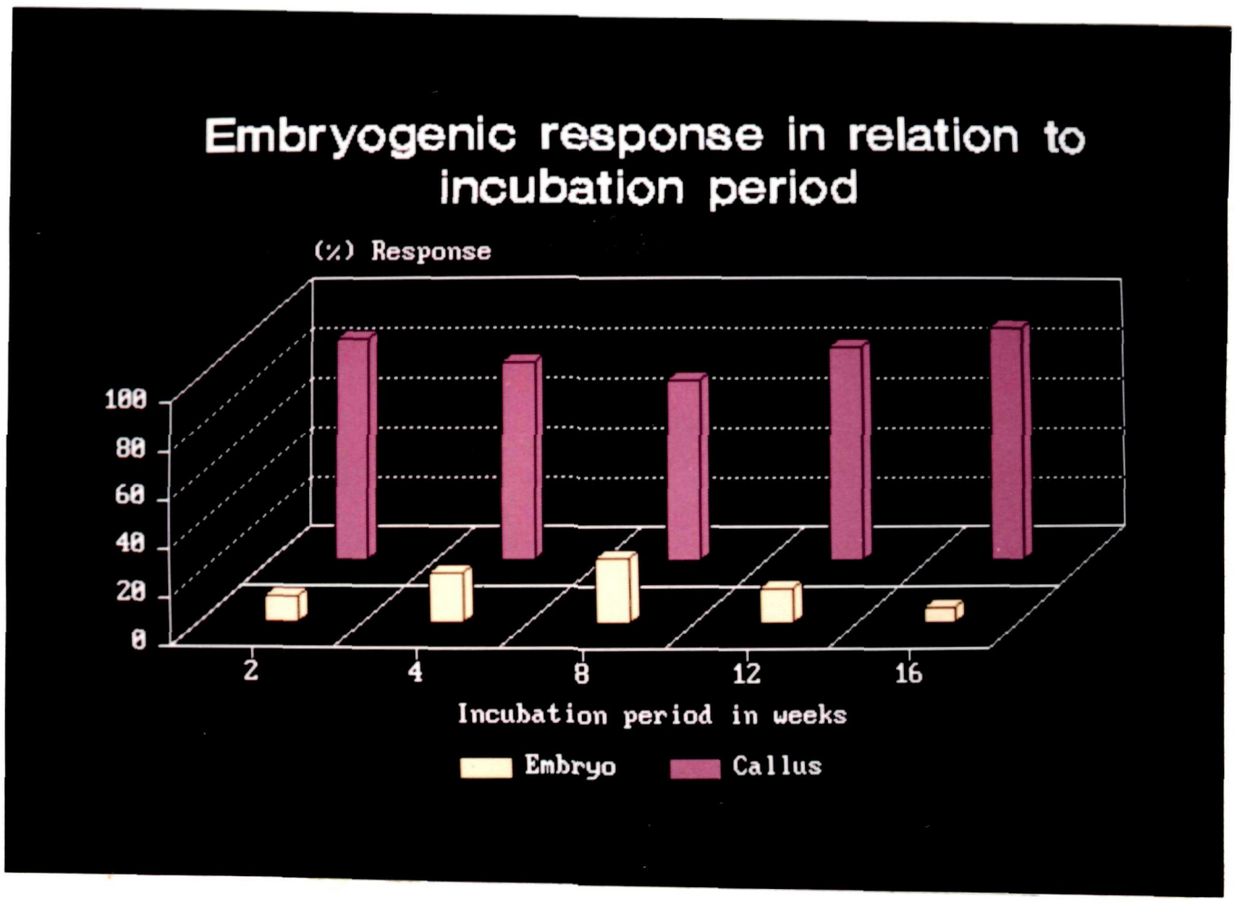


Figure-4.3

a rudimentary root **(Fig. 4.7)**. However, abnormal germination of the embryos,

Table 4.2: Effect of different concentrations and combinations of sucrose and glucose on embryo induction.

Sucrose (g/l)	Glucose (g/l)	% Embryogenesis
30	—	50
50	—	70
70	—	70
100	—	70
20	20	30
30	30	30
40	40	40
50	50	50

Medium: MS + IAA (0.5 mg/l) + BAP (1.0 mg/l) + CH (1000 mg/l).

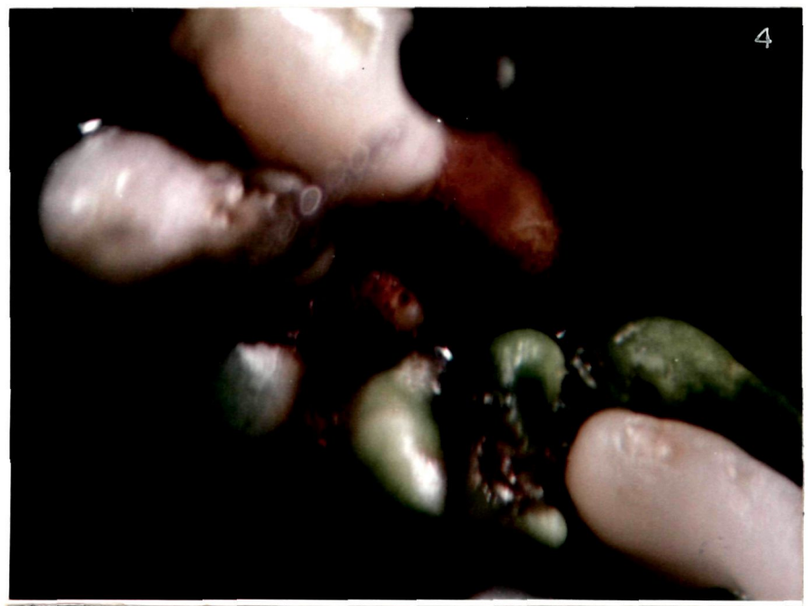
Figure 4.3 shows the effect of incubation period on embryo induction frequency. With increase in culture period upto 8 weeks there was increase in the number of explants showing embryogenesis (26%), after which there was a gradual decline in embryo differentiation. However, prolonged incubation on the initiation medium increased callusing with gradual decrease in embryo differentiation.

On the initiation medium callus gradually becomes nodular and compact. With the advancement of incubation period, globular shiny proembryogenic masses appeared on peripheral sectors of the callus. Various stages of defined embryos appeared after 40 days of incubation. Thus embryo development was non-synchronous **(Fig. 4.4, 4.5)**. Transfer of the embryogenic mass on fresh initiation medium after 21 days, resulted in further proliferation of embryos, but their subsequent development to plantlets was arrested. About 2-5% embryos germinated on the same medium **(Fig. 4.6)**. However, germinated plantlets were necrotic and fasciated. Therefore, the embryogenic masses were subcultured on different media for maturation and germination of the embryos **(Table 4.3)**. The cultures were transferred to light conditions under 16/8 hour photoperiod.

With increase in BAP concentration to 2.0 mg/l, keeping IAA (0.5 mg/l) and CH (1000 mg/l) constant, there was increase in the number of mature embryos. Some of the embryos even showed elongation of shoot axis and development of a rudimentary root **(Fig. 4.7)**. However, abnormal germination of the embryos,

Figure 4.4: Non-synchronous growth of embryos *from underlying* callus (10x).

Figure 4.5: Various stages of somatic embryos
a = globular stage (20x)
b = heart shaped (20x)
c = cotyledonary stage (16x)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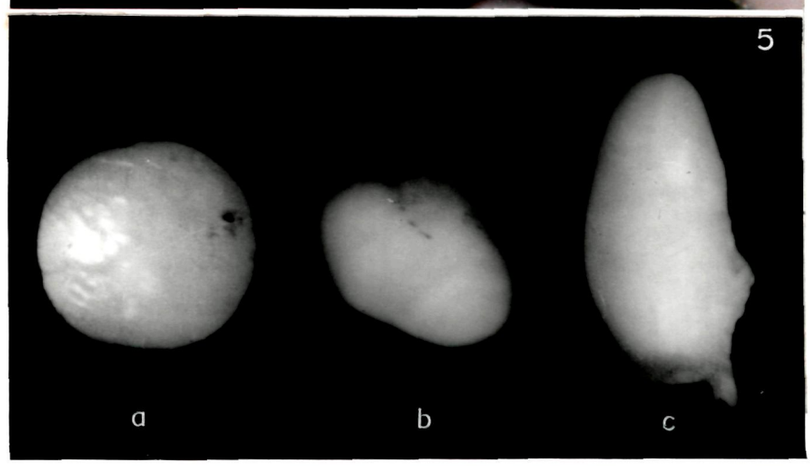
5
a
b
c

Figure 4.6: 2-5% germination on induction medium.

Figure 4.7: Germinating embryo showing shoot and rudimentary root (20x).

Figure 4.8: Abnormal germination of embryos (20x)
a = initiation of only shoot
b = initiation of only root


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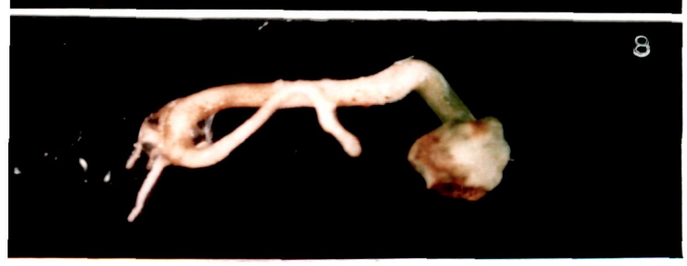
7


8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i.e., emergence of either shoot or root was also observed on this medium **(Fig. 4.8 a&b)**.

Reduction of CH (200 -500 mg/l) improved development of normal embryos but callusing of embryos persisted. Total omission of CH with increase in BAP to 2.0 mg/l concentration, had a promotory effect on embryo maturation and germination **(Table 4.3)**.

Table 4.3: Effect of BAP and CH on embryo maturation and germination

Growth hormones		CH (mg/l)	No. of Mature embryos/ culture	% Germination
IAA(mg/l)	BAP(mg/l)			
0.5	1.0	1000	65	1
		500	60	12
		200	63	28
		—	54	15
0.5	2.0	1000	70	45
		500	50	21
		200	45	12
		—	105	14
-	1.0	1000	31	25
		500	14	9.5
		200	3	NIL
		—	NIL	NIL

Medium: MS basal + sucrose (50 g/l)

On this medium 60-70% embryos enlarged and matured in about 20-30 days **(Fig. 4.9)**. These embryos were mainly in the late cotyledonary stage. An average of 105 embryos were obtained per explant on this medium. On total omission of IAA, very few embryos showed response but later turned black and died.

Even if the number of mature embryos obtained on MS basal medium supplemented with IAA (0.5 mg/l), BAP (2.0 mg/l) and sucrose (50 g/l), with total omission of CH was more, the germination percentage was only 14%. Therefore to increase the germination percentage, these embryos were transferred after 21 days to MS liquid medium supplemented with IAA (0.1 mg/l), GA_3 (0.01 mg/l), BAP (2.0 mg/l), CM (5%) and sucrose (20 g/l) **(Fig. 4.10, 4.11)**, where 97% of

Figure 4.9: 70% embryos mature on MS supplemented on IAA (0.5 mg/l), BAP (2.0 mg/l) and sucrose (50 g/l)

Figure4.10: Transfer of mass of mature embryos to liquid medium for proper development
Medium = MS liquid supplemented with IAA(0.5 mg/l), GA_3 (0.01 mg/l), BAP (2.0 mg/l), CM (5%) and sucrose (50 g/l).

Figure 4.11: Mature embryos separated from callus tissue.


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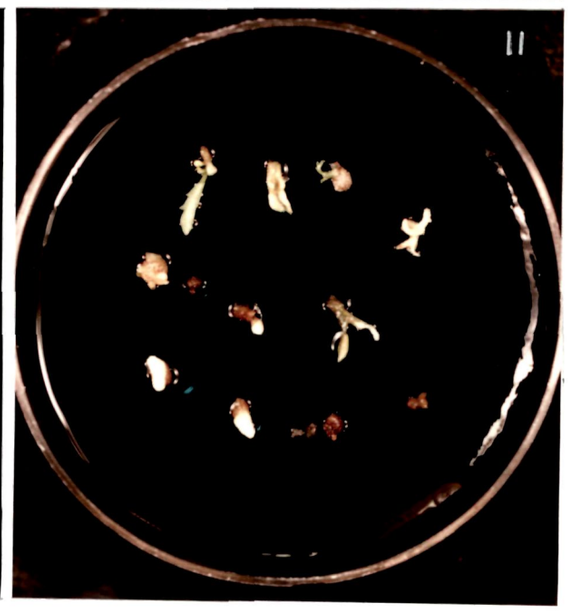

11

the embryos showed proper development and initiation of root and first leaf **(Fig. 4.12 a&b)**. Hardly 2-3% embryos showed abnormal growth.

The germinated embryos were then transferred to hormone free MS 1/2 strength liquid for further development into plantlets. On this medium the plantlets grew upto soil transferable height i.e. to a height of 2.5cm to 6.5cm within 3-7 days **(Fig. 4.13)**.

These plants were then transferred to polybags containing sand:soil (1:1) mixture and hardened under green house conditions **(Fig. 4.14)**. The fully acclimatized plants were then transferred to field conditions after three months **(Fig. 4.15)**. The morphogenetic characters of these plants were evaluated over the year, i.e., at sixth and 12th month. The data of control plants was taken only after 12 months.

It was observed **(Table 4.4)**, that the embryogenic plants undergo rapid growth as compared to control, right from the age of one month **(Fig. 4.14)**. The embryogenic plants at the age of six months were of 63cm height **(Fig. 4.15)** as compared to the control of 38.7cm after 12 months. The embryogenic plants showed all the desirable traits viz., rapid growth, canopy development, increase in number of branches and leaves and size of the leaves.

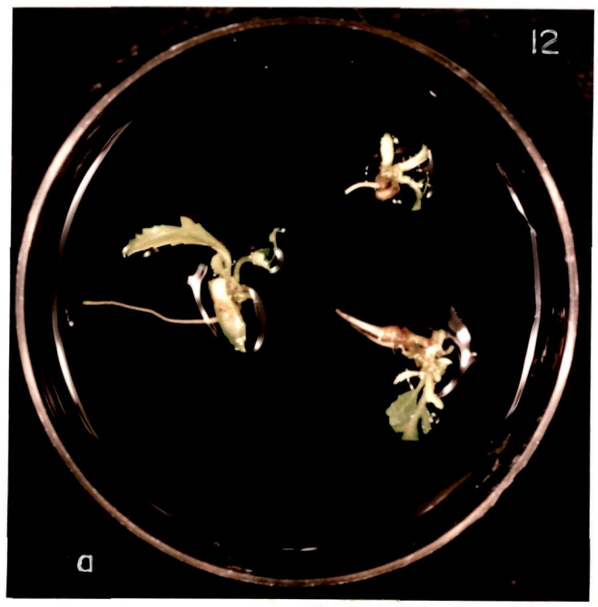
Table 4.4: Comparison of morphogenetic characters of embryogenic and control plants.

Character	Control	Embryogenic plants	
		At 6 months	At 12 months
Height(cm)	38.7±5.2	63±3.2	162.4±4.5
Number of branches	4±1.4	5±1.3	10±1.5
Height of first branch from ground (cm)	9±0.6	19±1.5	102.3±1.7
Number of leaves	44±4.5	60±5.1	81±2.3
Size of leaves (cm x cm)	1.2 x 3.1 ±0.15 ±0.4	1.4 x 3.5 ±0.3 ±0.11	1.8 x 4.3 ±0.18 ±0.5

Mean of 10 plants.

Figure 4.12: a&b Normal germination of embryos.

Figure 4.13: Growth of germinating embryos on
MS half strength medium
a = just transferred germinated embryo
b = after 3 days
c = after 7 days


12
a


12
b


13
BOROSIL
TEST TUBE
a
b
c

Figure 4.14: Plants to soil; Control and Experimental Age = one month.

Figure 4.15: 6 months old plant growing in field.


14


15

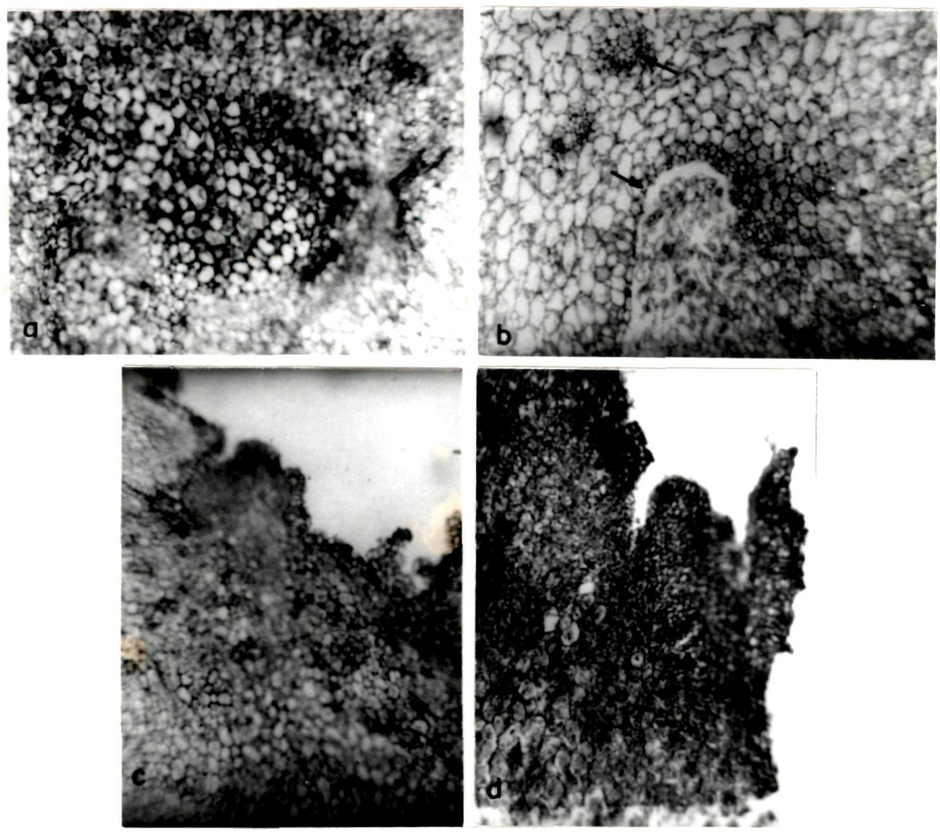
Figure 4.16

a: Transverse section of callus showing disorganized cells with meristematic pockets of small isodiametric cells (250 x).

b: Meristematic pockets demarked by fragmentation (160 x).

c: Meristematic pockets pushing towards periphery of the callus (160 x).

d: Defined embryo still attached to underlying callus (80 x).



Figure–4·16

Figure 4.17

a: Early heart shaped embryo, embryos in group attached to callus (32 x).

b: Late heart shaped embryo with defined suspensor (s) (25.6 x).

c: Torpedo stage embryo separated out from callus (25.6 x).

d: Fully grown embryo with defined cotyledons and short suspensor (25.6 x).

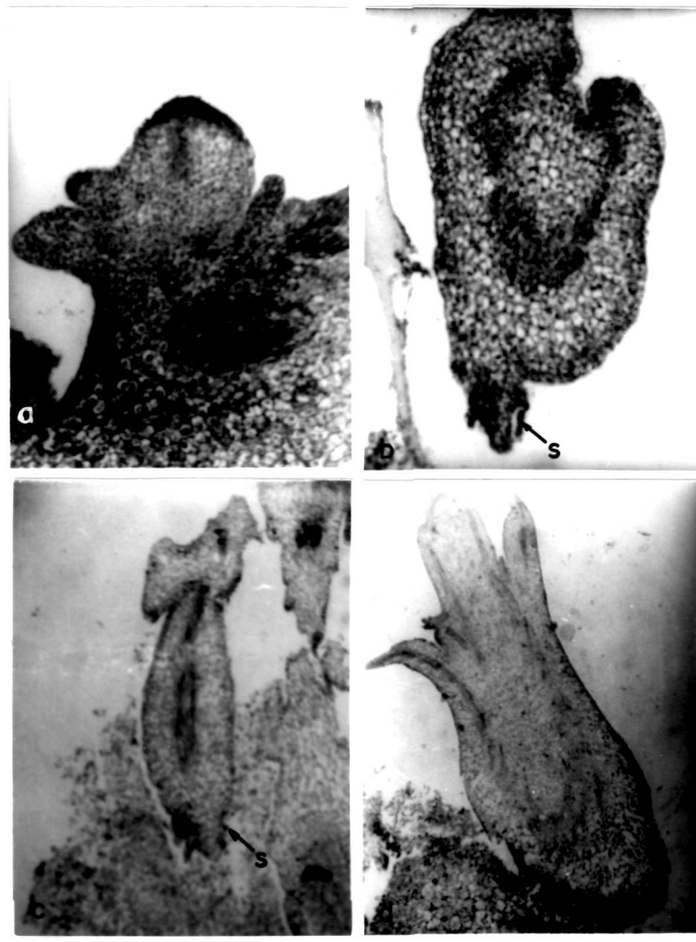


Figure – 4·17

4.3.2.Histological evidence

Histological sections of 15-20 day old cotyledonary explant cultures demonstrated callus proliferation. The callus cells were large, disorganized and highly vacuolated. Meristematic pockets comprising of compactly arranged small isodiametric cells, with dense cytoplasm were evident after 21 days **(Fig. 4.16a)**. The meristematic pockets had defined fragmentation lines which seem to be formed by thickened cell walls. These lines separate the smaller, meristematic cells from the surrounding larger, less meristematic and vacuolated cells **(Fig. 4.16b)**. These pockets were observed to push themselves towards the periphery of the callus to form embryos **(Fig. 4.16 c&d)**. The growth pattern of the embryos was non-synchronous. Therefore, histological sections of 30 day old culture show heart and torpedo stages **(Fig. 4.17 b&c)**.

