MICROPROPAGATION AND EVALUATION OF AZADIRACHTIN PRODUCTION IN THE PLANTLETS OF NEEM

(Azadirachta indica A. Juss.)

By

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THESIS

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Department of Tree Physiology and Breeding

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DECLARATION

I hereby declare that this thesis entitled "**Micropropagation and evaluation of azadirachtin production in the plantlets of neem (*Azadirachta indica* A. Juss.)**" is a bonafide record of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis, entitled "**Micropropagation and evaluation of azadirachtin production in the plantlets of neem (*Azadirachta indica* A. Juss.)**" is a record of research work done independently by **Ms. Roshni, A.J.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associate ship to her.

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We, the undersigned members of the Advisory Committee of **Ms. Roshni, A.J.**, a candidate for the degree of **Master of Science in Forestry**, agree that the thesis entitled "**Micropropagation and evaluation of azadirachtin production in the plantlets of neem (*Azadirachta indica* A. Juss.)**" may be submitted by Ms. Roshni, A.J., in partial fulfilment of the requirement for the degree.

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Dedicated to my loving Parents

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CONTENTS

ABBREVIATIONS

BA	-	Benzyl adenine
cm	-	centimeter
dia	-	diameter
DW	-	Dry Weight
$HgCl_2$	-	Mercuric chloride
HPLC	-	High performance liquid chromatography
IAA	-	Indole-3-acetic acid
IBA	-	Indole-3-butyric acids
Kn	-	Kinetin
m	-	meter
mg	-	milligram
min	-	minute
mm	-	millimeter
µm	-	micrometer
MS	-	Murashige and Skoog (1962) medium
½ MS	-	Half-strength MS medium
NAA	-	Napthalene acetic acid
ppm	-	part per million
spp	-	species
TLC	-	Thin layer chromatography
UV	-	Ultra Violet
v/v	-	volume by volume
w/v	-	weight by volume
WPM	-	Woody Plant Medium of Lloyd and McCown (1980)
yr	-	year
2, 4-D	-	2, 4-Dichlorophenoxy acetic acid

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

Introduction

1. INTRODUCTION

Neem, (*Azadirachta indica* A. Juss) is a hardy, medium – large, evergreen tree belonging to the family Meliaceae. It is native to the Indian sub continent (Hegde, 1992). Every part of the tree, *viz.*, leaf, flower, fruit, seed, kernel, bark, wood, twig, root etc. have been traditionally used for centuries for treatment of human ailments and to control pests. The wood is used for construction, furniture making and as firewood. Neem derivatives affect more than 200 insect and mite species, several species of nematodes, fungi including aflatoxin producing *Aspergillus flavus* (Sidhu and Behl, 1996). Thus, the tree has been held in high esteem because of its medicinal values and insecticidal properties of the derivatives from the plants. In rural areas of India the tree has been called as "the village pharmacy" (Shiva, 1995).

The neem tree has been increasingly attracting the interest of researchers from various fields. More than 300 compounds have been isolated and characterised from neem seed, one-third of which are tetranortriterpenoids (limonoids) (Kumar *et al.*, 1996). One of these limonoids, azadirachtin is considered to be the most important active principle, responsible for its various effects on insects (Schmutterer, 1990; Govindachari *et al.*, 1995).

A large number of manufacturing units using azadirachtin based formulations have come up in the country and abroad during the past few years. They require sizeable quantities of neem seeds. Though India produces an estimated five lakh tonnes of neem seed, currently only a third of it is collected due to various operational problems and quality concerns (Hegde, 1992). With growing quantum of neem based industries, the demand for quality neem seed is expected to rise sharply necessitating planting of neem for seed production. The azadirachtin content of the planting stock becomes an important parameter for such plantations. Therefore selection of high azadirachtin clones and development of suitable propagation methods assumes importance. Mass production of selected clones through *in vitro* techniques – 'micropropagation' – is of great importance in clonal forestry to over

come the constraints like scarce seed supplies, germination problems, long regeneration time etc. (Leaky, 1987). It has long been apparent that genetic gains can be captured by clonal propagation (Durzan, 1988). However, conventional methods of vegetative propagation are not feasible in most of these species. Under such circumstances *in vitro* propagation is known to be a possible method in many tree species (Bonga, 1982).

Saxena (1989) reported that the current supplies of bioactive compounds from the neem tree will not meet the increasing demands. Therefore an alternate method of product synthesis has to be thought off. Extraction of secondary metabolites from *in vitro* cultures is being attempted in many a crop plant and trees. In this context, cell cultures / *in vitro* plantlets of neem can possibly act as a source of azadirachtin. This technique offers potential beyond that of solely producing azadirachtin, as other bioactive principles, both known and novel may also be obtained. This can as well help to overcome the stringent biological constraints and environmental uncertainities.

In this backdrop the present study was carried out with the following objectives:

(1) To standardise the procedure for micropropagation of neem (*Azadirachta indica* A. Juss) through tissue culture.

(2) To evaluate the secondary metabolite production potential of *in vitro* produced plantlets and callus in neem (*Azadirachta indica* A. Juss).

Review of Literature

2. REVIEW OF LITERATURE

Azadirachta indica is an indigenous but under exploited tree, and the tree has been in use since time immemorial. For centuries the tree has been held in high esteem by Indian folk because of its medicinal and insecticidal value. To those millions, neem has always had miraculous powers and now scientists around the world are beginning to think they may be right. Two decades of research have revealed promising results in so many disciplines that this obscure species may be of enormous benefit to countries both poor and rich. Hence neem deserves to be called a 'wonder plant'.

Mass propagation of neem on a commercial scale has limitations of seed viability and it was observed that extensive cross pollination resulted in the segregation of characters in the progeny. Moreover, vegetative propagation of this tree using conventional methods is not possible (Narayan and Jaiswal, 1985). In view of the limitations of the traditional vegetative propagation methods for producing large quantity of planting stock from a single / few mother trees, micropropagation through tissue culture has been tried by many scientists.

A detailed review of the recent literature on *in vitro* propagation of neem, various factors affecting *in vitro* propagation, *in vitro* culture as a tool for secondary metabolite production etc. are presented below.

2.1 MICROPROPAGATION

Regeneration of *Azadirachta indica* shoots from embryos and decapitated seedlings, when cultured in MS media was reported as early as 1972 by Rangaswamy and Promila. Callus production in *A. indica* has been obtained from stem bark segments (Sanyal *et al.*, 1981), leaves, cotyledons and petioles (Narayan and Jaiswal, 1985; Rao *et al.*, 1990; Sanyal *et al.*, 1983; Schulz, 1983), stem segments (Jaiswal and Narayan, 1984) and young seedlings (Naina *et al.*, 1989).

In vitro morphogenesis in *A. indica* using cotyledons as explant was studied by Muralidharan and Mascarenhas (1987) and plantlets were obtained through organogenesis. Rao *et al.* (1988) reported that fresh cotyledons were the best source for producing calluses when cultured in MS medium supplemented with different combinations of 2,4-D and IAA.

Chalupa (1990) considered the regeneration of neem plants via somatic embryogenesis as an efficient approach for clonal plant propagation. Vieitez and Braciela (1990) reported that somatic embryogenesis provided the basis for the genetic improvement through somaclonal variation or enabling genetic manipulation. Adventitious shoot buds were obtained from the excised leaf discs of neem (*A. indica)* when cultured on Wood and Braun's medium supplemented with different concentrations of Kin and BA. Each isolated bud grown on medium containing GA_3 developed into a healthy shoot (Ramesh and Padhya, 1990).

Plantlets of neem were successfully regenerated, from nodal sections, when they were cultured on MS medium supplemented with different combinations of cytokinins. Rooting of shoots was achieved when the shoots were cultured on MS medium supplemented with 10 µm IBA. It was also observed that longer exposure to IBA, reduced rooting percentage, increased time to initiate roots and produced excess callus at the base of the explants (Drew, 1993).

Gautam *et al.* (1993) reported that highest per cent of cultures produced callus, derived from anthers of *A. indica* when cultured in Nitsch medium containing 1.0 µm IAA + 1.0 µm BA. Multiple shoots were induced from the callus when sub-cultured on MS medium augmented with 4.44 µm BA + 0.53 µm NAA + 18.75 µm polyvinyl pyrrolidone (PVP). Excised shoots induced rooting in MS medium containing 4.9 µm IBA + 18.75 µm PVP, which resulted in the development of complete plantlets. Multiple shoots were obtained when nodal explants collected from mature trees were cultured on MS medium supplemented with low concentration of cytokinin (0.05-0.3 mg l^{-1}) and rooting was induced in MS + 0.05 mg l^{-1} IBA (Joarder *et al.*, 1993).

Joshi and Thengane (1993) reported that somatic embryos were induced from callus and cotyledonary explants obtained from neem tree, when cultured on MS medium supplemented with auxins, IAA and 2, 4-D along with cytokinins, 6 BAP and Kin under different concentrations. Somatic embryogenesis was achieved when cotyledonary explants were cultured on MS medium supplemented with different concentrations of NAA. Embryogenic calli regenerated into shoots when they were sub cultured in MS medium supplemented with cytokinin (Islam *et al.*, 1993).

Eighty per cent of cultures showed callus induction when inter nodal explants of *A. indica* were cultured on MS medium supplemented with 2.0 mg l^{-1} BA (Nirmalakumari *et al.*, 1993). It was observed that leaf explants of *A. indica* induced callus when they were cultured in MS medium supplemented with 2, 4-D (2.0 mg l^{-1}) + Kin (0.5 mg l^{-1}) + sucrose (3.0 %). They observed the differentiation of callus when they were sub cultured on MS + BAP (2.0 mg l^{-1}) + IAA (0.5 mg l^{-1}) + sucrose (3.0%).

Successful *in vitro* regeneration of plantlets in *A. indica* from inflorescences and cotyledons through direct and indirect somatic embryogenesis and organogenesis was obtained (Shrikhande *et al.*, 1993). Thiagarajan and Murali (1994) reported that MS medium supplemented with sucrose (12%) + 0.01 mg l^{-1} NAA + 0.1 mg l^{-1} BAP was considered as the best medium for the regeneration of neem seed embryo and this was ready for transplanting within 75 days.

Maximum shoot proliferation and plant formation was achieved when stem nodes and stem segments taken from mature trees of *A. indica* were cultured on MS medium augmented with 0.5 µm thidiazuron (TDZ) and 0.5 µm NAA (Yassen, 1994). The use of peroxidase enzymes as biochemical markers for the callus organogenesis in *A. indica*, especially during the early stages of differentiation was reported by Preetha *et al.* (1995).

Venkateswarlu *et al.* (1995) reported the bud induction and multiplication of neem shoots when the nodal explants of juvenile neem tree were cultured on MS medium.

A micropropagation protocol was developed for *A. indica* and *Acacia auriculiformis* using axillary bud as explants. Multiple shoots were obtained on MS medium supplemented with 0.1 mg l^{-1}BA. Rooting of shoots occurred best on ½ MS + 0.1 mg l^{-1} IBA (Anaz and Vijayakumar, 1996).

Gill and Gosal (1996) successfully regenerated shoots from nodal segments of coppice shoots of mature tress of *Eucalyptus tereticornis, Dalbergia sissoo* and *A. indica* on MS medium supplemented with various concentrations and combinations of auxins and cytokinins. They reported that multiple shoot production was highest on medium containing MS + BAP (0.5 mg l^{-1}) for *Eucalyptus tereticornis*, MS + BAP (1.0 mg l^{-1}) + NAA (1.0 mg l^{-1}) + activated charcoal (0.2%) for *Dalbergia sissoo*. The regenerated plants developed roots on ½ MS medium, and were successfully planted under field conditions.

Organogenesis of germinating seeds of *A. indica* was observed when maintained in MS medium supplemented with various growth substances (Rier and Obasi, 1996). Eeswara *et al.* (1997) reported that shoots of *A. indica* could be induced from leaf explants when cultured on MS medium containing BA 1.0 mg l^{-1}, Kin 0.8 mg l^{-1} and Ads (Adenine sulphate) 6.0 mg l^{-1}. In 32 weeks, 80 shoots were produced from single leaf of 10 x 10 mm. Multiple shoots up to six were produced when shoot tips and stem explants were cultured on WPM supplemented with Kin and BA each at 0.5 and 1.0 mg l^{-1}. Tawfik (1997) observed that petiole segments induced callus when cultured on WPM containing BA, Kn or TDZ each at 0.5 mg l^{-1}. Shoots were regenerated from callus in MS medium supplemented with BA or Kin at 0.5 or 1.0 mg l^{-1} and rooting of the regenerated shoots were obtained in MS medium containing 0.5 or 1.0 mg l^{-1} IBA.

Zypman *et al.* (1997) successfully regenerated shoots from the nodal explants obtained from neem plants. They also observed the induction of callus and development of somatic embryos when the hypocotyl explants were cultured on MS medium supplemented with 0.5 mg l^{-1} IAA and 1.0 mg l^{-1} BA. Highest percentage of multiple shoots were obtained when the explants collected from neem, were cultured

on MS + 0.5 mg l^{-1} BAP + 0.05 mg l^{-1} NAA. The shoots were rooted in ½ MS + 0.05 mg l^{-1} IBA and 0.05 mg l^{-1} IAA (Khatun *et al.*, 1998).

Venkateswarlu *et al.* (1998) standardized the micropropagation protocol for a mature plus tree of neem. They reported the bud induction and multiplication of shoot when nodal explants were cultured in MS medium with suitable concentrations of cytokinin. Elongated shoots induced rooting in MS + 2.5 mg l^{-1} IAA.

Murthy and Saxena (1998) successfully regenerated neem plantlets from somatic embryos initiated from mature seeds using MS supplemented with TDZ (thidiazuron). Ruiz *et al.* (1999) obtained Somatic organogenesis and embryogenesis in neem by mixing gibberllic acid with EA and malt extract to MS medium. Rooting of shoots were obtained by combining IAA at levels between 0.1 and 3.0 mg l^{-1}. Salvi *et al.* (2001) reported that leaf explants from neem produced the highest average number of shoots per explant. The regenerated shoots, rooted when subcultured on MS medium supplemented with 1.0 mg l^{-1} IBA.

2.1.1 Controlling factors in micropropagation

2.1.1.1 Nutrient medium for in vitro cultures

Different nutrient combinations for successful culture establishment have been standardised and are in use. Murashige and Skoog (MS) (1962), White's medium, Gamborg *et al.* (1968), Linsmaier and Skoog (LS) (1965) medium, Woody Plant medium (WPM) (Llyod and Mc Cown, 1980) and Nitsch medium (1951) are some commonly used media in plant tissue culture (Narayanaswamy, 1997 and Gupta, 1995). Among them Murashige and Skoog medium (MS) is the most popular one.

A medium specially designed for tree species is the WPM of Llyod and McCown (1980). Compared with MS it is low in ammonium nitrate, potassium, chloride and high in sulphate. After 1980, the most popular media have been DCR (Gupta and Durzan, 1985) and WPM (Lloyd and McCown, 1980) especially for woody species.

In *A. indica* application of cytokinins has been commonly found effective in promoting direct or indirect shoot initiation (George and Sherrington, 1984). Kin and BAP are the most commonly used cytokinins, while 2ip and zeatin are used less frequently (Thorpe and Patel, 1984). Evans *et al.* (1981) found that for 75 per cent of the species forming shoots, Kin/BAP was used in the concentration of 0.05 μM - 46 μM.

Success in employing these various media in all probability lies in the fact that the ratios as well as concentrations of nutrients nearly match the optimum requirement with regard to the growth and differentiation of respective cell or tissue systems (Razdan, 1993).

2.1.1.2.1 Growth regulators

Growth regulators are organic compounds (other than a nutrient) which in small amounts promote, inhibit or qualitatively modify growth and development (More, 1979). Proper selection and addition of growth regulator at an optimum level is one of the important factors for successful plant tissue culture (Krikorian *et al.,* 1981). Plant growth regulators (PGR's) include naturally occurring hormones such as IAA, GA, BA, ABA etc. and also a number of synthetic chemicals that affect or control growth and development in plants (Minocha, 1987). It is generally necessary to add one or more of these PGRs to support good growth of tissues and organs (Bhojwani and Razdan, 1983). Kallak *et al.* (1997) working with carnation reported that, the addition of growth regulators into the callus regeneration media modified the genotypic effect on shoot formation frequency, more or less decreasing the genotypic differences and the number of shoots formed. Skoog and Miller (1957) proposed the concept of hormonal control. Their classic experiments on tobacco pith cultures showed that root and bud initiation were conditioned by balance between auxin and cytokinin. High concentration of auxin promoted rooting where as propotionately more cytokinin initiated bud or shoot formation.

Cytokinins and adenine derivatives are frequently used for shoot production, proliferation and elongation. In combination with auxin they produce callus (Yousef, 1997). However, extents of effects of these growth regulators vary from species to species (Bon *et al.,* 1998). Singh and Mangia (1998) reported that multiple shoot formation did not occur without growth regulators in *Acacia tortilis*. In general, it appears that BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed by, decreasing order kinetin and 2-ip (Bhojwani, 1980; Hasegawa, 1980; Lundergan and Janick, 1980; Kitto and Young, 1981). Too high a concentration of auxin may not only inhibit axillary bud branching, but also induce callus formation, especially when 2,4-D is used (Hasegawa, 1980).

2.1.1.2.2 Carbon energy sources

All media require the presence of a carbon source, as the source of energy. The early studies (Gautheret, 1941; Gautheret, 1945; Hilderbrandt and Riker, 1949) had documented that a number of carbohydrate could support growth, but over the years it has become apparent that sucrose is generally the best carbon and energy source (Street, 1969; Thorpe, 1982; Bhojwani and Razdan, 1983). Carbohydrates not only function as a carbon source in metabolism, but they also play an important role in the regulation of the external osmotic potential (Brown and Thorpe, 1980). It has been found that sucrose was the only sugar necessary for bud induction in *Pinus contorta* (Von and Eriksson, 1981). Despite the widespread use of sucrose, this compound is not always the most effective carbohydrates for shoot initiation. Fructose and glucose were found to be the best source for mulberry bud culture (Oka and Ohyama, 1982).

The choice and concentration of the sugar to be used depends mainly on the plant tissue to be cultured and the purpose of the explant (Dodds and Robert, 1985). In *Alnus crimastogyne* type and concentration of sugar used in the multiplication medium were observed to be a critical factors for both multiple shoot induction and bud elongation (Tang *et al.*, 1996).

2.1.1.3 Vitamins

It is necessary to supplement the medium with required vitamins and amino acids to achieve the best growth of the tissue. Some consider that thiamine (vitamin B_1) may be only essential vitamin for nearly all plant tissue cultures, where as nicotinic acid (niacin) and pyridoxine (vitamin B_6) may stimulate growth (Gamborg *et al.*, 1976). Some other vitamins that have been employed in tissue culture media include p-amino-benzoic acid (PABA; vitamin B_x), ascorbic acid (vitamin C), biotin (vitamin H), cyanocobalamine (vitamin B_{12}), folic acid (vitamin B_c), Calcium pantothenate and riboflavin (Huang and Murashige, 1977; Gamborg and Shyluk, 1981).

Linsmaier and Skoog (1965) demonstrated that most vitamins were not essential for callus growth in tobacco. Pyridoxine, biotin and nicotinic acid could be deleted from medium without serious impact on growth. Ascorbic acid which may be employed with other organic acids, is useful as an antioxidant to alleviate tissue browning (Reynold and Murashige, 1979).

2.1.1.4 Explant size and its position on the mother plant

The type of the explant varies with each plant species and the most suitable one should be determined for each species (Skirvin, 1980). Hussey (1983) quoted that, as a rule, larger the size of the explant more rapid is the growth rate and greater are the rates of survival. However, larger the explant size more will be the chance of harbouring contaminant microorganisms. In contrast, when eradication of viral infection is one of the culture objective, small explant should be used. When shoot tip explants of *Dianthus caryophyllus* were less than 2.0 mm only roots were induced. If explants measuring 7.5 mm long roots were used, virus could not be removed. So the optimum explants are 2-5 mm in length (Bhan, 1998). Pseudoterminal buds of *Betula uber*, approximate 5 mm long, opened after 4 to 5 days of culturing and produced 3 leaves in a week, whereas 3-4 mm axillary bud took 10-20 days to increase in the same size (Vijayakumar *et al.*, 1990).

The *in vitro* response may also vary with the position of the explant of the parent plant (Dhawan, 1993). Culture of buds taken from stem part located close to tip, yielded more callus than shoot whereas axillary buds at distant position from apical buds yielded more shoots (Periera *et al.*, 1995).

2.1.1.5 Age of explant

Explants are the potential source for *in vitro* cultures. Different explants have been reported to give stable cultures. Meristematic tissues generally have a high degree of morphogenic competence than older tissues. However, the ability of juvenile explants from hard-to-root species to multiply in cultures suggests that it should be possible to *in vitro* propagate their elite tress if they can be induced to develop juvenile shoots by suckering, coppicing or hormone treatment. To some degree rejuvenation of shoots also occurs after serial sub cultures (Zimmerman and Broone, 1981). However, *Betula tatewakiana* (Ide, 1995; Jones *et al.*, 1996). *Fagus sylvatica* (Meier and Reuther, 1994), *Fraxinus excelsa* L. (Hammatt, 1992), *Dalbergia sissoo* (Chauhan *et al.*, 1994), *Tectona grandis* (Gupta *et al.*, 1980; Devi *et al.*, 1994), *Azadirachta indica* (Eeswara *et al.*, 1997) etc. have been cloned *in vitro* successfully taking nodal and terminal bud explants from mature trees. Suryanarayanan and Pai (1998) observed that among various explants, florets, stem and shoot tips, florets yielded less callus than others in *Coleus forshohlii.*

2.1.1.6 Season of collecting the explant

The physiological state of the mother plant at the time of explant excision has a definite influence on the response of the buds. The physiological state of plant depends on season. The explants from actively growing shoots at the beginning of the growing season generally give best results (Anderson, 1980). The nodal explants harvested during March-April and August-September-October were found to be the best for establishment of cultures of *Capparis decidua* (Deora and Shekhawat, 1995). Yu (1991) observed that the test material taken after 10 continuous rainy days had a contamination rate of cent per cent and that taken after 15 continuous sunny days had a

low contamination rate of 20 per cent in *Litchi chinensis*. Meier and Reuther (1994) reported that February was the most beneficial month for the collection of explants having dormant buds since infection with endogenous bacteria was still low and *in vitro* growth of the plant material was the highest.

2.1.1.7 Genotype

Successful application of *in vitro* technology to the production of clone depends upon inducibility of growth and differentiation in tissues of woody plants and the regeneration of true to the type viable plants in selected genotypes. Great differences exist in organogenesis, embryogenesis and regeneration of plantlets among plant species, varieties and even individuals of the same varieties (Ahuja, 1983). McComb and Bennet (1982) observed that there was large difference in the capacity of explants from different selections of mature *Eucalyptus marginata* tree to survive in culture. Specific effect of genotype has been reported for *Sequoia sempervirens* (Sul and Korban, 1994). Explants from thornless tress of *Prosopis cineraria* produced 6-8 shoots per explant on MS medium containing 0.1 mg l^{-1} IAA, 5.0 mg l^{-1} BA and additives (Shekhawat *et al.*, 1993). Kallak *et al.* (1997) observed differences in shoot and root regeneration efficiency of carnation calli depending on genotype. The calli from different cultivars revealed significant differences in growth, colour and structure.

2.1.1.8 Surface sterilization

Surface of plant parts may harbour a wide range of microbial contaminants which when inoculated to a nutrient medium, contaminate the entire *in vitro* system. To avoid this, the tissue must be thoroughly surface sterilised before planting it on the nutrient medium. To disinfect the plant tissues various surface sterilizing agents have been used at varying durations of time. Kannan and Jasrai (1998) reported that washing of explants in ethanol (90% v/v) followed by soaking in mercuric chloride 0.1 per cent for one minute gave contamination free cultures in *Vitex negundo*. Srividya *et al.* (1998) reported that treating explants of *Azadirachta indica* in 70 per cent

ethanol for 30 seconds followed by treatment with 0.1 per cent mercuric chloride for 8 minutes gave contamination free cultures.

2.1.1.9 Systemic contaminants

Leifert and Woodward (1998) highlighted that surface sterilization is often inefficient. This problem may be due to the disinfectant being inactive or microorganisms being protected within the plant tissue used as the explant. Thus culture contamination control is extremely difficult and with many contaminants, impossible to control.

Use of various fungicides (Shields *et al.*, 1984) and antibiotics (Dodds and Roberts, 1985) in the culture medium to reduce systemic fungal and bacterial contamination respectively, has been suggested. Mallika *et al.* (1992) advocated growing stock plants under controlled conditions with regular sprayings using systemic and contact fungicides in order to avoid problem of contamination to some extent. Dodds and Roberts (1985) observed that it is preferable to avoid the use of antibiotics for sterilization because they or their degradation product may be metabolized by plant tissue with unpredictable result.

2.1.1.10 Culture environment

Physical conditions such as pH of the medium, light, temperature and relative humidity, season of culturing etc. have been found to have a profound influence on the rate of growth and extent of differentiation exhibited by the cultured cells.