In the early stages of embryo development, there was no evidence of procambium. The procambium appeared in the later stages of embryo development. The embryos at this stage were attached to the underlying callus. Further stages of embryo development are shown in **Fig. 4.17 a, b, c & d**. Heart shaped embryoids were observed to contain procambium, root meristem and rudimentary cotyledons **(Fig. 4.17b)**. These were either attached to the callus or separated from the callus mass **(Fig. 4.17 a&b)**. Late cotyledonary stage embryos seem to be separated from the callus mass. Presence of the suspensor was evident in the late heart and early cotyledonary stages **(Fig. 4.17 b&c)**. These stages showed presence of procambium, shoot and root primordia **(Fig. 4.17c)**. The histomicrograph of the fully grown embryos showed distinct cotyledons **(Fig. 4.17d)**.

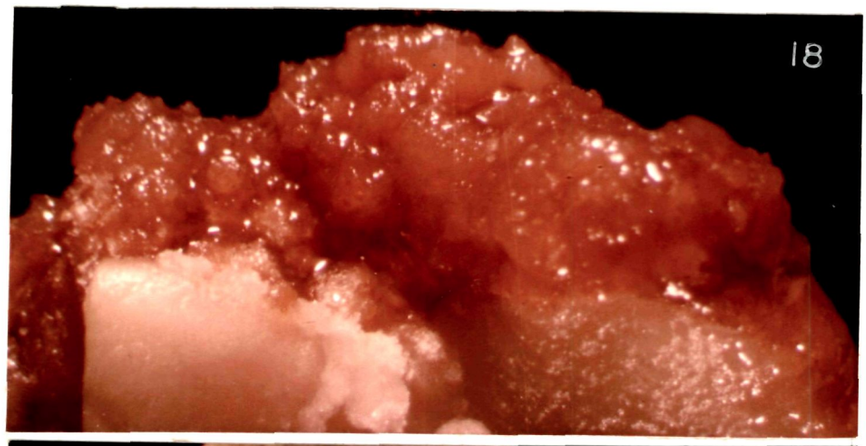
4.3.3.Direct Pathway

In the preliminary experiments, the cotyledons were incubated in dark on MS basal medium supplemented with 2,4-D (0.5 - 5.0 mg/l), BAP or KN (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l). All concentrations of 2,4-D (0.5-5.0 mg/l) alone and in combination with KN (1.0 mg/l), produced slimy brown callus **(Fig.4.18)**. 2,4-D at concentrations 0.5 mg/l and 1.0 mg/l in combination with BAP (1.0 mg/l) showed induction of tiny globular embryoids directly at the cut ends as well as on the surface of the cotyledons **(Fig. 4.19, 4.20)**. However, the

Figure 4.18: Slimy brown callus induced on MS basal medium supplemented with 2,4-D alone or in combination with KN (10x).

Figure 4.19: Initiation of embryogenesis at the cut ends of the cotyledons observed after 11 days on MS basal medium supplemented with 2,4-D (0.5mg/l) and BAP (1.0 mg/l) (16x)

Figure 4.20: Formation of tiny globular embryoids after 21 days (16x)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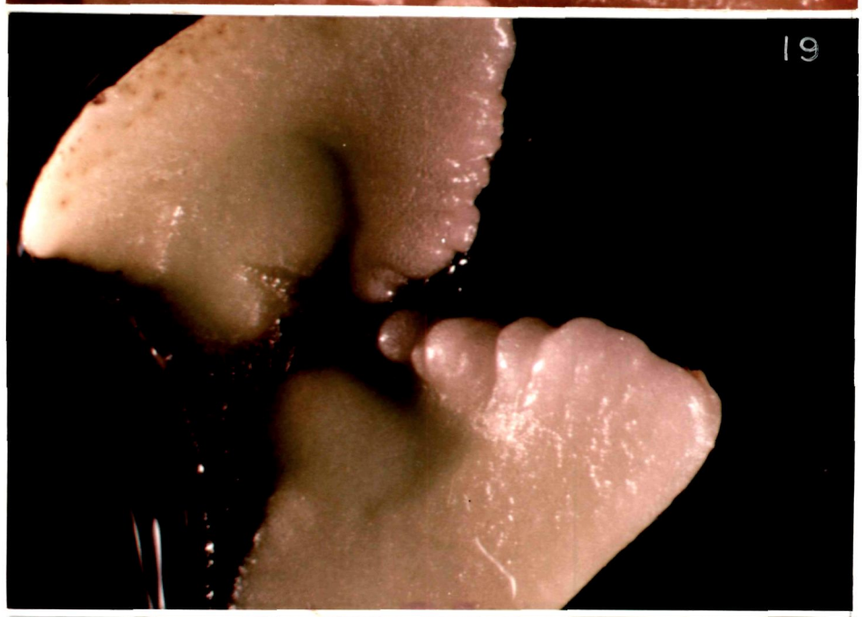
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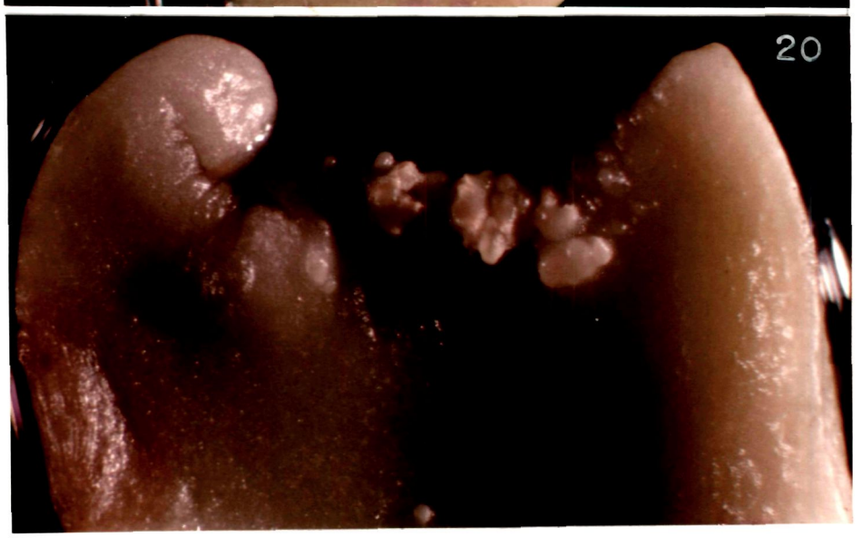
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embryos induced on MS basal medium supplemented with 2,4-D (1.0 mg/l) and BAP (1.0 mg/l) were abnormal and presence of slimy callus was also observed along with them **(Fig. 4.21)**.

Table 4.5: Effect of 2,4-D and BAP concentrations on embryo induction.

2,4-D (mg/l)	BAP (mg/l)	Percent embryogenesis	Number of embryos per culture
0.5	1.0	40	23±0.3
1.0	1.0	10	17±0.5
3.0	1.0	NIL	NIL
5.0	1.0	NIL	NIL
0.5	0.2	NIL	NIL
0.5	0.5	NIL	NIL
0.5	1.0	40	25±0.5
0.5	3.0	10	5±0.1
0.5	5.0	NIL	NIL

Medium: MS + CH (1000 mg/l) + Sucrose (50 g/l).
The embryos were counted after 15 days of incubation in dark.

The data shown in **Table 4.5**, confirms that the embryogenic induction was maximum (40%) with maximum number of embryos 23 per culture at 2,4-D (0.5 mg/l). Combination of 2,4-D (1.0 mg/l) with BAP (1.0 mg/l) also showed 10% embryogenesis with 17 embryos per culture, but with callus. With further increase in 2,4-D concentration upto 5.0 mg/l, induction of only white friable callus was observed **(Fig. 4.22)**.

To optimize the concentration of BAP keeping 2,4-D concentration constant (0.5 mg/l), the range of BAP concentrations (0.2 - 5.0 mg/l) were tested **(Table 4.5)**. On combination of 2,4-D (0.5 mg/l) with BAP (0.2 and 0.5 mg/l) only callus formation was observed. At BAP (1.0 mg/l), 40% of the cultures showed embryo induction. With increase in BAP (3.0 mg/l), embryo induction was only 10%. However, later these embryos turned brown and died. Further increase in BAP (5.0 mg/l) produced only callus.

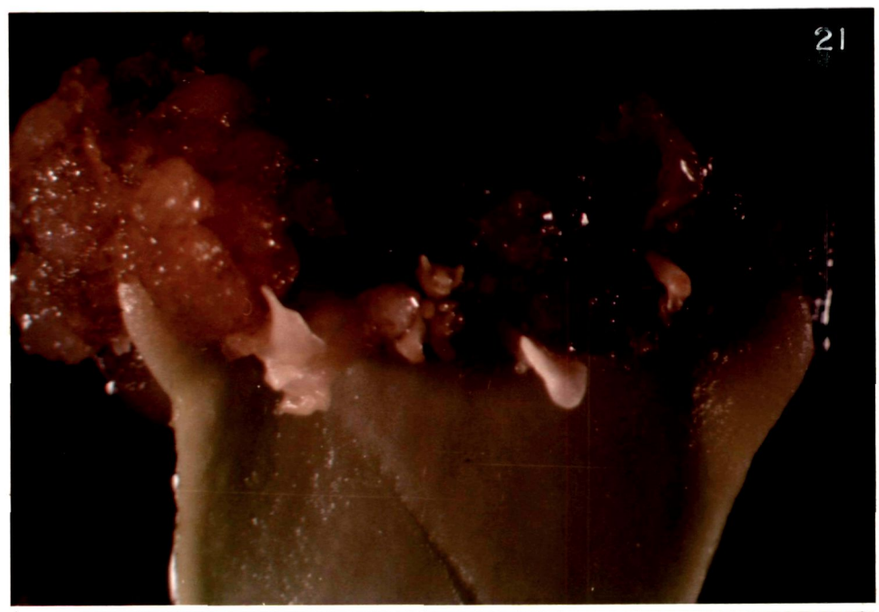
The embryogenic cultures initiated on MS basal medium supplemented with 2,4-D (0.5 mg/l), BAP (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l) were transferred to either fresh initiation medium or plain MS medium after 21 days

Figure 4.21: Abnormal embryos along with slimy brown callus induced on 2,4-D (1.0 mg/l) and BAP (1.0 mg/l) (16x)

Figure 4.22. Higher concentrations of 2,4-D induce white friable callus (16x)

Figure 4.23: Profuse but indistinct embryo formation on hormone free medium (16x)

Figure 4.24: Abnormal development of embryos when incubated on initiation medium for more than 21 days (20x)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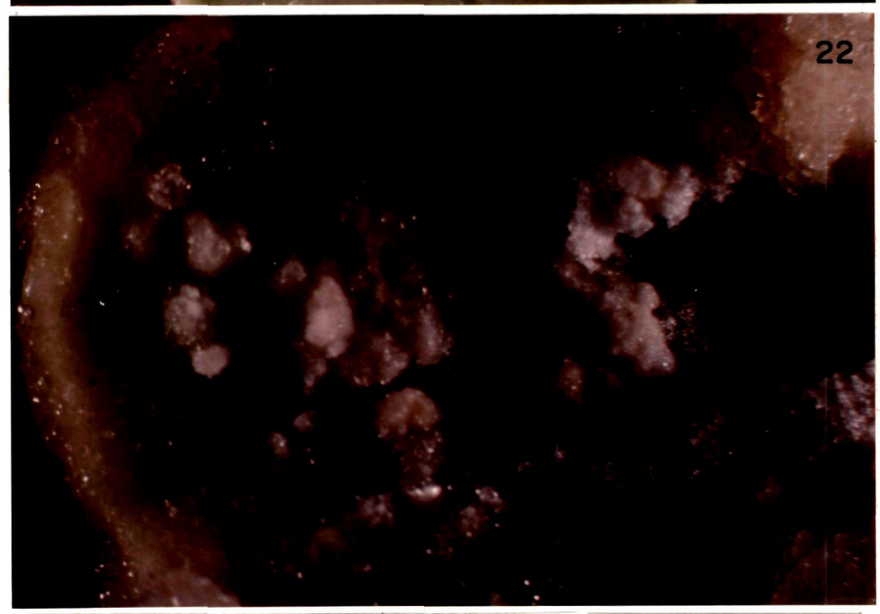
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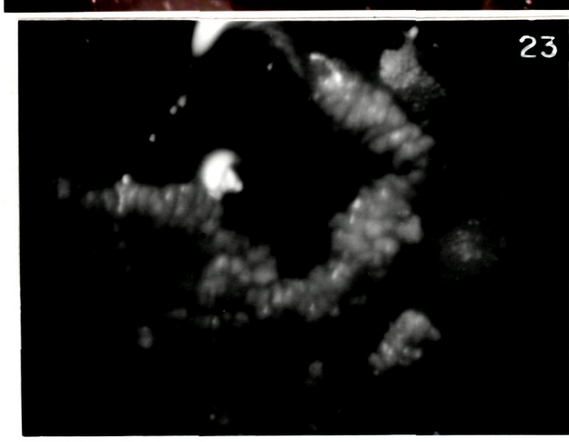
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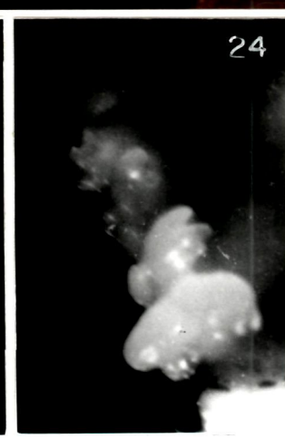
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with continued incubation in dark. When the cotyledons were transferred to MS plain medium, within 10 days 90% cotyledons showed initiation of profuse embryos which were not distinct **(Fig. 4.23)**. On fresh passage to initiation medium, 27% cultures showed abnormal development of embryos. The number of embryos varied from 2-65 **(Fig. 4.24)**.

Therefore, after incubation on the initiation medium for 17-20 days, the cotyledons were transferred to various media for maturation **(Table 4.7)**. When BAP concentration was increased to 2.0 mg/l and 2,4-D and CH were kept constant, the embryos turned brown and died. When BAP was increased to 2.0 mg/l and CH lowered to 500 mg/l, 18% of the cultures showed distinct embryos, but callusing was induced after 15 days.

Table 4.7: Various media tried for maturation of embryos induced on 2,4-D medium.

2.4-D mg/l	BAP mg/l	CH mg/l	Response
0.5	2.0	1000	C
	2.0	500	M,C
	2.0	200	C
	2.0	—	C
*0.5	*1.0	*1000	C
	1.0	500	C
	1.0	200	C
	1.0	—	C
0.4	1.0	1000	C
	1.0	500	C
	1.0	200	C
	1.0	—	C
0.3	1.0	1000	C
	1.0	500	M
	1.0	200	C
	1.0	—	C
0.2	1.0	1000	C
	1.0	500	M
	1.0	200	C
	1.0	—	C
0.1	1.0	1000	C
	1.0	500	C
	1.0	200	C
	1.0	—	C

* = Initiation medium; medium = MS basal + sucrose (50 g/l)
M = Maturation; C = Callusing

When CH was reduced to 500 mg/l, keeping 2,4-D and BAP concentrations constant, the embryos callused. Further reduction of CH to 200 mg/l or total omission of CH from the initiation medium also induced callusing of the embryos.

With lowering of 2,4-D concentration to 0.4mg/l, keeping BAP constant (1.0 mg/l), at all concentrations of CH tried, callusing of embryos persisted. Further lowering of 2,4-D to 0.3 and 0.2 mg/l, with BAP and CH concentration constant, also resulted in callusing of embryos. However, lowering of 2,4-D concentration to 0.3 mg/l and 0.2 mg/l and lowering of CH concentration to 500 mg/l, the embryos became distinct in 45% and 29% cultures respectively. On these media, only globular stage embryos were observed. Average number of embryos per explant at both the concentration was 25 and 38 respectively. Further lowering of CH concentration to 200 mg/l and on total omission of CH, resulted in callusing of embryos.

Drastic reduction in 2,4-D concentration to 0.1 mg/l or total omission of 2,4-D from the medium also lead to callusing or blackening of embryos.

To evaluate the number of days required for exposure to combination of 2,4-D (0.5 mg/) and BAP (1.0 mg/l), for induction of embryos, a separate experiment was conducted. After incubation on initiation medium for 7, 11, 15 and 21 days, the cotyledons were transferred to MS plain solid medium with or without activated charcoal (2.5 g/l) under light conditions. The results were recorded after 7 days of transfer to MS plain or MS plain with charcoal medium.

Table 4.6: Effect of incubation on embryo induction.

Incubation on initiation medium (days)	Percent cultures showing embryogenesis after transfer to	
	MS plain	MS plain + charcoal
7	9.2	7.3
11	37.5	51.2
15	65.7	37.5
21	21.4	19.1

Table 4.6 shows that the period of 11-15 days was critical as maximum number of the cultures showed induction of embryos. On MS plain medium, gradual increase was observed upto 15 days. Continued incubation upto 21 days

on the same medium induced callusing of the preformed embryos resulting in decrease in embryogenesis (21%). When MS plain medium was supplemented with charcoal, sudden increase in the percentage of embryogenesis was observed after 11 days (7.3% to 52.2%) which gradually reduced (37.5% to 19.1%) after 15 days incubation. In both the media 2,4-D does not promote embryo induction and development after 15 days. Most of the cultures after 17 days showed callusing of preformed embryos.

Findings of the above experiments revealed that all the media combinations tried so far could not improve embryogenesis with distinct embryo formation. Advanced stages of embryo development i.e. heart and torpedo stages were totally absent in any of the media compositions.

Another set of experiments on direct somatic embryogenesis was conducted where instead of BAP or KN, TDZ (0.5 mg/l) was used. Only 5% of the cultures showed embryogenesis, with white friable callus on MS basal medium supplemented with 2,4-D (0.5 mg/l) alone. However, the frequency of cultures showing embryogenesis increased to 55% when 2,4-D concentration was reduced to 0.1 mg/l **(Table 4.8)**.

Table 4.8: Embryogenic response on TDZ containing medium.

Growth Hormones		Percent Response		
2,4-D (mg/l)	TDZ	E	C	E+C
0.1	—	55	25	15
0.5	—	5	90	5
0.1	0.5	100	—	—
0.5	0.5	100	—	—

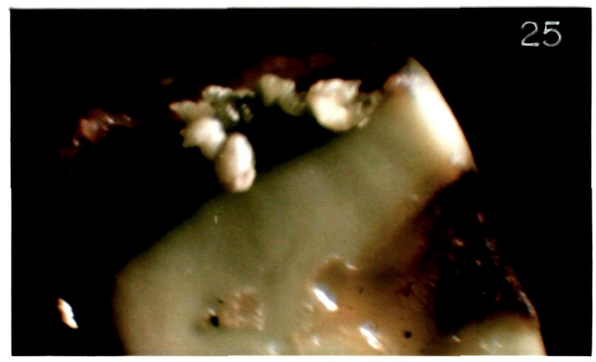
Medium: MS basal + sucrose (50 g/l)
E = Embryogenesis; C = Callusing.

Addition of TDZ (0.5 mg/l) to 2,4-D containing medium, irrespective of 2,4-D concentration, induced 100% embryogenesis within 11 days **(Fig. 4.25)**. However, the embryos induced on 2,4-D (0.5 mg/l) and TDZ (0.5 mg/l), formed white friable callus after 15 days in culture **(Fig. 4.26)**. Since the embryos induced on 2,4-D (0.1 mg/l) and TDZ (0.5 mg/l) did not callus even after 15 days of

Figure4.25: Formation of tiny embryoids on MS basal medium supplemented with 2,4-D and TDZ within 11 days. (16x)

Figure 4.26: The embryos formed white friable callus after 15 days on MS basal medium supplemented with 2,4-D (0.5 mg/l) and TDZ (0.5 mg/l) (10x)

Figure 4.27: Formation of profuse embryos on MS basal medium supplemented with 2,4-D (0.1 mg/l) and TDZ (0.5 mg/l) (16 x).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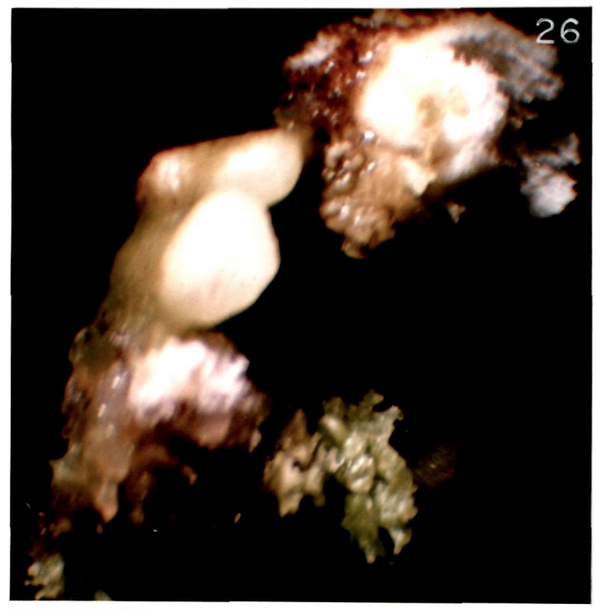
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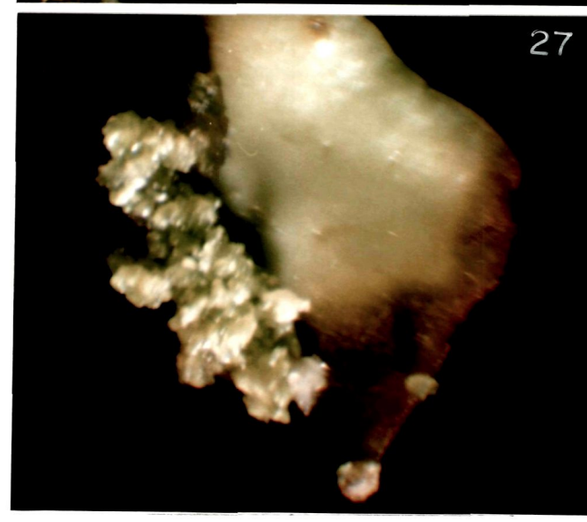
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incubation, this medium was taken as optimum for the direct embryo induction. **(Fig. 4.27)**.

For maturation of these embryos the cultures were transferred to respective initiation media with reduction in TDZ (0.05, 0.1 mg/l) after 11 days **(Table 4.9)**.

Table 4.9: Effect of reduced TDZ concentration on embryo maturation.

2,4-D (mg/l)	TDZ	Response
0.5	0.1	Blackening of explant
0.5	0.05	Callusing of embryos
—	—	No increase in size
0.1	0.1	Blackening of embryos
0.1	0.05	Callusing of embryos
—	—	Precocious germination

Medium: MS basal + sucrose(50 g/l)

The embryogenic cultures initiated on MS basal medium supplemented with 2,4-D (0.5 mg/l) and TDZ (0.5 mg/l), were transferred to MS basal medium supplemented with reduced TDZ (0.1 mg/l or 0.05 mg/l), keeping 2,4-D concentration constant (0.5 mg/l). On these media, callusing of embryos **(Fig 4.28)**, and blackening of the explant **(Fig. 4.29)** was observed within 7 days . On transfer to hormone free medium also there was no indication of maturation **(Fig. 4.30)**.

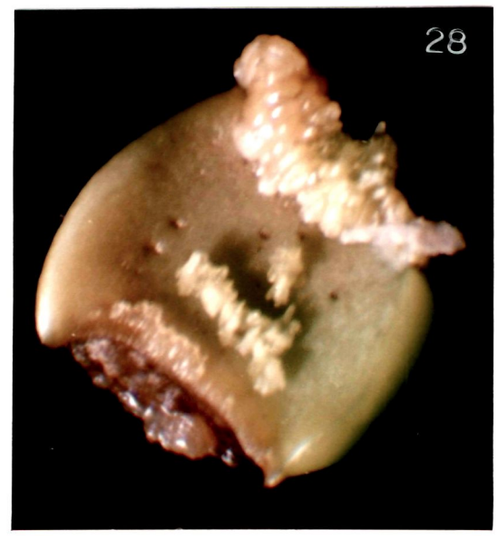
The embryogenic cultures initiated on MS basal medium supplemented with 2,4-D (0.1 mg/l) and TDZ (0.5 mg/l), when transferred to medium containing low concentrations of TDZ i.e. 0.1 mg/l or 0.05 mg/l, keeping 2,4-D concentration constant, also showed blackening and callusing of embryos within 7 days. However, transfer of these cultures to hormone free MS medium, led to precocious germination within 7 days **(Fig. 4.31)**. Distinct stages of embryogenesis were not observed on this medium also. However, embryos with underdeveloped or fused cotyledonary leaves were observed among them **(Fig. 4.31 a&b)**.

Based on the earlier studies conducted on direct embryogenesis for the number of days needed for the exposure to auxin, and the fact that the embryos

Figure 4.28: *Callusing of embryos; production of* slimy brown callus from preformed embryos. (1.6x)

Figure 4.29: Blackening of the explant (1.6x)

Figure 4.30: No increase in size (1.6x)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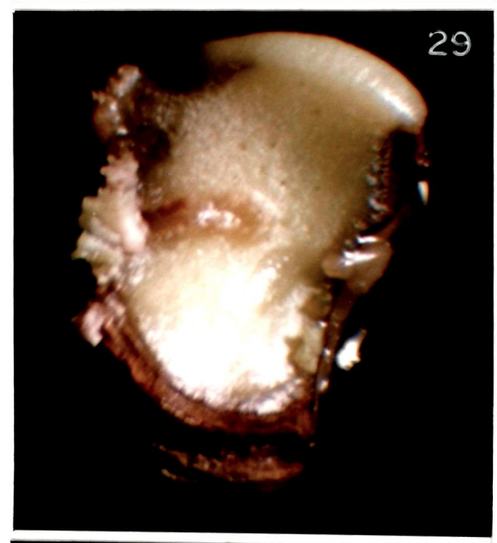
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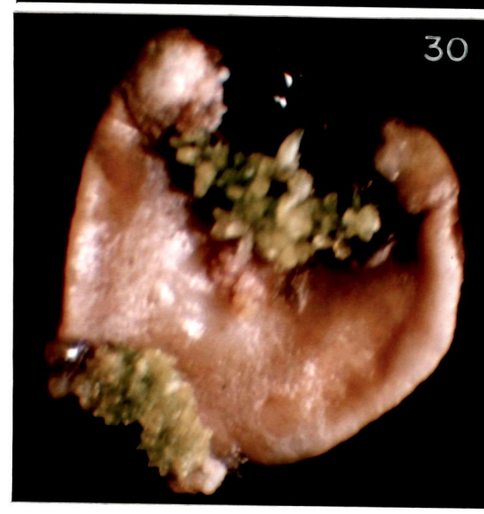
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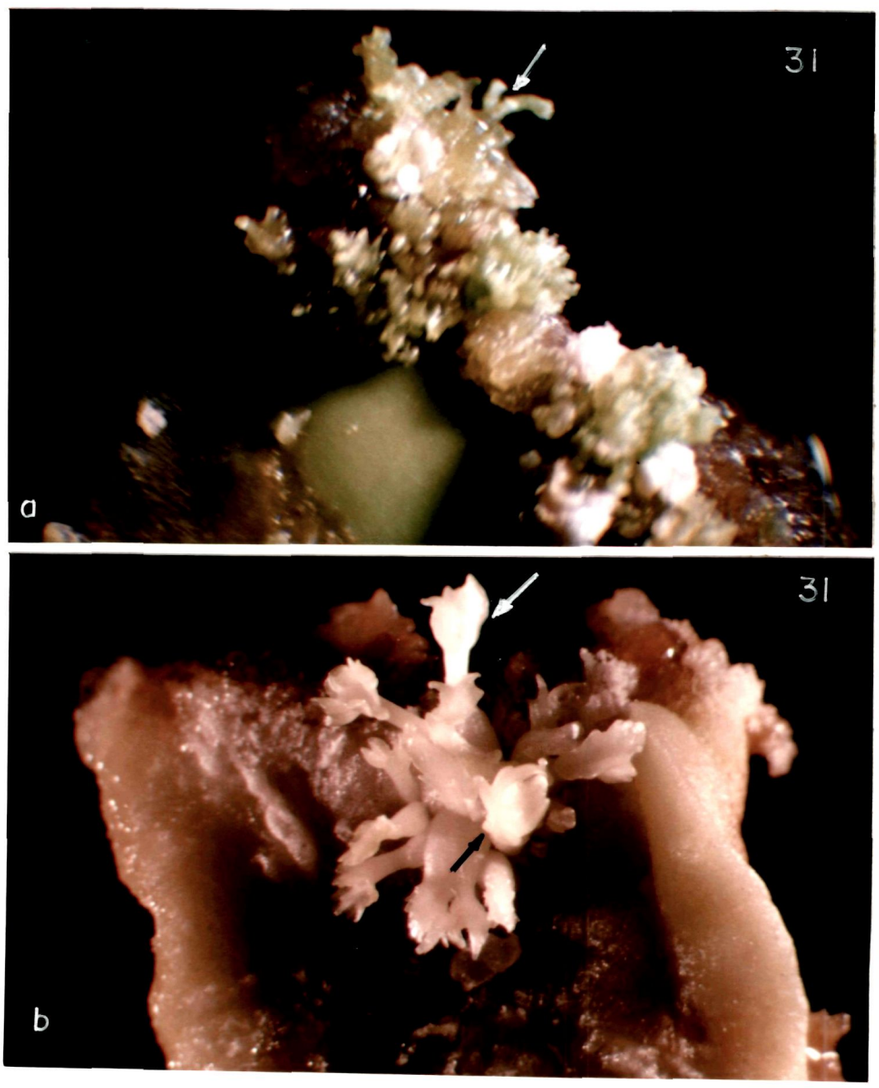
Figure 4.31: Precocious germination on hormone free medium(16x).
a = Late cotyldonary stage embryo in the mass of embryos
b = Precociously germinating fused embryos.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a
31
b

on transfer to hormone free media induce precocious germination, a separate experiment was conducted for the maturation of the embryos induced directly on the cotyledonary surface.

Table 4.10: Effect of BAP on maturation.

BAP (mg/l)	RESPONSE			
	7 days		11days	
	E	C	E	C
0.5	30	80	5	100
1.0	5	95	NIL	100
3.0	15	75	NIL	100
5.0	NIL	85	NIL	100

Medium = MS basal medium + sucrose (50 g/l).
E = embryogenesis; C= callusing

In this, the cotyledons were incubated on initiation medium in dark for 11 days. After 11 days these were transferred to hormone free MS medium supplemented with charcoal. After 4 days (i.e. 15 days incubation from the date of inoculation), these were transferred to MS basal medium supplemented with BAP (0.5 - 5.0 mg/l). Transfer of the embryogenic cultures to BAP supplemented medium also did not have a promotory effect on the growth of the embryos. At all concentrations of BAP, the embryos revert back to callusing within 7 days resulting in decreased percentage of embryogenesis.

4.3.4. Histological evidence

The initiation of the somatic embryos was observed directly from the sub-epidermal layer, after 11 days of incubation in dark. A distinct pattern of a 'budding' process was observed in the sub-epidermal layer **(Fig. 4.32a)**. A clump of small isodiametric cells appeared from this layer of the cotyledonary surface after 11-15 days **(Fig. 4.32b)**. These globular stage embryos did not undergo further development. Therefore, heart, torpedo and cotyledonary stages were not observed.

However, these embryos showed precocious germination on transfer to

Figure 4.32

a. Initiation of budding process from the sub-epidermal layer (200 x).

b. Globular stage embryos observed after 11 to 15 days of incubation (200 x).

c. Abnormal late heart shaped embryo cut out from the sub-epidermal layer (125 x).

d. Profuse abnormal embryos (160 x).

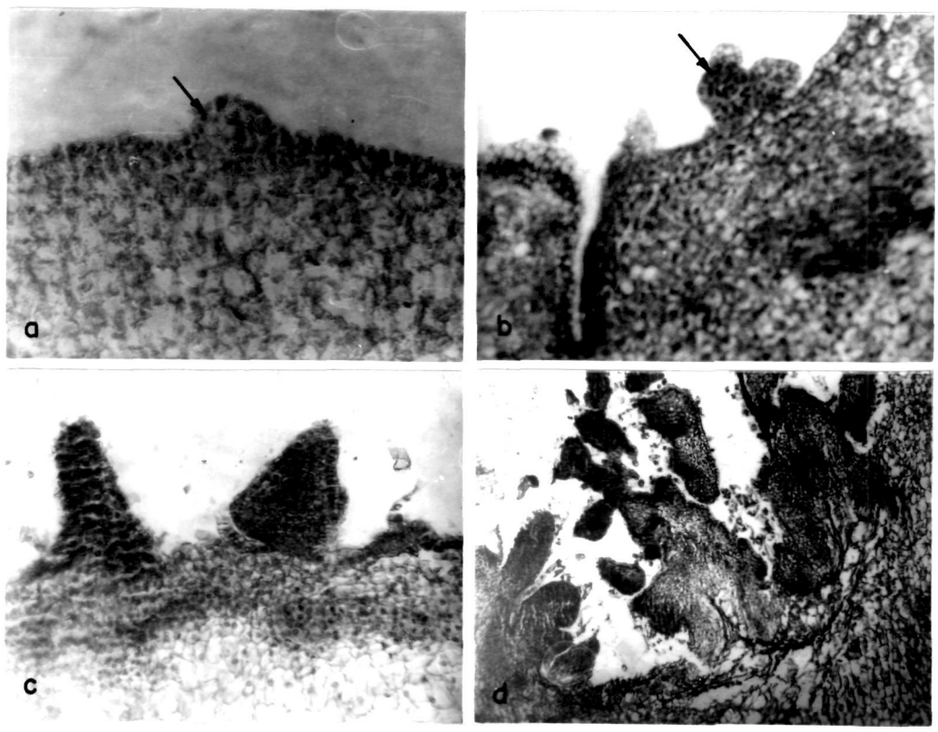


Figure – 4·32

hormone free medium. **Fig. 4.32b** shows the presence of globular stage embryos only. Abnormal embryo formation was more prominent in the histological section without any procambium, shoot and root primordia. These embryos do not show clear stages of development **(Fig. 4.32 c&d)**.

4.4. DISCUSSION

To date, shoot culture is the most successful method of propagation for hardwood trees. However, somatic embryogenesis is being viewed as an alternative method for several species. Once a protocol for somatic embryogenesis is defined, it can prove to be a more effective method for the rapid propagation of selected trees. Somatic embryos can be induced directly on a variety of treated explants or obtained indirectly by manipulating non-embryogenic callus *in vitro*. The sequence of steps which leads to plantlet formation includes: induction, tissue maintenance, maturation, germination of somatic embryos and acclimatization of plantlets before field trials.

The most important factor contributing to the success of somatic embryogenesis is perhaps the choice of explant. According to Sharp *et al*, 1980, more juvenile the material, more easy to induce somatic embryogenesis. Immature embryos, nucellar tissue and immature cotyledons have proven to be the most responsive source of explant. Williams and Maheswaran (1986) have postulated that in juvenile material, the cells are 'determined' to follow embryo development. They further reported that explants derived from tissue associated with reproduction (e.g. nucellar and anther tissue) and hypocotyls or cotyledons of young seedlings tend to be more easily induced to an embryogenic state than do cells from mature tissue (Williams *et al*, 1990). Similarly, age of the explants also plays an important role in the success of somatic embryogenesis in woody species (Rao and Lakshmi Sita, 1996; Gingas and Lineberger, 1989).

In neem, the choice of explant for induction of somatic embryogenesis was the immature cotyledons. This was because the neem seeds are the best source for neem oil as well as the important active principles like azadirachtin, salannin, gedunin, etc. Moreover, the immature cotyledons were more responsive in culture as compared to the mature ones.

Sharp *et al* (1982), described two routes of somatic embryogenesis. The first is the direct embryogenesis, where embryos initiate directly from the tissue in the absence of callus proliferation. This occurs through pre determined embryogenic cells (PEDC), where the cells are already committed to embryogenic development. In walnut (Gharyal and Maheshwari, 1981) and carrot (Smith and Krikorian, 1989), these cells were observed to form somatic embryos on hormone free media. Such cells are usually found in tissues like immature zygotic embryos, nucellar tissue, etc., where the tissue is involved in zygotic embryogenesis. When more differentiated plant tissue i.e. cotyledons, etc., are used for somatic embryogenic development program, several cell division cycles in the presence of auxin are required before somatic embryogenic pathway is expressed. The term induced embryogenic determined cells (IEDC) was used to identify such cells (Sharp *et al*, 1982).

In the present study, both the pathways were induced from cotyledons of immature seeds. Similar results were obtained in *Juglans regia* (Tulecke and McGranahan, 1985), where cotyledons from immature zygotic embryos form somatic embryos more easily than those from mature embryos. Tulecke (1987), however reported that embryogenesis in cultures of angiosperm trees is achieved with mature embryos also.

Other criteria involved in the induction of somatic embryogenesis are effective conditioning medium and suitable environmental conditions (Tautorus, 1991). Thus, after transfer from the conditioning to the inductive medium, embryogenic centers may develop which can be used to develop embryogenic lines. Alternatively, callus cultures may be directed to embryogenesis.

In the present work, for induction of the somatic embryogenesis pathway, Murashige and Skoog's (MS) basal medium supplemented with CH, auxins like IAA and 2,4-D, cytokinins like BAP, KN and TDZ and high concentration of sucrose was used.

For the induction of somatic embryos, the use of a range of basal media from relatively low salt concentration (White's medium, 1963), to the more concentrated formulations of Murashige and Skoog's medium (MS, 1962) has been reported. Evans *et al* (1981), reported that 70% of the reported somatic

embryogenesis protocols were on MS basal medium or modification of MS.

A key element of the MS medium is the presence of high levels of nitrogen in the form of NH_4NO_3. In somatic embryogenesis, the embryo initiation and maturation is benefited by reduced nitrogen. The source of reduced nitrogen in addition to nitrate in the medium can be in the form of complex additives such as CM (Steward and Shantz, 1956), CH (Ammirato and Steward, 1971), a mixture of amino acids (Kato and Takeuchi, 1963), a single amino acid (Wetherell and Dougall, 1976) or the presence of ammonium ion (Halperin, 1966). In the present study, CH (1000 mg/l) was used as reduced nitrogen source in the initiation media for both the pathways of embryogenesis.

The important factor which influences the induction of somatic embryogenesis has essentially turned out to be the auxin used in the culture medium. The importance of auxin in the induction of somatic embryogenesis is well established for many species (Ammirato, 1983; Kochba and Spiegel-Roy, 1977; Guimaraes *et al*, 1988; Gleddie *et al*, 1983; Lakshmi Sita *et al*, 1979). Moreover, the effectiveness of an auxin type is very species and tissue specific (Bano *et al*, 1991; Meijer and Brown, 1987; Hazra *et al*, 1989). In neem, the type of auxin is the deciding factor for the pathway of embryogenesis. Use of IAA induces embryos via callus phase, whereas the use of 2,4-D induces embryos directly on the cotyledonary surfaces. Laurain *et al*, 1996, observed that type of growth regulators decided the pathway of somatic embryogenesis in *Gingko*. Direct embryogenesis was induced on the hypertrophic cotyledons only in the presence of benzyladenine. Indirect embryogenesis was seen to be correlated with the presence of exogenous auxin like NAA.

2,4-D has been observed to be the most commonly used auxin for the induction of somatic embryogenesis. Dudits *et al* (1991), indicated that synthetic auxins, typically 2,4-D are key factors for induction of embryogenesis. Kysley and Jacobsen (1990). observed that out of picloram, 2,4-D and NAA, 2,4-D induced the best response. Eapen and George (1993), have tested 11 different auxins for direct somatic embryo induction from immature cotyledons of peanut. They found 2,4-D to be the most favorable as it produced the highest average number of embryos.