Changes in the physical condition of the medium is brought about by changing the concentration of the gelling agent in the medium. Importance of physical conditions on culture is evident, in case of *Dalbergia latifolia* cultures (Mahato, 1992). At higher pH conditions precipitation of the nutrient results. pH also affects nutrient and hormone uptake. Bonga (1982) remarked that pH of the medium is usually set at

about 5.0 for liquid cultures and 5.8 for semi solid cultures. In plant cell media the pH is generally adjusted to 5.7-5.8.

In neem the optimum temperature of 28 ± 2° C with a light intensity of 3000 lux was found suitable for the growth of culture (Srividya *et al.,* 1998). Relative humidity is rarely a problem except in climates, where rapid drying occurs. The humidity of air is rarely controlled and when controlled, 70 per cent has been found to be the most frequent setting (Hu and Wang, 1983).

2.1.1.11 Rooting of in vitro produced shoots

In vitro produced shoots can be successfully rooted through *in vitro* methods. A low salt medium is found satisfactory for rooting of shoots in a large number of plant species. Often where shoot multiplication was induced on full-strength MS medium, the salt concentration was reduced to half (Garland and Scoltz, 1981; Zimmerman and Broone, 1981) or a quarter (Skirvin and Chu, 1979) for rooting. It is the endogenous auxin : cytokinin balance that is the key factor in the initiation of the rooting (Thorpe, 1980).

Generally, auxin favours root formation. Among the auxins, NAA has been the most effective one for induction of rooting (Ancora *et al.,* 1981). Sometimes a combination of auxins may give a better response (Gupta *et al.*, 1980). Drew (1993) reported that longer exposure to IBA reduced rooting percentage in *Azadirachta indica*, as they took longer time to initiate roots and produced excess callus at the base of the explant. Venkateswarlu *et al.* (1998) obtained rooting in *Azadirachta indica* on full strength MS with 3.0 mg l^{-1} IAA.

2.1.1.12 Hardening and planting out

Tissue culture plantlets are tender and their transfer from the artificial environment of the culture vessel to the self dependent green house or similar environment makes their existence and establishment tough. The plantlets formed in

the culture are deficient in photosynthetic efficiency and mechanism to control water loss. The humidity inside the culture vessel is very high (close to 95%) thus the plants lack the protective cuticle. Success in acclimatization depends upon not only post transfer condition but the pre-transfer culture conditions also (Ziv, 1986).

Joarder *et al.* (1993) transferred rooted planlets of *A. indica* into the mixture of garden soil and compost (1:1) and were covered with glass beaker for 7–10 days there after they were transferred to sandy soil. Similar reports were quoted by Venkateswarlu *et al.* (1998) when the plantlets of *A. indica* were transferred to soil + vermiculite mix and grown under 90 per cent humidity.

2.2 UTILISING *IN VITRO* CULTURES FOR SECONDARY PRODUCT SYNTHESIS

Plant secondary products are compounds present in plants which are believed to have no role in the basic life process but have secondary non essential roles. They are characterized by their extreme chemical diversity. With the observation that plant cells can be cultured *in vitro*, strategies have been developed for improving the productivity of plant cell cultures. Presently there has been considerable interest in investigating the potential of plant cell cultures as an alternative to traditional agriculture for industrial production of secondary metabolites (Fowler, 1980). Many recent reviews emphasize the biotechnological potential of plant cell cultures (Misawa *et al.*, 1985).

Presented below is a comprehensive review on the biosynthetic potential of cell cultures with major emphasis on medicinal plants, the methodology adopted for *in vitro* synthesis of secondary products and their impact in pharmacy.

Production of shikonin from *Lithospermum erythrorhizon* cultures was the first successful case of *in vitro* production of any secondary metabolite on commercial scale. This was a remarkable break through, as conventionally the plant roots took 3-4 years to grow and then yielded 1-2 per cent shikonin, while the cultures yielded 15-20 per cent shikonin in just 23 days (Fujitha, 1988).

High metabolite production was also observed from *Coptis* for berberine (Sato and Yamada, 1984) and Coleus for rosmarinic acid (Ulbrich *et al.*, 1985).

Bhalsing and Maheswari (1998) have listed the plants which give yield of secondary metabolites in *in vitro* cultures (Table 1).

Table 1. *In vitro* yields of certain secondary metabolites

Secondary metabolites	Source plant	Yield (% of dry weight)
Alkaloids		
Benzophenanthridine alkaloids	*Escholtzia californica*	1.70
Protoberberine alkaloids	*Berberis stolonifera*	10.00
Berberine	*Coptis japonica*	8.20
	Thalictrum minnus	12.10
Steriods and Terpenoids		
Diosgenin	*Dioscorea deltoidea*	7.80
Sterols	*Delphinium ajacis*	8.10
Solasodine	*Solanum khasianum*	2.07
Ferruginol	*Solvia miltiorrhiza*	1.30
Quinones		
Shikonin	*Lithospermum*	12.40
Anthraquinones	*erythrorhizon*	27.00
Napthaquinones	*Galium spp.*	12.30
	Echium lycopsis	

(Bhalsing and Maheswari, 1998)

Sanyal *et al.* (1981) detected nimbin through TLC from the differentiating callus when the young stem bark of *A. indica* was cultured in MS + 0.5 mg l^{-1} IBA. Sanyal *et al.* (1988) reported that addition of glycine to the culture media increased the biosynthesis of nimbin and decreased β- sitosterol.

Huang and Morgan (1990) detected azadirachtin from the crude extract of neem seeds using supercriticalfluid chromatography, with UV absorption at 210-220 nm and using CO_2 – methanol as mobile phase. They reported that the least detectable amount of azadirachtin was 10 ng at 212 nm.

Azadirachtin content in neem extracts or in commercially available neem – base pesticides can be estimated by HPLC (Sundaram and Curry, 1993; Azam *et al.*, 1995; Yamaski *et al.*, 1986). A protocol had been developed to analyse the azadirachtin in neem seeds using reversed – phase HPLC. It was observed that the azadirachtin content in seed appeared only after ninth week and gradually reached the maximum of 0.38 ± 0.06 per cent (w/w, on a dry weight basis) around 17^{th} week and decreased to 0.29 ± 0.03 per cent (w/w) by 19^{th} week. However, storage of neem seeds reduced the azadirachtin content to 68 per cent of the original level in a period of 4 months in the dark and to 55 per cent in day light (Yakkundi *et al.*, 1995).

Seasonal variation on azadirachtin content in neem seeds was reported by Sidhu and Behl (1996). It was observed that the seeds produced in monsoon yielded higher (1.54%) azadirachtin as compared to the winter season seeds (1.26%).

The principal compound for which extensive structural and biological studies have been carried out is azadirachtin A (Rembold, 1989). Rembold isolated six related compounds (azadirachtins B-G) while Govindachari *et al.* (1992) reported five compounds (azadirachtin A, B, D, H and E) from neem kernels. Presently, nine azadirachtins have been isolated from neem seed extracts (Rembold and Puhlmann, 1995). Isolation and purification of azadirachtin have been perfected by flash chromatography, normal-phase and reverse – phase preparative and analytical HPLC (Govindachari *et al.*, 1992).

David and Dayanandan (1997) observed the formation of callus, when cotyledons obtained from neem seeds were cultured on MS medium supplemented with different combinations of growth regulators. They observed, the differentiation of secretory cells or terpenoid storage cells at the cut ends of the hypocotyl segments.

Azadirachtin content in bark, stem, root and leaf explants were estimated and it was reported to have 0.40, 0.50, 0.57 and 0.63 mg l^{-1} dry weight respectively (Fu *et al.*, 1998). They also observed that best callus growth rate was observed in low ammonium salt B_5 medium and high azadirachtin content in callus grown in MS medium.

Sanker (1998) reported significantly higher yield of ephedrine from *in vitro* cultures (0.02%) than from field grown plants (0.008%) of *Sida spp.*

Srividya *et al.* (1998) reported that callus was developed with 100 per cent response from immature embryos of *A. indica*, when cultured on MS medium supplemented with 2.0 mg l^{-1} NAA + 0.5 mg l^{-1} BA, 2.0 mg l^{-1}BA and 1.0 mg l^{-1} IAA + 0.5 mg l^{-1}BA. Maximum multiple shoots occurred on medium containing 2.0 mg l^{-1} BA and 0.5 mg l^{-1} BA + 0.1 mg l^{-1} NAA. Rooting of shoots was observed in ½ MS + 0.5 mg l^{-1} IBA. Based on HPLC analysis the azadirachtin content in *in vitro* roots and shoots were reported, which had 0.004 mg l^{-1} and 0.008 mg l^{-1} azadirachtin and 0.003 mg l^{-1} and of nimbin respectively. Callus had negligible amount of azadirachtin.

Azadirachtin content of callus cultures varied depending on the cell line, the nutrient medium and the carbohydrate source employed (Wewetzer, 1998). She reported that the azadirachtin content was three times higher on media supplemented with 15 g l^{-1} sucrose (<0.5-64 µg g^{-1} DW) as compared with those with 30 g l^{-1} sucrose (<0.5-22 µg g^{-1} DW). However, the highest concentrations were detected in completely undifferentiated cells. Kuruvilla *et al.* (1999) reported that 10 mg l^{-1} of azadirachtin was secreted from callus cultures of *A. indica* when Triton X - 100 (at 150 ppm) was used as a permiabilizing agent.

A colorimetric method was developed for the determination of total azadirachtin-related limonoids (AZRL) in neem seed kernel extracts (Dai *et al.*, 1999). The method employed acidified vanillin solution in methanol for the colorization of the standard azadirachtin or NSKE in dichloromethane, and the absorbance at 577 nm was found to be optimum.

The azadirachtin content in the seeds of micropropagated neem plants (0.82 ± 0.03%) was more or less similar to its mother tree (0.85%) (Venkateswarlu and Mukhopadhaya, 1999). Jarvis and Morgan (2000) developed a method, which is particularly useful for the small samples available from tissue culture explants and is equally applicable for the analysis of seed extracts. They quantified the azadirachtin following the solid phase extraction procedures and the individual compounds was accurately quantified using supercritical fluid chromatography or HPLC.

Lokanatha *et al.* (2000) quantified the azadirachtin content in neem kernels using HPLC from different agro climatic zones of Andhra Pradesh and reported as 0.070 to 0.5213 per gram dry weight of kernel.

Dai *et al.* (2001) developed two- component and multivariate techniques for the quantification of total aza-related limonoids (AZRL) and simple terpenoids in neem extracts using vanillin assay. They reported that the relative content of limonoids was much higher than that of the terpenoids in all parts of the neem plant.

2.2.1 Factors regulating synthesis of secondary products *in vitro*

2.2.1.1 Carbon energy source

Dougall (1980) reported that with increase in concentrations of sucrose from 1 to 5 per cent, the yield of shikonin increased in *Lithospermum erythrorhizon.* Wijnsma *et al.* (1986) reported that the anthraquinone yield was maximum at 8 per cent sucrose in Gamborg B_5 medium for *Cinchona ledgeriana.* In *Datura stramonium,* highest hyoscyamine production (upto 7.4 mg l^{-1} day^{-1}) was recorded in root cultures on full strength B_5 medium containing 5 per cent sucrose (Hilton and Rhodes, 1993).

Asaka *et al.* (1994) reported that ginseng embryoids produced maximum amount of saponins when raised in media containing 30 g sucrose and 30 g glucose l^{-1}. According to Jeon *et al.* (1995) high yield of Ginkgolid B was obtained in MS medium supplemented with 30 g sucrose in *Ginkgo biloba.* The azadirachtin content of callus cultures was three times higher on MS media supplemented with 15 g l^{-1} sucrose as compared with those of 30 g l^{-1} sucrose (Wewetzer, 1998).

2.2.1.2 Hormonal regime

Shrivastava and Padhya (1995) reported that increase in the IAA level from 0.5 to 1.0 and 2 µM l^{-1} in the medium, reduced the number of roots produced as well as alkaloid accumulation (0.05% to 0.15%) in the regenerated roots of *Boerhaavia diffusa.* It has been found that production of anthraquinones was inhibited by addition

of 2, 4-D, whereas NAA induced its production in *Morinda citrifolia* cultures in B_5 medium (Vander *et al.*, 1995). Content of Colchicine was favourably attained in cultures of *Gloriosa* containing 0.2 mg l^{-1} Kin and 0.5 mg l^{-1} 2, 4-D (Ramamurthy and Reddy, 1997).

In *Sida spp.* NAA and Kin at 1.0 mg l^{-1} each, resulted in high ephedrine yield whereas incorporation of 2, 4-D resulted in complete inhibition of alkaloid production (Sanker, 1998). Sindhu (1999) observed that MS medium at half strength with phosphate ions reduced to 25 per cent supplemented with IAA 2.0 mg l^{-1} and BA 1.0 mg l^{-1} gave high callus index for immature fruit explants of *Coscinium fenestratum.*

2.2.1.3 Temperature

The release of benzyl isoquinoline alkaloid, berberine from cultured cells of *Thalictrum minus* into the medium proved to be temperature dependent showing a marked decrease at low temperature. At 5 °C the release was only 37 per cent of that at 25 °C during a period of 12 hours incubation (Yamamoto *et al.*, 1987). Levels of alkaloid accumulation of hairy root cultures of *Catharanthus roseus* showed a clear increase with lowering of temperature (Toivonen *et al.*, 1992).

2.2.1.4 Photoperiod

Yamada and Sato (1981) found that tissue culture of *Coptis japonica* in a solid medium followed by successive liquid cultures produced friable cell lines with high berberine content and that light inhibited berberine production in cell lines. Treating callus cultures of *Datura* with amino acids and incubating them in dark resulted in alkaloid production (Dattagupta and Datta, 1984). Kim *et al.* (1988) found that maximum dry cell weight was 20.4 g l^{-1} with continuous illumination compared to 17.9 g l^{-1} without light. Sanker (1998) and Sindhu (1999) observed higher percentage of callusing under illumination in *Sida spp.* and *Coscinium fenestratum* respectively.

2.2.2 Morphological differentiation

Artemisin, an antimalarial drug isolated from *Artemisia annua* was not accumulated in undifferentiated *in vitro* cultures, possibly because the expression of secondary metabolism was closely correlated with morphological differentiation (Paniego *et al.*, 1995).

Punarnavine profile of regenerated roots of *Boerhaavia diffuse* was studied by Shrivastava and Padhya (1995). In the presence of 2,4-D, leaf segments produced callus which regenerated roots that contained trace amounts of punarnavine. Callus obtained from cotyledonary leaves differentiated to shoots when adenine was added to the culture (Purohit *et al.*, 1997). Amount of xanthotoxin in such regenerating cultures was more than that in undifferentiated callus. Jaggi and Singh (2001) observed that differentiated leaf calli have higher potential for producing solasodine (0.101% dry weight) in *Solanum platanifolium.*

Successful reports on *in vitro* propagation of neem and extraction of azadirachtin from seeds, callus and *in vitro* grown plants has been reported by several workers. It is well known that plants produce and accumulate endogenous chemicals that function in many different ways. Most of these natural products, called 'secondary metabolites', are restricted in their distribution to specific tissues or organs of the plants. Tremendous efforts have been directed to increase the production of these metabolites, either by improved plant type or through *in vitro* techniques. Hence it has been found that plant tissue culture offers fascinating solutions to either of the approaches.

Materials and Methods

3. MATERIALS AND METHODS

The present investigation was carried out at the Plant Tissue Culture Laboratory, College of Forestry, and the Biochemistry Laboratory, College of Horticulture, Kerala Agricultural University (KAU), Vellanikkara, Thrissur, from 2000 to 2002. Details regarding the experimental materials used and methodology adopted for various experiments under the study are presented here.

3.1 MICROPROPAGATION

Axillary buds from 12-15 year old neem trees, maintained in the KAU campus were used as explants.

3.1.1 Collection and preparation of explants

Stem segments with 3-4 nodes were excised from mother plant with the help of secateurs. The stem cuttings were brought to the laboratory defoliated and converted into nodal segment of different dimension (1.0-2.5 cm). The segments were then washed under running water for 2-3 minutes. After this, they were washed thoroughly in a frothing solution of detergent (Teepol 0.01%) to remove all extraneous materials adhered to the segments. The explants were dipped in a mixture of the systemic fungicide, Bavistin 50 per cent W.P. (Carbendazim) and contact fungicide, Indofil M-45 (Mancozeb) each at 0.1 per cent for 30 min. The material was later washed under running water and taken for surface sterilization.

3.1.2 Surface sterilization of explants

The process of surface sterilization was carried out in a "Klenzaids" laminar airflow cabinet, which was made sterile by the incessant exposure of germicidal U.V. rays for half an hour before use. The pretreated explants were taken in sterilized conical flask and immersed in the solution of mercuric chloride of different concentrations (0.10 and 0.20 %) and for different duration (6, 8, 10 and 15 minutes).

A drop of teepol was added to the mercuric chloride as a wetting agent. The liquid was stirred by swirling to give proper contact of the chemical to the explant. The explants were washed five times with sterile water after they were taken out of the mercuric chloride solution. After thorough wash with sterile water the explants were spread on the pre-sterilized petridishes lined with blotting paper for drying. Explant free from adhering water were trimmed at edges to remove all drying-off tissues.

3.1.3 Culture media used

3.1.3.1 Composition of culture medium

The explants were cultured on agar solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), and Woody Plant Medium (WPM) (Llyod and McCown, 1980). In certain experiments the basal medium used was ½ MS. The chemical compositions of the different media are given in Table 2.

Different plant growth regulators were added to the basal MS medium. The stock solutions of growth regulators at 25 mg / 50 ml were prepared and stored under refrigeration and aliquots were taken from them for use after dilution. These aliquots were added to the medium, before the pH was adjusted.

The growth regulators used were auxins (2,4–D, NAA, IAA and IBA) and cytokinins (BA and Kinetin).

3.1.3.2 Preparation of culture medium

Standard procedure as given by Gamborg and Shyluk (1981) was followed for the preparation of media. The chemicals used were of analytical grade from SISCO Research Laboratories (S.R.L), Merck and Sigma. Stock solutions of major and minor nutrients were prepared by dissolving required quantity of the chemicals in distilled water and were stored in amber coloured bottles under refrigerated conditions. The stock solutions of nutrients were prepared freshly every four weeks and that of vitamin and growth regulators once every week.

Table 2. Chemical composition of Murashige and Skoog (MS) medium and Woody Plant Medium (WPM)

Compound	Quantity ($mg\ l^{-1}$)	
	Murashige and Skoog (MS)	Woody Plant Medium (WPM)
Inorganic		
Ammonium nitrate	1650.00	400.00
Boric acid	6.20	6.20
Calcium chloride-2-hydrate	440.00	96.00
Calcium nitrate-4-hydrate	0.00	556.00
Cobalt chloride-6-hydrate	0.025	0.00
Copper sulphate-5-hydrate	0.025	0.025
Ferrous sulphate-6-hydrate	27.80	27.80
Manganese sulphate-1-hydrate	22.30	22.30
Manganese sulphate-7-hydrate	370.00	370.00
Na_2 EDTA-2-hydrate	37.30	37.30
Potassium dihydrate phosphate	170.00	170.00
Potassium iodide	0.83	0.00
Potassium nitrate	190.00	0.00
Potassium sulphate	0.00	990.00
Sodium molybdate-2-hydrate	3.25	0.25
Zinc sulphate-7-hydrate	0.60	8.60
Organic		
Inositol	100.00	100.00
Nicotinic acid	0.50	0.50
Thiamine HCl	0.10	0.10
Pyridoxine HCl	0.50	0.10
Glycine	2.00	2.00
Others		
Sucrose (in per cent w/v)	3.00	2.00
Agar (in per cent w/v)	0.70	0.70

½ MS denotes half amounts of the inorganic constituents, full amount of organic and other constituents per liter of MS medium.

Stock solutions in the required quantity were pipetted out into a steel vessel containing distilled water. Inositol (100 mg l^{-1}) and sugar (30 g l^{-1}) were then dissolved in the solution in appropriate concentrations. The whole solution was made upto the required volume. The pH of the solution was adjusted to the range 5.7 – 5.8 (using 1 N NaOH or 1 N HCl) with the help of pH meter. Weighed quantity of agar (7 to 8 g l^{-1}) was added to this and mixed well.

The solution was then heated on a gas burner or in a microwave oven to dissolve the agar. The hot media (approximately 20 ml / tube) was poured into the well washed, oven dried culture tubes (150 mm x 25 mm). The culture tubes were then plugged tightly with non-absorbent cotton wool plugs.

3.1.3.3 Sterilization of culture medium

Sterilization of the culture medium was carried out in an autoclave at pressure of 1.06 Kg cm^{-2} for 15 minutes at 121° C (Dodds and Roberts, 1985).

After sterilization, the culture tubes were stored in an air-conditioned culture room until further use.

3.1.3 Sterilization of equipment

All metal and glass instruments and other accessories used in the laminar air flow cabinet were wrapped in aluminium foil and sterilized in an autoclave at 1.06 Kg cm^{-2} for 15-20 minutes at 121° C temperature. Scalpels, scissors, forceps etc. used were again dipped in alcohol and flamed on a spirit lamp at the time of use.

3.1.4 Inoculation of explants

The inoculation was carried out under strict aseptic condition inside the transfer cabinet. The floor of the work-bench, inside the chamber was wiped thoroughly with 100 per cent ethanol to remove any traces of dirt. Sterilized forceps, petridishes, surgical blades, knives and blotting papers were kept inside the chamber and the whole chamber was sterilized with UV light for 30 minutes.

The cotton plug of the culture tube was removed in front of the flame of a gas burner, kept inside the cabinet. The tube neck was thoroughly flamed. The sterile explant was quickly transferred into the medium with the help of a sterile forceps. One explant per test tube was placed horizontally on the surface of medium. The neck of the culture tube was again flamed and the cotton plug replaced. In order to curtail contamination during drying and inoculation only few explants were handled at a time.

3.1.5 Culture conditions

The cultures were incubated at 26 ± 2°C in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by fluorescent tubes. Humidity in the culture room varied between 60 and 80 per cent according to the climatic conditions prevailed. To find the effect of dark condition on callus induction, one treatment was maintained under dark condition.

3.1.6 Shoot induction and growth

For induction of shoots various treatments combinations (116) were tried. Details of the combinations are given in the Table 3.

3.1.8 Rooting of shoots:

3.1.8.1 In vitro rooting

The *in vitro* regenerated shoots were cultured in certain selected growth regulator combinations in full MS and half MS. The rooting treatments attempted are listed in Table 4.

3.1.9 Acclimatization

In vitro raised plantlets were carefully taken out of the test tubes without damaging their roots. The roots were washed gently under running tap water to remove the adhering medium. The roots were dipped in 0.1 per cent solution of Bavistin and Indofil M-45 to avoid fungal attack and plantlets were transferred into pots, containing different potting media such as sand, coco peat, vermiculite, soil rite, sand + vermiculite (1:1) and sand + vermiculite + coco peat (1:1:1). They were then

Table 3. Growth regulator combinations tried for bud break and shoot growth from axillary buds of *Azadirachta indica* in MS

Basal Media	Growth regulator combination in mg l^{-1}				
	BA	Kn	NAA	IAA	IBA
MS	0.5 1.0 1.5 2.0				
MS		0.5 1.0 1.5 2.0			
MS			0.5 1.0 1.5 2.0		
MS				0.5 1.0 1.5 2.0	
MS					0.5 1.0 1.5 2.0
MS	0.5 1.0 1.5 2.0		0.5 1.0 1.5 2.0		
MS	0.5 1.0 1.5 2.0			0.5 1.0 1.5 2.0	
MS	0.5 1.0 1.5 2.0				0.5 1.0 1.5 2.0
MS		0.5 1.0 1.5 2.0	0.5 1.0 1.5 2.0		
MS		0.5 1.0 1.5 2.0		0.5 1.0 1.5 2.0	
MS		0.5 1.0 1.5 2.0			0.5 1.0 1.5 2.0

Table 4. Media combinations tried for *in vitro* root induction from microshoots of *Azadirachta indica*

Basal Media	Auxins (mg l^{-1})			Cytokinin (mg l^{-1})
	NAA	IAA	IBA	Kinetin
½ MS and full MS	0.5 1.0 1.5	0.5 1.0 1.5	0.5 1.0 1.5	
½ MS		1.0		1.0

covered with polythene bags to maintain humidity. After one week, holes were made in polythene bags to reduce the humidity. Subsequently the plantlets were gradually exposed to natural milieu by keeping them open (without polythene cover) for few hours a day. Later the polythene covers were completely removed.

3.1.10 Observations

Each trial was carried out with minimum 25 tubes replicated three times. The observations were recorded for 2 months after inoculation. The data were calculated based on cultures that remained uncontaminated after the required period of incubation. The following observations were recorded from various experiments.

1. Number of cultures uncontaminated

This was recorded for different surface sterilization treatments. Numbers of cultures free from contamination were expressed as percentage of total number of cultures.

2. Number of explants showing bud break

Number of cultures showing bud initiations were expressed as percentage of total number of surviving cultures. A culture was said to have bud initiation when the dormant axillary bud has just emerged.

3. Time taken for bud break

The time taken (in days) for bud initiation was noted.

4. Number of explants showing leaf production

Number of cultures that produced leaves was expressed as percentage of total surviving culture that produced bud.

5. Time taken for leaf production

This was recorded in days.

6. Number of explants with shoot development

Number of explants with shoot development were expressed as percentage of total number of surviving cultures.

7. Number of shoots per culture

Average number of shoots per culture were expressed as average of the number of shoots produced in different replications.