Induction of somatic embryogenesis using other auxins like NAA (Ammirato and Steward, 1971; Rugini, 1995), IBA (Tulecke and McGranahan, 1985; Ostrolucka and Pretrova, 1991) and IAA (Sussex and Frei, 1968; Bapat and Rao, 1984) are very few in woody species. IAA has been reported to stimulate embryogenesis at low concentrations (Epstein *et al*, 1977; Rao and Bapat, 1995). Moreover, it was observed that these auxins are mostly involved in the organogenesis pathway (Litz and Jaiswal, 1991).

In neem, the lower concentrations of auxin induce embryogenesis from the cotyledonary explant, while higher concentrations of auxin induce only callus. The requirement of auxin must be determined empherically as the optimal concentration of growth regulator for induction of somatic embryogenesis is species as well as genotype specific (Kao and Michayluk, 1981).

It is generally observed that when higher concentrations of 2,4-D are involved, somatic embryos are formed via callus stage and when lower concentrations of 2,4-D are involved embryogenesis is seen to be induced directly on the explant surface (Stuart *et al*, 1985; Cruz *et al*, 1990). Similar results have been reported by Cruz *et al*(1990) in *Feijoa sellowiana* cultures. Epstein *et al* (1977), observed embryogenic callus from shaumouti orange cultures growing on IAA containing medium. They also showed that low levels of IAA are required for the induction of embryogenic callus from cotyledons when incubated in dark.

In neem, Islam *et al* (1996), reported the induction of compact nodular callus from the cotyledons of immature seeds. When cultured on MS medium supplemented with NAA (3 or 5 mg/l) alone, embryogenic calli were induced. These on transfer to MS medium supplemented with either BAP or KN, with or without an auxin led to further development of embryoids into shoots.

However, in the present study, since auxin alone induced only callus or rhizogenesis, combination of auxin and cytokinin were tested to induce embryogenesis. It was confirmed that the cytokinin was necessary for induction of somatic embryos via either of the pathways. Preliminary experiments showed that the auxin alone or in combination with KN induced callus, whereas auxin in combination with BAP induced embryogenic callus (when IAA is involved) or embryos (when 2,4-D is involved) from the cotyledonary surface. Out of the two cytokinins tried, BAP proved to be more effective in inducing either embryogenic

callus or direct embryogenesis. The presence of cytokinin in the medium during the early phases i.e. commitment and expression phases of embryogenesis was reported to stimulate somatic embryogenesis (Eapen and George, 1993). Cytokinins BAP and KN are most commonly used for induction of somatic embryogenesis, in the range 0.5 - 5.0 µM (Ammirato, 1983). Cytokinins are important in the process of somatic embryo maturation (Fujimura and Komamine, 1980), and especially for cotyledon development (Ammirato and Steward, 1971). Kiss *et al*(1992), have observed that the number and size of somatic embryos is directly related to concentration of BAP present in the medium.

In walnut, Tulecke and McGranahan (1985), have reported embryo induction via callus from immature cotyledons on medium containing BAP, IBA, KN and L-glutamine. A transfer to hormone free medium was necessary for induction of globular stage embryos. Rao and Lakshmi Sita (1996) have also reported induction of somatic embryos directly on immature cotyledons of rosewood on MS basal medium supplemented with low 2,4-D concentration(1.0 mg/l) and KN, and maturation on BAP containing medium. However, these embryos did not show conversion into complete plantlets.

According to the present investigations, the embryogenic potential of the callus growing on IAA and BAP medium decreased after eight weeks of incubation. Similar observations have been reported by Kochba *et al* (1972), where nucellar callus of *Citrus sinensis* initially required the presence of IAA and KN for growth and differentiation. However, in repeated subcultures the callus showed a gradual decline of embryogenic potential. According to them after prolonged incubation, the tissue gets habituated and thus even very low concentration (0.001 mg/l) of auxin inhibits embryogenesis.

It is evident that recovery of plants from somatic embryos is essential for the ultimate success of somatic embryogenesis system. Although auxin is used to induce somatic embryos, continued exposure to auxin like 2,4-D has been known to be the determining factor on development of apical meristem of embryos (Halperin and Wetherell, 1964). Exposing the explant tissue to an auxin for a short period followed by transfer to hormone free basal medium has led to the recovery of somatic embryos and plants from alfalfa (Walker and Sato, 1981), pecan (Wetzstein *et al*, 1989) and black locust (Merkle and Wiecko, 1989).

Germination of mature somatic embryos is sometimes problematic but can be improved with prevalent methods like cold treatment (Tulecke and McGranahan, 1985); desiccation of embryos (Deng and Cornu, 1992); use of ABA (Attree *et al*, 1991) and high osmoticum (Litz *et al*, 1995).

In the present studies also, when CH was omitted from the medium, the neem embryos arising from callus underwent rapid growth and different stages of embryo development were observed. Presence of CH in the medium also inhibits germination and prolongs embryogenic growth (Zeibur *et al*, 1950). This response is brought about by the combined effect of amino acids, phosphate ion and sodium chloride. The inhibition of germination is mainly due to the high osmotic pressure of CH, which is produced mainly by sodium chloride and the amino acids. However, in the direct pathway, reduction or omission of CH from the medium resulted in callusing of the preformed embryos and further developmental stages could not be observed in any of the media tried.

In the present study, the embryogenic mass arising from callus was transferred to MS basal liquid medium supplemented with IAA (0.1 mg/l), GA_3 (0.01 mg/l), CM (5%), BAP (2.0 mg/l) and sucrose (20 g/l), to increase germination percentage. On this medium, 97% of the embryos showed proper development and initiation of root and first leaf. Later these germinated embryos were transferred to MS 1/2 strength liquid medium, where rapid development to plantlet stage was observed within 7 days.

Islam *et al* (1996), also found that frequency of conversion of the somatic embryos to shoots was very low in neem. To increase the frequency of somatic embryo conversion, they isolated the embryoids and cultured them on MS basal medium supplemented with BAP or KN with or without GA_3. However, only a few well developed embryos grew into complete plantlets, while others produced secondary embryogenesis.

There are few reports of somatic embryogenesis using thidiazuron (TDZ) in hardwoods. TDZ has been used in the range of 0.5-10 µM to stimulate somatic embryogenesis from cotyledons of white ash (Bates *et al*, 1992), eastern black walnut (Neuman *et al*, 1993).

Direct embryogenesis from neem immature cotyledons was observed on two media, viz., MS basal supplemented with 2,4-D (0.5 mg/l), BAP (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l) and MS basal supplemented with 2,4-D (0.1 mg/l), TDZ (0.5 mg/l) and sucrose (50 g/l). Similarly, somatic embryos were reported in walnut on woody plant medium supplemented with 2,4-D (0.1 mg/l) and TDZ (0.5 mg/l).

Prolonged exposure to TDZ was found to cause hyperhydricity, abnormal shoot growth and difficulty in rooting (Lu, 1993). A transfer to other cytokinin like BAP or KN or 2iP, after exposure to TDZ for the required time was recommended. However, transfer of these embryos to BAP containing medium after 11 days of exposure to TDZ also resulted in callusing of the preformed embryos.

Transfer to hormone free medium either led to callusing (in case of 2,4-D + BAP) or precocious germination (in case 2,4-D + TDZ). Trigiano *et al* (1988) have reported precocious germination of somatic embryos initiated on immature zygotic embryos of redbud. They attributed the failure of the embryos to undergo proper development pattern to the fact that there may be a presence of a non-functional meristem. Similar observations were reported by Kysley *et al* (1987). However, they emphasized the requirement of a complex regime of growth regulators and media.

HISTOLOGICAL EVIDENCE

In neem, the somatic embryos induced directly on the cotyledonary surface were found to be arising from the epidermal layer. These embryos did not show all the development stages, but germinated precociously. In case of the indirect pathway, meristematic pockets were observed in the callus, which later on developed into full fledged embryos.

Somatic embryos have been shown to arise either directly from single epidermal layer as in *Eugenia* (Litz, 1984), *Ilex* (Hu and Sussex, 1971), *Ranunculus*(Konar *et al*, 1972) and *Trifolium*(Maheswaran and Williams, 1985) or indirectly from groups of meristematic cells usually located at the periphery of callus mass, like *Corylus* (Radjovic *et al*, 1975) and *Coffea*(Sondahl *et al*, 1979; Pierson, 1983), *Theobroma* (Kononowicz *et al*, 1984)..

According to Spahlinger *et al*, 1977, embryogenic tissue consists of clumps of cells which are characterized by their small size and dense cytoplasm. In the present studies also such highly stained tissue of small cells was evident in the histological sections **(Fig. 4.16a)**.

The embryo development was seen to arise from such clumps of embryogenic cells. Furthermore, the meristematic activity was seen in the callus as early as within 20 days. This phenomenon is interpreted as High Frequency Somatic Embryogenesis (HFSE) induction, by Sondahl *et al*, 1979. Somatic embryos in various stages of growth were observed on the callus surface after 40 days of incubation.

4.5. CONCLUSION

The application of tissue culture techniques to semi-tropical and tropical trees is not common and success in plant production through organogenesis or embryogenesis in these tree species is limited.

In neem, what is needed today, is large scale research and development efforts for sustainable neem-based technology. The ever increasing demand for its products needs major emphasis on planned propagation of elite germplasm. Somatic embryogenesis in neem can hasten the process of increasing genetic diversity, thereby, increasing the chances of isolating plants with desirable traits. In addition, variants with wide adaptability can be isolated.

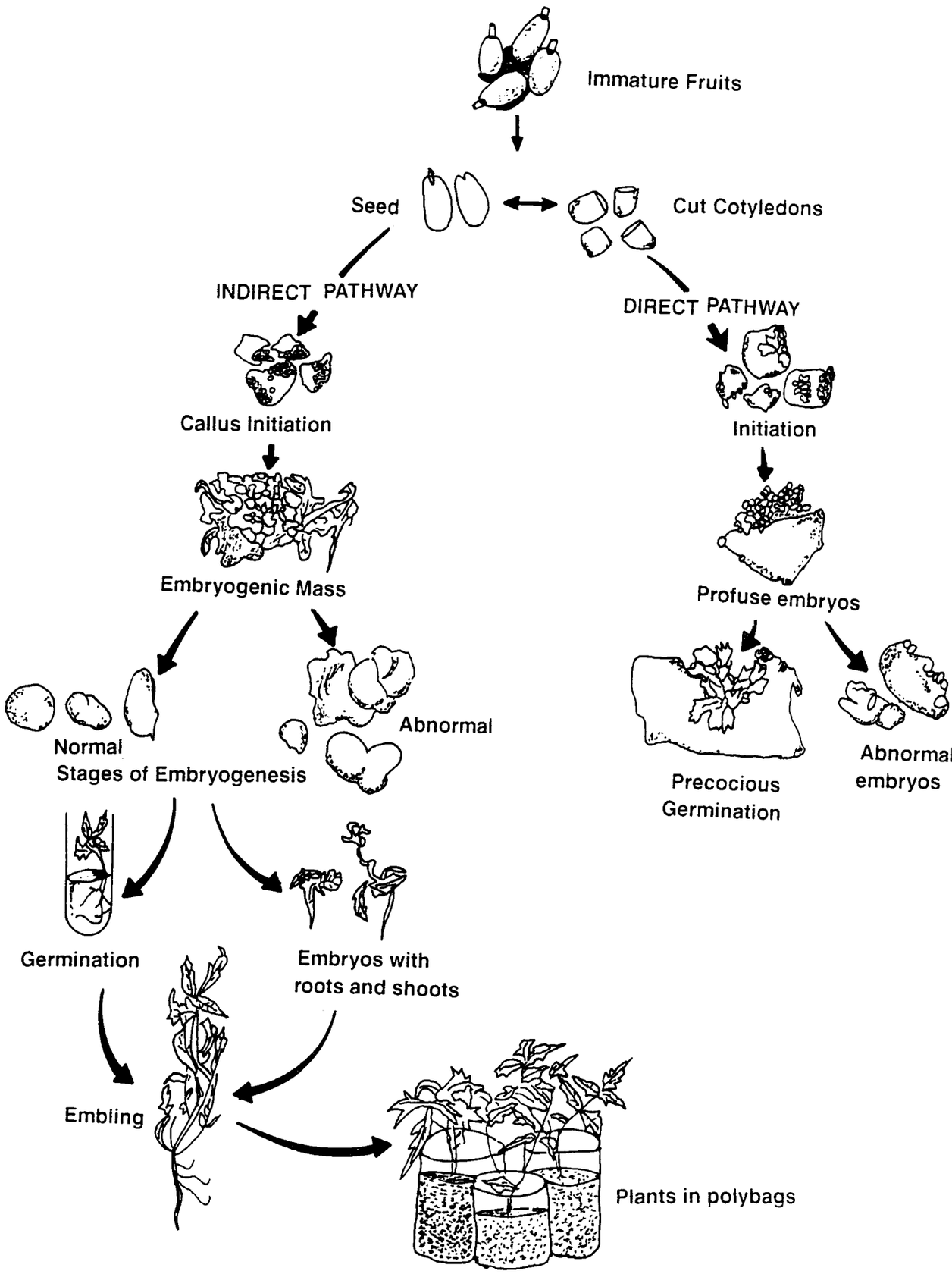


Figure 4.33. Schematic representation of plant regeneration via somatic embryogenesis in neem.

CHAPTER 5

SECONDARY METABOLITE ANALYSIS

5.1. INTRODUCTION

Plants are a valuable source of a large range of chemical compounds which are used as raw materials for medicines, agrochemicals, perfumes, flavoring agents, dyes and gums. Generally, the plant products of commercial interest are the secondary metabolites (Staba, 1980; Fowler, 1983; Whitaker and Hashimoto, 1986). These metabolites are the products of secondary metabolism i.e., synthesis, metabolism and catabolism of endogenous compounds by specialized enzymes (Luckner *et al*, 1977), and differ from primary compounds in:

i) an apparently limited taxonomic distribution
ii) synthesis occurring only under certain conditions, especially stress
iii) an apparent lack of vital functions, and
iv) an apparent necessity for life.

There are three main categories of these products:

Essential oils: consisting of mixtures of terpenoids and are used as flavoring agents, perfumes and solvents.

Glycosides: which include flavonoids, saponins, phenolics, tannins, etc., used as food, colors, dyes and medicinals.

Alkaloids: a diverse group of compounds and are of strong interest to suppliers of medicines e.g., morphine, strychnine, atropine, etc.

These secondary metabolites are thus an important source of complex organic compounds which are difficult to synthesise by conventional chemical methods. A number of the plant species commonly sought for their secondary products are native to remote and sometimes politically unstable geographic areas. The plants that produce valuable products are subjected to a variety of biotic and abiotic stresses that can determine the level and quality of production.

There are two approaches to overcome these problems:

1. Large scale cultivation of these plants which can help to provide a steady supply of raw material for secondary metabolite extraction.
2. Exploitation of cell culture techniques.

The concept of using plant cell cultures to produce various products was started with Rourtier and Nickell in 1956. Nickell (1962), stated that 'In growing plant cells in culture it should be theoretically possible to produce any compounds that are produced normally by the plant from which the culture has been obtained'. Over the last three decades, a wide range of plant products have been detected in cell cultures (Fowler, 1983; Sahai and Knuth, 1985).

The main reasons for using plant cell cultures as an alternative route to natural production are:

1. Useful compounds can be produced under controlled environmental conditions independent of agroclimatic differences.
2. Cultured cells are free of microbes and insects.
3. The cells of any plants, tropical or alpine, can easily be multiplied to yield their specific metabolites.
4. Automated control of cell growth and rational regulation of metabolic processes can contribute to the reduction of labour costs and improvement of productivity.

To propel tissue culture production of secondary metabolites into the realm of economic feasibility, two major challenges must be met. First is to grow the tissues in culture conditions that produce the desired products and secondly to select variant cultures capable of synthesising high levels of the compound (Whitaker and Hashimoto, 1986).

The basic technique of initiating cell cultures are well known. Plant cell cultures with doubling times of 20 hours are classified as rapidly growing and such rates can only be achieved in suspension cultures. Therefore, the only culture system of biotechnological relevance for the production of secondary metabolites are suspension cultures that can be grown in shake flasks as well as in large bioreactors.

A main pre-requisite for establishing a good producing culture is that the desired product should accumulate spontaneously in typical growth or production medium. Over the years the *de novo* synthesis of many commercially important

compounds have been reported (Callebaut *et al*, 1990; Nair *et al*, 1986).

Improvements in growth rate of cells in suspension cultures are generally essential for their exploitation. This involves optimising the nutritional components of the medium, in particular carbon, nitrogen, and phosphorous components and provide a suitable combination of hormones to promote a high rate of growth, and encourage the cells after division to separate and maintain a highly dispersed culture (Ikeda *et al*, 1977; Park *et al*, 1989). To increase the yield, a two stage process was suggested by Zenk (1978). The first stage is the induction medium optimised for growth and second stage is for the production. For this, the initial cultures are obtained from high yielding strains.

Due to the high production costs involved in suspension culture systems, the immobilization technique is fast gaining importance in the field of secondary metabolite production. Immobilized plant cells in a variety of polymeric materices e.g. alginate, agar, agarose, carragenum, etc., have been used for a wide range of reactions (Scragg *et al*, 1988).

Exogenous supply of a biosynthetic precursor to the culture may also increase the yield of the final product (Tabata *et al*, 1972; Merli *et al*, 1987). In addition, production of valuable plant products by biotransformation from cheap precursors is another possibility. This method can be used to obtain novel compounds from substrates that are not normally available to plants and also to transform natural intermediates of important active plant products. Biotransformation studies have been reported in a variety of compounds viz., cardiac glycoside digoxin (Alfermann *et al*, 1985), acetyltropine (Hiraoka and Tabata, 1974), benzylisoquinoline (Viel *et al*, 1993), etc.

Another approach to the secondary product synthesis is that of the elicitor mediated induction. It has already been postulated that the secondary metabolites are produced in response to external stimuli such as stress, attack of pathogens, etc. The accumulation of phytoalexins against microbial attack is one of the defense responses in higher plants (Eilert, 1988). Recently, there are several reports on elicitor-mediated formation of secondary products (Cramer *et al*, 1985; Hakamatsuka *et al*, 1994).

In the course of time, scientists working on secondary metabolite production from plant cell cultures, found out that the production of a particular compound is also dependent on differentiation (Constabel *et al*, 1974). Many of the secondary products are formed only in highly specialized tissues i.e. roots, leaves, flowers, etc., e.g. the cardiac glycosides of *Digitalis* are formed in leaves, quinine and quinidine in the bark of *Cinchona* trees, tropane alkaloids in roots of Solanaceous spp., etc. Therefore, during growth, it seems that cells not only undergo a morphological specialization, but also chemically differentiate in their capacity to produce specific chemicals. This has led to the exploitation of *Agrobacterium* transformed cultures for secondary metabolite production (Flores and Filner, 1985; Hamill *et al*, 1986).

In those plant species, in which roots constitute a main site of synthesis of secondary metabolites, root cultures are now being considered as an alternative to cell suspension cultures. *Agrobacterium rhizogenes* is capable of transforming plant cells and this transformed material when grown in the absence of phytohormones give rise to 'hairy root' cultures (Toivonen and Rosenquist, 1995).

5.1.1.Tissue Culture Studies on Neem Secondary Metabolites.

There are numerous potential applications of *in vitro* techniques towards improvement of neem. The recently held International Neem Conference (February, 1996), has pointed out that the interest lies in:

1. Mass propagation of neem on a commercial scale
2. Development of high yielding lines with improved seed quality
3. Production of important phytochemicals using tissue culture techniques

Initially, the focus was on nimbin, ß-sitosterol, etc (Sanyal *et al*, 1981, 1983, 1986, 1988), but now the focus has shifted to most active compound found in neem i.e. azadirachtin (Holowach-Keller, 1994; Wewetzer, 1996) and other compounds such as flavonoids (Ramesh and Padhya, 1996); gedunin (Rajagopal and Ramaswamy, 1996), etc.

Sanyal *et al* (1986, 1988), have reported the synthesis of nimbin and ß-sitosterol from neem bark callus and cotyledonary callus. They observed the

presence of both the metabolites in mainly the cotyledonary callus. They also observed that the concentration of nimbin in the callus cultures decreased with the age of the callus. Redifferentiation of callus into roots resulted in accumulation of nimbin (0.25%) and decrease in accumulation of ß-sitosterol. Addition of glycine in the culture medium triggered the biosynthesis of nimbin, blocking the ß-sitosterol pathway.

Ramesh and Padhya (1996), have reported selection of stable cell lines producing higher levels of compounds particularly the flavonoids. In neem, the tetranortriterpenoids are the lipid associates and constitute 1/4th of the neem oil. There are more than 80 tetranortriterpenoids in the neem seed; out of which the important ones are the azadirachtins, gedunin, epoxy azadiradione, nimbin, salanin, de-acetyl nimbin, etc. Holowach-Keller *et al* (1994) have patented a method for producing azadirachtin. The method comprises of:

1. Generating callus cell cultures of *A. indica* tissues inoculated with *A. tumefaciens* in a cell culture medium.
2. Forming a cell suspension culture
3. Maintaining the suspension culture such that azadirachtin is produced.
4. Recovering the azadirachtin from cell suspension cultures.

The azadirachtin content varies considerably due to environmental factors and genetic reasons. Therefore, in order to obtain constant amounts of standard quality, it seems appropriate to employ tissue culture techniques for its production. Wewetzer (1996), investigated azadirachtin content in callus cultures derived from leaf or bark from trees of different geographical origins (Nigeria, Nicaraqua, Cuba). He found highest yields of azadirachtin in callus cultures derived from Nicaraguan leaves.

Similarly, Mordue *et al* (1996), reported that azadirachtin content in callus cultures derived from seeds of Sri Lankan trees vary with different climatological zones. Moreover, the azadirachtin content of seeds and that of callus did not correlate with one another nor with the climatological zone.

Rajagopal and Ramaswamy (1996) studied two genotypes, for the presence of azadirachtin and other compounds. Two explants, bark and root, were used

for callus induction, cell suspension and isolation of secondary metabolites. The bark callus of one genotype (G1) was found to contain azadirachtin (0.12%), nimbin (0.21%) and gedunin (0.13%).

The present study was undertaken to induce *in vitro* production of secondary metabolites and oil. The important active principles of neem are tetranortriterpenoids, which are mainly present in the seeds. Out of the different tetranortriterpenoids, azadirachtin, salanin, gedunin, epoxy-azadiradione, etc., are important insect control agents. The neem oil obtained from seeds is equally important for pharmaceuticals and toiletries. Callus was induced on various combinations of auxins and cytokinins using cotyledonary explants. Effect of basal medium modifications on growth of callus was studied. As production of active principles *in vitro* is associated with differentiation processes, differentiation experiments were also undertaken, to evalute its effect on chemical differentiation.

5.2. MATERIALS AND METHODS:

5.2.1. Preparation of explant:

Immature fruits of neem, collected from the NCL campus, were used to prepare cotyledonary explants. The fruits were surface sterilized, the cotyledons were aseptically removed, cut horizontally into two pieces (0.5 cm each) and inoculated abaxially onto the medium (see Chapter 2, Section 2.8).

5.2.2. Callus induction:

Murashige and Skoog's (MS, 1962) basal medium supplemented with MS vitamins, auxins like NAA, IAA, IBA, and 2,4-D at concentrations 0.5, 1.0, 3.0 and 5.0 mg/l alone or in combination with either BAP or KN at concentration 1.0 mg/l, casein hydrolyste (CH, 1000 mg/l) and sucrose (50g/l) as carbon source, was used for callus initiation.

The medium was solidified with 0.4% agar-agar (Qualigens, India), after adjusting the pH to 5.7. The auxins and cytokinins were incorporated into the medium prior to autoclaving. The media were autoclaved at 120°C and 15 psi for 20 minutes. Cultures were incubated at 25±2°C in dark.

To study the secondary metabolite production in non-differentiated and differentiated calli, a set of experiment was carried out. In this, the calli induced on MS basal medium supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l); NAA (0.5 mg/l) and KN (1.0 mg/l) were chosen.

Differentiation in the form of somatic embryogenesis has been obtained on MS basal medium supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l) after 21 days (Refer Chapter 4). To obtain differentiation, the callus induced on combination of NAA (0.5 mg/l) and KN (1.0 mg/l) was subcultured on medium supplemented with glycine (0.3 mg/l); $AdSO_4$ (10 mg/l) and reduced concentrations of NAA (0.1 mg/l) and KN (0.1 mg/l), after 21 days of incubation on the initiation medium.

When these calli were subjected to TLC analysis prominent spots were observed only in non-differentiated calli. Therefore, to study the effect of media modifications both the combinations of growth hormones were used to induce calli on MS basal medium with the following modifications:

1. Omission of phosphate.
2. 1/4 concentration of phosphate.
3. 1/2 concentration of phosphate.
4. 3/4 concentration of phosphate.
5. Normal medium.
6. Omission of both the nitrates (KNO_3, NH_4NO_3).
7. Omission of both the nitrates and phytohormones.

Growth of callus tissue was measured on fresh weight basis. The tissue was weighed before inoculation and after 7 and 15 days incubation and there after 30-60 days with the interval of every 15 days.

5.2.3. Extraction from callus:

Ten grams of calli induced on various media were used for the extraction after 30 days of incubation in dark. Differentiated (after 40 days) and non differentiated (after 20 days) calli were extracted and analysed separately. The calli induced on various modified basal media were analysed after 60 days. Before extraction of each calli the medium adhered to the calli was removed and surface dried. They were then extracted with acetone in a Soxhlet unit for three hours. Acetone was evaporated and the dry extract treated with acetone (10ml x 3). From the combined solubles, acetone was evaporated to yield acetone soluble

fraction which was a mixture of lipid and secondary metabolites produced by the callus.

5.2.4. TLC of the callus extract:

Acetone fractions of callus extract were spotted on a TLC plate (silica gel H; 200 mesh, 1mm thick, activated at 100°C for 1 hour) along with ß-sitosterol, nimbin, de-acetyl-nimbin and epoxy-azadiradione as reference samples and using acetone pet-ether 60:80 (25:75) as developing system. Spots were visualized by spraying with nitrating mixture and heating the plate at 150°C for 10 minutes.

To isolate the compound, the acetone fraction was separated first by column chromatography (CC) using silica gel (60-120 mesh size) and acetone:pet-ether mixture as elution gradient. For this 50 gm of the callus was processed as described earlier and the acetone fraction was separated by CC. Total seven fractions were collected. Out of which the fraction showing spot with Rf value corresponding to nimbin were combined and separated further by preparative TLC (SiO_2 (400 mesh) ; 13% anhydrous $CaSO_4$; 2mm thick; activated at 100°C for 1 hour). The preparative TLC was also developed with acetone pet-ether (25:75) and spots were visualized with iodine spray. Silica gel corresponding to major spots, as developed in iodine, were scrapped and eluted using acetone. Acetone was evaporated to yield the compound. ^{1}H-NMR (200 MHz) in $CDCl_3$ was carried out to find out the exact nature of the compound.

In the whole course of work, one set of experiment comprising nearly 70 samples were analysed for secondary metabolites. Each experiment was repeated thrice.

5.3. RESULTS

5.3.1. Induction, differentiation and growth of callus.

Non-differentiating versus differentiating callus

The effect of auxin and cytokinin combinations on callus quality and texture is described in **Table 5.1.** For the secondary product analysis the friable type of callus is considered suitable (Sakuta and Komamine, 1987). Such calli were induced on MS basal medium supplemented with MS vitamins and auxins like

IAA or IBA or NAA (0.5, 1.0, 3.0 and 5.0 mg/l) in combination with either BAP (1.0 mg/l) or KN (1.0 mg/l).

Table 5.1. Effect of various combinations of auxins and cytokinins on callus induction from cotyledons.

Auxin	Cytokinin	Response
NAA	—	Profuse rhizogenesis
NAA	KN	Brown friable callus
NAA	BAP	Brown friable callus
IBA	—	Slimy brown callus
IBA	KN	Mixture of friable and slimy brown callus
IBA	BAP	Mixture of friable and slimy brown callus
IAA	—	Profuse rhizogenesis
IAA	KN	Brown friable callus
IAA	BAP	Brown nodular embryogenic callus
2.4-D	—	Slimy brown callus
2.4-D	KN	Slimy brown callus
2,4-D	BAP	Direct somatic embryogenesis

Medium = MS basal + MS vitamins + CH (1000 mg/l) + sucrose (50 g/l)

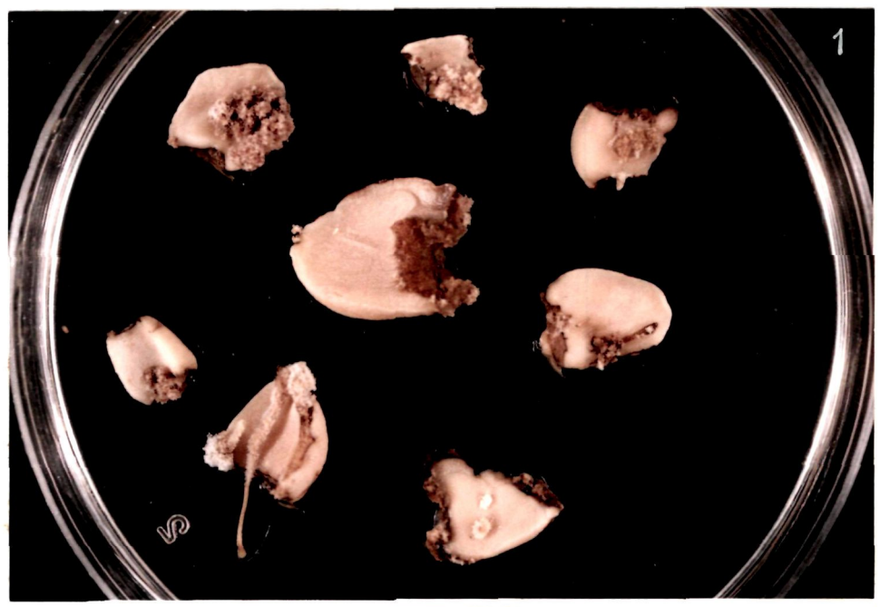
The initiation of callus was noted after 10-15 days incubation in dark **(Fig. 5.1)**. Any auxin present in the medium singly, either produce rhizogenesis (due to IAA/ NAA) or slimy brown callus (due to IBA /2,4-D) **(Fig. 5.2 a&b)**.

Fig. 5.3. shows the comparison between the types of calli as well as the rate of growth of callus after 21 days on various media compositions. The callus induced on IBA and BAP was a mixture of friable as well as slimy brown callus and was very slow growing. However, the calli obtained on MS basal medium supplemented with NAA and KN; IAA and BAP were compact and friable. The IAA and BAP callus was fast growing as compared to NAA and KN callus. Supplementation of 2,4-D with KN induced slimy brown callus. Moreover, on 2,4-

Figure 5.1: Cotyledonary explants showing initiation of callus after 15-20 days of incubation.

Figure 5.2a: Induction of only rhizogenesis observed on MS basal medium supplemented with auxin (IAA or NAA) only.

Figure 5.2b: Induction of slimy brown callus on MS basal supplemented with auxin (IBA or 2,4-D) alone.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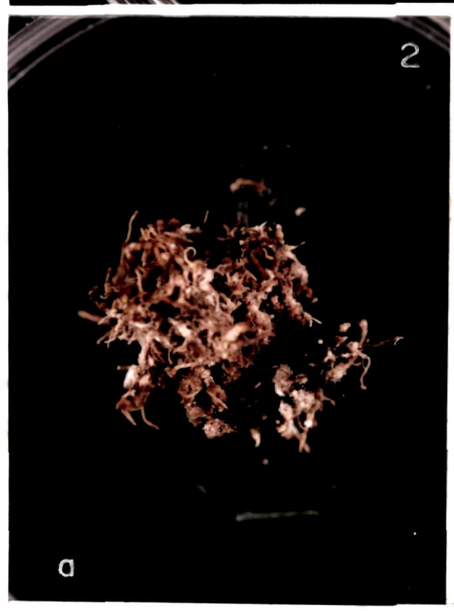
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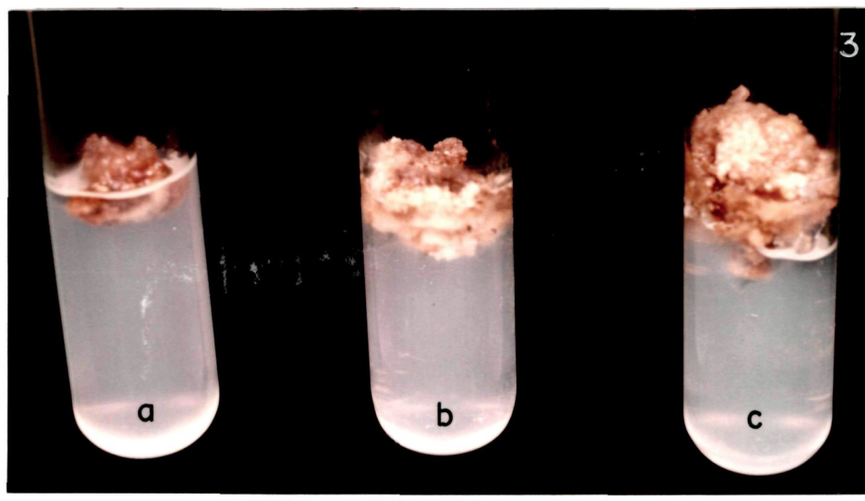
a


2
b

Figure 5.3: Comparison of type of callus induced on various combinations, a = IBA + BAP; b = NAA + KN; c = IAA + BAP

Figure 5.4: Induction of somatic embryos directly on the cotyledonary surface on MS basal medium supplemented with 2,4-D and BAP.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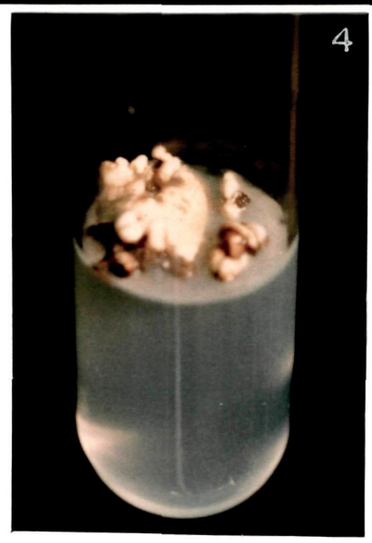
a
b
c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D and BAP supplemented medium direct somatic embryogenesis was obtained within 21 days **(Fig. 5.4)**.

The TLC analysis of the calli induced on combinations of IAA (0.5 mg/l) and BAP (1.0 mg/l); NAA (0.5 mg/l) and KN (1.0 mg/l), revealed prominent spots indicating presence of secondary metabolites. To compare between nondifferentiated and differentiated calli for production of secondary metabolites, these calli were subjected to differentiation and subsequent TLC analysis.

Indirect somatic embryogenesis was noticed on MS basal medium supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l) after about 20-25 days. Incubation of the callus in dark on the initiation medium upto 40-45 days, induced non-synchronous growth of somatic embryos. The callus initiated on MS basal medium supplemented with NAA (0.5 mg/l) and KN (1.0 mg/l) was transferred to various media for differentiation after 21 days. On transfer to medium containing glycine, the callus turned black after 15 days of incubation **(Fig. 5.5)**. On addition of $AdSO_4$, initiation of rhizogenesis was observed after 15 days, however, on further incubation, the callus turned black **(Fig. 5.6)**. Transfer of the callus to MS basal medium supplemented with NAA (0.1 mg/l) and KN (0.1 mg/l), induced profuse rhizogenesis **(Fig. 5.7)**. The TLC analysis of non-differentiated and differentiated calli revealed the presence of secondary metabolites in non-differentiated calli only.

Effect of MS basal modifications

To study the effect of basal media modifications on callus growth and secondary metabolites , calli were initiated on modified basal media containing IAA (0.5 mg/l) and BAP (1.0 mg/l) ; NAA (0.5 mg/l) and KN (1.0 mg/l) (*PGR* combinations for non-differentiated callus). The data on growth of calli on these media is presented in **Table 5.2.** and **Table 5.3.**

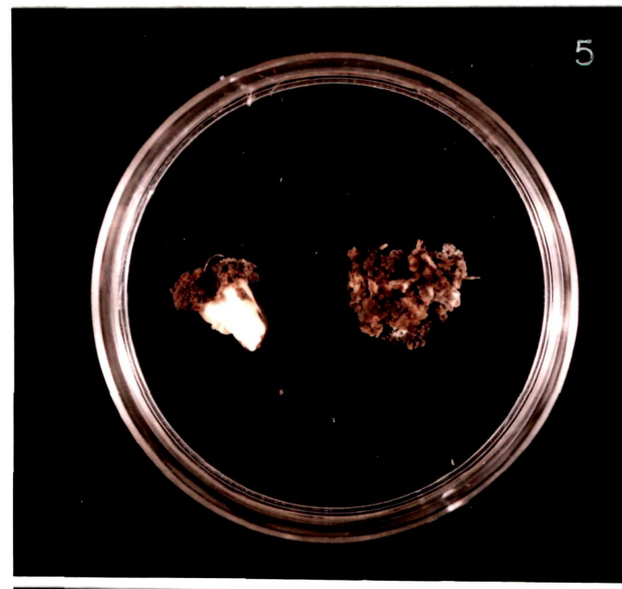
The calli induced on these modifications were observed to be very slow growing as compared to those on the respective normal medium (Treatment no. 5). A gradual increase in growth was noted in all the modification from day 7 to day 60. On comparison between the auxins, IAA proved to be the one to induce faster growth as mentioned earlier **(Fig. 5.3)**.

Effect of transfer of callus induced on MS + NAA (0.5 mg/l) + KN (1.0 mg/l)

Figure 5.5: Transfer to glycine containing medium.

Figure 5.6: Transfer to $AdSO_4$ containing medium.

Figure 5.7: Profuse rhizogenesis observed on MS basal medium supplemented with NAA (0.1 mg/l) and KN (0.1 mg/l).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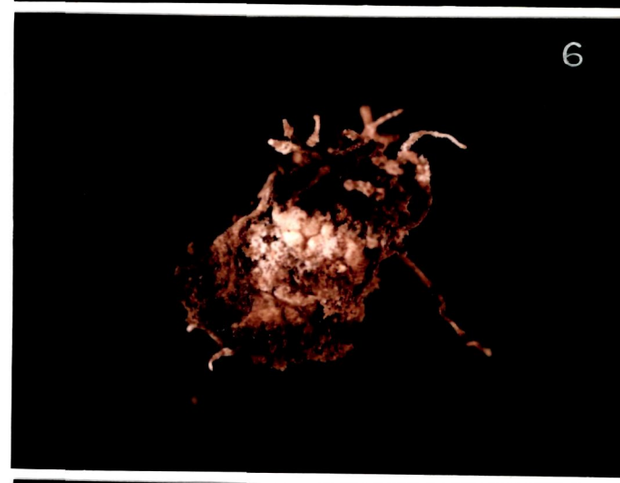
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In medium supplemented with IAA and BAP, phosphates at 1/4 concentrations induced better growth than at 1/2 and 3/4 concentrations (Treatment 2,3,4). However, no such difference on callus growth was noticed in callus induced on NAA and KN. In both the media, omission of nitrates however, seem to have promotory effect on callus growth. The growth of callus on modification number 7, **(Table 5.2, 5.3)** was the least in both the media, indicating positive effect of the phytohormones on callus growth.

Table 5.2. Effect of media modifications on callus growth *.

Days	Treatment						
	1	2	3	4	5	6	7
0	0.028	0.028	0.028	0.028	0.028	0.028	0.028
7	0.420	0.510	0.640	0.630	1.030	0.680	0.240
15	1.314	0.970	1.147	0.875	1.380	1.420	0.342
30	1.521	1.300	1.592	1.128	2.342	1.942	0.584
45	1.568	1.711	1.721	1.410	2.645	2.187	1.120
60	1.598	2.086	1.855	1.830	4.888	2.395	1.580

Medium = MS basal + IAA (0.5 mg/l) + BAP (1.0 mg/l) + CH (1000 mg/l) + sucrose (50 g/l)

Modifications: 1 = Omission of phosphate; 2 = 1/4 concentration of phosphate;
3 = 1/2 concentration of phosphate; 4 = 3/4 concentration of phosphate;
5 = normal medium; 6 = omission of both the nitrates;
7 = omission of nitrates and phytohormones.
* = Growth measured in grams quantity.