8. Shoot length

This was expressed as average of all shoots produced in different replications.

9. Number of cultures rooted *in vitro*

Number of cultures that produced roots *in vitro* was expressed as percentage of total cultures in a particular combination tried.

10. Number of acclimatized plantlets

Number of acclimatized plantlets were expressed as a percentage of total plantlets kept for acclimatization.

3.2 CALLUS INDUCTION AND GROWTH

Nodal segments collected from the mature neem trees of 12–15 years old were used as explant. For induction of callus various treatment combinations (36) were tried. Details of the combinations are given in the Table 5.

Sub-culturing of calli was done every 4 weeks in fresh medium, having respective composition. Approximately one gram of callus was cub-cultured in each tube.

3.2.1 Observations on growth of callus

The observations taken for calli cultures were:

1) Percentage of cultures initiating calli

Of all the inoculated tubes, those which showed signs of callusing, were counted and were expressed as per cent total number of inoculated tubes.

Table 5. Growth regulator combinations tried for callus induction from nodal explants collected from *Azadirachta indica* in MS

Basal media	IAA (mg l^{-1})	IBA (mg l^{-1})	2,4- D (mg l^{-1})
MS	1.0 1.5 2.0		
MS		1.0 1.5 2.0	
MS			1.0 1.5 2.0
MS	1.0 1.5 2.0	1.0 1.5 2.0	
MS	1.0 1.5 2.0		1.0 1.5 2.0

2) Number of days for callus initiation

The time interval between the day of inoculation to the day when first visible signs of callus growth were seen, was counted as number of days for callus initiation.

3) Percentage of tubes producing full-tube callus

Of all the inoculated tubes, those which produced full or more callus, were counted and this was expressed as percentage of total number of inoculated tubes.

4) Number of days to produce full tube callus

The time taken for callus to grow and cover the complete surface of medium in test tube was recorded.

5) Callus index

Observations were recorded for callus induction, growth rate and morphology, after six weeks on culture tube. Callus index (CI) was worked out as below:

$$C.I. = P \times G$$

Where, P is the percentage of callus initiation and G is the growth score. Scoring was made based on the spread of the calli, as shown in Table 6 and Fig.1.

Table 6. Scale for scoring of callus growth in *A. indica*

Extent of callus growth at end of culturing period	Growth score (G)
No callus initiated	0
Very little callus	1
Callus covering about $1/4^{th}$ surface	2
Callus covering about 1/2 surface	3
Callus covering full tube	4

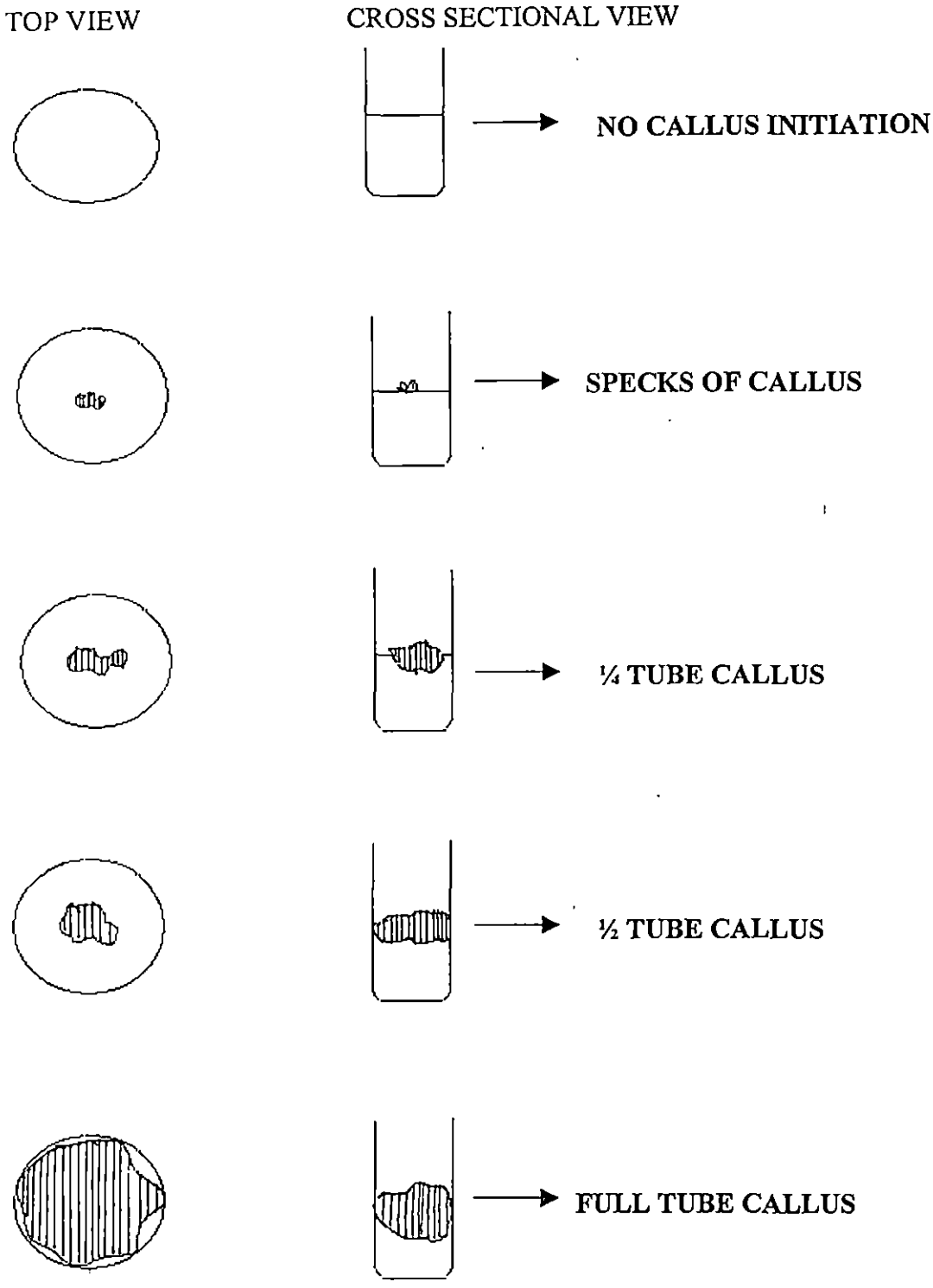


Fig. 1. DIFFERENTIAL GROWTH OF CALLUS OBSERVED FROM *IN VITRO* CULTURE OF NEEM

6) Callus morphology

The colour of the calli was recorded.

3. 3 EXTRACTION AND ESTIMATION OF AZADIRACHTIN

Total azadirachtin was extracted and estimated from shoot, root and calli grown *in vitro* using two different methods. These methods are described below.

3.3.1 Thin layer chromatography method

3.3.1.1 Preparation of the crude extract

Extraction method as suggested by Wewetzer (1998) was adopted with minor modification for preparation of the crude extract.

Freeze dried sample (0.5 g each) of shoot, root and callus were ground separately in a mortar and pestle, by slowly adding a mixture of equal quantity of petroleum ether and water. The final volume was made to 20 ml with the above solvent mixture. The ground sample were filtered and the filterates were centrifuged at 10,000 rpm for 10 minutes. The supernatants were evaporated to dryness by placing the samples in an exhaust chamber. The solvent free samples were re-dissolved in 5 ml methanol and stored at 4° C until analysis.

3.3.1.2 Preparation of TLC plates

Forty grams of silica gel G (Merck) was added to 85 ml of distilled water. Immediately, it was shaken vigorouly for 30 seconds to get a homogenous slurry which was poured in the trough of the TLC plate gel applicator of CAMAG brand. The glass plates of 20 x 20 cm size were passed one by one beneath the trough and each was coated with a 300 μm thick coat of silica gel G. Eight plates were coated with a slurry made from 40 grams of silica gel G. The plates were coated quickly, within 2 minutes, to avoid setting of the silica within the applicator itself. The plates were allowed to dry for about ten minutes, after which they were placed in aluminium racks and kept in chromatographic ovens for heating at 110°C for 30 min. This desiccated the plates and activated them for further use.

3.3.1.3 Spotting on TLC plates

Samples were spotted on activated TLC plates with calibrated capillary tubes. Usually, 2.5 and 5 µl volume were spotted from each sample. Spotting was done 2.0 cm above the lower edge of the plate in a straight line parallel to the lower edge. A distance of 1.5 cm was maintained between two consecutive spots. After spotting, the solvent was evaporated from the point of application with a hair drier.

3.3.1.4 Standardisation of running solvent systems

The composition of running solvent system is a very crucial factor in the resolution of azadirachtin spots, many different running solvent systems were tried with the following objectives:

a) To get a single condensed spot having all azadirachtin to aid quantification and
b) To fractionate the azadirachtin in maximum number of spots to get a profile for characterization.

The solvent systems tried are listed in Table 7. For evaluating the performance of these running solvent systems, samples were eluted in each of them and the chromatograms were compared.

Table 7. Running solvent systems tried for suitability to elute the azadirachtin from shoot, root and callus of *Azadirachta indica* on thin layer chromatograms.

Solvent system number	Running solvent systems	Ratio of solvent
1	Hexane : Ethyl Acetate	50 :50
2	Methanol : Water	50 : 50
3	Methanol : Water	60 : 40
4	Methanol : Water	40 : 60
5	Methanol : Water	30 : 70

Different running solvent systems were prepared by mixing solvents in desired ratios. About 100 ml of the solvent system was poured in the tank and lid was placed tightly. Adjacent to the walls of the tank were placed filter paper sheets. The tank was shaken once vigorously. It was retained as such for 30 min to saturate with the vapours of the solvent system. Then the spotted plate was placed in the tank such that the edge of the plate below the spots was immersed in the solvent system. The lid was closed tightly to avoid the loss of volatile solvents. All chromatograms were eluted between 29 to 30°C and at 72 to 75 per cent relative humidity, to maintain uniformity in the elution pattern. In approximately 30 minutes, the solvent eluted the spots vertically upto 2/3 rd length of the plate. Then the plate was removed for further processing.

3.3.1.5 Preparation of the spray reagent

To detect the azadirachtin on the TLC plates, one per cent vanillin in concentrated sulphuric acid was used as spray reagent.

3.3.1.6 Developing the chromatogram

The eluted plates were placed under an exhaust flow of air to evaporate the solvents. When the reverse side (uncoated) of the plate became dry, each gel was sprayed with a minimum of 10 ml of spray reagent using "Vensil" reagent sprayer. The eluted chromatogram was uniformly sprayed all over, with fine droplets of spray reagent. Care was taken to avoid use of excess force in spraying, as it would scrape out the silica gel coat, which would result in loss of a part of the chromatogram. The sprayed plate was kept at 110°C for 15 minute in a chromatographic oven to develop coloured spots. The plate was removed and observations were documented.

The standard azadirachtin gave a brown spot at 365 nm with appropriate solvent system. Colours of characteristic main zones were described, Rf values were calculated and compared with that of standard azadirachtin.

$$\text{Rf values} = \frac{\text{Distance travelled by the compound}}{\text{Distance traveled by the solvent}}$$

3.3.1.7 Screening of azadirachtin from in vitro cultures by TLC method

Azadirachtin was quantified using UV Spectrometer. The brown spot at 365 nm in the gel was scraped and dissolved in 10ml of methanol. It was centrifuged at 10,000 rpm for 10 minutes. The supernatant was taken in the UV spectrometer at 577 nm against the reagent blank. Azadirachtin was estimated by following the procedure as mentioned in colorimetry method.

The absorbance of azadirachtin from different extracts were recorded. The standard graph (Fig.2) was used for estimating the concentration of azadirachtin from samples of various treatments.

3.3.1.8 Observations on the azadirachtin production from the in vitro cultures

The following observations were recorded for extracts from shoot, root and callus cultures.

a) Rf values of azadirachtin spots

The Rf values of the coloured azadirachtin spots were recorded for each sample.

b) Colour of azadirachtin spots

The colour of the azadirachtin spots were recorded. The azadirachtin were identified by light brown, light green colurs.

c) Azadirachtin yields

From the TLC plates, azadirachtin were quantified and expressed as µg of azadirachtin per gm of shoot, root and callus.

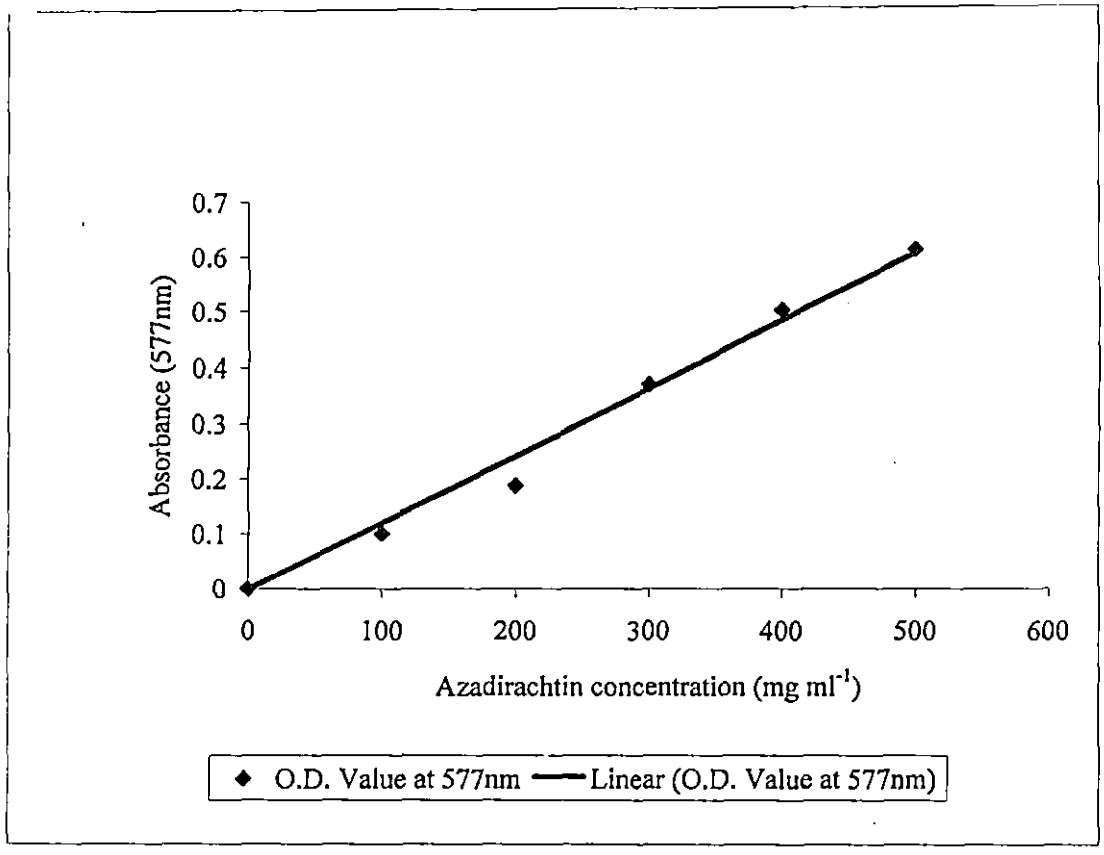


Fig. 2. Absorbance vs concentration (mg ml^{-1}) of standard azadirachtin solution

3. 3. 2 Colorimetric determination of total azadirachtin

3.3.2.1 Extraction of crude azadirachtin from in vitro cultures.

The shoot, root and calli grown *in vitro* were collected from test tube and all adhering pieces of nutrient media were removed. The cleaned shoot, root and calli were stored in the refrigerator at 3°C until analysis.

Extraction method as suggested by Dai *et al.* (1999) was used with minor modification for determination of the crude extract.

Sample (containing shoot, root and callus) of 0.5 g was taken separately in a mortar and pestle. To this methanol was added little by little and ground into a fine paste. The final volume was made up to 20 ml with methanol. The methanol extract was filtered and the filterate was taken in a beaker. To this 20 ml of hexane was added and shaken well. It was then transferred into a separating funnel. Two layers were formed, botton layer containing methanol was collected, and used for further analysis.

3.3.2.2 Preparation of colouring reagent

A mixture of methanol: concentrated H_2SO_4 (100:1) was used to prepare 5.00 per cent vanillin solution. This was used as a colouring reagent for the quantification of azadirachtin in the plant extract using colorimetric method.

3.3.2.3 Preparation of standard

Ten mg of standard azadirachtin (obtained from EID Parry India Ltd.) was dissolved in 100 ml methanol and stored in the refrigerator.

3.3.2.4 Determination of total azadirachtin

1) 0.1 ml of standard and 0.2 ml vanillin were taken, mixed well, and kept at room temperature for 2 minutes.

2) 0.3 ml concentrated H_2SO_4 was added and mixed thoroughly.
3) 0.7 ml of methanol was added and the volume was made upto 10 ml.
4) The solution was kept at room temperature for 5 minutes.
5) The light blue-green colour developed was measured at 577 nm using a UV spectrophotometer.
6) The blank was read by substituting the test solution with the same quantity of distilled water.

A standard curve of azadirachtin (Fig.2) was prepared using different concentrations of azadirachtin ranging from 100 to 600 mg^{-1} ml, which were read in the UV Spectrometer at 577 nm against the reagent blank.

The absorbance of azadirachtin from different extracts were recorded in a similar manner as described for the preparation of the standard curve. The standard graph was used for estimating the concentrartion of azadirachtin from various treatments.

Total Azadirachtin was calculated as follows:

Total Azadirachtin (μg^{-1} g)

$$= \frac{\text{Conc. of the std. azadirachtin}}{\text{OD value of the standard}} \times \frac{\text{OD sample value} \times \text{dilution factor}}{\text{Quantity of the sample taken (ml)} \times \text{weight of the sample}}$$

3.4 STATISTICAL ANALYSIS

Wherever necessary the data were statistically analysed by CRD (Snedecor and Cochron, 1967) with one factor or two factors as the case may be. Treatment means were compared using Duncan's Multiple Range Test (Duncan, 1955).

Results

4. RESULTS

The results of various experiments on micropragation of *Azadirachta indica* A. Juss, and the estimation of azadirachtin from the *in vitro* produced plantlets and callus in neem, conducted at the Tissue Culture Laboratory of College of Forestry and Biochemistry laboratory of College of Horticulture, Vellanikkara during 2000-2002 are presented in this chapter.

4.1 SURFACE STERILIZATION OF EXPLANTS

The nodal segments, which were used as the explants during the present study were subjected to various surface sterilization treatments to reduce the culture contamination. The results obtained are presented in Table 8. It was found that the treatment effects differed significantly. A 15 minute sterilization treatment in 0.10 per cent mercuric chloride preceded by fungicidal dip of 0.1 per cent Bavistin and 0.1 per cent Indofil for 30 minute to the explant was found to be significantly superior to all other treatments. The culture contamination was as high as 83.54 per cent after surface sterilization with 0.20 per cent $HgCl_2$ for 6 minutes.

It was observed that the size of explants had a pronounced effect on culture contamination. The percentage of contamination in different size of explants, treated with 0.10 per cent mercuric chloride for 15 min is presented in Table 9 and Fig.3. The contamination was found to be higher (79.22 %) with 1.0 cm long explants when compared to 2.0 cm long explants which recorded the least contamination (49.11%).

4.2 EFFECT OF DIFFERENT BASAL MEDIA ON CULTURE ESTABLISHMENT AND GROWTH IN AXILLARY BUDS.

Two basal media, namely, Murashige and Skoog (MS) and Woody Plant Medium (WPM) were used in the present study to evaluate their suitability for culture establishment in *A. indica*. The data is presented in Table 10 and Fig 4. The treatments differed significantly from each other. The percentage of bud initiation in MS (48.71 %) was found to be significantly superior to WPM (27.40 %).

Table 8. Effect of various surface sterilization treatments on culture contamination of *Azadirachta indica*

Duration of treatment	Percentage of contamination with different treatments			
	$HgCl_2$ (0.10)	$HgCl_2$(0.20)	Bavistin (0.1%) + Indofil (0.1%)	
			0.10 $HgCl_2$	0.20 $HgCl_2$
6	72.24 [a]	83.54 [a]	60.33 [a]	69.83 [a]
8	67.25 [b]	81.03 [a]	47.39 [b]	68.41 [a]
10	55.55 [c]	67.52 [b]	44.34 [bc]	60.09 [b]
15	50.67 [d]	65.19 [b]	40.69 [c]	66.39 [a]
SEm±	1.19	1.63	1.82	1.43
CD (0.05)	S	S	S	S

Figures with the same alphabet do not differ significantly.

Table 9. Effect of mercuric chloride (0.10%) on culture contamination of two different sizes of explants of *Azadirachta indica*

Size and characteristics of explants	Percentage of contamination
1.0 cm long	79.22 [a]
2.0 cm long	49.11 [b]
SEm±	4.56
CD (0.05)	S

Figures with the same alphabet do not differ significantly.

Table10. Effect of different basal medium on culture establishment and growth in axillary buds of *Azadirachta indica*

Basal media	Percentage of		Period (days)		Mean No. of leaves
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation	
WPM	27.40 [b]	11.33 [b]	7.70 [a]	18.65 [a]	2.67
MS	48.71 [a]	49.89 [a]	5.88 [b]	9.43 [b]	3.89
SEm±	5.37	3.21	0.37	0.59	0.69
CD (0.05)	S	S	S	S	NS

Figures with the same alphabet do not differ significantly.

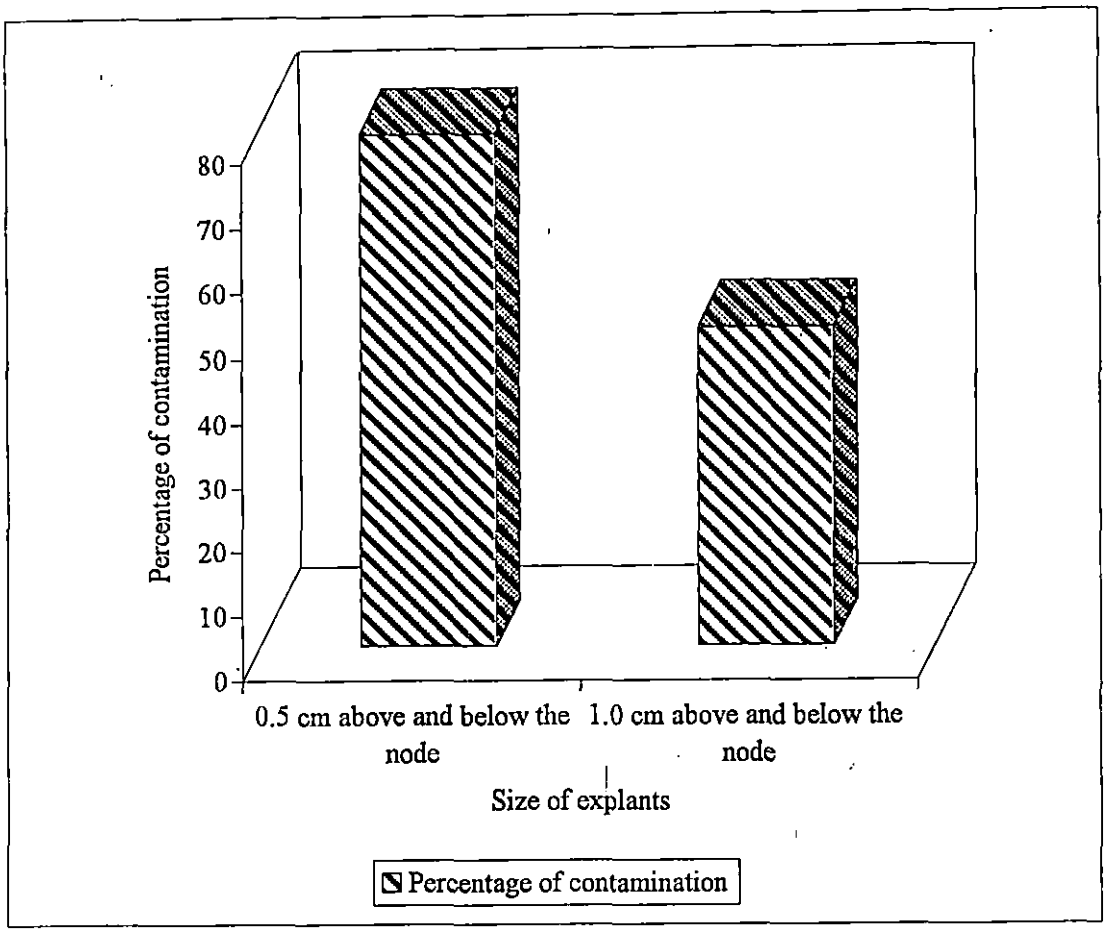
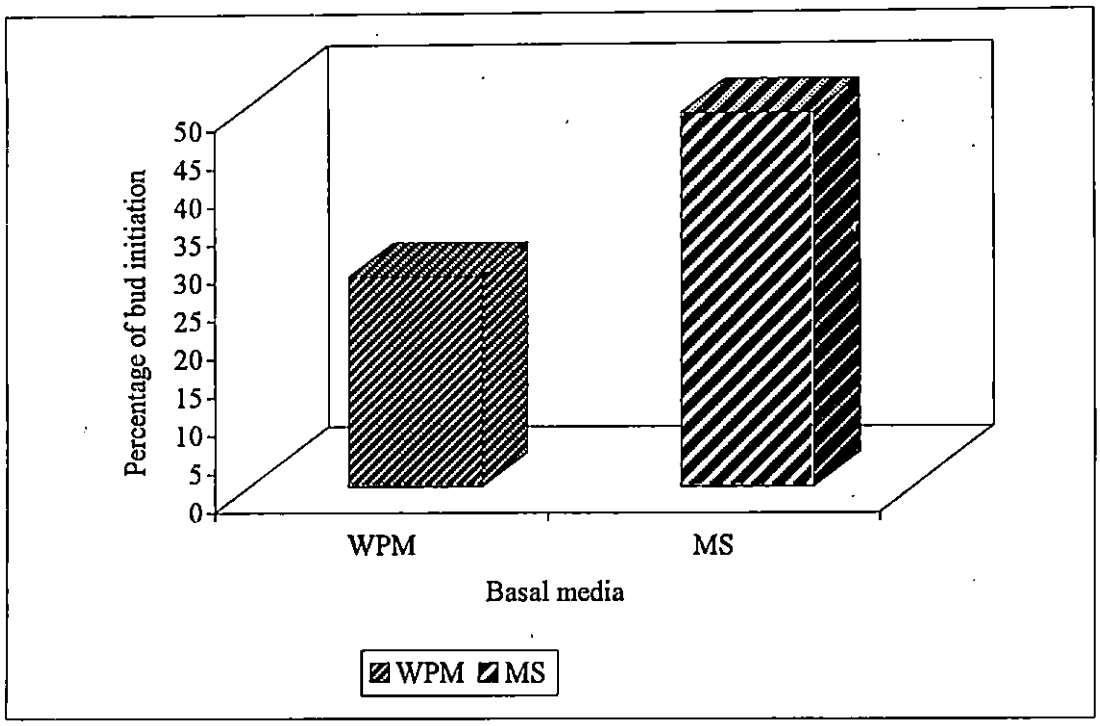


Fig. 3. Effect of mercuric chloride (0.01 %)on culture contamination of two different sizes of explants of *A. indica* in MS media



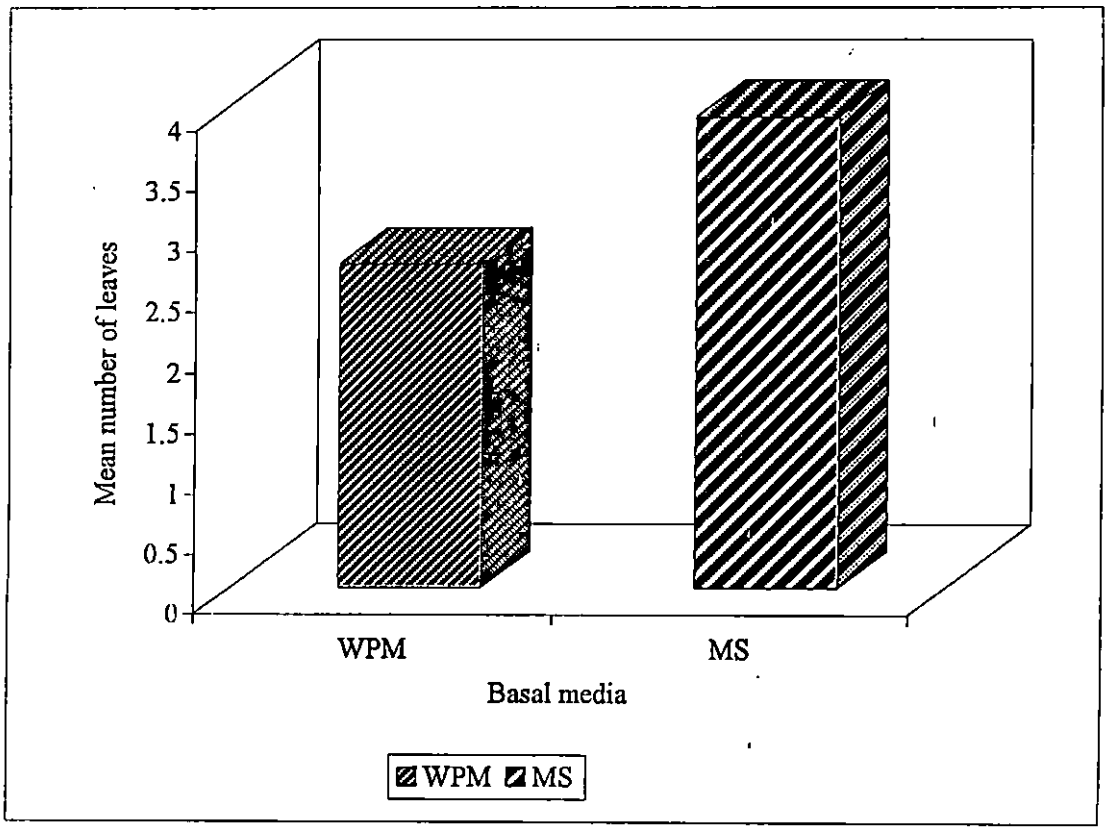


Fig. 4. Effect of different basal medium on culture establishment and growth in axillary buds of *A. indica*

Percentage of leaf initiation was also higher in MS (49.89) followed by WPM (11.33). The bud initiation was quite early in the explants cultured on MS (5.88 days) followed by WPM (7.70 days). Same trend was observed for leaf initiation, were the time taken for leaf initiation was early for the explants cultured on MS (9.43 days) and 18.65 days for WPM. Maximum mean number of leaves (3.89 leaves per explant) was recorded on MS, whereas it was 2.67 leaves per explant in case of WPM. After a comparative perusal of various growth parameters in two different media, MS media has been found to be superior to WPM, for tissue culture in neem (Plates 1, 2 and 3).

4.3 EFFECT OF GROWTH REGULATORS

MS medium was selected for further studies. This was supplemented with various cytokinins and auxins at different concentrations either separately or in combination to find out the best media combination for maximum culture response in *A. indica*.

4.3.1 Effect of different concentration of BA on culture establishment and growth in MS media

The MS medium was supplemented with four different concentrations of BA to study their effect on different growth parameters. The results obtained are presented in Table 11.

Percentage of bud initiation (89.00%) and leaf initiation (61.00%) was found to be maximum in media supplemented with 0.5 mg l^{-1} BA. However, in general bud and leaf initiation percentage was higher, when MS medium was supplemented with low concentrations of this growth regulator.

Treatment effect on the time taken for bud initiation was found nonsignificant. It was observed that with higher concentration of BA, explant took generally less time for bud initiation as compared to explants on media with low

Table 11. Effect of different concentration of BA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

BA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	89.00	61.00	9.53	14.34	2.45	1.47	2.28 [ab]	2.00
1.0	76.00	57.00	15.64	23.35	4.38	1.01	2.48 [ab]	2.70
1.5	78.67	57.67	11.63	16.83	3.15	1.27	1.78 [b]	3.00
2.0	84.00	59.33	9.02	21.00	2.69	1.46	2.84 [a]	2.00
SEm±	5.80	5.35	1.86	2.94	0.69	0.36	0.22	0.53
CD (0.05)	NS	NS	NS	NS	NS	NS	S	NS

Figures with the same alphabet do not differ significantly.

concentration of BA. Days taken for bud initiation were as high as 15.64 days when MS medium was supplemented with 1.0 mg l^{-1} BA and as low as 9.02 days in media with higher concentration of 2.0 mg l^{-1} BA. Leaf initiation was delayed upto 23.35 days at 1.0 mg l^{-1} BA followed by 21 days when MS medium was supplemented with 2.0 mg l^{-1} BA. With reference to leaves, the mean number of leaves per explant was more (4.38) in media contained BA at 1.0 mg l^{-1} BA, whereas at 0.5 mg l^{-1} BA it recorded 2.45 leaves per explant. Treatment effect on mean number of leaves was, however, nonsignificant.

The treatment effect on mean number of shoots was found to be nonsignificant. The media with 0.5 mg l^{-1} BA produced a mean number of 1.47 shoots per explant. Maximum shoot length (2.84cm) was observed in 2.0 mg l^{-1} BA which was significantly superior to MS + 1.5 mg l^{-1} BA (1.78 cm). Treatment effect on mean number of shoots was found to be nonsignificant. The media with 2.0 mg l^{-1} BA produced a maximum number of 3.0 shoots per explant and callusing at the base of the explant was observed, which resulted in the formation of globular structure after three subcultures with a growth period of 17 days each (Plate 4).

4.3.2 Effect of different concentration of Kn on culture establishment and growth in MS media

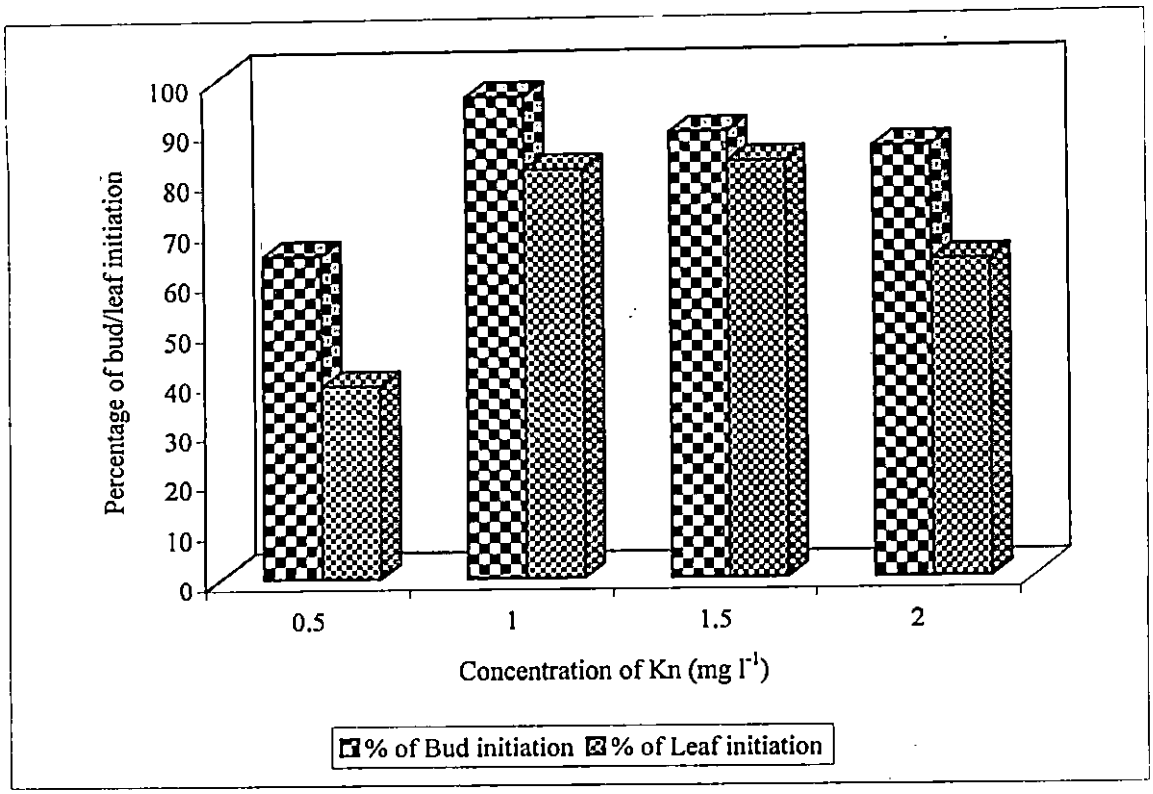
Four different levels of Kn were added to MS. Their effect on culture establishment and growth parameters are presented in Table 12 and Fig. 5. Higher percentage of bud initiation were recorded in media containing 1.0 mg l^{-1} Kn (96.00%), 1.5 mg l^{-1} Kn (88.67%) and 2.0 mg l^{-1} Kn (85.33%) which was statistically superior to media containing 0.5 mg l^{-1} Kn (64.67%). It was observed that media supplemented with 1.5 mg l^{-1} Kn produced highest percentage of leaf initiation, which was found significantly superior to media containing 0.5 mg l^{-1} Kn, which recorded 38.67 per cent.

Treatment effect on time taken for bud initiation and leaf initiation was found nonsignificant. It was observed that MS + 1.5 mg l^{-1} Kn was superior to MS +

Table 12. Effect of different concentration of Kn on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

Kn (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	64.67 [b]	38.67 [c]	11.64	20.45	4.28	1.28	3.49	2.00
1.0	96.00 [a]	81.33 [b]	12.40	21.14	3.28	0.55	3.90	1.00
1.5	88.67 [a]	82.67 [a]	10.84	15.69	3.57	1.09	3.55	3.00
2.0	85.33 [a]	63.00 [b]	13.23	23.94	3.19	0.85	3.99	1.00
SEm±	3.95	4.69	1.74	3.43	0.63	0.23	0.39	0.50
CD (0.05)	S	S	NS	NS	NS	NS	NS	NS

Figures with the same alphabet do not differ significantly.



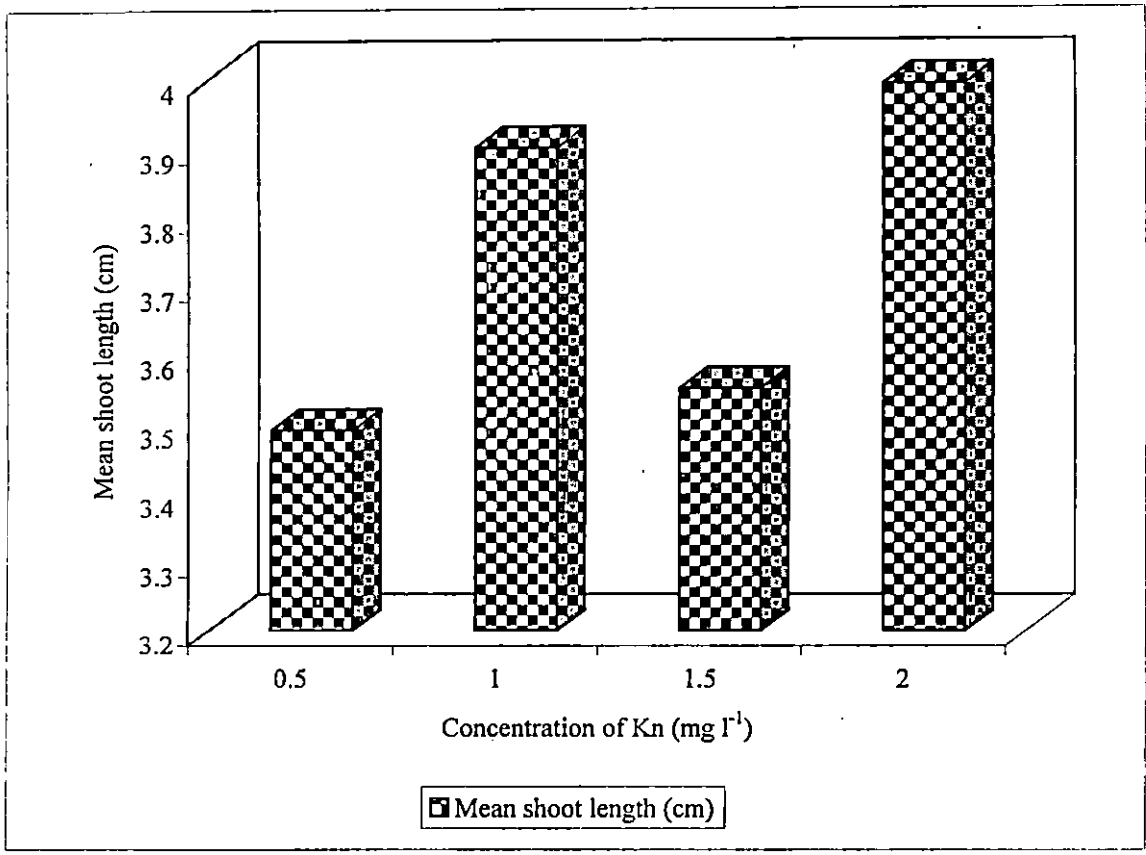


Fig. 5. Effect of different concentration of Kn on bud/leaf initiation and shoot elongation in MS medium

2.0 mg l^{-1} Kn. As, the former produced bud and leaf in 10.84 and 15.69 days. It was delayed upto 13.23 days for bud initiation and 23.94 days for leaf initiation in the latter media.

The effect of treatment on mean number of leaves per explant, mean number of shoots, mean shoot length and maximum number of shoots was found non significant. However, highest number of leaves i.e., 4.28 were recorded on media containing 0.5 mg l^{-1} Kn which produced a maximum of 1.28 shoots. Multiple shoot (3.00) was recorded on media supplemented with 1.5 mg l^{-1} Kn (Plate 5). Mean height of the shoot was recorded highest (3.99 cm) in cultures on media containing 2.0 mg l^{-1} Kn. Callusing at the base of the explant was observed on MS + 1.0 mg l^{-1} Kn, 1.5 mg l^{-1} Kn and 2.0 mg l^{-1} Kn.

4.3.3 Effect of different concentration of IAA on culture establishment and growth in MS media

The data on the effect of different concentrations of IAA on culture establishment is given in Table13. Higher percentages of bud initiation, 95.00 and 90.00 per cent, were recorded on media supplemented with 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} IAA respectively. These treatments were at par and significantly superior to the other two. Same trend was noted in leaf initiation. Maximum number of explants (71.66 %) exhibited leaf production on media containing 0.5 mg l^{-1} IAA. Explants on media containing 0.5 and 1.5 mg l^{-1} IAA produced buds in 12.62 and 11.17 days, respectively. While leaf initiation was observed after 17.62 days on media with 1.0 mg l^{-1} IAA, it took 25.65 days in explants cultured on media containing 0.5 mg l^{-1} IAA.

Effect of treatment on mean number of leaves per explant was found non significant. Highest numbers of leaves i.e., 4.73 were recorded on media containing 0.5 mg l^{-1} IAA. Maximum of 0.94 shoots were produced per explant on media containing 1.5 mg l^{-1} IAA. Maximum of 2.0 multiple shoots were recorded in MS +

Table 13. Effect of different concentrations of IAA on culture establishment and growth in axillary bud of *Azadirachta indica* in MS media

IAA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	95.00 [a]	71.667	12.62	25.67	4.73	0.88	3.30	1.00
1.0	85.33 [ab]	62.662	11.59	17.62	2.25	0.81	4.43	1.00
1.5	90.00 [a]	69.00	11.17	18.74	2.67	0.94	4.66	2.00
2.0	80.67 [b]	57.00	12.43	22.74	2.74	0.80	3.30	1.00
SEm±	3.13	6.34	1.25	2.22	1.13	0.13	0.44	0.00
CD (0.05)	S	NS	NS	NS	NS	NS	NS	NS

Figures with the same alphabet do not differ significantly.

1.5 mg l^{-1} IAA. Mean height was highest (4.66 cm) in cultures on media containing 1.5 mg l^{-1} IAA. It was observed that in MS + 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} IAA maximum number of explants produced single leaf which were emerging directly from the bud portion showing the symptom of chlorosis and they lacked the elongation of stem (Plate 6).

4.3.4 Effect of different concentration of NAA on culture establishment and growth in MS media

The MS media was supplemented with four different concentrations of NAA to study their effect on different growth parameters. The result obtained is presented in Table14.

Percentage of bud initiation was 46.67 per cent when MS medium was supplemented with 2.0 mg l^{-1} NAA, this was not significantly different from the rest of the treatments. In general, bud initiation percentage was higher when MS medium was supplemented with higher concentration, whereas it decreased to 34.33 per cent when MS was supplemented with 1.0 mg l^{-1} NAA.

Percentage of leaf initiation also followed the same trend as bud initiation. It was 27.00 per cent in MS + 2.0 mgl^{-1} NAA, where as at lower concentration of NAA (1.0 mgl^{-1} NAA) the percentage of leaf initiation was 15.67.

Treatment effect on the time taken for bud initiation was found significant and it was as high as 24.25 when supplemented with 1.0 mg l^{-1} NAA and as low as 11.88 days at the levels of 0.5 mg l^{-1} NAA. Leaf initiation was delayed upto 31.12, when MS media was supplemented with 1.0 mg l^{-1} NAA followed by 24.71 and 30.67 days at 1.5 and 2.0mg l^{-1} NAA, respectively. With reference to leaves, the mean number per explant was more when NAA was added to media at lower concentration. Treatment effect on mean number of shoots was found significant. The media with 0.5 mg l^{-1} NAA produced a mean number of 1.37 shoots per shoot. Maximum mean height (3.17 cm) was found on media containing 0.5 mg l^{-1} NAA and maximum

Table 14. Effect of different concentration of NAA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

NAA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	39.00	23.67	11.88 [b]	16.67 [b]	1.40	1.37 [a]	3.17	1.00
1.0	34.33	15.67	24.25 [a]	31.12 [a]	1.43	0.63 [b]	1.87	1.00
1.5	40.33	24.33	13.09 [b]	24.71 [a]	1.20	0.83 [ab]	2.13	1.00
2.0	46.67	27.00	20.50 [ab]	30.67 [a]	1.30	0.57 [b]	1.70	1.00
SEm±	6.08	3.95	2.60	2.28	0.24	0.09	0.39	0.00
CD (0.05)	NS	NS	S	S	NS	S	NS	NS

Figures with the same alphabet do not differ significantly.

number of shoots per explant was recorded as one in all the four treatments. Direct rooting was observed in all the treatment at the base of the explants.

4.3.5 Effect of different concentration of IBA on culture establishment and growth in MS

The data on effect of different concentration of IBA in MS is given in Table 15. Effect of the treatment on percentage of bud initiation, percentage of leaf initiation and days taken for bud initiation differed significantly. Among the four media combinations the percentage of bud initiation was found to be highest (42.67) in media containing 1.5 mg l^{-1} IBA and the least (22.67) was 2.0 mg l^{-1} IBA. Maximum leaf initiation (30.00) was observed on media with 0.5 mg l^{-1} IBA. In media supplemented with 2.0 mg l^{-1} IBA, only 14.67 per cent cultures exhibited leaf initiation. It was observed that the time taken for bud initiation was 9.21 days on media containing 2.0 mg l^{-1} IBA as compared to 22.22 days on media with 1.0 mgl^{-1} IBA.

The treatment effect on time taken for leaf initiation was non-significant. It was observed that after 37.89 days leaf initiation occurred on media containing 1.5 mg l^{-1} IBA. Maximum mean number of leaves per explant (5.22) were observed on media with 0.5 mg l^{-1} IBA.

The mean number of shoots per explant was recorded to be maximum (0.80) when 2.0 mg l^{-1} IBA was supplemented to the media. Maximum mean height (3.90 cm) was found on MS + 2.0 mg l^{-1} IBA. Multiple shoots were not produced by any of the treatment combinations. Few explants induced direct rooting from the explants along with shoot morphogenesis.