Table 5.3. Effect of media formulations on callus growth .*

Days	Treatment						
	1	2	3	4	5	6	7
0	0.028	0.028	0.028	0.028	0.028	0.028	0.028
7	0.520	0.620	0.460	0.280	1.018	0.320	0.240
15	0.860	0.636	0.646	0.622	1.840	0.526	0.674
30	1.016	0.938	1.476	0.762	2.920	0.756	0.720
45	1.038	1.224	1.572	1.580	3.070	1.814	1.177
60	1.370	1.554	1.720	1.840	3.660	2.184	1.306

Medium = MS basal + NAA (0.5 mg/l) + KN (1.0 mg/l) + CH (1000 mg/l) + sucrose (50 g/l)

Modifications: 1 = Omission of phosphate; 2 = 1/4 concentration of phosphate;
3 = 1/2 concentration of phosphate; 4 = 3/4 concentration of phosphate;
5 = normal medium; 6 = omission of both the nitrates;
7 = omission of nitrates and phytohormones.
* = Growth measured in grams quantity.

5.3.2 TLC Analysis.

The acetone soluble fractions isolated from the calli raised on MS basal medium supplemented with MS vitamins, IAA or IBA or NAA (0.5, 1.0, 3.0 and 5.0 mg/l) in combination with either BAP (1.0 mg/l) or KN (1.0 mg/l) were subjected to TLC analysis separately. The data on amount of total extract and weight of acetone soluble fractions obtained from the calli raised on IAA or IBA or NAA (0.5 mg/l) in combination with BAP (1.0 mg/l) or KN (1.0 mg/l) are given in **Table 5.4**.

Table 5.4. Amount of total extract and acetone soluble fraction of calli initiated on various media.

Phytohormones	Weight of total extract (in grams)	Weight of acetone soluble fraction (in milligrams)
IAA + KN	0.70	40
IAA + BAP	1.13	40
IBA + KN	0.48	30
IBA + BAP	0.50	30
NAA + KN	0.27	40
NAA + BAP	0.38	20

Medium = MS + auxin (0.5 mg/l) + cytokinin (1.0 mg/l)

Only the calli induced on either MS basal medium supplemented with IAA(0.5 mg/l) and BAP(1.0 mg/l) or NAA (0.5 mg/l) and KN (1.0 mg/l), showed significant spots **(Fig. 5.8)**. From the TLC it was clear that two spots having Rf values = 0.15 and 0.42 were observed in case of callus initiated on MS basal supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l). Similarly, six prominent spots having Rf values = 0.09, 0.22, 0.26, 0.30, 0.52 and 0.57 were observed in case of callus initiated on MS basal supplemented with NAA (0.5 mg/l) and KN (1.0 mg/l). Comparing with de-acetyl nimbin (Rf value = 0.5) as reference, compound having Rf value 0.42 (more polar than de-acetyl nimbin) was subjected to further analysis.

Comparison between non-differentiated and differentiated callus

The TLC analysis of non-differentiated, compact nodular callus (20 day old) and 40 day old embryogenic callus **(Fig. 5.9)**, obtained on IAA (0.5 mg/l) and BAP (1.0 mg/l), exhibited a prominent spot at Rf value = 0.24. This is more polar

Figure 5.8:

TLC of calli initiated on MS basal medium supplemented with various combinations of auxin (0.5 mg/l) and cytokinin (1.0 mg/l).

S1 = ß-sitosterol; S2 = MS + IAA + KN; S3 = IAA + BAP;
S4 = NAA + KN; S5 = NAA + BAP; S6 = IBA + KN; S7 = IBA + BAP;
S8 = de-acetyl nimbin; SF = Solvent Front.

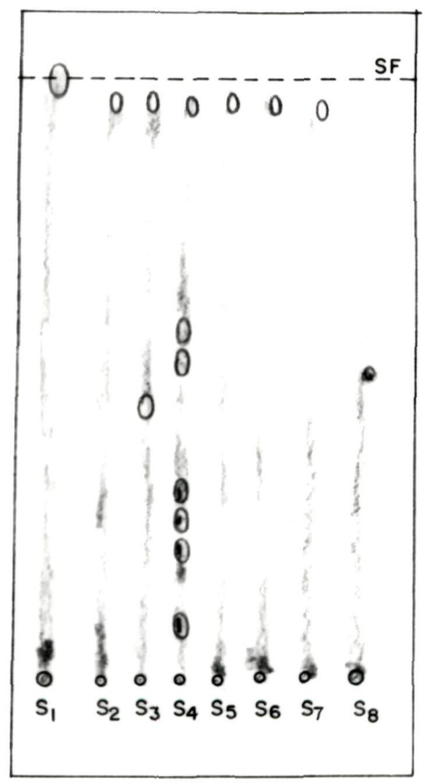

Figure-5·8

Figure 5.9: Induction of somatic embryos via underlying callus on MS basal medium supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l).

Figure 5.10: TLC plate showing spots; S = ß-sitosterol;
A = epoxy -azadiradione; N = deacetyl nimbin;
S1 = embryogenic callus;
S2= non-embryogenic callus;
S3 = callus on MS + NAA (0.5 mg/l) + KN (1.0 mg/l).

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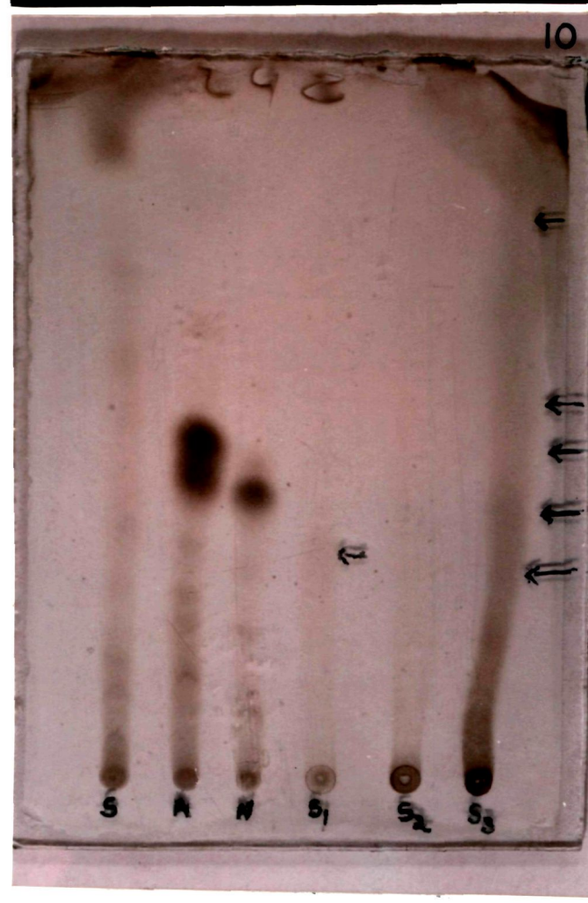

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than de-acetyl nimbin of Rf value = 0.30. For the isolation of this compound, 50 gm of callus was processed to obtain 1.13 gm acetone soluble fraction which was separated by CC to collect broad fractions. Fraction 4 (66.4 mg) which showed Rf value corresponding to that of de-acetyl nimbin, was subjected to preparative TLC seperation to isolate the compound (2.1 mg). However, this compound did not exhibit TLC charring pattern corresponding to that of standard, de-acetyl nimbin by acid spray and heat treatment **(Fig. 5.10, spot S1)**. ^{1}H-NMR (200 MHz) of this fraction in $CDCl_3$ showed signals at δ = 0.90, 1.30, 1.70, 2.35 and 3.75. The NMR data **(Fig. 5.11)**, was very close to that of reported for glycerine-1-monostearate (Hopkins, 1968). Hence the identified compound could be a mixture of glycerine-1-mono stearate and glycerine-1-mono palmitate.

Another **non-differentiated** callus which showed spots on TLC with the Rf value close to de-acetyl nimbin and epoxy-azadiradione was the one induced on MS medium supplemented with NAA (0.5 mg/l) and KN (1.0 mg/l). Acetone fraction of this particular callus showed spots corresponding to the Rf values 0.30, 0.39, 0.47 and 0.54 respectively as compared to Rf values of the references: de-acetyl nimbin = 0.42 and epoxy-azadiradione = 0.48 **(Fig. 5.10, spot S3)**. These spots did not show typical color of neem tetranortriterpenoids on acid spraying and heating probably due to the complexity and dilution of the spots. The TLC of the **differentiated** callus induced on MS basal medium supplemented with NAA (0.1 mg/l) and KN (0.1 mg/l) does not show any prominent spots, indicating that secondary product formation is not related to redifferentiation. Only a spot corresponding to ß-sitosterol (Rf value = 0.83; Rf value of ß-sitosterol = 0.88) was observed **(Fig. 5.12, spot S3)**.

Effect of media modifications

Calli induced on modified MS basal medium supplemented with either of the combinations of IAA (0.5 mg/l) and BAP (1.0 mg/l) or NAA (0.5 mg/l) and KN (1.0 mg/l) were subjected to analysis using TLC. The weights of the total extract and of their acetone soluble fractions are presented in **Table 5.5 and 5.6**, respectively. The acetone soluble fraction was subjected to TLC using ß-sitosterol and de-acetyl nimbin as reference.

Figure 5.11: ^{1}H-NMR spectrum of the compound isolated from 40 day old embryogenic mass.

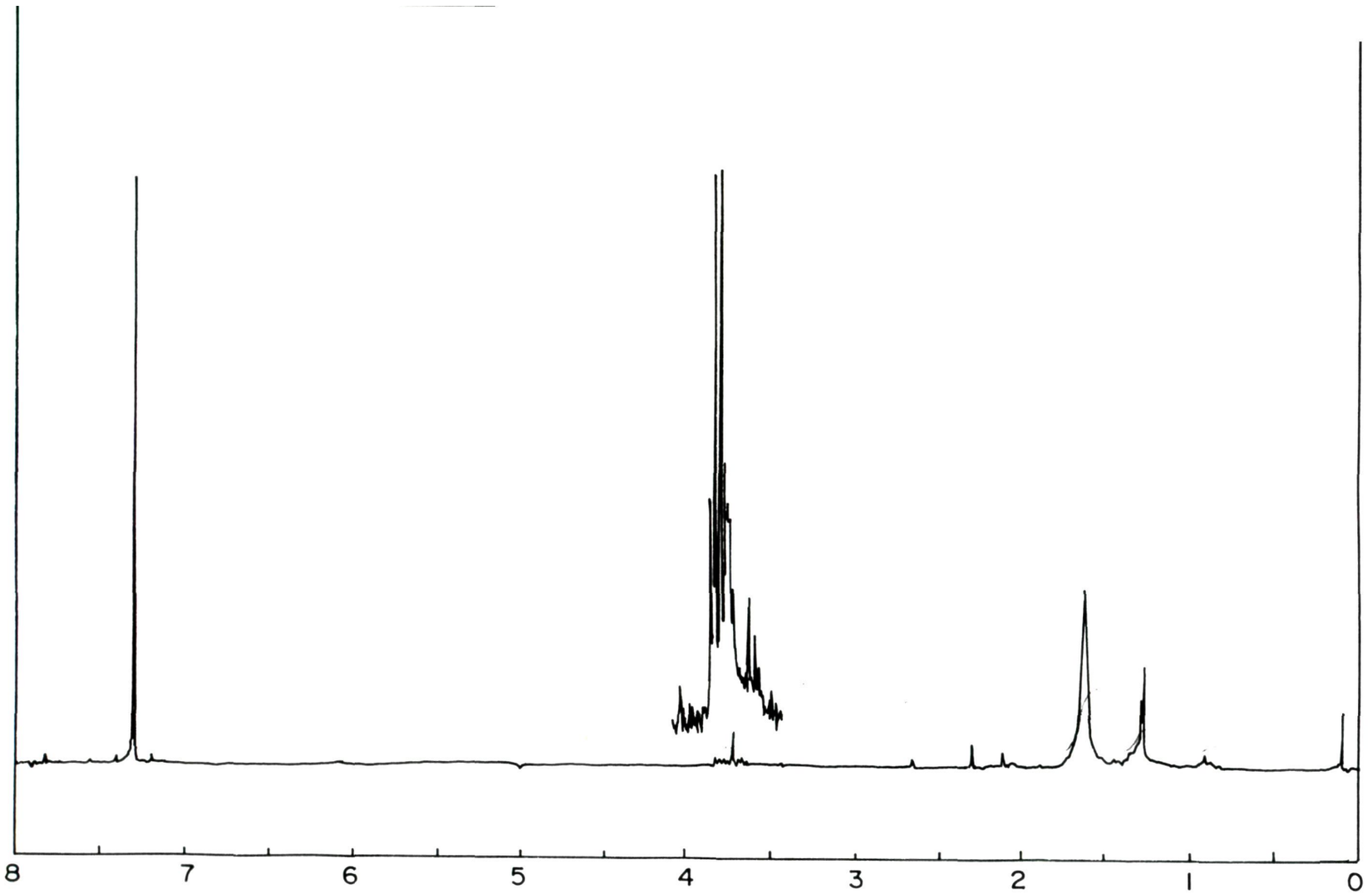

Figure - 5.11

Table 5.5: Effect of media modifications on recovery of fractions and its TLC analysis.

Treatment number	Weight of total extract (in gms)	Acetone soluble (in mgs)	Rf values
1.	0.35	30	0.84
2.	0.56	30	0.81
3.	0.72	20	0.86
4.	0.66	20	0.86
5.	1.03	40	0.81
6.	0.78	30	0.80
7.	0.58	30	0.77

Medium: MS basal + IAA (0.5 mg/l) + BAP (1.0 mg/l).

Table 5.6: Effect of media modifications on recovery of fractions and its TLC analysis.

Treatment number	Weight of total extract (in gms)	Acetone soluble (in mgs)	Rf values
1.	0.27	20	0.91
2.	0.38	30	0.87
3.	0.48	20	0.90
4.	0.56	40	0.90
5.	0.93	30	0.91
6.	0.71	20	0.93
7.	0.68	40	0.91

Medium: MS basal + NAA (0.5 mg/l) + KN (1.0 mg/l)

The calli were analysed after 60 days of dark incubation on the respective media. From the TLC of these calli **(Fig. 5.13, 5.14)**, it was observed that none of the treated calli show any presence of the important active principles. The spots observed had Rf values corresponding to that of ß-sitosterol, Rf value = 0.95 **(Table 5.5, 5.6)**. A very faint spot was observed on S6 column **(Fig. 5.13)** which has Rf value = 0.34 as compared to that of de-acetyl nimbin = 0.45. This confirms the presence of secondary metabolites in non-differentiated callus induced on MS basal medium supplemented with IAA (0.5 mg/l) and BAP (1.0 mg/l) only.

Figure 5.12:

TLC analysis of non-differentiated and differentiated callus.

S1 = ß-sitosterol; S2 = NAA (0.5 mg/l) + KN (1.0 mg/l);
S3 = NAA (0.1 mg/l) + KN (0.1 mg/l); S4 = salanin;
S5 = de-acetyl nimbin; SF= Solvent Front.

Figure 5.13:

TLC of calli induced on MS modified supplemented with
IAA (0.5 mg/l) and BAP (1.0 mg/l).

S1 = ß-sitosterol; S2 = omission of phosphate;
S3 = 1/4 concentration of phosphate; S4 = 1/2 concentration of phosphate;
S5 = 3/4 concentration of phosphate; S6 = normal medium;
S7 = omission of nitrates; S8 = omission of nitrates and phytohormones;
S9 = de-acetylnimbin.SF= Solvent Front.

Figure 5.14:

TLC of calli induced on MS modified supplemented with
NAA (0.5 mg/l) and KN (1.0 mg/l).

S1 = ß-sitosterol; S2 = omission of phosphate;
S3 = 1/4 concentration of phosphate; S4 = 1/2 concentration of phosphate;
S5 = 3/4 concentration of phosphate; S6 = normal medium;
S7 = omission of nitrates; S8 = omission of nitrates and phytohormones;
S9 = de-acetylnimbin.SF= Solvent Front.

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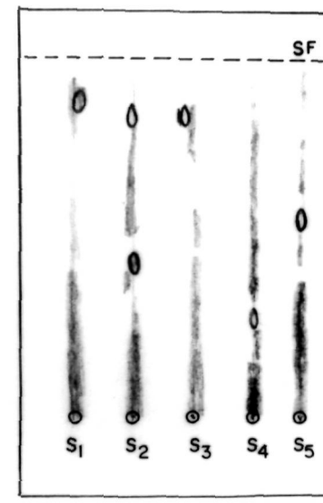
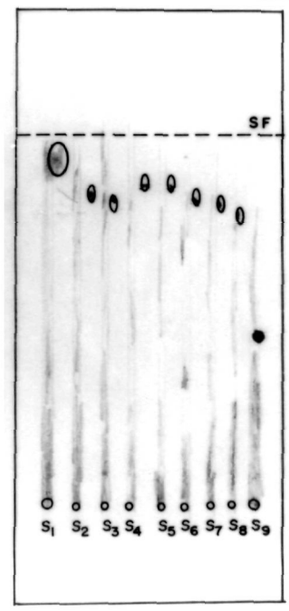

SF
S1
S2
S3
S4
S5

13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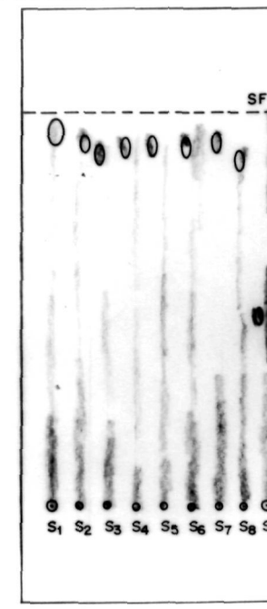
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5.4. DISCUSSION

Higher plants are remarkable in their ability to produce a wide array of diverse metabolites varying in chemical complexity and biological activity (McChesney, 1993). These are called as the secondary metabolites and many of these are used as medicines, pharmaceuticals, insecticides, etc. At least 2000 species of plants are known to have insecticidal properties (Mishra, 1994). The use of insecticidal plants is prevalent especially in the developing countries, where the plants grown locally are cheaper for subsistence farmers to use, than are synthetic pesticides.

The most promising botanicals of today and even for future are the limonoids. This class of compounds are confined to the Cneoraceae, Meliaceae, Rutaceae and Simaroubaceae families (Ahmed and Grainge, 1986). The limonoids occurring in Meliaceae family are also known as Meliacins. Out of over 300 limonoids known today, about one-third are accounted by neem (*Azadirachta indica* A. Juss) and Chinaberry (*Melia azedarach*) alone. Moreover, the limonoids of *Azadirachta indica* A. Juss (neem) are the most outstanding of them all, due to their non-toxicity to animals and human beings and their effectiveness towards more than 250 insect species.

The biologically active compounds of neem are protolimonoids, limonoids or tetranortriterpenoids, pentanortriterpenoids, hexanortriterpenoids and nontriterpenoidal constituents (Siddiqui *et al*, 1988). The tetranortriterpenoids, isolated from the seeds, are lipid associated and constitute 1/4th of the neem oil. More than 80 tetranortriterpenoids have been isolated, out of which the class of compounds known as Azadirachtins (0.3%) are most active as insect antifeedant and growth inhibitory against more than 200 insect species both agricultural pests and vectors (Warthern, Jr., 1989). Azadirachtin (A) and its derivatives are active at 0.1-100 ppm (Rembold, 1990). However, due to its complex structure, the production of Azadirachtin by synthetic methods is non-economical. Even though several scientists the world-over are trying to synthesize the Azadirachtin molecule, the use of natural azadirachtin from neem is more advisable. The crude extracts of neem, are more safe as well as economical due to the synergistic effect of all the compounds (Parmar and Datta, 1986). However, the percentage of

Azadirachtins changes from source to source, thus affecting the quality of the product.

Along with azadirachtins, neem seeds also contain other tetranortriterpenoids, like gedunin, epoxy-azadiradione (0.15%), nimbin (0.5%), salanin (1.4%) and de-acetyl nimbin (0.5%), which are comparatively less oxidised and biogenetic precursors of Azadirachtins (Govindacharri *et al*, 1996). These compounds are also active as insect antifeedant and growth inhibitory agents but at a higher dosage.

Today neem oil is being used for various industrial purposes including *manufacture of soap. Neem oil after the removal of bitter principles by alcohol-*extraction is a good source of stearic and oleic acids. It is reported to contain triglycerrides, diglycerrides, monoglycerrides and free fatty acids like myristic, stearic, palmitic, arachidic, oleic and linoleic acids (Ketkar, 1982).

Production of secondary metabolites from tree species often depends on differentiated cells or structures, such as glandular hairs, lactifers and resin ducts (Yeoman, 1987). However, these cannot be induced in cell suspension cultures. Occasional formation of ducts or glands that produce the products such as terpenes and volatile oils are more common in callus cultures of tree species (Wiermann, 1981).