4.3.6 Effect of BA and IAA on culture establishment and growth in MS

Effect of various concentrations of BA with IAA on culture establishment and growth is presented in Table 16 and Fig. 6. Percentage of bud initiation was

Table 15. Effect of different concentration of IBA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

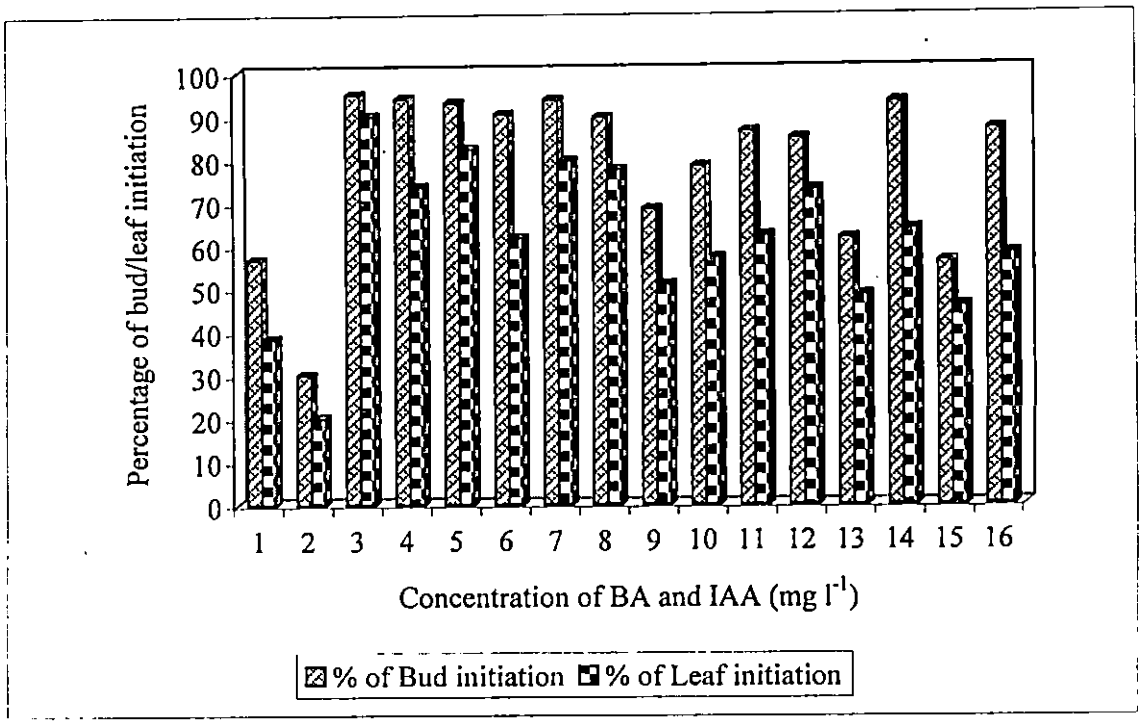
IBA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
	Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	42.00 [a]	30.00 [a]	18.19 [ab]	32.64	5.22	0.39	2.73	1.00
1.0	40.67 [a]	27.33 [a]	22.22 [a]	35.47	5.19	0.22	2.43	1.00
1.5	42.67 [a]	19.33 [ab]	10.63 [b]	37.89	3.78	0.51	3.16	1.00
2.0	22.67 [b]	14.67 [b]	9.21 [b]	33.17	3.75	0.80	3.90	1.00
SEm±	4.04	3.51	2.69	8.93	2.43	0.15	0.54	0.00
CD (0.05)	S	S	S	NS	NS	NS	NS	NS

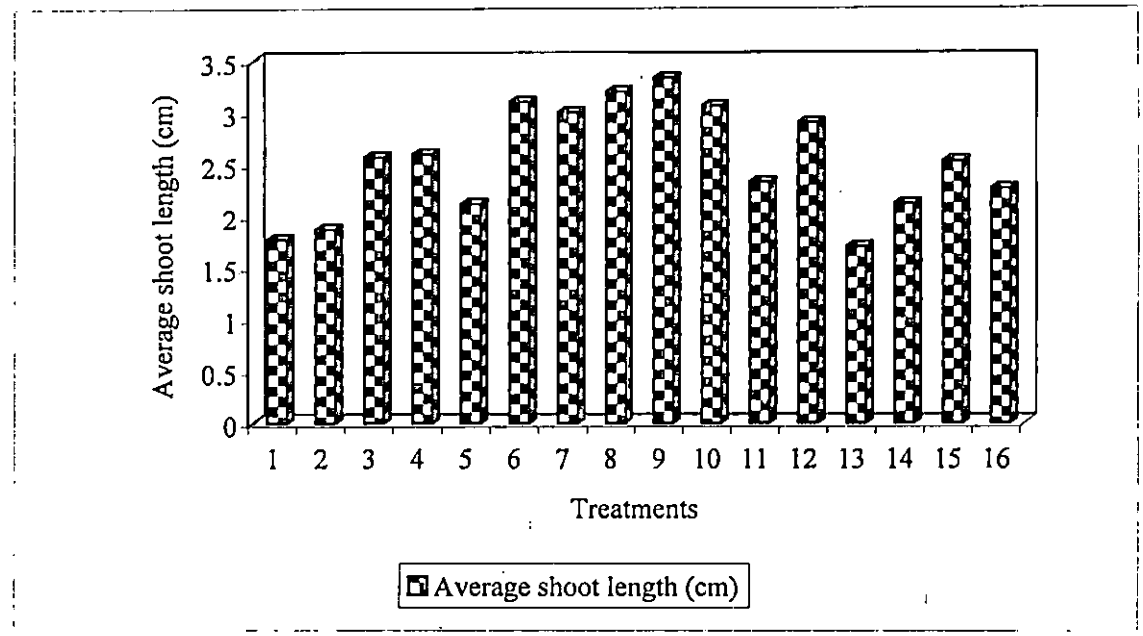
Figures with the same alphabet do not differ significantly.

Table 16. Effect of different concentration of BA and IAA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

BA (mg l^{-1})	IAA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	56.67	38.33 ^{fg}	11.83 ^{a}	20.33	1.53	0.57 ^{d}	1.77	1.00 ^{c}
0.5	1.0	30.00	20.00 ^{g}	11.67 ^{a}	18.00	1.23	0.64 ^{cd}	1.87	1.00 ^{c}
0.5	1.5	95.00	90.00 ^{a}	4.77 ^{d}	17.13	1.99	1.63 ^{a}	2.57	2.00 ^{a}
0.5	2.0	94.00	73.67 ^{abcd}	6.83 ^{bcd}	16.99	2.30	1.30 ^{ab}	2.60	2.00 ^{a}
1.0	0.5	93.00	82.33 ^{ab}	6.16 ^{cd}	13.40	2.17	1.31 ^{ab}	2.12	2.00 ^{a}
1.0	1.0	90.33	61.67 ^{bcde}	5.10 ^{d}	13.80	1.70	1.21 ^{abc}	3.10	2.00 ^{a}
1.0	1.5	93.67	79.65 ^{abc}	9.12 ^{abc}	13.21	1.93	1.24 ^{abc}	3.00	2.00 ^{a}
1.0	2.0	89.57	77.57 ^{abcd}	8.10 ^{ab}	16.68	2.34	1.21 ^{abc}	3.20	2.00 ^{a}
1.5	0.5	68.47	50.75 ^{ef}	8.54 ^{abc}	16.75	2.12	1.46 ^{ab}	3.33	2.00 ^{a}
1.5	1.0	78.33	57.11 ^{def}	8.92 ^{abc}	16.09	1.72	1.08 ^{abcd}	3.06	2.00 ^{a}
1.5	1.5	86.33	62.33 ^{bcde}	9.13 ^{ab}	15.64	1.72	1.30 ^{ab}	2.33	1.70 ^{ab}
1.5	2.0	84.67	73.00 ^{abcd}	10.54 ^{abc}	17.13	1.70	1.17 ^{abc}	2.90	1.70 ^{ab}
2.0	0.5	61.67	48.33 ^{ef}	9.00 ^{abc}	16.48	1.70	0.99 ^{bcd}	1.70	1.30 ^{bc}
2.0	1.0	93.00	63.78 ^{bcde}	9.85 ^{ab}	18.58	1.95	1.19 ^{abc}	2.12	1.70 ^{ab}
2.0	1.5	56.11	45.68 ^{ef}	4.80 ^{d}	13.23	5.78	1.63 ^{a}	2.53	2.00 ^{a}
2.0	2.0	86.67	58.00 ^{cdef}	9.73 ^{ab}	18.17	2.17	1.38 ^{ab}	2.27	1.7 ^{ab}
SEm±		5.62	6.66	1.01	1.69	1.01	0.17	0.29	0.19
CD (0.05)		NS	S	S	NS	NS	S	NS	S

Figures with the same alphabet do not differ significantly.





1- MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA
2- MS + 0.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA
3- MS + 0.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA
4- MS + 0.5 mg l^{-1} BA + 2.0 mg l^{-1} IAA
5- MS + 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA
6- MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA
7- MS + 1.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA
8- MS + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} IAA
9- MS + 1.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA
10- MS + 1.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA
11- MS + 1.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA
12- MS + 1.5 mg l^{-1} BA + 2.0 mg l^{-1} IAA
13- MS + 2.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA
14- MS + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA
15- MS + 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA
16- MS + 2.0 mg l^{-1} BA + 2.0 mg l^{-1} IAA

Fig. 6. Effect of different concentration of BA and IAA on bud/leaf initiation and shoot elongation in MS medium

highest (95.00) on media containing 0.5 mg l^{-1} BA + 2.0 mg l^{-1} IAA. Least bud initiation (30.00 %) was recorded on MS + 0.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA. Maximum leaf initiation (90.00 %) was observed in media supplemented with 0.5 mg l^{-1} BA +1.5 mg l^{-1} IAA which was found to be significantly superior when compared to MS + 0.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA, which recorded a minimum of 20.00 per cent leaf initiation.

The treatment effect on time taken for leaf initiation was non significant. However, after 13.21 days leaf initiation was observed on media containing 1.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA. While leaf initiation was prolonged upto 20.33 days on media containing 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA.

Effect of treatment on mean number of leaves was non significant. However, media containing 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA produced more leaves (5.78 leaves) per explant as compared to 1.23 leaves per explant on MS + 0.5 mg l^{-1} BA +1.0 mg l^{-1} IAA.

For mean number of shoots per explant treatment MS + 0.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA and MS + 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA were found significantly superior to MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA, on which only 0.57 shoots (2.00) were recorded on media containing, 0.5 mg l^{-1} BA + 1.5mg l^{-1} IAA, 0.5 mg l^{-1} BA + 2.0 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 1.5 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 2.0 mg l^{-1} IAA, 1.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA, 1.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA and 2.0 mg l^{-1} BA + 1.5mg l^{-1} IAA, which were significantly superior than the media supplemented with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA and 0.5 mg l^{-1} BA + 1.0 mg l^{-1} IAA.

Maximum mean height (3.33 cm) was observed on MS + 1.5 mg l^{-1} BA + 0.5 mg l^{-1} IAA and least was recorded (1.70 cm) on MS + 2.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA. It was found that sub-culturing for 2-3 times resulted in more number of shoot production, when the explants were transferred and maintained in the same media combinations.

4.3.7 Effect of BA and NAA on culture establishment and growth in MS media

Sixteen combinations of BA and NAA (four levels of BA and four levels of NAA) were used to find their effect on culture establishment and growth. The result obtained is tabulated in Table 17. The results revealed that the percentage of bud initiation differed significantly. It was observed that the medium supplemented with 2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA was superior to all other treatments, as it produced 83.39 per cent of bud initiation. And it was on par with the media containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA (46.78 %), 1.0 mg l^{-1} BA + 2.0 mg l^{-1} NAA (49.46 %) and 1.5 mg l^{-1} BA + 1.0 mg l^{-1} NAA (45.54 %) which showed the minimum percentage of bud initiation. Treatment effect on percentage of leaf initiation was recorded as non significant. Maximum percentage of leaf initiation (56.00) was recorded in MS + 2.0 mg l^{-1} BA + 0.5 mg l^{-1} NAA and a minimum of 22.07 per cent in media containing 1.5 mg l^{-1} BA + 1.5 mg l^{-1} NAA.

Treatment effect on time taken for bud initiation and leaf initiation was found significant. The time taken for bud initiation and leaf initiation was minimum in media containing MS + 1.5 mg l^{-1} BA + 1.0 mg l^{-1} NAA. In former media the time taken for bud initiation and leaf initiation were recorded as 3.57 and 7.96 days respectively, whereas in latter they were 20.21 and 29.30 days, respectively.

Maximum mean number of leaves per explant (2.93 leaves) were found on MS + 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA and minimum of 1.35 leaves were recorded on MS + 1.5 mg l^{-1} BA + 1.0 mg l^{-1} NAA. Effect of treatment on mean number of shoots / explant and maximum number of shoots per test tube was found significant. Multiple shoots (3.67) were observed in 1.5 mg l^{-1} BA + 1.0 mg l^{-1} NAA, which was found significantly superior to MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA (1.0 shoot / culture tube). Mean number of shoots per explant was recorded maximum (3.29) in media containing 1.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA, which was found significantly superior to media containing 0.5 mg l^{-1} BA + 1.5 mg l^{-1} NAA (0.99), 1.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA (0.96) and 2.0 mg l^{-1} BA + 2.0 mg l^{-1} NAA (0.97). Maximum height per shoot (2.93 cm) was found on MS + 1.5 mg l^{-1} BA + 2.0 mg l^{-1} NAA.

Table 17. Effect of different concentration of BA and NAA culture establishment and growth in axillary bud culture of *Azaditachta indica* in MS media

BA (mg l^{-1})	NAA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	63.85 ^{abcd}	24.93	12.51 ^{bc}	27.98 ^{ab}	2.93	1.57 ^{cde}	1.80	2.33 ^{bcd}
0.5	1.0	70.93 ^{abc}	33.75	13.80 ^{b}	22.13 ^{bcde}	1.83	1.29 ^{de}	1.90	1.67 ^{cde}
0.5	1.5	77.22 ^{ab}	55.21	11.49 ^{bcd}	18.52 ^{cd}e	2.22	0.99 ^{e}	2.23	1.33 ^{de}
0.5	2.0	76.89 ^{ab}	34.38	8.11 ^{cdef}	16.26 ^{de}	2.23	1.14 ^{de}	2.47	1.33 ^{de}
1.0	0.5	46.78 ^{d}	33.83	11.12 ^{bcde}	20.90 ^{cde}	2.83	2.09 ^{bcd}	2.13	2.33 ^{bcd}
1.0	1.0	59.68 ^{bcd}	35.42	11.91 ^{bcd}	21.86 ^{bcde}	2.31	0.96 ^{e}	2.63	1.00 ^{e}
1.0	1.5	64.10 ^{abcd}	33.57	8.30 ^{cdef}	15.67 ^{de}	2.60	1.93 ^{bcde}	2.23	2.00 ^{bcde}
1.0	2.0	49.46 ^{d}	38.09	11.43 ^{bcd}	20.17 ^{cde}	2.36	1.71 ^{bcde}	2.70	2.00 ^{bcde}
1.5	0.5	54.03 ^{cd}	37.22	7.74 ^{defg}	14.93 ^{e}	2.06	3.29 ^{a}	1.67	4.00 ^{a}
1.5	1.0	45.54 ^{d}	25.09	3.57 ^{g}	7.96 ^{f}	1.35	2.39 ^{bc}	2.13	2.67 ^{abc}
1.5	1.5	54.39 ^{cd}	22.07	6.95 ^{efg}	16.04 ^{de}	2.10	1.72 ^{bcde}	2.00	2.67 ^{abc}
1.5	2.0	72.82 ^{abc}	45.12	11.57 ^{bcd}	24.12 ^{abc}	2.03	1.39 ^{de}	2.93	2.00 ^{bcde}
2.0	0.5	60.35 ^{bcd}	56.00	20.21 ^{a}	29.30 ^{a}	2.27	2.63 ^{ab}	1.73	3.00 ^{ab}
2.0	1.0	65.32 ^{abcd}	46.05	11.57 ^{bcd}	22.79 ^{abcd}	2.02	1.62 ^{cde}	1.53	2.00 ^{bcde}
2.0	1.5	83.39 ^{a}	33.71	6.61 ^{fg}	16.64 ^{de}	1.78	1.45 ^{cde}	2.13	2.00 ^{bcde}
2.0	2.0	74.22 ^{abc}	42.21	7.78 ^{defg}	16.32 ^{de}	2.10	0.97 ^{e}	2.47	1.33 ^{de}
SEm±		6.04	8.36	1.35	2.16	0.30	0.29	0.25	0.35
CD (0.05)		S	NS	S	S	NS	S	NS	S

Figures with the same alphabet do not differ significantly.

4.3.8 Effect of BA and IBA on culture establishment and growth in MS media

The data on the effect of different concentrations of BA and IBA on culture establishment and growth in MS media is presented in Table 18. Effect of treatment on percentage of bud initiation and leaf initiation, time taken for bud initiation and leaf initiation, mean number of leaves per explant, mean number of shoots per explant, maximum shoot length and maximum number of shoots produced were found to be significant.

Highest percentage of bud initiation was recorded on media supplemented with 0.5 mg l^{-1} BA + 1.0 mg l^{-1} IBA (69.81), 0.5 mg l^{-1} BA +1.5 mg l^{-1} IBA (71.14) and 1.0 mg l^{-1} BA + 1.5 mg l^{-1} IBA (67.11) which was significantly superior to media supplemented with 1.5 mg l^{-1} BA + 1.0 mg l^{-1} IBA (30.93) and 2.0 mg l^{-1} BA +1.5 mg l^{-1} IBA (31.00). The effect of treatment on leaf initiation, showed that MS + 0.5 mg l^{-1} BA + 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} BA + 1.5 mg l^{-1} IBA were statistically superior to MS + 2.0 mg l^{-1} BA +1.5 mg l^{-1} IBA.

The time taken for bud initiation containing MS + 0.5 mg l^{-1} BA +1.5 mg l^{-1} IBA was found significantly inferior to MS + 1.0 mg l^{-1} BA +1.0 mg l^{-1} IBA, MS + 1.0 mg l^{-1} BA + 2.0 mg l^{-1} IBA and MS + 1.5 mg l^{-1} BA + 2.0 mg l^{-1} IBA. In former media, the time taken for bud initiation were recorded as 12.20 days, whereas in latter they were 5.02, 4.49 and 4.57 days respectively. For leaf initiation media combination MS + 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IBA was found significantly superior (9.66 days), while it was delayed upto 24.71 days when MS + 1.5 mg l^{-1} BA + 1.0 mg l^{-1} IBA was used.

Media containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IBA produced more leaves (3.01 leaves) per explant as compared to 1.23 leaves per explant on MS + 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IBA. Mean number of shoots per explant was found to be higher (2.27) in media containing 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IBA and least in media containing 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IBA (0.65) and 1.5 mg l^{-1} BA + 2.0 mg l^{-1} IBA

Table 18. Effect of different concentration of BA and IBA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

BA ($mg\ l^{-1}$)	IBA ($mg\ l^{-1}$)	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	41.67 ^{bc}	29.59 ^{bc}	10.29 ^{ab}	21.78 ^{abc}	1.33 ^{de}	0.65 ^{e}	1.69 ^{cd}	1.00 ^{c}
0.5	1.0	69.81 ^{a}	67.56 ^{a}	9.56 ^{abc}	21.28 ^{abc}	2.36 ^{abc}	1.06 ^{de}	3.21 ^{ab}	1.70 ^{abc}
0.5	1.5	71.14 ^{a}	66.59 ^{a}	12.20 ^{a}	19.63 ^{abc}	1.96 ^{bcde}	1.14 ^{cde}	2.13 ^{bcd}	1.70 ^{abc}
0.5	2.0	63.67 ^{ab}	42.30 ^{abc}	5.85 ^{cd}	18.27 ^{abc}	1.78 ^{cde}	1.85 ^{abc}	2.73 ^{abc}	2.00 ^{abc}
1.0	0.5	50.22 ^{abc}	48.43 ^{ab}	5.90 ^{cd}	16.75 ^{bcd}	3.01 ^{a}	2.27 ^{a}	3.40 ^{a}	3.00 ^{a}
1.0	1.0	58.67 ^{ab}	48.05 ^{ab}	5.02 ^{d}	9.66 ^{e}	1.38 ^{cde}	1.85 ^{abc}	2.08 ^{bcd}	2.30 ^{ab}
1.0	1.5	67.11 ^{a}	43.26 ^{abc}	5.64 ^{cd}	18.42 ^{abc}	2.76 ^{ab}	1.53 ^{bcd}	2.77 ^{abc}	2.00 ^{abc}
1.0	2.0	57.59 ^{ab}	38.95 ^{bc}	4.49 ^{d}	11.60 ^{de}	2.26 ^{abcd}	2.17 ^{ab}	3.03 ^{ab}	2.00 ^{abc}
1.5	0.5	47.94 ^{abc}	45.91 ^{abc}	7.74 ^{bcd}	22.21 ^{ab}	2.18 ^{abcde}	1.45 ^{bcd}	2.17 ^{bcd}	1.70 ^{abc}
1.5	1.0	30.93 ^{c}	24.05 ^{bc}	6.05 ^{cd}	24.71 ^{a}	2.18 ^{abcde}	1.04 ^{de}	2.40 ^{abcd}	1.30 ^{bc}
1.5	1.5	61.59 ^{ab}	35.84 ^{bc}	8.69 ^{abcd}	15.13 ^{cde}	1.89 ^{bcde}	0.91 ^{de}	2.70 ^{abc}	1.70 ^{abc}
1.5	2.0	60.78 ^{ab}	28.44 ^{bc}	4.57 ^{d}	17.08 ^{bcd}	1.85 ^{bcde}	0.60 ^{e}	1.63 ^{cd}	1.00 ^{c}
2.0	0.5	47.85 ^{abc}	34.96 ^{bc}	7.13 ^{bcd}	16.91 ^{bcd}	1.70 ^{cde}	1.24 ^{cde}	2.97 ^{ab}	2.00 ^{abc}
2.0	1.0	60.33 ^{ab}	29.33 ^{bc}	10.44 ^{ab}	20.52 ^{abc}	1.35 ^{de}	1.34 ^{cde}	2.37 ^{abcd}	2.00 ^{abc}
2.0	1.5	31.00 ^{c}	18.33 ^{c}	7.87 ^{bcd}	17.36 ^{bcd}	1.23 ^{e}	1.23 ^{cde}	2.90 ^{ab}	3.00 ^{a}
2.0	2.0	56.67 ^{ab}	34.24 ^{bc}	7.85 ^{bcd}	17.55 ^{bcd}	1.69 ^{cde}	1.23 ^{cde}	1.43 ^{d}	2.30 ^{ab}
SEm±		7.47	8.38	1.25	1.97	0.29	0.23	0.34	0.35
CD (0.05)		S	S	S	S	S	S	S	S

Figures with the same alphabet do not differ significantly.

(0.60) respectively. Maximum mean height was found to be 3.40 cm on 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IBA as compared to 2.0 mg l^{-1} BA + 2.0 mg l^{-1} IBA which recorded a mean height of 1.43 cm. Maximum number of shoots (2.7) was observed on two media combination viz., 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IBA and 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IBA. However, least (1.0) was observed on media containing 0.5 mg l^{-1} BA + 0.5 mg l^{-1} IBA and 1.5 mg l^{-1} BA + 2.0 mg l^{-1} IBA respectively

4.3.9 Effect of Kn and IAA on culture establishment and growth in MS media

Different combinations of Kn and IAA were used to find their effect on growth. The result obtained is presented in Table 19. Percentage of bud initiation was highest (79.78) on MS + 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} IAA. In contrast, least was recorded (49.89) in MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} IAA. Maximum bud initiation (73.67 %) was observed on MS + 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} IAA and a minimum (32.33 %) was recorded in 1.0 mgl^{-1} Kn + 1.5 mg l^{-1} IAA.

Treatment effect on time taken for bud initiation and leaf initiation was found significant. For bud and leaf initiation MS + 1.5 mg l^{-1} Kn + 1.0 mg l^{-1} IAA (3.39 days for bud initiation and 7.89 days for leaf initiation) were found significantly superior to MS + 2.0 mg l^{-1} Kn + 1.5 mg l^{-1} IAA (8.07 days for bud initiation and 14.83 days for leaf initiation).

The effect of treatment on maximum mean number of leaves per explant, mean number of shoots per explant, mean shoot length and maximum number of shoot was found non significant. The maximum number of leaves (2.58 leaves) were observed on MS + 1.5 mg l^{-1} Kn + 1.5mg l^{-1} IAA and no multiple shoots were observed. Maximum of 1.01 shoots were recorded in media supplemented with 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} IAA and multiple shoots were observed in the explant after subsequent sub culturing. Maximum mean height per shoot was found to be 3.30 cm on MS + 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} IAA and minimum (1.87 cm) on MS + 2.0 mg l^{-1} Kn + 0.5 mg l^{-1} IAA. Callusing was observed at the base of the explant in all the treatment combinations.

Table 19. Effect of different concentration of Kn and IAA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

Kn (mg l^{-1})	IAA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	56.00	44.00	5.35 ^{bcdef}	13.81 ^{ab}	1.87	0.75	1.97	1.00
0.5	1.0	68.96	48.89	7.00 ^{abcd}	10.33 ^{def}	1.57	1.00	2.60	1.00
0.5	1.5	70.00	46.67	5.4 ^{bcdef}	13.57 ^{abc}	2.00	1.00	2.10	1.00
0.5	2.0	78.00	56.44	5.00 ^{cdef}	8.80 ^{ef}	1.86	0.85	2.03	1.00
1.0	0.5	60.00	40.43	7.67 ^{ab}	13.84 ^{ab}	2.07	1.01	2.00	1.00
1.0	1.0	79.79	43.00	7.39 ^{aoc}	12.73 ^{abcd}	1.43	0.87	2.40	1.00
1.0	1.5	49.89	32.33	5.33 ^{bcdef}	10.03 ^{def}	1.80	0.95	2.10	1.00
1.0	2.0	70.78	49.55	5.59 ^{bcdef}	11.52 ^{bcde}	1.95	0.95	2.35	1.00
1.5	0.5	52.67	43.43	5.57 ^{bcdef}	11.74 ^{bcd}	1.86	0.66	2.77	1.00
1.5	1.0	60.11	58.09	3.39 ^{f}	7.89 ^{f}	1.53	0.91	2.53	1.00
1.5	1.5	56.73	37.91	6.20 ^{abcde}	12.59 ^{abcd}	2.58	0.96	2.53	1.00
1.5	2.0	66.67	73.67	5.73 ^{abcdef}	13.00 ^{abcd}	1.80	0.63	3.30	1.00
2.0	0.5	56.55	51.51	4.31 ^{ef}	10.69 ^{cdef}	1.90	0.88	1.87	1.00
2.0	1.0	66.67	50.00	4.80 ^{def}	11.10 ^{bcde}	2.31	0.63	3.20	1.00
2.0	1.5	70.81	69.78	8.07 ^{a}	14.83 ^{a}	1.43	0.78	2.87	1.00
2.0	2.0	70.74	46.66	4.69 ^{def}	12.87 ^{abcd}	1.68	0.93	2.47	1.00
SEm±		6.76	9.03	0.75	0.89	0.29	0.11	0.34	0.00
CD (0.05)		NS	NS	S	S	NS	NS	NS	NS

Figures with the same alphabet do not differ significantly.

4.3.10 Effect of Kn and NAA on culture establishment and growth in MS media

Effect of various concentrations of Kn with NAA on culture establishment and growth is presented in Table 20 and Fig 7. Percentage of bud initiation was highest (91.11%) on media containing 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA. Least bud initiation (49.75%) was recorded on MS + 1.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA. Maximum leaf initiation (71.11%) was observed on MS + 2.0 mg l^{-1} Kn + 2.0 mg l^{-1} NAA which was found to be statistically superior when compared to MS + 2.0 mg l^{-1} Kn + 1.0 mg l^{-1} NAA which recorded a least percentage of 15.55.

Treatment effect on time taken for bud initiation and leaf initiation was found to be significant. For bud initiation, media containing 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} NAA was found to be significantly superior to MS+ 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} NAA and MS + 2.0 mg l^{-1} Kn + 1.5 mg l^{-1} NAA. In the former treatment the time taken for bud initiation was found to be 3.75 days, while in latter treatments that was 16.81 and 15.83 days respectively. Leaf initiation was delayed upto 30.18 days, when 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} NAA media combination was used.

Treatment effect on mean number of leaves per explant was found to be non significant. Maximum mean leaves (3.06) were observed in media containing 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} NAA and least (1.36) on MS + 1.0 mg l^{-1} Kn + 1.0 mg l^{-1} NAA. For maximum mean number of shoots per explant treatment MS + 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} NAA on which only 0.57 shoots were recorded. Multiple shoots were observed in media containing 1.0 mg l^{-1} Kn + 0.5mg l^{-1} NAA. Maximum mean height (3.63cm) was observed on MS+ 1.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA which was found statistically superior to MS+ 0.5 mg l^{-1} Kn + 2.0 mg l^{-1} NAA (1.30 cm).

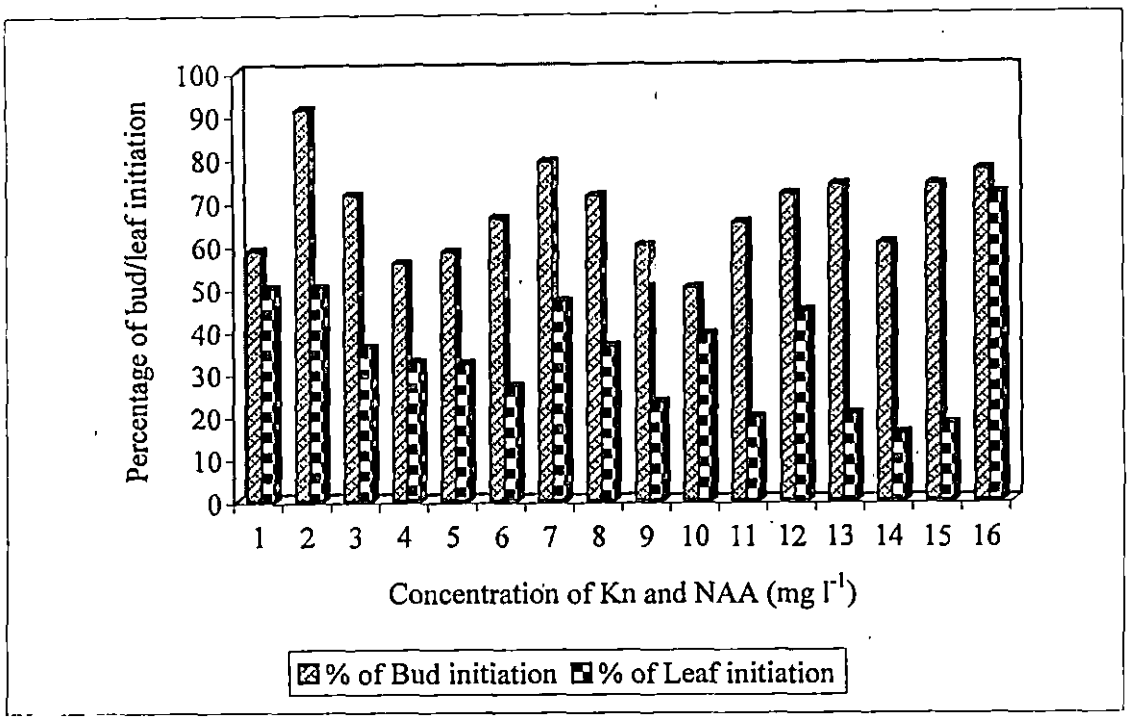
4.3.11 Effect of Kn and IBA on culture establishment and growth in MS media

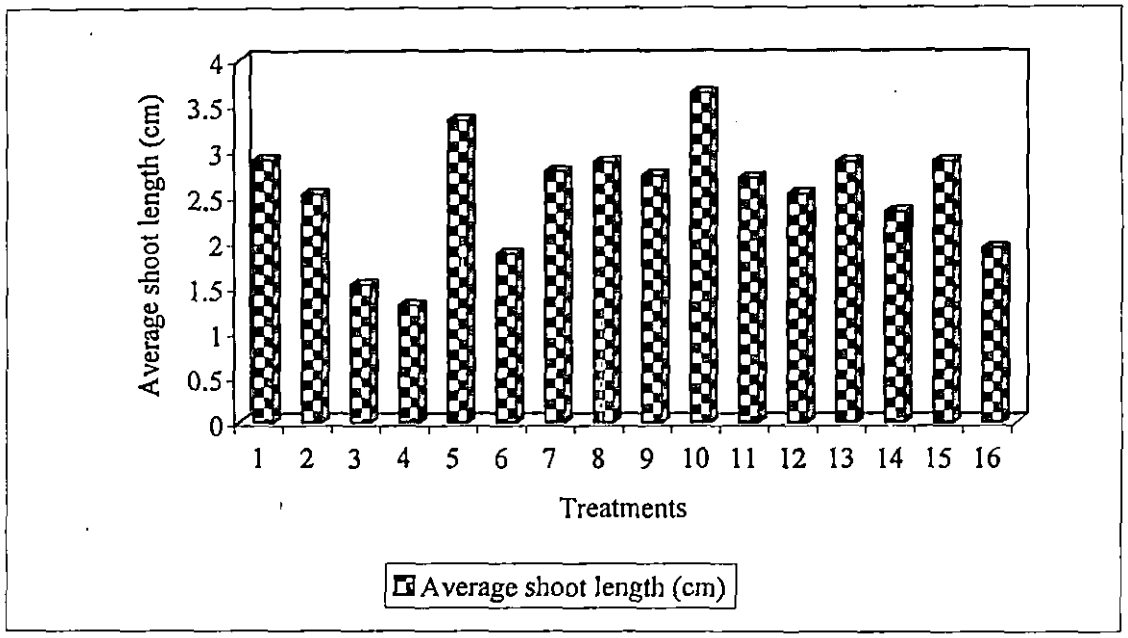
Various combinations of Kn and IBA were used to find out their effect on growth and culture establishment. The result obtained are presented in Table 21.

Table 20. Effect of different concentration of Kn and NAA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

Kn ($mg\ l^{-1}$)	NAA ($mg\ l^{-1}$)	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	58.52	49.89 ^{ab}	8.23 ^{bcdef}	15.38 ^{c}	2.08	0.74 ^{bcd}	2.90 ^{abc}	1.00
0.5	1.0	91.11	49.99 ^{ab}	9.42 ^{bcde}	13.84 ^{c}	1.75	0.71 ^{bcd}	2.53 ^{bcd}	1.00
0.5	1.5	71.29	35.88 ^{bcd}	6.39 ^{defg}	13.91 ^{c}	1.78	0.91 ^{bc}	1.53 ^{de}	1.00
0.5	2.0	55.56	32.41 ^{bcd}	7.81 ^{bcdefg}	14.57^{c}	1.39	0.77 ^{bcd}	1.30 ^{e}	1.00
1.0	0.5	58.18	31.98 ^{bcd}	5.53 ^{efg}	11.87 ^{c}	3.06	1.54 ^{a}	3.33 ^{ab}	2.00
1.0	1.0	65.92	26.75 ^{bcd}	7.50 ^{cdefg}	13.54 ^{c}	1.36	0.99 ^{b}	1.87 ^{cde}	1.00
1.0	1.5	78.83	46.69 ^{abc}	7.74 ^{bcdefg}	14.10 ^{c}	2.01	0.96 ^{b}	2.77 ^{abc}	1.00
1.0	2.0	71.11	36.12 ^{bcd}	4.75 ^{fg}	10.46 ^{c}	2.10	0.99 ^{b}	2.87 ^{abc}	1.00
1.5	0.5	59.61	22.99 ^{bcd}	3.75 ^{g}	12.72 ^{c}	2.97	1.00 ^{b}	2.73 ^{abc}	1.00
1.5	1.0	49.75	38.87 ^{bcd}	4.25 ^{fg}	13.30 ^{c}	2.14	0.90 ^{bc}	3.63 ^{a}	1.00
1.5	1.5	64.77	19.38 ^{cd}	11.27 ^{bc}	22.62 ^{b}	1.87	0.97 ^{b}	2.70 ^{abc}	1.00
1.5	2.0	71.19	44.35 ^{abcd}	16.81 ^{a}	30.18 ^{a}	2.01	0.76 ^{bcd}	2.53 ^{bcde}	1.00
2.0	0.5	73.33	20.00 ^{cd}	11.70 ^{b}	24.33 ^{b}	1.70	0.66 ^{cd}	2.87 ^{abc}	1.00
2.0	1.0	59.99	15.55 ^{d}	10.00 ^{bcd}	23.53 ^{b}	1.53	0.57 ^{d}	2.33 ^{bcd}	1.00
2.0	1.5	73.33	17.78 ^{cd}	15.83 ^{a}	25.07 ^{ab}	1.47	0.92 ^{bc}	2.88 ^{abc}	1.00
2.0	2.0	76.67	71.11 ^{a}	4.67 ^{fg}	9.71 ^{c}	1.79	0.84 ^{bcd}	1.93 ^{cde}	1.00
SEm±		8.73	8.71	1.25	1.90	0.40	0.09	0.32	0.25
CD (0.05)		NS	S	S	S	NS	S	S	NS

Figures with the same alphabet do not differ significantly.





1- MS + 0.5 mg l^{-1} Kn + 0.5 mg l^{-1} NAA
2- MS + 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA
3- MS + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA
4- MS + 0.5 mg l^{-1} Kn + 2.0 mg l^{-1} NAA
5- MS + 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} NAA
6- MS + 1.0 mg l^{-1} Kn + 1.0 mg l^{-1} NAA
7- MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} NAA
8- MS + 1.0 mg l^{-1} Kn + 2.0 mg l^{-1} NAA
9- MS + 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} NAA
10- MS + 1.5 mg l^{-1} Kn+ 1.0 mg l^{-1} NAA
11- MS + 1.5 mg l^{-1} Kn + 1.5 mg l^{-1} NAA
12- MS + 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} NAA
13- MS + 2.0 mg l^{-1} Kn + 0.5 mg l^{-1} NAA
14- MS + 2.0 mg l^{-1} Kn + 1.0 mg l^{-1} NAA
15- MS + 2.0 mg l^{-1} Kn + 1.5 mg l^{-1} NAA
16- MS + 2.0 mg l^{-1} Kn + 2.0 mg l^{-1} NAA

Fig. 7. Effect of different concentration of Kn and NAA on bud/leaf initiation and shoot elongation in MS medium

Treatment effect on percentage of bud initiation was found to differ significantly from each other. Maximum percentage of bud initiation (76.27%) was recorded in media containing 1.5 mg l^{-1} Kn + 1.5 mg l^{-1} IBA which was found statistically superior when compared to media containing 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} IBA (45.00%), 0.5 mg l^{-1} Kn + 2.0 mg l^{-1} IBA (40.27%),1.5 mg l^{-1} Kn + 1.0 mg l^{-1} IBA (46.25%), 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} IBA (41.09%) and 2.0 mg l^{-1} Kn + 1.0 mg l^{-1} IBA (45.23%) respectively.

Maximum percentage of leaf initiation (48.47) was observed on MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} IBA and only 20.00 per cent leaf initiation was recorded when 0.5 mg l^{-1} Kn + 0.5 mg l^{-1} IBA were supplemented to MS medium.

Treatment effect on time taken for bud initiation, leaf initiation, mean number of leaves per explant, mean number of shoots per explant, mean shoot length and maximum number of shoots per culture tube were found significant. Maximum time taken for bud initiation was recorded as 18.71 and 19.15 days on media containing 1.5 mg l^{-1} Kn + 1.5 mg l^{-1} IBA and 2.0 mg l^{-1} Kn + 1.5 mg l^{-1} IBA which was found significantly inferior to MS + 0.5 mg l^{-1} Kn + 2.0 mg l^{-1} IBA and 1.0 mg l^{-1} Kn + 2.0 mg l^{-1} IBA which took 4.18 and 4.29 days for bud initiation, respectively. Media supplemented with 1.0 mg l^{-1} Kn + 2.0 mg l^{-1} IBA took only 14.08 days for leaf initiation, which was delayed upto 31.37 days on MS + 0.5 mg l^{-1} Kn + 1.5 mg l^{-1} IBA. Maximum mean number of leaves (3.75) per explant was recorded on 1.5 mg l^{-1} Kn + 1.5 mg l^{-1} IBA which was significantly superior to media containing 1.0 mg l^{-1} Kn + 0.5 mg l^{-1} IBA, 1.5 mg l^{-1} Kn + 1.0 mg l^{-1} IBA, 2.0 mg l^{-1} Kn + 2.0 mg l^{-1} IBA respectively. Maximum of 2.25 shoots were found (one from each bud) on MS + 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} IBA. Maximum mean height per shoot was found to be 3.83 cm on MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} IBA which was statistically superior to MS + 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} IBA (1.70cm) and MS + 1.5 mg l^{-1} Kn + 2.0 mg l^{-1} IBA (1.70cm) respectively. Maximum number of shoots per culture was recorded on MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} IBA (2.33), MS + 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} IBA (2.67).

Table 21. Effect of different concentration of Kn and IBA on culture establishment and growth in axillary bud culture of *Azadirachta indica* in MS media

Kn (mg l^{-1})	IBA (mg l^{-1})	Percentage of		Period (days)		Mean No. of leaves per explant	Mean No. of shoots/bud sprouts per explant	Mean shoot length (cm)	Maximum No. of shoots
		Bud initiation	Leaf initiation	Bud initiation	Leaf initiation				
0.5	0.5	63.33 ^{abcd}	20.00	7.93 ^{cdef}	16.67 ^{fg}	1.82 ^{cd}	0.94 ^{c}	2.73 ^{bcd}	1.33 ^{b}
0.5	1.0	45.00 ^{e}	24.23	10.69 ^{cde}	25.51 ^{abcd}	1.69 ^{cd}	0.53 ^{c}	1.70 ^{e}	1.00 ^{b}
0.5	1.5	48.60 ^{de}	31.90	16.85 ^{ab}	31.37 ^{a}	1.78 ^{cd}	1.08 ^{c}	3.03 ^{abc}	1.33 ^{b}
0.5	2.0	40.27 ^{e}	31.21	4.18 ^{f}	21.00 ^{def}	1.50 ^{cd}	1.00 ^{c}	3.07 ^{abc}	1.00 ^{b}
1.0	0.5	67.92 ^{ab}	36.72	8.78 ^{cdef}	24.33 ^{bcde}	1.42 ^{d}	1.07 ^{bc}	2.33 ^{cde}	1.00 ^{b}
1.0	1.0	48.89 ^{de}	29.72	9.48 ^{cdef}	23.76 ^{bcde}	1.77 ^{cd}	0.60 ^{c}	3.45 ^{ab}	1.00 ^{b}
1.0	1.5	54.03 ^{bcde}	48.47	5.65 ^{ef}	21.27 ^{def}	2.20 ^{cd}	1.67 ^{b}	3.83 ^{a}	3.00 ^{a}
1.0	2.0	49.70 ^{cde}	27.00	4.29 ^{f}	14.08 ^{g}	3.49 ^{ab}	0.91 ^{c}	3.10 ^{abc}	1.00 ^{b}
1.5	0.5	64.65 ^{abc}	31.34	9.07 ^{cdef}	25.34 ^{abcde}	1.78 ^{cd}	2.25 ^{a}	2.03 ^{de}	3.00 ^{a}
1.5	1.0	46.25 ^{e}	44.25	11.20 ^{cd}	22.97 ^{cdef}	1.30 ^{d}	0.75 ^{c}	2.43 ^{cde}	1.00 ^{b}
1.5	1.5	76.27 ^{a}	31.37	18.71 ^{a}	28.30 ^{abc}	3.75 ^{a}	0.77 ^{c}	2.35 ^{cde}	1.00 ^{b}
1.5	2.0	41.09 ^{e}	37.44	6.63 ^{cdef}	18.47 ^{efg}	1.87 ^{cd}	0.69 ^{c}	1.70 ^{e}	1.00 ^{b}
2.0	0.5	54.25 ^{bcde}	47.45	5.60 ^{def}	22.16 ^{cdef}	1.81 ^{cd}	0.93 ^{c}	2.02 ^{de}	1.00 ^{b}
2.0	1.0	45.23 ^{e}	40.98	5.63 ^{def}	23.93 ^{bcde}	1.93 ^{cd}	0.91 ^{c}	1.90 ^{de}	1.00 ^{b}
2.0	1.5	55.57 ^{bcde}	41.47	19.15 ^{a}	30.22 ^{ab}	2.63 ^{bc}	0.94 ^{c}	2.30 ^{cde}	1.00 ^{b}
2.0	2.0	74.81 ^{a}	35.73	12.06 ^{bc}	20.49 ^{def}	1.33 ^{d}	0.85 ^{c}	2.45 ^{cde}	1.00 ^{b}
SEm±		4.72	10.08	1.67	2.04	0.34	0.21	0.27	0.26
CD (0.05)		S	NS	S	S	S	S	S	S

Figures with the same alphabet do not differ significantly.

4.4 ROOT INDUCTION FROM IN VITRO PRODUCED SHOOTS.

4.4.1 *In vitro* rooting on ½ MS

Ten different treatment combinations were tried for root induction in the *in vitro* produced shoots. Results obtained are presented in Table 22 and Fig 8. Maximum percentage of rooting (93.33) was observed on ½ MS + 1.5 mg l^{-1} IAA and least (23.72) was observed on ½ MS + 0.5 mg l^{-1} NAA (Plate 7). It was found that callus was observed at the base of the shoot when the media was supplemented with different combinations of NAA. As a result of this, a mass of roots were observed arising from the callus (Plate 8).

Treatment effect on mean number of roots per explants was found non significant. Highest mean number of roots (4.33) were observed on ½ MS + 1.5 mg l^{-1} IAA, ½ MS+ 1.0 mg l^{-1} NAA and ½ MS + 1.5 mg l^{-1} NAA least number of roots (2.33) were observed on ½ MS + 0.5 mg l^{-1} NAA and ½ MS +1.0 mg l^{-1} NAA respectively. However, maximum number of roots per shoot (6.17) were found on ½ MS + 0.5 mg l^{-1} IBA and least (2.67) was recorded on media supplemented with ½ MS + 0.5 mg l^{-1} NAA and ½ MS + 1.0 mg l^{-1} NAA. Mean length of roots was found significant. Maximum mean length of roots (4.00 cms) were observed on ½ MS + 1.0 mg l^{-1} IAA and lowest (1.63 cm) were found on ½ MS + 1.0 mg l^{-1} IBA and (1.27 cm) ½ MS + 1.5 mg l^{-1} IBA.

4.4.2 *In vitro* rooting on full MS

The data on the effect of different concentrations of IAA, NAA and IBA on growth and rooting of shoots in MS media is presented in Table 23 and Fig. 9.

It was observed that full MS resulted in maximum callus development at the base of the shoot which was kept for rooting. Highest rooting percentage (25.66) were found on media containing 0.5 mg l^{-1} IAA. Mean number of roots per explant differed significantly. It was found that MS + 0.5 mg l^{-1} IAA showed maximum number of roots (4.33), whereas media supplemented with 1.5 mg l^{-1} IAA, 1.0 mg l^{-1}

Table 22. Effect of various growth regulator combinations on *in vitro* rooting of microshoots on of *A. indica* in half MS

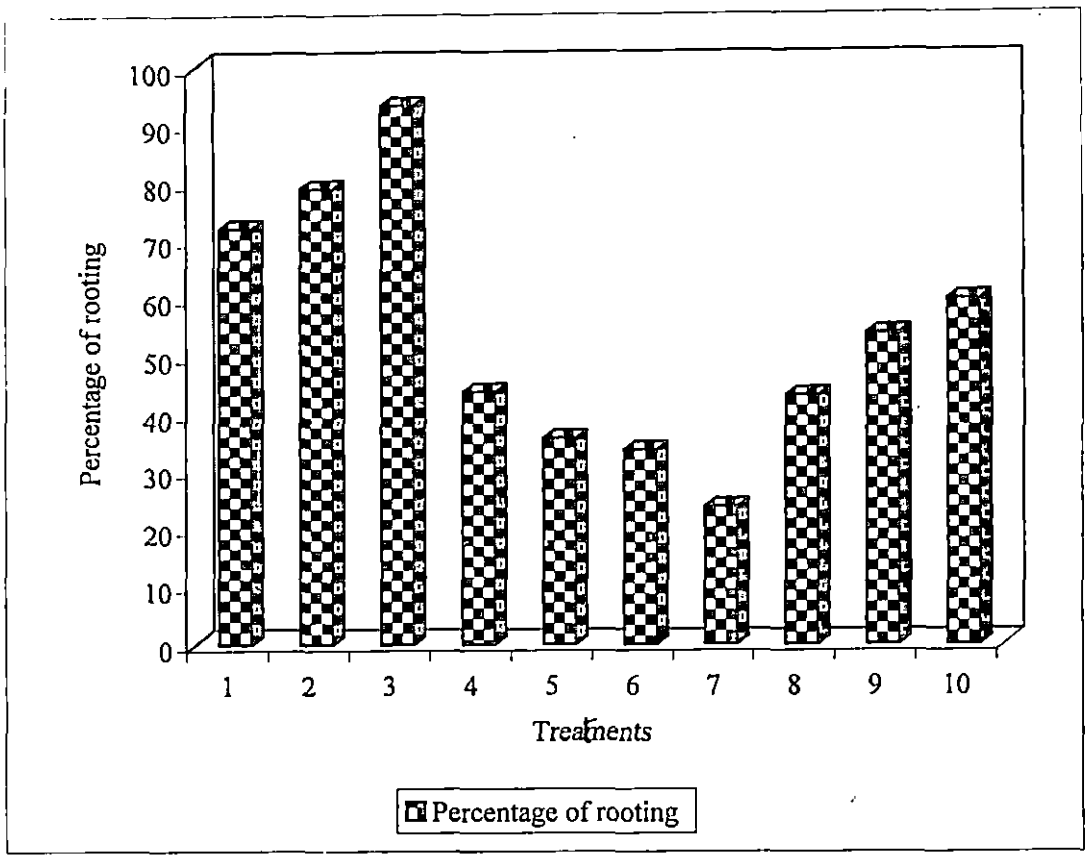
Treatment (mgl^{-1})	Percentage of rooting	Mean no. of roots / explants	Maximum no. of roots (cm)	Mean length of roots (cm)	Maximum length of roots (cm)
0.5 IAA	72.09	3.00	4.67	2.00 ^{cd}	3.40
1.0 IAA	78.97	4.00	5.33	4.00 ^{a}	4.20
1.5 IAA	93.33	4.33	5.00	3.30 ^{ab}	3.30
0.5 IBA	43.85	3.85	6.17	2.20 ^{cd}	2.30
1.0 IBA	35.78	3.00	3.33	1.63 ^{d}	1.80
1.5 IBA	33.68	3.33	3.50	1.27 ^{d}	1.50
0.5 NAA	23.72	2.33	2.67	1.90 ^{cd}	1.90
1.0 NAA	43.13	2.33	2.67	2.00 ^{cd}	2.10
1.5 NAA	53.69	4.33	5.00	2.73 ^{bc}	2.80
1.0 IAA + 1.0 Kn	59.53	4.33	5.33	2.97 ^{bc}	3.10
SEm±		0.75		0.33	
CD (0.05)		NS		S	

Figures with the same alphabet do not differ significantly.

Table 23. Effect of various growth regulator combinations on *in vitro* rooting of microshoots on of *A. indica* in full MS.