In the present study, callus cultures were initiated on wide combinations of media from immature cotyledons, as seeds contain the important secondary metabolites of neem. Secondary metabolite production in tissue culture has been reported to be influenced by the nature and levels of auxins, cytokinins, vitamins, carbon source and mineral content of media (Knobloch *et al*, 1982; Fernandes-Ferriera *et al*, 1991). Sanyal *et al* (1983), also studied the production of secondary metabolites of neem from cotyledonary callus using auxins like NAA/IAA in combination with BAP/KN. They reported that the contents of nimbin decreased, but that of ß-sitosterol increased with the age of callus (after 2 months of incubation on the induction medium). They attributed this to the fact that mevalonic acid (a common precursor) gets utilised in biosynthesis of one or the other compound according to the tissue.

Lindsay and Yeoman (1983), showed that the fast growing, soft friable calli usually accumulate low levels of secondary metabolites than slow growing compact friable calli. In our experiments we have chosen compact friable calli for the secondary metabolite analysis **(Table 5.1)**. Such calli were found to be induced on combinations of IBA/NAA/IAA with BAP/KN. 2,4-D in combination with KN or BAP induced slimy brown callus or direct somatic embryogenesis respectively.

The cultures were incubated in dark due to the photosensitive nature of the neem oil and its associated, especially, azadirachtin (Isman *et al*, 1990). Hence, non-differentiating and differentiating calli were incubated in dark for 20 and 40 days respectively. Since embryo maturation and germination require incubation in light (16/8 hour photoperiod), these stages were not analyzed.

It was expected to isolate lipids and the associated tetranortriterpenoids. For this acetone, a polar solvent was used for extraction as it extracts both lipids as well as the lipid associates. Moreover, azadirachtin being a sensitive molecule is reported to undergo degradation in presence of less polar solvents like benzene (Ley *et al*, 1993).

According to Yeoman *et al* (1982), accumulation of secondary products occur maximally when the growth rate of the culture decreases and when the cultures exhibit some structural differentiation such as root, shoot and embryoids. The relation between organogenesis and secondary product formation has been studied in great detail (Tabata and Hiraoka, 1976; Roja *et al*, 1987). Embryogenic cultures (Ozeki and Komamine, 1981; Schuchmann and Wellmann, 1983; Luckner and Diettrich, 1985), have also been reported to produce high levels of secondary products as compared to their respective cell cultures.

From the analysis of the calli, it is evident that the embryogenic mass produces polar lipids. The non-embryogenic callus showed the presence of ß-sitosterol alone, whereas the embryogenic callus showed the presence of a compound identified as glycerine-1-monostearate. From the high resolution NMR , the upfield position of the proton indicated that the compund is a mono-glyceride. This was confirmed by compairing the NMR to that of synthetically prepared glycerine -1- monosterate (Hopkins, 1968) . Absence of the downfield signal

around δ = 5.2, indicated absence of unsaturation in the acid moiety. Thus, this indicates the presence of either stearate or palmitate. As the secondary metabolites of neem are associated with the lipids, further manipulation of the growth conditions may induce the desired compounds from the callus cultures.

Naturally occuring seed oils contain triglycerrides as major component followed by other components like di- and mono-glycerrides, free fatty acids, sterols, sterol esters, phospholipids, with traces of hydrocarbons and sulphur containing hydrocarbons and lipid associates. Sepertation of this complex mixture is time as well as money consuming . Commercial use mono-glycerrides are prepared by molecular or short-path distillation. Thus, production of mono-glycerrides exclusively in culture would be a good source of pure mono-glycerrides, which are used as emulsifiers and in preperation of cosmetics, pigments, floor waxes, synthetic rubbers, coatings, textiles, etc.

In most cases secondary metabolite synthesis is uncoupled from cell growth and division. Therefore, the factors which enhance growth rate tend to lower product yield. Nutrient medium is one of the obvious factors which affects growth rate. The level and the nature of nitrogen source (Fowler, 1988) or phosphate content (Sakano *et al*, 1995) have been reported to influence callus growth.

In the present study, 1/4 concentration of phosphate and omission of nitrate were found to have promotory effects on callus growth **(Table 5.5. 5.6)**. However, the other modifications which induced slow growth did not have a promotory effect on secondary metabolite production. All the calli showed the presence of ß-sitosterol only.

Sanyal *et al* (1986, 1988), have observed the production of nimbin and ß-sitosterol from the cotyledonary callus cultures of neem. They studied the effect of low concentration of cytokinin and auxin; addition of $AdSO_4$ and glycine on redifferentiation of the callus. They found that redifferentiation to roots induced 0.25% *nimbin* and to shoots produce 0.028% nimbin.

However, from the present studies there is an indication of production of de-acetylnimbin and epoxy-azadiradione from dedifferentiated callus. Whereas differentiation caused only induction of a compound identified as glycerine-1-

mono stearate and glycerine-1-mono palmitate. Moreover, when experiments based on the observations of Sanyal *et al* (1986, 1988), were conducted, the callus transferred to low NAA and KN concentration induced profuse rhizogenesis **(Fig. 5.13)**. Addition of $AdSO_4$ and glycine had adverse effect on the growth of callus.

5.5. CONCLUSION

From the present studies it appears that differentiation of tissue has no correlation with chemical differentiation. The unorganized tissue like callus was found to produce the specific active principles. The neem oil devoid of the bitter principles is nowadays of great commercial value. Moreover, the exclusive production of monoglycerrides of palmitate or stearate, using *in vitro* technique has tremendous value commercially. To confirm the presence of the acid moiety, saponification and analysis and estimation of the acid moiety by GC analysis is needed. To exploit cell culture systems of neem for the production of the extracts enriched with desired properties requires directive efforts. However present studies indicate that by using specific ingredients in the growth medium, it is possible to induce specific compunds among the wide array of mixture of compunds.

SUMMARY

Currently, *Azadirachta indica* A. Juss (Neem), has attained a pride of place in international scientific research and literature. The recent spurt of interest in this tree is due to the presence of about 80 terpenoidal compounds having spectacular biological activity against a wide range of insects, pests and nematodes. Azadirachtin and other terpenoids are natural, environmentally compatible products which could be promising candidates for pest control. Its products are also used in toiletries, cosmetics, pharmaeceuticals, plant and animal nutrition, energy generation and other areas.

Mass propagation of neem on a commercial scale has limitations of seed viability. Moreover, vegetative propagation of this tree using conventional methods is not possible. Tissue culture offer a reliable method for mass production of plants in a shorter time and without seasonal constraints. Plantlet regeneration is best achieved with adventitious shoots which are then rooted or from somatic embryogenesis. The advantages of somatic embryogenesis over the conventional two-stage method has drawn much attention. A large number of plants having an in-built root and shoot system can be produced. Somatic embryos are amenable to automatic handling and their encapsulation with synthetic gels for use as artificial seeds has added advantages. Tissue culture also offers fascinating solution for the production of natural products called as secondary metabolites. During the last few decades, tremendous efforts have been directed to increase the production of these metabolietes, either by improved plant type or through *in vitro* techniques.

In the present investigation, **micro propagation** was carried out on MS basal medium supplemented with KN (1.0 mg/l) and BAP (0.5 mg/l). An increase in multipliction frequency of shoots per explant (45-50) and average shoot length (4.4 cm) was obereved upon **dilution of MS macronutrients with reduction in cytokinins and addition of CP and B**, after 14-16 weeks. Rooting of these shoots was achieved when the shoots were transferred to hormone free medium or on half strength MS liquid medium supplemented with IAA (2.0 mg/l). The rooted plantlets were washed with water to remove agar/medium sticking to them and kept in tap water for one week, under room conditions and then transplanted to sand:soil mixture and later shifted to green house where they were maintained under a very high humidity. Plants acclimatized under these conditions were

shifted to field conditions after three months. The field evaluation data taken after 1 year showed that the *in vitro* derived plants were superior to the control ones in terms of growth, number of branches and leaves and also in the size of the leaves. Part of this investigation has been published in Indian Journal of Experimental Biology (Joshi and Thengane, 1996).

Studies were also initiated to optimize the conditions for both the pathways of somatic embyrogenesis. The choice of explant was the immature cotyledons as the seed is the main source of the important active principles of neem. The induction of somatic embryos was achieved directly on the explant or from callus. The type of auxin used was found to be the determining factor for embryo induction pathway.

The complete protocol for the **indirect pathway of somatic embryogenesis**, is presented. MS medium supplemented with IAA (0.5 mg/l), BAP (1.0 mg/l), CH (1000 mg/l) and sucrose (50 g/l), induced compact nodular callus which showed initiation of globular shiny masses after two subcultures of 20 days each. Total omission of CH, with increase in BAP was effective for maturation, where 60-70 embryos enlarged and germinated within 20 days. To increase the germination percentage, the mature embryos were transferred to MS basal liquid medium supplemented with IAA (0.1 mg/l), GA_3 (0.01 mg.l), BAP (2.0 mg/l) and sucrose (20 g/l). Germinated embryos, when transferred to MS half strength medium, showed elongation and development of complete plantlet with well-developed root and shoot. A part of this investigation is published in journal In Vitro Cellular and Developemental Biology (Shrikhande *et al*, 1993).

For **direct embryogenesis**, two types of media were tried, viz., MS medium supplemented with 2,4-D (0.5 mg/l) and BAP (1.0 mg/l) or TDZ (0.5 mg/l). Induction of 20-25 embryos (in case of BAP) and profuse but not distinct embryos (in case of TDZ) per explant were observed. Germination of these embryos was found to be very difficult. Transfer to hormone free medium induced callusing of embryos (in case where BAP is involved) or precocious germination (in case wher TDZ is involved) was observed.

Studies were also undertaken to isolate the lipids and the associated tetranortriterpenoids. For this, acetone, a polar solvent was used for extraction as it extracts both lipids as well as the lipid associates. Callus cultures were initiated on wide combinations of media from immature cotyledons, as seeds contain the important secondary metabolites of neem. The slow growing compact friable calli were chosen for the secondary metabolite analysis. The calli initiated on MS basal medium supplemented with IAA/IBA/NAA in combination with either BAP/KN were used.

From the analysis of the non-differentiated and differentiated calli, it was evident that the embryogenic calli produces monoglycerrides exclusively. The non-differented callus showed the presence of ß-sitosterol alone. Production of monoglycerrides in culture has great commercial value. This is because, the monoglycerrides are used as emulsifiers and in preparation of cosmetics, pigments, floor waxes, synthetic rubbers, coatings, textiles, etc. Moreover, for the commerical purposes pure monoglycerrides have to be obtained by molecular or short-path distillation. Thus, the production of monoglycerrides alone, has tremendous value. To confirm the presence of the acid moiety, saponification, analysis and estimation of the methyl esters of these acid moieties by GC analysis can be carried out. As the secondary metabolites of neem are associated with the lipids, further manipulation of the growth conditions may induce the desired compounds from the callus cultures.

Even though woody species are recalcitrant to *in vitro* culture, neem appears to be very responsive. The mass multiplication protocol can be used for rapid reforestation programmes and also multiplication of selected elites. Somatic embryogenesis protocols have tremendous potential in isolation of variants and in transformation studies. Similarly, work can be directed for the production of neem oil and its associates, especially the tetranortriterpenoids.

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AUTHOR'S PUBLICATIONS

LIST OF PUBLICATIONS:

1. Effect of auxin on growth and differentiation in Sunflower (*Helianthus annuus* L.): A genotype response. S. R. Thengane, **M. S. Joshi** and R. J. Thengane. Paper accepted for publication in In Vitro Cell. Dev. Biol. Plant, July 1997.

2. Correlation of metabolic differentiation with somatic embryogenesis in *Azadirachta indica* A. Juss. **M. S. Joshi** and S. R. Thengane. Paper communicated to Plant Science, December, 1996.

3. *In vitro* techniques for 'Wonder Tree' more wonderful. S. R. Thengane and **M. S. Joshi.** In: Sulebkha K. P. (ed.) Proceedings of the VII All India Meeting of Women in Science, Ajay Printers and Publ., Roorkee, India, September, 1996. pp 9-13.

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SOMATIC EMBRYOGENESIS AND PLANT REGENERATION IN *AZADIRACHTA INDICA* A. JUSS

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SUMMARY

Media for induction of somatic embryogenesis from immature cotyledonary tissues of *Azadirachta indica* (Neem) were determined. Callus was initiated on Murashige and Skoog medium supplemented with 0.5 mg · liter^{-1} of indol-3 acetic acid, 1.0 mg · liter^{-1} of 6-benzyl amino purine, and 1000 mg · liter^{-1} of casein hydrolysate. Effect of kinetin was also studied for embryo induction. Carbohydrate source in the form of sucrose and glucose alone and in combination was tested for embryogenic efficiency. Seventy percent embryos showed germination. Healthy plants were potted in sand and soil. Histologic studies confirmed indirect somatic embryogenesis.

Key words: Azadirachta indica; cotyledon; somatic embryogenesis; glucose; plant regeneration.

INTRODUCTION

In vitro techniques are gradually being included in tree-improvement programs due to limitations in the conventional methods. Considerable progress in the development of tissue culture methodologies for trees with possible application to forestry has already been made (2). Conventionally, enhanced proliferation of meristems followed by rooting, in a two-stage process is followed for obtaining high multiplication rates (11). More recently attention has been focused on the production of plantlets by somatic embryogenesis (4). The advantages of somatic embryogenesis are mainly in the production of large numbers of individual plants having an in-built root and shoot system. Encapsulation of embryos with synthetic gels for use of artificial seeds has added advantages.

Azadirachta indica, commonly known as "Neem," is one of the most valuable arid zone trees belonging to the family Meliaceae. A native of dry forest areas of India and the subcontinent, it is widely cultivated in the arid, nutrient-deficient regions of India and Africa. Recently, Neem has achieved a new identity from a mere avenue tree to a more versatile tree having multifarious properties and uses in agriculture, as an insecticide and nematocide, and in the pharmaceutical and soap industries (8).

As a forest tree it has its own advantages. It is used as fuel in Africa. The wood resembles teak in its strength and properties. It is moderately resistant to fungi and relatively immune to termite attack.

The present paper describes the conditions for in vitro regeneration of Neem from immature cotyledonary tissue by indirect somatic embryogenesis.

MATERIALS AND METHODS

Immature cotyledons of *A. indica*, 3 to 5 mm in diameter were used as the source of explant for the induction of callus. Unripe mature fruits were washed with liquid detergent (10% savlon), followed by thorough washing under running tap water. Fruits were surface sterilized with 0.15% mercuric chloride for 10 min and finally washed 4 to 5 times with sterile distilled water.

Cotyledons were removed aseptically and the embryo axis excised. The cotyledon was cut horizontally into two pieces and inoculated in a culture tube with its abaxial surface in contact with the medium. Each culture tube contained 20 ml of the medium. For preliminary experiments, Murashige and Skoog (MS) (12) basal medium was supplemented with MS vitamins, 1000 mg · liter^{-1} of casein hydrolysate (CH) (DIFCO, Detroit, MI), 5% sucrose, 0.5 to 5.0 mg · liter^{-1} of indol-3-acetic acid (IAA) alone, and in combination with 1.0 mg · liter^{-1} of 6-benzyl amino purine (BAP) or 1.0 mg · liter^{-1} of kinetin (KN). The medium was solidified with 0.4% agar (bacteriological grade) after adjusting pH to 5.7.

All growth regulators were incorporated into the medium before autoclaving. The media were autoclaved at 120° C and 15 psi for 20 min. The cultures were maintained at 25 ± 1° C and incubated in the dark. The passage interval for cultures was 20 days.

The effect on embryo induction of different concentrations (0.2 to 3.0 mg · liter^{-1}) of cytokinins, BAP, or KN was evaluated. In addition, the effect of sucrose alone (3 to 10%) and in combination with glucose (2 to 5%) on embryo induction was also studied.

In media used for maturation and germination of somatic embryos the concentration of CH (0 to 1000 mg · liter^{-1}) and BAP (1 to 2 mg · liter^{-1}) was tested. The cultures were incubated in 16/8 hr photoperiod at 3000 lux light intensity.

Well-rooted plantlets derived through somatic embryogenesis were planted in plastic pots containing a sand:soil mixture (1:1 ratio) and were transferred to a glass house for further growth.

Data were taken from three replicates of each experiment (10 cultures/replicate). The percent normal embryo was counted on the basis of 400 embryos from randomly selected cultures.

Histological preparation. Thirty- to forty-day-old cultures were used for histologic studies. Embryos at various stages of development were isolated and fixed. Tissues were fixed in formalin:acetic acid and alcohol (1:1:18) followed by dehydration through ethanol-xylol series and embedded in paraffin wax [melting point (M. P.) 59 to 60° C], according to Sharma and Sharma (16). Serial sections cut at 10 μm were stained with hematoxylin-eosin, mounted with DPX-4 189-(2-chloro-*N*- (4-methoxy-6-methyl-1,3,5-triazin-2-yl amino carbonyl) benzene sulfonamide (BDH) and observed microscopically.

RESULTS AND DISCUSSION

Induction of callus and development of somatic embryos. With 1 to 2 wk of incubation, swelling and callusing of the cotyledonary

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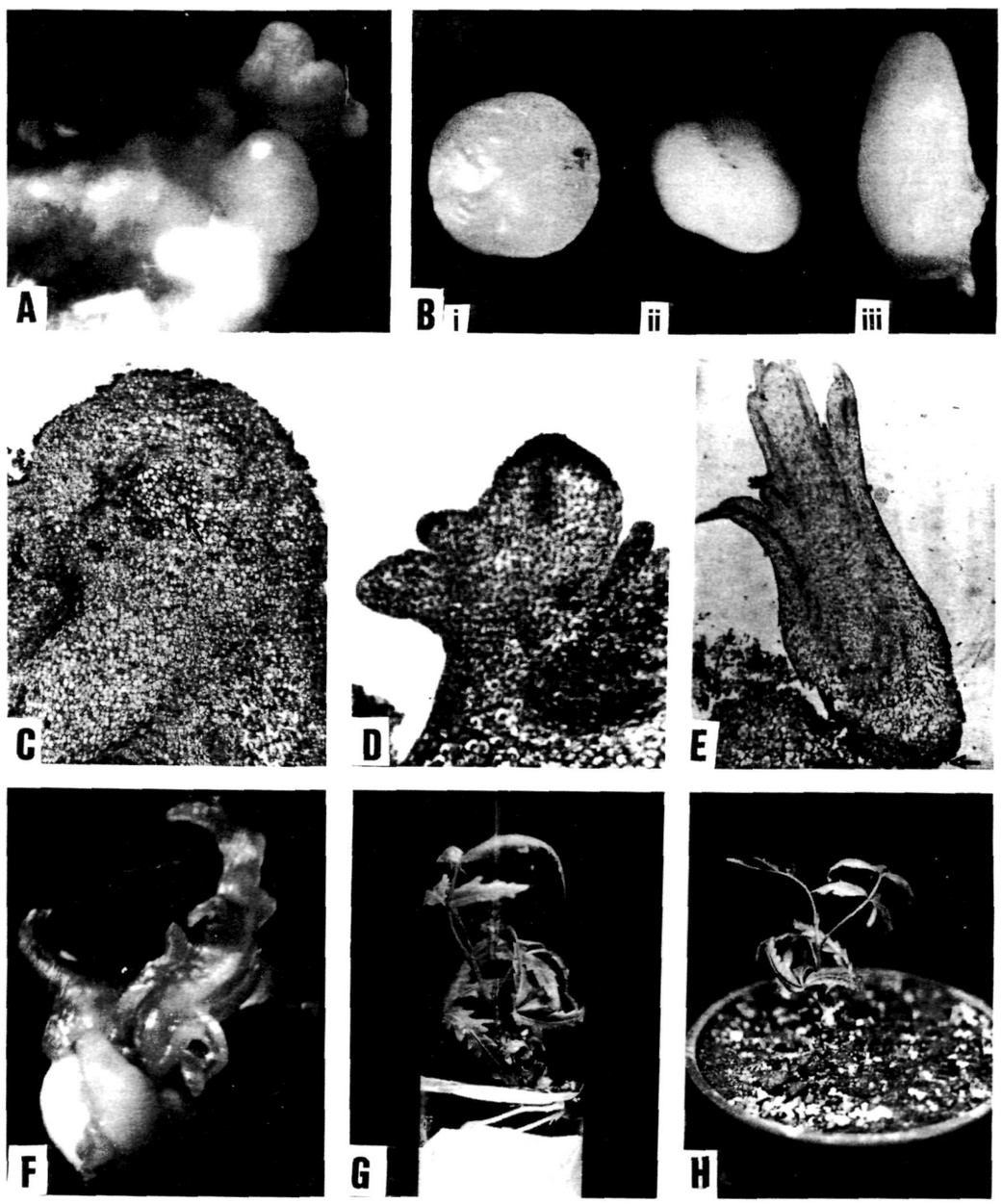

FIG. 1. Stages in somatic embryogenesis in Neem. *A*, callus showing shiny globular masses (magnification = ×10); *B*, *i*, globular embryo (×16); *ii*, heart shaped (×16); *iii*, mature embryo with suspensor (×10); *C*, transverse section through callus showing meristematic pockets (×25); *D*, proembryoid emerging from callus (×50); *E*, germinating embryo still attached to callus (×25). Plantlet formation in Neem. *F*, single germinating embryo with development of shoot (×10); *G*, complete plantlet on MS half medium; *H*, plantlet transferred to soil.

tissue at the cut surfaces were observed. In the preliminary experiments, explants growing on medium with IAA (0.5 to 5.0 mg · liter^{-1}) alone and in combination with KN (1.0 mg · liter^{-1}) showed callusing and rhizogenesis, where the callus was smooth and friable. All combinations of IAA with BAP (1.0 mg · liter^{-1})induced compact nodular callus. Upon transfer of these calluses to their respective media, there was further increase in callusing. However after two subcultures, 60% of the cultures on medium with IAA (0.5 mg · liter^{-1}) and BAP (1.0 mg · liter^{-1}) showed initiation of globular shiny masses (Fig. 1 *A*). The compact nodular callus obtained on other combinations of IAA and BAP did not show any embryogenic response even after repeated (five to six) subculture

TABLE 1

EFFECT OF DIFFERENT CONCENTRATIONS OF CYTOKININS ON INDUCTION OF SOMATIC EMBRYOGENESIS[a]

Media with		
BAP mg · liter^{-1}	KN mg · liter^{-1}	Response, %
0.2	—	10
0.5	—	40
1.0	—	60
3.0	—	30
—	0.2	—
—	0.5	10
—	1.0	10
—	3.0	—

[a] Medium used: MS basal medium + IAA (0.5 mg · liter^{-1}) + CH (1000 mg · liter^{-1}) + sucrose (5%). Concentration of cytokinins varied. Number of cultures studied = 10/replicate; number of replicates studied = 3; precentage response = embryogenic response/10 cultures.