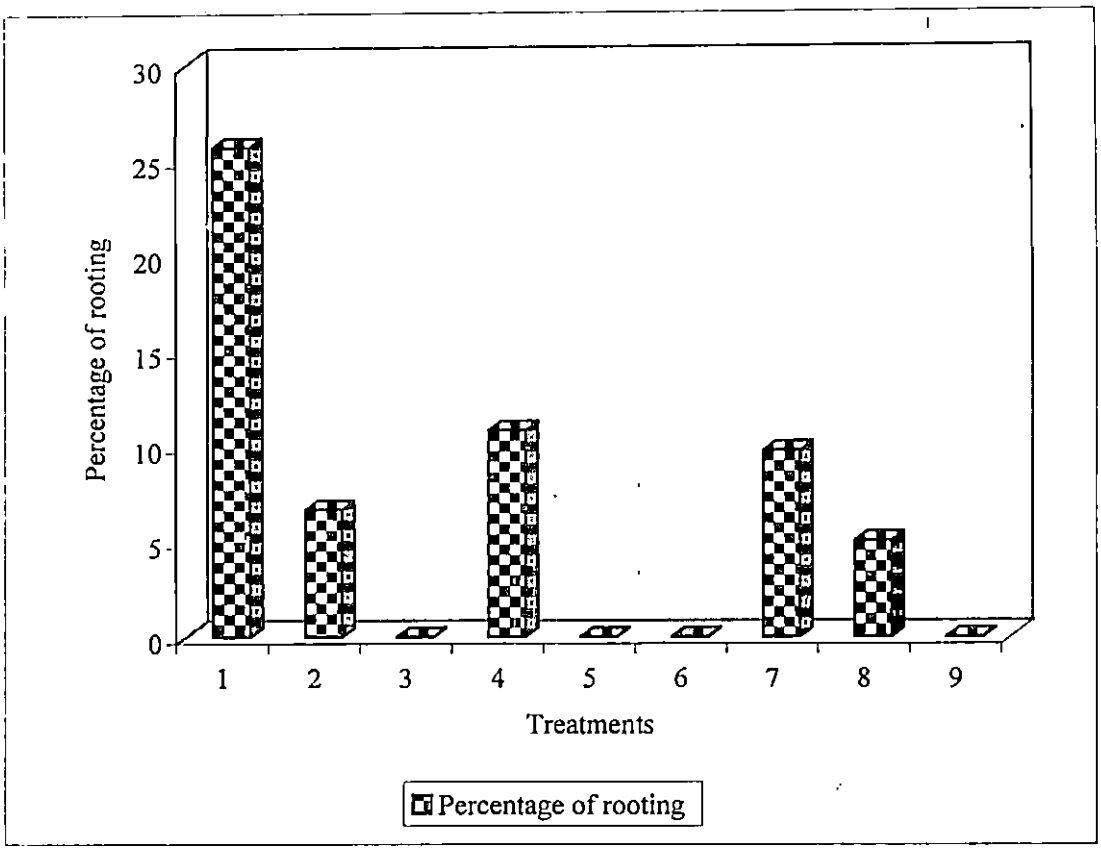
Treatment (mgl^{-1})	Percentage of rooting	Mean no. of roots / explants	Maximum no. of roots (cm)	Mean length of roots (cm)	Maximum length of roots (cm)
0.5 IAA	25.66	4.33 ^{a}	4.33	2.13 ^{a}	2.73
1.0 IAA	6.67	1.00 ^{bc}	1.33	0.83 ^{ab}	0.87
1.5 IAA	0.00	0.00 ^{c}	0.00	0.00 ^{b}	0.00
0.5 IBA	10.79	2.33 ^{abc}	2.67	1.30 ^{ab}	1.47
1.0 IBA	0.00	0.00 ^{c}	0.00	0.00 ^{b}	0.00
1.5 IBA	0.00	0.00 ^{c}	0.00	0.00 ^{b}	0.00
0.5 NAA	9.73	3.00 ^{ab}	4.00	2.40 ^{a}	2.67
1.0 NAA	5.00	1.67 ^{abc}	2.00	1.00 ^{ab}	1.07
1.5 NAA	0.00	0.00 ^{c}	0.00	0.00 ^{b}	0.00
SEm±		0.84		0.51	
CD (0.05)		S		S	

Figures with the same alphabet do not differ significantly.



1- 1/2 MS + 0.5 mg l^{-1} IAA
2- 1/2 MS + 1.0 mg l^{-1} IAA
3- 1/2 MS + 1.5 mg l^{-1} IAA
4- 1/2 MS + 0.5 mg l^{-1} IBA
5- 1/2 MS + 1.0 mg l^{-1} IBA
6- 1/2 MS + 1.5 mg l^{-1} IBA
7- 1/2 MS + 0.5 mg l^{-1} NAA
8- 1/2 MS + 1.0 mg l^{-1} NAA
9- 1/2 MS + 1.5 mg l^{-1} NAA
10- 1/2 MS + 1.0 mg l^{-1} IAA +1.0 mg l^{-1} Kn

Fig. 8. Effect of various media combinations on *in vitro* rooting of microshoots of *Azadirachta indica* in half MS media



1- MS + 0.5 mg l^{-1} IAA
2- MS + 1.0 mg l^{-1} IAA
3- MS + 1.5 mg l^{-1} IAA
4- MS + 0.5 mg l^{-1} IBA
5- MS + 1.0 mg l^{-1} IBA
6- MS + 1.5 mg l^{-1} IBA
7- MS + 0.5 mg l^{-1} NAA
8- MS + 1.0 mg l^{-1} NAA
9- MS + 1.5 mg l^{-1} NAA

Fig. 9. Effect of various media combinations on *in vitro* rooting of microshoots of *Azadirachta indica* in full MS media

IBA, 1.5 mg l^{-1} IBA and 1.5 mg l^{-1} NAA did not showed any response for the induction of rooting. Maximum number of roots (4.33) were observed on media containing 0.5 mg l^{-1} IAA, which had a mean length of 2.13 cm. Mean length of roots was recorded as significant. It was found that MS + 0.5 mg l^{-1} IAA and MS + 0.5 mg l^{-1} NAA produced the highest mean length of roots and maximum length of roots respectively.

4.4.3 Planting out and acclimatization

Various media combinations were tried for acclimatization of *in vitro* produced plantlets. The results are given in Table 24. The *in vitro* raised plantlets were transferred to pots containing different potting media. The pots were then covered with polythene bags to maintain the humidity (Plate 9). After one week some holes were made in the polythene bags to reduce the humidity. The plantlets were gradually exposed to ambient condition by keeping them open (without polythene covers) for a few hours a day, later polythene covers were completely removed.

All the plants survived on vermiculite and vermiculite + sand (1:1) after one month (Plate 10). In all other treatments the plantlets died due to fungal infection at the basal portion.

4.5 CALLUS INDUCTION AND PROLIFERATION

4.5.1 Effect of auxins in MS on callus growth of *A. indica*

Different concentrations of three auxins, namely, IAA, IBA and 2,4-D were supplemented to MS medium to study their effect on callus induction and proliferation in nodal segments of *A. indica*. The data are presented in Table 25.

Among the auxins, highest percentage of callus initiation was recorded in media supplemented with 1.5 mg l^{-1} IAA. This treatment, however was on par with other treatments except 0.5 mg l^{-1} IBA (30.00%) and 1.0 mg l^{-1} IBA (26.67%). It was

Table 24. Effect of various media on acclimatization of the *in vitro* regenerated plantlet of *A. indica.*

Media	Survival percentage
Sand	0.00
Coco peat	0.00
Vermiculite	100.00
Soil rite	0.00
Sand + Vermiculite (1:1)	100.00
Sand + Vermiculite + Cocopeat (1:1:1)	0.00

observed that with increasing concentrations of IAA, percentage of callus initiation also increased, whereas no such trend was observed with respect to IBA and 2, 4-D. Treatment effect on time taken for callus initiation was found to be nonsignificant.

The average performance of all the levels of each auxin showed that 1.0 mg l^{-1} IBA in MS medium needed least time (21.83 days) to initiate callus, whereas it was delayed upto 43.57 days when the media was supplemented with 0.5 mg l^{-1} IBA. It was found that the growth performance of the callus was better, when the media was supplemented with 1.5 mg l^{-1} 2,4-D, which recorded a maximum growth score of 2.00 (Plate 11), when compared to the medium supplemented with 1.0 mg l^{-1} IBA. Cultures which were kept under dark condition failed to produce any callus.

Maximum callus index (93.10) was observed on media with 1.5 mg l^{-1} IAA when compared to media containing 1.0 mg l^{-1} IBA, which recorded a minimum callus index of 17.60. It was found that an increase in the values of callus index occurred with an increase in the concentration of different auxins, when supplemented to the media.

The callus morphology recorded at 50 days showed that the media containing 2,4–D produced more of light yellow callus, whereas media with IAA, IBA produced brown and light green colours, respectively.

4.5.2 Effect of combinations of auxins

4.5.2.1 Effect of IAA and IBA on callusing in MS media.

The data on the effect of various concentrations of IAA and IBA on callus initiation and growth are presented in Table 26.

The percentage of cultures initiating calli varied significantly. Percentage of callus initiation was highest on media containing 1.0 mg l^{-1} IAA + 1.5 mg l^{-1} IBA, 1.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA and 1.5 mg l^{-1} IAA + 1.5 mg l^{-1} IBA. Lack of callus initiation was noticed in media containing 0.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} IAA + 1.0 mg l^{-1} IBA.

Table 25. Effect of different concentrations of auxins on callus growth in *A. indica* in MS media

Treatment (mg l^{-1})	Percentage of cultures initiating calli (P)	No. of days for callus initiation	Growth Score (G)	Callus index	Callus morphology
MS + 0.5 IAA	50.00 ^{abc}	25.42	1.33	66.50	Brown
MS +1.0 IAA	63.33 ^{ab}	23.25	1.33	84.22	Brown
MS +1.5 IAA	70.00 ^{a}	25.76	1.33	93.10	Brown
MS +0.5 IBA	30.00 ^{c}	43.57	1.00	30.00	Light green
MS +1.0 IBA	26.67 ^{c}	21.83	0.66	17.60	Light green
MS +1.5 IBA	46.67 ^{abc}	28.88	1.00	46.67	Light green
MS +0.5 2,4 - D	36.67 ^{bc}	23.61	1.33	48.77	Light yellow
MS +1.0 2,4 - D	46.67 ^{abc}	25.39	1.66	77.47	Light yellow
MS +1.5 2,4 - D	43.33 ^{abc}	22.00	2.00	86.66	Light yellow
SEm±	8.39	4.32			
CD (0.05)	S	NS			

Figures with the same alphabet do not differ significantly.

Table 26. Effect of different concentrations of IAA and IBA on callus growth in *A. indica* in MS media

Treatment (mg l^{-1})		Percentage of cultures initiating calli (P)	No. of days for callus initiation	Growth Score (G)	Callus index	Callus morphology
IAA	IBA					
0.5	0.5	0.00 ^{d}	0.00 ^{d}	0.00	0.00	Light green
0.5	1.0	0.00 ^{d}	0.00 ^{d}	0.00	0.00	Light green
0.5	1.5	56.67 ^{ab}	23.84 ^{bc}	1.66	94.07	Light green
1.0	0.5	20.00 ^{c}	18.98 ^{c}	1.00	20.00	Light green
1.0	1.0	46.67 ^{ab}	23.97 ^{bc}	1.66	77.47	Light green
1.0	1.5	63.33 ^{a}	31.48 ^{a}	2.00	126.66	Light green
1.5	0.5	63.33 ^{a}	24.60 ^{b}	2.30	145.65	Light green
1.5	1.0	40.00 ^{b}	19.46 ^{c}	2.30	92.00	Light green
1.5	1.5	60.00 ^{a}	24.72 ^{b}	1.66	99.60	Light green
SEm±		5.88	1.56			
CD (0.05)		S	S			

Figures with the same alphabet do not differ significantly.

Treatment effect on time taken for callus initiation was found to be significant. The results revealed that the media containing 1.0 mg l^{-1} IAA + 0.5 mg l^{-1} IBA and 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} IBA was found to be significantly superior to MS + 1.0 mg l^{-1} IAA + 1.5 mg l^{-1} IBA. In the former treatment the time taken for callus initiation was found to be 18.98 days, and 19.46 days while in latter treatment that was 31.48 days.

Media containing 1.5 mg l^{-1} IAA + 1.0 mg l^{-1} IBA recorded the maximum growth score of 2.30. Callus morphology for all the treatment combination was recorded as light green. Maximum callus index was observed on MS + 1.5 mg l^{-1} IAA + 0.5 mg l^{-1} IBA (145.65). Rhizogenesis was observed in all the media combinations (Plate 13).

4.5.2.2 Effect of 2,4-D and IAA on callusing in MS media

Different combinations of 2,4-D and IAA were used to find out their effect on callus initiation and proliferation. The result obtained is presented in Table 27 and Fig.10.

Treatment effect on percentage of callus initiation and time taken for callus initiation was found significant. Percentage of callus induction was highest (83.33 %) on MS + 1.5 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IAA which was found to be significantly superior to MS+ 1.0 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IAA (43.33%). In case of time taken for callus initiation, media containing 1.0 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IAA (13.73 days) were found significantly superior to media containing 0 .5 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IAA which delayed the callus initiation upto 23.05 days.

Maximum growth score (3.00) and callus index (240.99) were recorded on media containing 1.5 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IAA. The morphology of callus was observed to be light brown in colour for all the treatment combinations.

Media containing 1.5 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IAA showed the differentiation of callus, which resulted in the development of leaf (Plate 12A and 12B).

Table 27. Effect of different concentrations of 2, 4 –D and IAA on callus growth in *A. indica* in MS media

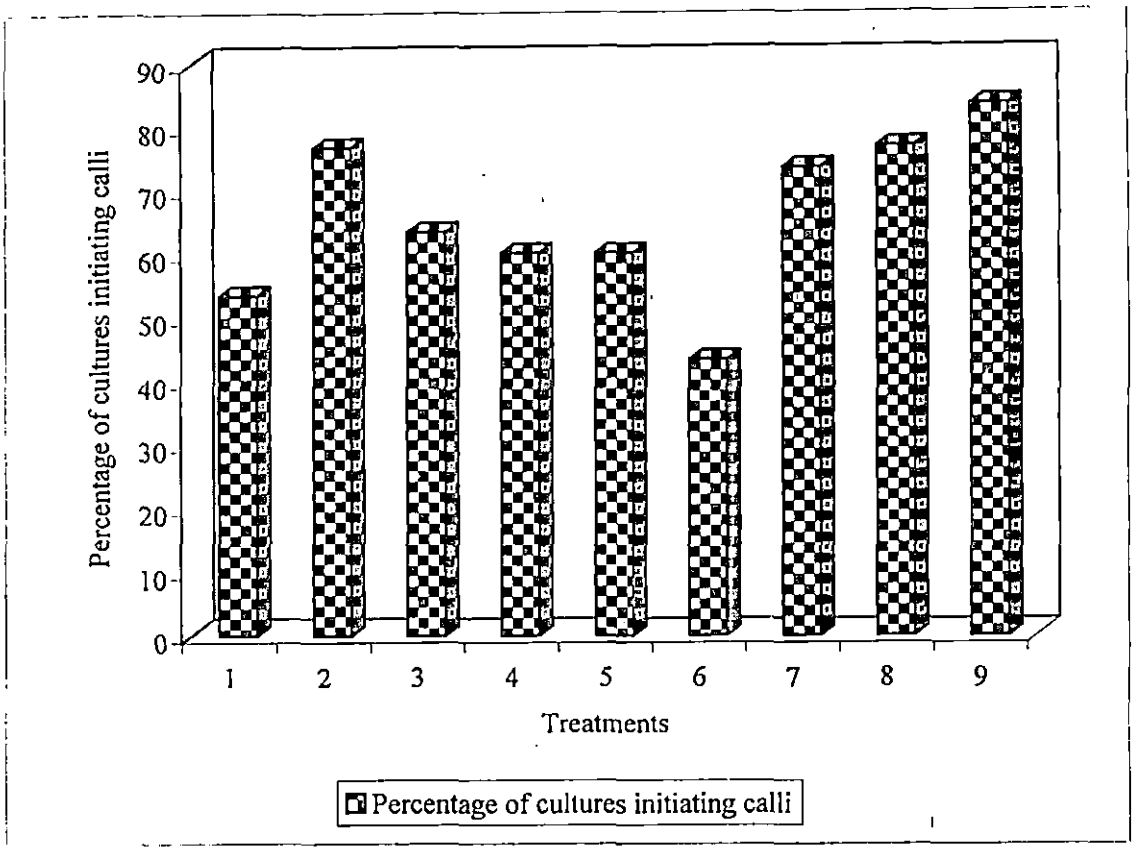
Treatment (mg l^{-1})		Percentage of cultures initiating calli (P)	No. of days for callus initiation	Growth Score (G)	Callus index	Callus morphology
2,4 - D	IAA					
0.5	0.5	53.33 ^{cd}	17.56 ^{abc}	1.66	88.52	Light brown
0.5	1.0	76.67 ^{ab}	23.05 ^{a}	1.33	101.97	Light brown
0.5	1.5	63.33 ^{bc}	20.05 ^{ab}	1.66	105.12	Light brown
1.0	0.5	60.00 ^{bcd}	16.62 ^{bc}	2.66	159.60	Light brown
1.0	1.0	60.00 ^{bcd}	12.48 ^{c}	2.33	139.80	Light brown
1.0	1.5	43.33 ^{d}	14.34 ^{bc}	1.66	17.92	Light brown
1.5	0.5	73.33 ^{ab}	16.36 ^{bc}	2.33	170.85	Light brown
1.5	1.0	76.67 ^{ab}	15.64 ^{bc}	2.66	203.94	Light brown
1.5	1.5	83.33 ^{a}	13.73 ^{c}	3.00	249.99	Light brown
SEm±		5.21	1.83			
CD (0.05)		S	S			

Figures with the same alphabet do not differ significantly.

Table 28. Effect of different concentrations of 2, 4 –D and IBA on callus growth in *A. indica* in MS media

Treatment (mg l^{-1})		Percentage of cultures initiating calli (P)	No. of days for callus initiation	Growth Score (G)	Callus index	Callus morphology
2,4 - D	IBA					
0.5	0.5	33.33 ^{bc}	22.62	1.66	55.32	Light brown
0.5	1.0	43.33 ^{b}	17.18	2.33	100.95	Light brown
0.5	1.5	16.67 ^{c}	12.30	1.33	22.17	Light brown
1.0	0.5	20.00 ^{bc}	13.25	1.66	33.20	Light brown
1.0	1.0	70.00 ^{a}	17.83	2.33	163.10	Light brown
1.0	1.5	73.33 ^{a}	16.23	2.00	146.66	Light brown
1.5	0.5	83.33 ^{a}	12.55	2.00	166.66	Light brown
1.5	1.0	73.33 ^{a}	15.02	2.66	195.05	Light brown
1.5	1.5	80. 00 ^{a}	16.25	2.66	212.80	Light brown
SEm±		7.70	3.32			
CD (0.05)		S	NS			

Figures with the same alphabet do not differ significantly.



1- MS + 0.5 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IAA

2- MS + 0.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} IAA

3- MS + 0.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IAA

4- MS +1.0 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IAA

5- MS + 1.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} IAA

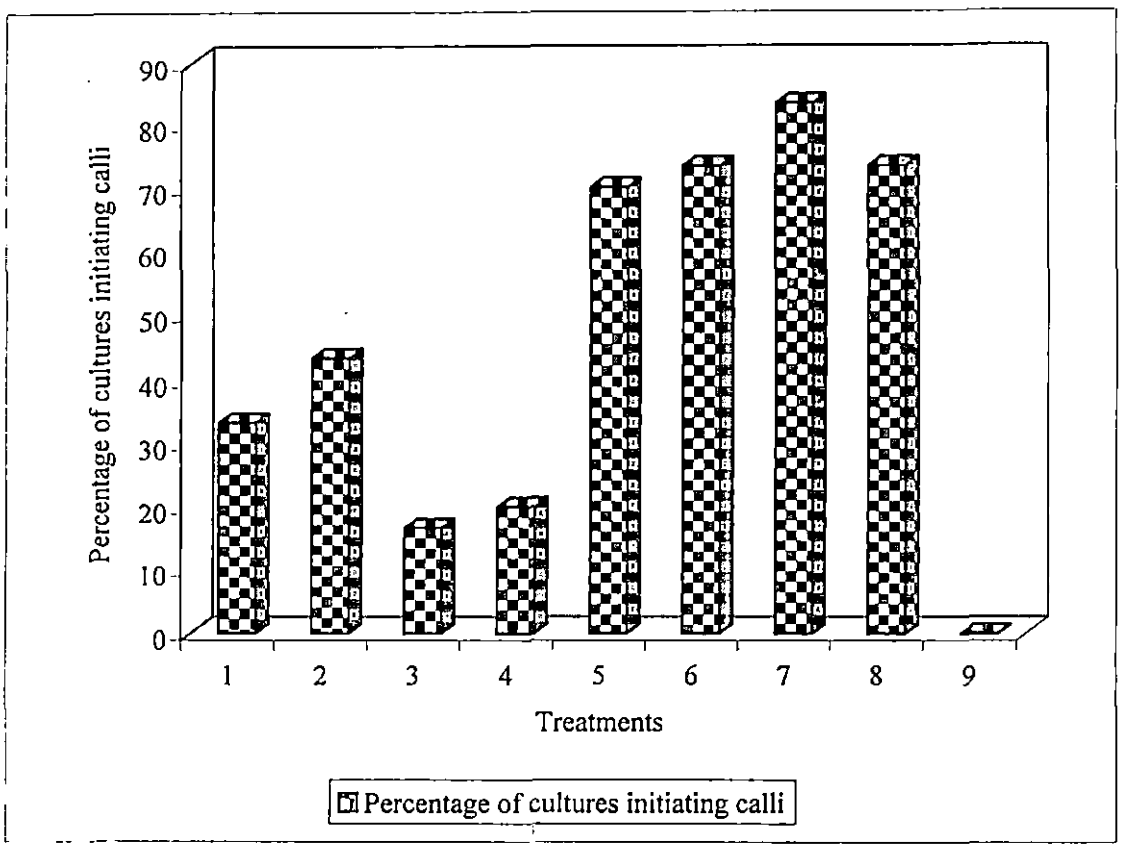
6- MS + 1.0 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IAA

7- MS + 1.5 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IAA

8- MS + 1.5 mg l^{-1} 2, 4-D +1.0 mg l^{-1} IAA

9- MS + 1.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IAA

Fig. 10. Effect of different concentrations of 2, 4-D and IAA on callus growth in *A. indica* in MS media



1- MS + 0.5 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IBA

2- MS + 0.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} IBA

3- MS + 0.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IBA

4- MS +1.0 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IBA

5- MS + 1.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} IBA

6- MS + 1.0 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IBA

7- MS + 1.5 mg l^{-1} 2, 4-D + 0.5 mg l^{-1} IBA

8- MS + 1.5 mg l^{-1} 2, 4-D +1.0 mg l^{-1} IBA

9- MS + 1.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IBA

Fig. 11. Effect of different concentrations of 2, 4-D and IBA on callus growth in *A. indica* in MS media

4.5.2.3 Effect of 2,4-D and IBA on callusing in MS media

Various combinations of 2,4-D and IBA were used to find out their effect on callus induction and proliferation. The result obtained are presented in Table 28 and Fig.11.

The treatment effect on percentage of callus initiation was found to be significant. Maximum percentage of callus initiation was found on media containing 1.0 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IBA (70.00%), 1.0 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IBA (73.33 %), 1.5 mg l^{-1} 2,4–D + 0.5 mg l^{-1} IBA (83.33 %), 1.5 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IBA (73.33%) and 1.5 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IBA (80.00%). Whereas, on MS + 0.5 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IBA recorded the minimum percentage of (16.67%) of callus initiation and it recorded the least number of days for initiating the callus growth. But it was delayed upto 22.62 days on MS + 0.5 mg l^{-1} 2,4–D + 0.5 mg l^{-1} IBA.

Highest callus growth score (2.66) was recorded on media containing 1.5 mg l^{-1} 2,4–D + 1.0 mg l^{-1} IBA and 1.5 mg l^{-1} 2,4–D + 1.5 mg l^{-1} IBA. In latter media it was observed that it recorded the highest callus index of 212.80. It was found that the entire callus produced were light brown in colour.

4.6 ESTIMATION OF AZADIRACHTIN FROM *IN VITRO* CULTURES

In the present study azadirachtin estimated from *in vitro* produced shoots, roots and callus. In case of shoots and roots the estimation was done on 18th and 30th day after initiation. For callus it was done at 21st and 50th day of growth. All the cultures were initiated and maintained in MS medium. Different levels of auxins and cytokinins were supplemented to the medium to find out its effect on azadirachtin production.

4.6.1 Estimation of azadirachtin using TLC method

4.6.1.1 Standardization of the procedure

Optimum quantity of sample for spotting in the TLC plates was standardized as 2.5 µl which gave better elution and separated compared to 5 µl.

Table 29. Pattern of chromatogram developed for *in vitro* and field grown samples of *A. indica* screened for the presence of azadirachtin using different solvent systems

Source	Growth regulators (mg l^{-1})	Solvent system (Methanol: water)	Rf value		Colour of the spot
			18th day	30th day	
Shoot	1.0 Kn	50: 50	0.85	0.85	Brown
	1.5 Kn		0.85	0.85	Brown
	0.5 IAA		0.85	0.84	Brown
	1.0 IAA		0.85	0.85	Brown
	1.5 IAA		0.85	0.85	Brown
	1.0Kn	60: 40	0.82	0.82	Brown
	1.5 Kn		0.82	0.82	Brown
	0.5 IAA		0.82	0.82	Brown
	1.0 IAA		0.82	0.82	Brown
	1.5 IAA		0.82	0.82	Brown
	1.0Kn	40:60	0.67	0.67	Brown
	1.5 Kn		0.67	0.67	Brown
	0.5 IAA		0.67	0.67	Brown
	1.0 IAA		0.67	0.67	Brown
	1.5 IAA		0.67	0.67	Brown
	1.0Kn	30:70	0.60	0.60	Brown
	1.5 Kn		0.60	0.59	Brown
	0.5 IAA		0.60	0.59	Brown
	1.0 IAA		0.60	0.60	Brown
	1.5 IAA		0.60	0.60	Brown
	Young leaves		0.60	0.60	Brown
	Mature leaves		0.60	0.60	Brown
Root	1.5 IAA	50: 50	-	-	-
		60:40	-	-	-
		40: 60	-	0.67	Light brown
		30:70	-	0.60	Light brown
			21st day	50th day	
Callus	1.0 IAA + 1.5 2, 4- D	50: 50	0.85	0.84	Brown
		60:40	0.82	0.82	Brown
		40: 60	0.67	0.67	Brown
		30:70	0.60	0.60	Brown

Different running solvent systems were evaluated for their suitability to elute azadirachtin from extracts of *in vitro* cultures. The elution pattern of the compound as indicated by their Rf values varied depending on the solvent system used (Table 29). While hexane: ethyl acetate (50:50) failed to elute the sample, methanol: water system exhibited separation of the compound with different Rf values. Under UV at 365 nm brown colour spots similar to standard azadirachtin were clearly visible from the eluted samples (Plate 14). A perusal of the data in Table 29 indicates that the movement of the compound varies with proportion of the chemicals in the solvent system. At higher levels of water the compound moved slowly as indicated by the low Rf values. This is equally applicable to all the samples tested.