TABLE 2

EFFECT OF DIFFERENT CONCENTRATION AND COMBINATIONS OF SUCROSE AND GLUCOSE ON EMBRYO INDUCTION[a]

Sucrose, %	Glucose, %	Embryogenesis, %	Normal Embryos, %
3	—	50	85
5	—	70	95
7	—	70	60
10	—	70	25
2	2	30	70
3	3	30	60
4	4	40	30
5	5	50	15

[a] Medium used: MS basal medium + IAA (0.5 mg · liter^{-1}) + BAP (1.0 mg · liter^{-1}) + CH (1000 mg · liter^{-1}). Number of cultures studied = 10/replicate; number of replicates studied = 3; *percentage response* = embryogenic response/10 cultures.

on the respective media. According to Jaiswal and Narayan (5), friable callus can be induced on a variety of explants like leaf, axillary buds, stem, on MS medium supplemented with NAA.

Supply of reduced nitrogen is generally desirable for embryogenesis to occur in callus cultures (3). According to Rangaswamy (13), reduced nitrogen is essential, especially at the induction phase of somatic embryogenesis. Different sources of reduced nitrogen, e.g., coconut water, casein hydrolysate, mixture of amino acids, or a single amino acid such as L-glutamine or L-alanine, has been used (1). According to Murashige and Skoog (12), presence of CH allows vigorous organ development over a broader range of growth hormones. Maheswari and Lal (9) observed extremely rapid growth and maturation of embryos on Nitsch medium supplemented with 1000 mg · liter^{-1} of casein hydrolysate. It was therefore decided to supplement the medium with 1000 mg · liter^{-1} of CH.

According to the work done previously in our laboratory, 5% sucrose gave best callus initiation and growth (unpublished data). Sanyal et al. (15) also obtained callus development on MS basal medium supplemented with IAA (0.1 to 0.5 mg · liter^{-1}), BAP (0.1 to 0.5 mg · liter^{-1}), and 5% sucrose.

Thus preliminarily, medium supplemented with IAA (0.5 mg · liter^{-1}), BAP (1.0 mg · liter^{-1}), CH (1000 mg · liter^{-1}), and sucrose (5%) was used for induction of somatic embryos via callus.

To optimize these conditions, BAP and KN concentrations were varied from 0.2 to 3.0 mg · liter^{-1} keeping IAA constant (0.5 mg · liter^{-1}). In the presence of KN (0.5 to 1.0 mg · liter^{-1}) only 10% of the cultures showed induction of embryos (Table 1). All combinations of BAP with IAA were effective, and maximum response (60%) was observed with BAP 1.0 mg · liter^{-1}.

The most commonly used carbohydrate for plant tissue or cell culture is sucrose (18). There are few critical reports on the use of alternative carbohydrate sources for somatic embryogenesis, such as galactose, mannose, raffinose, stachyose, maltose, and corn syrup (19); lactose, glucose, fructose, and galactose (14); or maltose, maltotriose, and soluble starch (17). In general, sucrose and glucose are the preferred carbon sources for growth of cultures (10).

Table 2 shows the effect of different concentrations of sucrose (3 to 10%) alone and in combination with glucose (2 to 5%) on embryogenic efficiency. At 5 to 10% sucrose concentration, 70% of the cultures showed embryo induction from callus, where normal as well abnormal shapes of embryos were noted. The normal embryos resembled the zygotic embryos grown in vivo. These were oblong shaped with well-developed suspensor (Fig. 2 *B iii*). The abnormal embryos were formed by union of two to three embryos during the early developmental stages. These varied in shape, e.g., kidney shape, and did not form the suspensor.

In *Theobroma cacao*, Kononowicz and Janick (7) observed that higher concentrations of sucrose were inhibitory for embryo growth. They attributed this to increased osmotic potential of the medium or to oxygen limitation. They also noted that embryos cultured in the presence of glucose did not enter or complete the phase of embryo development characteristic of zygotic embryos grown in vivo.

A similar phenomenon was observed in the present study, where the number of normal embryos decreased with increasing sucrose concentration (Table 2). In the combinations of sucrose with glucose, an increase in callusing but reduction in embryo differentia-

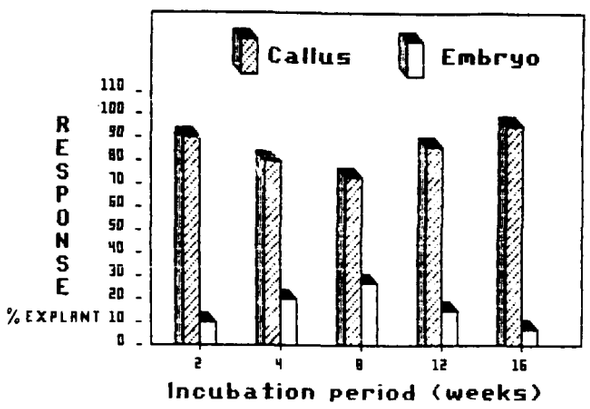

FIG. 2. Embryogenic response in relation to incubation period.

TABLE 3

EFFECT OF BAP AND CH ON EMBRYO MATURATION AND GERMINATION[a]

Growth Hormones				
IAA	BAP	CH	No. of Mature Embryos	Germination, %
mg · liter^{-1}		mg · liter^{-1}		
0.5	1.0	1000	65	1.0
		500	60	12.0
		200	63	28.5
		—	54	50.0
0.5	2.0	1000	70	45.0
		500	50	21.0
		200	45	12.0
		—	75	74.0
—	1.0	1000	31	25.0
		500	14	9.5
		200	3	NIL
		—	NIL	NIL

[a] Number of mature embryos. 100/experiment: number of replicates. 3.

tion was noticed. Moreover, with increase in glucose concentration, the percentage of normal embryos reduced drastically (15%). From the conditions tested. 5% sucrose seems to be optimal for embryo induction.

The effect of the incubation period on embryogenic response was studied by subculturing the callus on the induction medium up to 16 wk. With an increase in the incubation period there was increase in embryogenic response (26%) up to the 8th wk. Only 6% of the cultures showed embryogenic response after 16 wk incubation on the same medium. After that time, cultures did not produce any embryos (Fig. 2).

Histologic studies confirmed the origin of the embryos from callus tissue. On the induction medium, callus showed differentiation of meristematic pockets which developed into proembryonic masses at the periphery. These later differentiated into embryos (Fig. 1 *C,D*). Differentiation of embryos was continuous and nonsynchronous. therefore embryos at different developmental stages could be observed (Fig. 1 *B i,ii,iii*).

About 1 to 2% embryos germinated on the same medium when still attached to the callus (Fig. 1 *E*). However, the resulting plantlets were necrotic and fasciated. Subculture of callus with embryos of different developmental stages in the same medium resulted in proliferation of embryos to a good extent, but their subsequent development to plantlets was arrested. Further experiments were therefore carried out for the maturation and normal germination of embryos.

Maturation and germination of somatic embryos. Different media were tested for maturation and germination of embryos. With an increase in BAP concentration (2.0 mg · liter^{-1}) keeping IAA (0.5 mg · liter^{-1}) and CH (1000 mg · liter^{-1}) constant, there was an increase in the number of cotyledonary stage embryos, within 10 to 15 days (Table 3). Some of the embryos even showed elongation of the shoot axis and development of a rudimentary root. However, the development of all embryos was not normal. Different abnormal shapes of embryos could be observed which later showed callusing. Reduction of CH (200 to 500 mg · liter^{-1}) improved development of normal embryos, but callusing of embryos persisted. Total omission of CH with an increase in BAP (2.0 mg · liter^{-1}) had a promotory effect on maturation and germination of embryos. (Table 3). Thus medium containing IAA (0.5 mg · liter^{-1}), BAP (2.0 mg · liter^{-1}), and sucrose (5%) showed maximum response where development of embryo with suspensor and initiation of germination was noticed. On this medium 60 to 70% of the embryos enlarged and germinated in about 20 to 30 days. (Fig. 1 *F*).

Kapoor (6), reported a tendency toward cleavage in embryos, when higher concentration of CH (800 to 1000 mg · liter^{-1}) was used. Zeibur et al. (20) observed inhibition of germination of *Hordeum* embryos in high CH containing medium, which they attributed to high osmotic pressure. According to them amino acids and phosphate of CH promote early embryo growth. This supports our findings in which high CH concentration (1000 mg · liter^{-1}) promoted induction of embryos but was found to be inhibitory for maturation and germination.

On total omission of IAA, embryos turned black. Germinated embryos when transferred to MS half strength medium with 2% sucrose showed elongation and development of complete plantlets with well-developed root and shoot (Fig. 1 *G*). Rooted plantlets were transferred to pots filled with sand:soil (1:1) mixture (Fig. 1 *H*). Out of 87 plants transferred to soil, survival of the plants was 80 to 90%.

The present study describes the conditions for induction of somatic embryos via callus. The method can be useful for propagation of superior Neem trees and also for isolation of variant lines with higher medicinal value.

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In vitro propagation of *Azadirachta indica* A. Juss (neem) by shoot proliferation

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Clonal propagation of *A. indica* (neem), was achieved on MS medium supplemented with BAP (0.5 mg/l) and KN (1.0 mg/l). Two to three shoots per explant were observed after 20 days. An increase in multiplication frequency of shoots per explant (45-50) and average shoot length (4.7 cm) was observed after 14-16 weeks on medium containing 3/4 dilution of MS macronutrients, full strength MS micronutrients, KN (0.05 mg/l), BAP (0.1 mg/l), calcium pantothenate and biotin (CP; B; 0.05 mg/l). Rooting of these shoots was achieved on half strength MS liquid medium supplemented with IAA (2 mg/l). The regenerated plantlets were successfully established in soil.

Azadirachta indica A. Juss (neem), is a tree, having multifarious properties and wide adaptability to various ecological environments. It is particularly valuable in forestry, land reclamation and conservation, revegetation of wastelands and for biomass production. It is also of great commercial importance due to its nitrogen fixing, pesticidal, medicinal and pharmacological properties[1]. In spite of the wide adaptability of the tree, its propagation is limited by low seed viability. Moreover, the propagation by root suckers, shoot cuttings, air layering, and grafting is difficult[2]. Thus *in vitro* techniques provide an alternative means of propagation.

Most of the earlier work in tissue culture of neem, comprises studies on organogenesis from callus cultures of anther[3], cotyledon[4], embryo/seedling[5,6] and stem tissue[7-11]. Organogenesis from callus was obtained either for the de-novo production of secondary metabolites, as chemo-differentiation was seen to be related to morpho-differentiation[7-11], or for regeneration studies.

Joarder *et al.*[12], reported the formation of 1-3 shoots/culture from axillary buds of woody trunks of 5-40 years old trees. They observed an inverse relation between age of the tree and number of shoots per explant.

In our preliminary report[13], about 8-10 plantlets were obtained from a single axillary bud in about 2 months period.

We report here a refined protocol for the mass production of neem plantlets using shoot proliferation where about 50 plantlets can be produced from a single bud within 4 months.

Non-woody branches of young, non-flowering (2-5 year old) trees of *Azadirachta indica*, were used as explant. The leaves were excised and the branches were cut into segments (1.5 cm) comprising one single node. These explants were washed thoroughly under running tap water for 5 min and then with 10% savlon (antiseptic detergent) for 2 min followed by thorough rinsing with running tap water. Explants were surface sterilized with 70% ethanol for 2 min followed by 0.1% mercuric chloride for 10 min in the laminar unit. Finally the explants were rinsed (5-6 times) with sterile distilled water.

Murashige and Skoog's (MS) salts[14], supplemented with MS vitamins, cytokinins such as 6-benzylamino purine (BAP) and kinetin (KN) (0.5-3 mg/l) and sucrose (2 g/l) as carbon source, was used for bud sprouting. Many combinations of media, for enhancing proliferation of shoots, were based on low cytokinin concentration, BAP and KN (0.05-0.5 mg/l), additives like calcium pantothenate (CP) and biotin (B) (0.05 and 0.1 mg/l) and dilutions of MS salts (full, 1/2, 3/4 strength). Combinations used were—medium (A) – MS full strength + BAP and KN (0.2 mg/l); (A1) – MS 1/2 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l); (A2) – MS 3/4 strength + BAP and KN (0.2 mg/l) + CP and B (0.05 mg/l); (B) – MS full strength + BAP (0.1 mg/l) + KN (0.05 mg/l); (B1) MS 1/2 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l); and (B2) – MS 3/4 strength + BAP (0.1 mg/l) + KN (0.05 mg/l) + CP and B (0.05 mg/l).

MS half strength liquid medium, supplemented with indole 3-acetic acid (IAA; 0.5-2.0 mg/l) filter sterilzed before autoclaving was used for rooting.

All the growth hormones were incorporated into the medium before autoclaving unless otherwise stated. After adjusting the *p*H to 5.7, the media were autoclaved at 15 psi and 120°C.

Glass tubes (150 × 25 mm) plugged with cotton were used for all the experiments. Each tube contains 20 ml of medium. In case of liquid medium, to avoid submersion of cultures, Whatman No. 1 filter paper

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supports were used. Cultures were maintained under 16/18 hr photoperiod at 25° ± 1°C. Illumination was provided by white fluorescent tubes (~ 35 μE/m²/s).

For statistical analysis two criteria were studied simultaneously, i.e. average shoot number and average shoot length. The response was studied for four different media. Ten explants were studied for each medium. In each experiment three replicated were used.

Bud growth from nodal explants of mature, flowering trees (15-20 yr old) was negligible, and most of the explants turned brown and died. Only 9% of the buds showed initiation after 20 days of incubation on initiation medium. Continued incubation did not improve the number of sprouts. The explants became thick and developed callus at the base of sprouted shoots.

Explants from young trees (2-5 yr old) showed 85% initiation within 7 days (Fig. 1A). Combination of BAP (0.5 mg/l) and KN (1 mg/l) produced maximum sprouting (82%) and number of sprout per explant (2-3). Higher concentrations of cytokinins (1.5, 2 and 3 mg/l) favoured only callusing of buds from explant base.

At lower cytokinin concentration, BAP and KN (0.05-0.5 mg/l) rapid shoot elongation was observed. For further multiplication shoots were separated after 15-20 days, cut into sections of 1-2 nodes and re-inoculated on these two media A and B. 2 shoots per explant with shoot length 3.9 cm was observed on medium A after 15-20 days and 3 shoots per explant with shoot length 3 cm was observed on medium B.

Addition of vitamins (CP and B) at 0.05 mg/l concentration gave better response than at 0.1 mg/l concentration. The best results were obtained on medium B supplemented with CP and B (0.05 mg/l), where shoot number increased to 8 and shoot length to 5 cm. Shoots were green, sturdy and healthy as observed earlier[15].

For further multiplication, the shoots were subcultured on different MS dilutions after 3 weeks (Table 1). MS dilution at 3/4 strength was more effective than 1/2 strength, irrespective of the cytokinin concentration used. Shoots of various sizes (0.5-5 cm) developed on these media (Fig. 1B). The shoots more than 1.5 cm in length were isolated, cut (1-2 nodes) and used as inoculum for further multiplication. While the shoots of sizes less than 1.5 cm were inoculated in bunches of 3-4 shoots. On all media, initiation of lateral shoots from axils of lower leaves as well as from base of inoculated shoot section was observed. The B2 medium proved to be the best for mass production of neem plantlets as rapid increase (nearly double) in shoot number was observed at every subculture (Table 2).

In-vitro rooting—For rooting, shoots (more than 2.5 cm long) were subcultured on the media

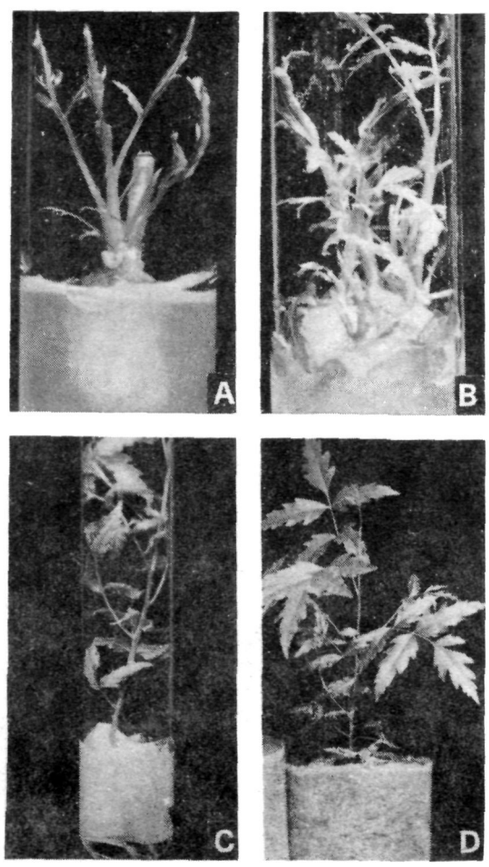

Fig. 1—(A)—Sprouting of buds; (B)—multiplication of shoots in various sizes; (C)—*in vitro* rooted plantlet; and (D)—plant in polybag.

Table 1—Effect of MS dilutions on shoot multiplication after two subcultures

MS dilution (strength)	Mean number of shoots	Average shoot length (cm)
	Medium A	
Full	4	2.1
3/4	6	2.5
1/2	4	2.0
	Medium B	
Full	5	3.5
3/4	8	4.2
1/2	6	2.5

Table 2—Effect of subculture and media compositions on shoot proliferation
[Values are mean ±SE of 3 replications]

Medium	Average number of shoots			Average shoot length (cm)		
	*S3	S4	S5	S3	S4	S5
A1	12.9 ±0.8	12.0 ±0.7	15.3 ±1.4	1.9 ±0.1	2.4 ±0.1	1.8 ±0.1
A2	15.7 ±1.1	23.1 ±0.6	31.2 ±1.2	3.6 ±0.2	3.2 ±0.2	3.5 ±0.1
B1	15.5 ±0.9	18.9 ±0.4	10.8 ±1.1	2.9 ±0.1	2.2 ±0.1	3.2 ±0.3
B2	17.2 ±0.4	27.6 ±1.3	49.5 ±2.1	3.6 ±0.1	4.1 ±0.1	4.7 ±0.2

*Subculture number

supplemented with various concentrations of IAA (0.5-2 mg/l). Maximum rooting (80%) was observed within five days of incubation at 2 mg/l concentration of IAA (Fig. 1C). The rooted plants were transferred to polybags containing sand and soil mixture (1:1) and later shifted to green house where 100% survival was obtained (Fig. 1D).

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