The elution pattern of azadiractin with different solvent system is presented in Fig.12.

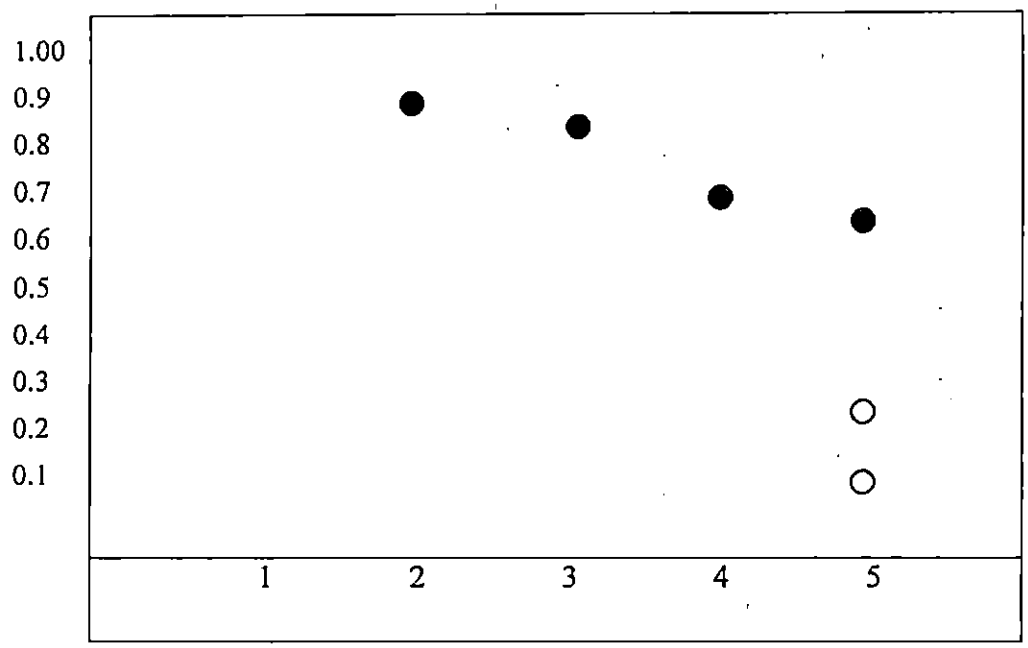
4.6.1.2 Screening for azadirachtin from in vitro samples of A. indica by TLC method

Azadirachtin content of the test samples under the influence of growth regulators as obtained from standard graph (Fig. 2) are given in Table 30. It is seen that shoot contains higher amounts of azadirachtin than callus and root. At higher maturity level of the tissue the azadirachtin content increases. This is found to be more evident in the case of callus, where at 21 days the azadirachtin content was 0.32 $\mu g\ g^{-1}$ while at 50th day the content was increased to 2.24 $\mu g\ g^{-1}$. In the case of roots azadirachtin could not be detected at young age (18 days). While the relatively mature roots (30 days) exhibited an average of 0.32 $\mu g\ g^{-1}$ of the compound.

Azadirachtin content in the shoot varied depending on the nutrient medium also. The highest amount of 3.51 and 6.71 $\mu g\ g^{-1}$ was estimated at 18th and 30th day, respectively, in MS medium containing 1.0 mg l^{-1} IAA. Auxin IAA in general appeared to induce higher production of azadirachtin in comparison to Kn.

4.6.2 Estimation of azadirachtin using colorimetric method

The data on the estimation of azadirachtin from *in vitro* produced shoot, root and callus of *A. indica* are presented in Table 31.



1. Hexane: Ethyl acetate (50:50)
2. Methanol: water (50:50)
3. Methanol: water (60:40)
4. Methanol: water (40:60)
5. Methanol: water (30: 70)

Fig. 12. Elution pattern of azadirachtin using different running solvent systems

Table 30. Screening for azadirachtin from *in vitro* samples of *A. indica* by TLC method

Source	Growth regulator ($mg\ l^{-1}$)	Azadirachtin content ($\mu g\ g^{-1}$)	
		18^{th} day	30^{th} day
Shoot	0.5 Kn	1.59	2.55
	1.0 Kn	0.96	1.59
	1.5 Kn	0.32	1.28
	1.0 IAA	3.51	6.71
	1.5 IAA	2.87	6.39
Root	1.5 IAA	-	0.32
Callus	1.0 IAA + 1.5 2,4 - D	21^{st} day	50^{th} day
		0.32	2.24

Among all the samples, shoot recorded the highest amount of azadirachtin (6.81µg g^{-1}) followed by callus (2.34 µg g^{-1}) and root (0.32 µg g^{-1}). It was observed that media supplemented with different growth regulators contributed the variation in the production of azadirachtin. The best media combination was found to be 1.0 mg l^{-1} IAA, followed by 1.5 mg l^{-1} IAA. In case of Kn, it was observed that at low concentration (0.5 mg l^{-1}), shoots yielded higher amount of azadirachtin (2.45 µg g^{-1}) when compared to higher concentration (1.5 mg l^{-1} Kn), which yielded 1.92 µg g^{-1} of azaidrachtin.

4.6.3 Estimation of azadirachtin from *in vivo* plant sample

In the present study azadirachtin was estimated from young as well as mature leaves from field grown plants. Results obtained based on the colorimetry and thin layer chromatography methods are presented in the Table 32.

It was observed that in both the methods mature leaves recorded higher azadirachtin content when compared to young leaves.

Table 31. Azadirachtin content estimated by colorimetric method in shoot, root and callus cultures of *A. indica* grown in MS medium.

Source	Growth regulator (mg l^{-1})	Azadirachtin content (µg g^{-1})	
		18th day	30th day
Shoot	0.5 Kn	1.76	2.45
	1.0 Kn	0.75	1.71
	1.5 Kn	1.28	1.92
	1.0 IAA	3.09	6.81
	1.5 IAA	3.10	6.49
Root	1.5 IAA	0.11	0.32
Callus	1.0 IAA + 1.5 2,4 - D	21st day	50th day
		0.75	2.34

Table 32. Estimation of azadirachtin from leaf samples of field grown plants of *A. indica*

Methods to estimate azdirachtin	Young fresh leaves	Mature leaves
Colorimetry method	9.58	12.45
TLC method	9.42	11.81

Discussion

5. DISCUSSION

The results of the present investigation in *A. indica,* undertaken to develop a workable protocol for *in vitro* propagation and evaluation of azadirachtin production in the plantlets and callus of neem, by using explants from mature tree are discussed below.

5.1 CULTURE CONTAMINATION

Microbial contamination is the single most important cause of losses in commercial and scientific plant tissue culture laboratories (Leifert and Woodward, 1998). All cultures will end up with contamination if the explant used is not properly disinfected. At times control of contamination is extremely difficult and with many contaminants it is impossible (Leifert and Woodward, 1998). Hence more emphasis will have to be placed on early detection and prevention of contamination at source itself.

In the present study, culture contamination was a serious problem since the mother plant were growing in the open field. It was observed that there was almost 100 per cent contamination during rainy season. This may be due to the congenial and conducive weather conditions which favour the rampant proliferation of microbial innoculam in the field.

Among the various measures taken to control culture contamination during the present study, a substantial reduction in contamination could be obtained by immersing the explants in 0.10 per cent mercuric chloride for 15 minutes. A further enhancement in the number of contamination free culture was noticed when the explants were dipped in a solution of Bavistin and Indofil M-45 (both at 0.1%) for 30 min and finally surface sterilized by using 0.10 per cent mercuric chloride for 15 min. Use of mercuric chloride as a surface sterilant was reported by a number of authors in *A. indica* (Joarder *et al.*, 1993; Nirmalakumari *et al.*, 1993; Thiagarajan and Murali, 1994; Anaz and Vijayakumar, 1996; Srividya *et al.*, 1998; Eeswara *et al.*, 1999).

The size of explants appears to have its effect on manifestation of culture contamination. Hussey (1983) quoted that, as a rule, larger the size of the explant more rapid is the growth rate and greater are the rates of survival. However, larger the explant size more will be the chance of harbouring contaminant microorganisms. If the explant size is small, the cut surface: volume ratio is high and there will be difficulty in the survival of the explant (Hussey, 1978). However in the present study it is seen that smaller explants (1.0 cm long) showed a higher contamination (79.22%) in comparison to the 2.0 cm long nodal explants (49.11%). Chavan *et al.* (1996) have also documented that larger explants (20mm) were better than smaller explants (10mm), when the axillary buds of jackfruit were cultured on MS media supplemented with various growth regulators.

It is always desirable for economic feasibility of tissue culture technique to have minimum percentage of contamination. In many cases the contamination was found as late as after two weeks of culturing and also with bud sprout. This may be due to the presence of latent spores of fungus or other micro organisms deep inside the tissues which easily get survived in the surface sterilization treatment and later when metabolic process starts their growth also is favoured and get expressed after a long gap.

5.2 BASAL MEDIA AND EFFECT OF VARIOUS GROWTH REGULATORS ON CULTURE ESTABLISHMENT AND GROWTH

The most extensively used culture media for micropropagation in trees are MS (Murashige and Skoog, 1962) and WPM (Llyod and Mc Cown, 1980). A comparative study of the two media on culture establishment and growth of axillary bud was carried out. Through initial screening it was found that MS medium showed significantly higher performance for the culture characters tested in comparison with WPM. In some of the earlier studies also Murashige and Skoog medium was reported as the best basal media for shoot production in *A. indica* (Drew, 1993; Yassen, 1994; Venkateswarlu *et al.*, 1998; Salvi *et al.*, 2001).

Considering the above observations, more detailed studies were carried out by supplementing the MS medium with various combinations of plant growth regulators for axillary bud cultures of *A.indica*

Ahmed (1990) reported that, in general BA has been frequently used to induce better shoot growth and multiplication than other cytokinins particularly in tree species. In the present study it was observed that kinetin yielded better results in comparison to BA. Lower concentration of Kn lead to the best growth of shoots and leaves when compared to BA (Table 12). Similar reports had been documented by many authors in species like *Hevea brasiliensis* Muell. Arg. (Mendanha *et al.*, 1998), *Jatropa curcas* (Sujatha and Mukta, 1996), *Syzygium cumini* (Roy *et al.*, 1996). Excess callus formation was observed at the base of the explant and the callus production increased with Kn concentration. It was observed that the shoot multiplication rate increased with the increasing of sub-cultures. Cultures of *Syzgium cumini* (Roy *et al.*, 1996) and *A. indica* (Joarder *et al.*, 1993) have also shown similar type of response to sub culturing. Callusing at the base of the explant was observed when the medium was supplemented with higher concentration of BA (2.0 mg l^{-1}), which showed the initiation of globular structure after 3 sub cultures of 17 days each (Plate 4). Similar results in *A. indica* were obtained by Joshi and Thengane (1993), when MS medium was supplemented with BA + IAA.

In plant tissue culture, auxins are extensively used to exploit its potentiality for stimulation of cell division and elongation. The requirement of these growth hormones vary considerably with reference to the types and its concentration. The response of the tissues to the various auxins is believed to vary depending upon their endogenous levels in the tissue. The best shoot growth of *Murraya koenigii* was obtained when the nodal segments were cultured on MS medium supplemented with various concentrations of NAA and IBA (Mathew *et al.*, 1999).

In the present study four levels of IAA, NAA and IBA were supplemented to MS. A comparision between the three auxins, points out that IAA has a better

response with reference to bud and leaf initiation. The superior response of IAA was evident in case of the average shoot length and produced a maximum of 2.00 multiple shoot in the media containing 1.5 mg l^{-1} IAA. The cotyledonary node and nodal explants of *Leucaena leucocephala* also recorded the same response, when IAA at different concentrations were supplemented to the MS medium (Nangia *et al.*, 1996).

It was observed that the maximum number of explants produced single leaf, emerging directly from the bud portion when the media was supplemented with 0.5 mg l^{-1} IAA and 1.5 mg l^{-1} IAA. The leaves exhibited chlorosis. Stem elongation was lacking in these media combinations. In case of NAA and IBA all treatments produced thickened roots at the base of the explant. Similar results had been reported in *A. indica* by Drew (1993).

The interaction of auxin and cytokinin is complex. Kaur *et al.* (1998a) reported that in *Acacia senegal* maximum number of shoot buds were found in MS medium supplemented with auxin and cytokinins. The inhibitory effect of higher concentration of cytokinin alone in media have been observed in earlier experiments. Lundergan and Janick (1980) reported that the auxin can nullify the suppresive effect of high cytokinin. A synergistic effect of cytokinin and auxin combination on shoot production has been reported in *Syzygium alternifolium* by Khan *et al.* (1997).

In the present study, a combination of BA and IAA (0.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA) were found to have synergistic effects in bud as well as leaf initiation from the nodal segments. It was observed that sub culture resulted in rapid shoot multiplication. BongHee *et al.* (1997) have reported that best shoot multiplication of *Ficus benjamina* was obtained on a medium containing cytokinin (BA) and auxins (IAA), compared with those containing BA alone. Species such as *A. indica* also exhibited similar results (Salvi *et al.*, 2001).

Combination of other two auxins, namely IBA and NAA along with BA seemed to be not as effective as the IAA combination, with respect to percentage of

bud and leaf initiation. However combination of Kn and NAA, was found to be effective when compared to other two auxins (IAA, IBA).

Media containing Kn and NAA has induced upto 91.11 per cent of bud initiation in MS + 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA. However, leaf initiation was found to be better at higher levels of cytokinin and auxins (Table 20). Callusing at the base of the explant was observed in all the above treatment combination.

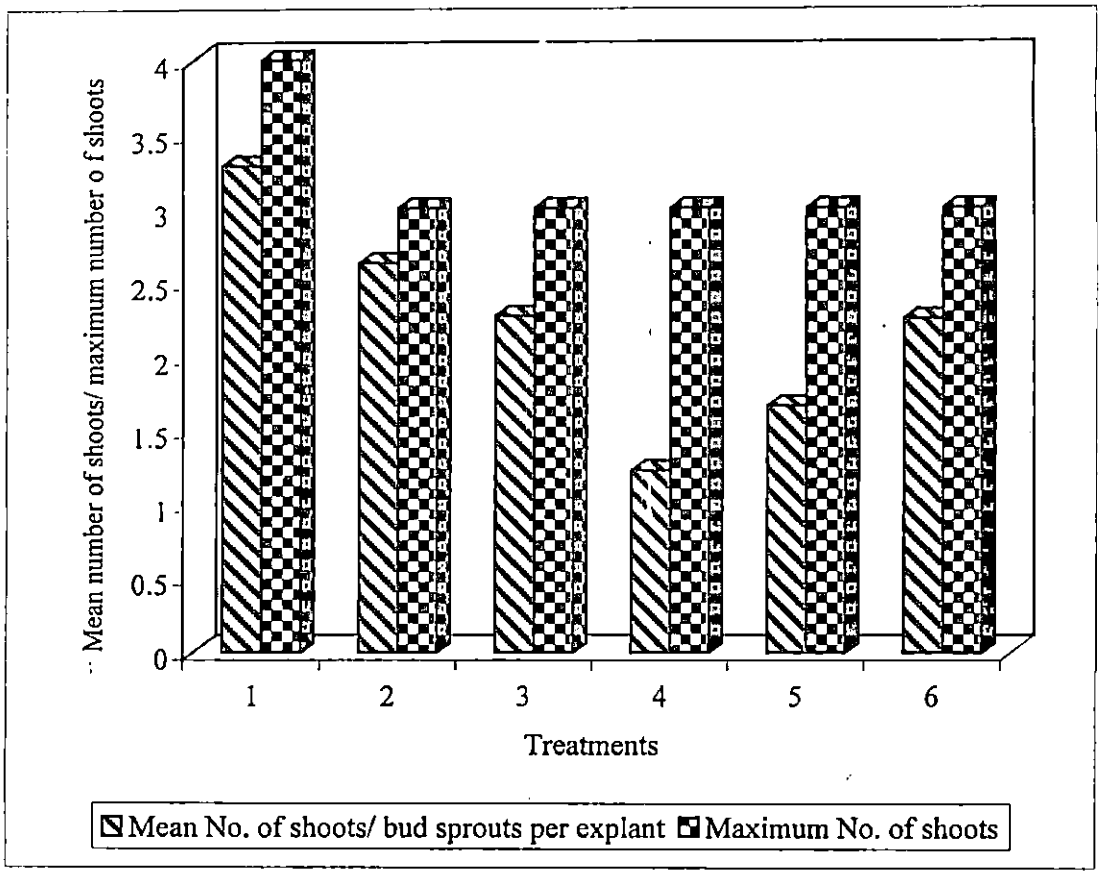
5.2.1 Multiple shoot

In commercial tissue culture, production of one shoot from one bud is neither economical nor desirable. So to decrease the cost of production of tissue cultured plants and to produce large number of planting material from scarce resource available, it is imperative to develop a procedure to obtain multiple shoots. These shoots can be obtained simultaneously or by subsequent sub culturing of single explant.

A normal explant (nodal segment) of neem has one bud owing to the alternate leaf arrangement. Thus one shoot from one explant was considered as normal (Plate 2). However, in some of the growth regulator combinations multiple shoots were obtained (Table 33 and Fig.13). Highest number of shoots per explant (4.00) was found in MS media supplemented with relatively high cytokinin concentration of 1.5 mg l^{-1} BA along with 0.5 mg l^{-1} NAA, indicating a quantitative interaction between NAA and BA on shoot differentiation. Such a quantitative interaction between auxin and cytokinin is also reported to occur in other tissue systems (Bhojwani and Johri, 1970). Multiple shoots were also recorded in the media combination containing Kn along with NAA and IBA. Similar results were obtained in *Moringa ptrerygosperma* (Vandina *et al.*, 1995). Combination of Kn and IAA on the other hand, failed to produce multiple shoots. Induction of multiple shoots have been reported in many species like *Dalbergia sissoo* (Gulati and Jaiswal,1996), *A. indica* (Srividya *et al.*, 1998; Khatun *et al.*, 1998), *Dalbergia lanceolaria* (Dwari and Chand, 1996), *Juniperus excelsa* (Negussie, 1997), *Acacia catechu* (Kaur *et al.*, 1998b).

Table 33. Some promising media combination which induced multiple shoots

Basal Media	Growth regulators (mg l^{-1})					Mean shoot length (cm)	Mean No. of shoots/ bud sprouts per explant	Maximum No. of shoots
	BA	Kn	IAA	IBA	NAA			
MS	1.5	-	-	-	0.5	1.67	3.29	4.00
	2.0	-	-	-	0.5	1.73	2.63	3.00
	1.0	-	-	0.5	-	3.40	2.27	3.00
	2.0	-	-	1.5	-	2.90	1.23	3.00
	-	1.0	-	1.5	-	3.83	1.67	3.00
	-	1.5	-	0.5	-	2.03	2.25	3.00



1- MS + 1.5 mg l^{-1}BA + 0.5 mg l^{-1} NAA
2- MS + 2.0 mg l^{-1} BA + 0.5mg l^{-1}NAA
3- MS + 1.0 mg l^{-1}BA+ 0.5 mg l^{-1} IBA
4- MS + 2.0 mg l^{-1} BA + 1.5 mg l^{-1} IBA
5- MS + 1.0 mg l^{-1} Kn + 1.5 mg l^{-1} IBA
6- MS + 1.5 mg l^{-1} Kn + 0.5 mg l^{-1} IBA

Fig. 13. Some promising media for multiple shoot induction

5.2.2 Rooting

Generally auxins promote rooting of micro shoots especially at lower concentrations (Minocha, 1987). A low salt concentration is found satisfactory for rooting of shoots in large number of plant species. Often where shoot multiplication was induced in full strength MS medium, the salt concentration was reduced to half when shoots were to be rooted. (Garland and Scholtz, 1981; Suwai *et al.*, 1988; Kumar and Kumar, 1997).

In our study, nineteen different treatments (ten in MS medium and nine in ½ MS) were tried for induction of *in vitro* rooting. Zaheer Ahmed *et al.* (1990) and Nirmalakumari *et al* (1993) reported that in *A.indica* auxins IAA and NAA supplemented in ½ MS had no effect on root induction. Whereas it was observed that the regenerated shoots showed rooting in ½ MS containing various concentrations of IBA. In the present study it was observed that treatment combinations containing lower levels of IAA and NAA in ½ MS induced lower percentage of rooting in comparison to IBA at lower concentration. Highest per cent of rooting (93.33) with average length of 3.30 cm was obtained on ½ MS + 1.5 mg l^{-1} IAA. Use of half strength basal media with various concentration of IAA as rooting medium is reported by many authors in a number of tree species such as *Syzygium travancoricum* (Anand *et al.*, 1999), *Syzygium alternifolium* (Khan *et al.*, 1997). *Acacia mangium* (Bhaskar and Subhash, 1996).

Full MS medium was not a favourable medium for inducing rooting of neem. At all levels of auxin supplementation in this medium, per cent of root induction, number of roots per shoot as well as length of roots were relatively less than that obtained in ½ MS media combinations. Probable reason may be that higher salt concentration in MS medium in comparison to ½ MS favoured the development of callus at the cut end of shoots.

5.2.3 Acclimatization and planting out

Acclimatization is critical to any micropropagation scheme since shoots and plantlets produced *in vitro* must be re adapted to the less humid environmental

conditions outside the culture vessels. A period of humidity acclimatization is considered necessary for the newly transferred plantlets to adapt to the outside environment. During this period the plantlets undergo morphological and physiological adaption enabling them to develop typical terrestrial plant water control mechanism (Hu and Wang, 1983; Sutter *et al.*, 1985). In the present investigation, high relative humidity was maintained during the initial period of planting out by covering the plantlets with polythene bags and spraying water regularly. The plantlets were gradually exposed to ambient conditions and later the covering was removed completely (Plate 10). Similar method of covering plantlets with plastic tent or glass followed by misting for the first few weeks in soil, subsequently removing the cover in a gradual process was successfully adopted by Murashige (1977). Plants are believed to develop their stomatal control mechanism during the period under high humidity, which helps them to reduce excessive water loss when planted out. Application of 5 to 10 ml nutrient solution containing MS organic salts at half concentration on alternate days enhanced the survival and promoted normal growth of the plantlets once they were planted out from the rooting medium.

Various media combination were tried (Table 24) for hardening. All the plantlets survived on vermiculite as well as on vermiculite + sand (1:1). Such hardening techniques have been documented in *Robinia pseudoacacia* (Wang *et al.*, 1985), *Morus alba* (Kanwar *et al.*, 1991) and *Tectona grandis* (Sharma, 2000). Use of vermiculite + sand (1:1) mix was also reported in *A. indica* (Venkateswarlu *et al.*, 1998).

5.3 CALLUS PRODUCTION

Callus, which shows stable characteristics under specific conditions after subculture through many successive passages, is a suitable material for cytodifferentiation. The advantage of using such callus is that it is composed of a fairly homogeneous mass of cells and can be proliferated in large amounts under known culture conditions (Razdan, 1993). The most serious drawback in the use of

callus culture is the possible genetic instability of the cells. However, successful micropropagation technique following this route have been reported in several species including *Sesbania bispinosa* (Sinha and Mallick, 1991), *Bauhinia purpurea* (Kumar, 1992), *Cephalotaxus harringtonia* (Wickremesinhe and Arteca, 1993), *Populus trichocarpa* x *Deltoides* cv. Hunnegem (Jehan *et al.*, 1994).

Callus cultures were successfully initiated from nodal segments of *A. indica* (Plate 11) when cultured on MS medium supplemented with various concentrations of auxins (IAA, IBA and 2,4-D) individually or in combination.

Mendanha *et al.* (1998) reported that leaf explants and axillary buds of *Hevea brasiliensis* Mull. Arg. formed calluses on MS medium supplemented with different combinations of IBA, IBA, 2,4-D, NAA and IAA. In the present study, it was observed that MS medium containing 1.5 mg l^{-1} IAA induced maximum percentage of callus when compared to other two auxins (IBA and 2,4-D). Schulz (1983) achieved callus induction using young leaf explants of *A. indica* in MS medium supplemented with IAA. However, the growth performance of the callus was better when the media was supplemented with 1.5 mg l^{-1} 2,4-D, which recorded a maximum growth score of 2.00. Medium MS supplemented with 2, 4-D is reported to be widely used in a number of species like *Taxus mairei* (Shuttwa *et al.*, 1996), *Bauhinia variegata* (Sumita *et al.*, 1995) and *Emblica officinalis* (Kant *et al.*, 1999). Sanyal and Datta (1986) reported that maximum initiation of callus from young stem base of *A. indica* was observed on medium supplemented with 0.5 mg l^{-1} IBA. However, in the present study, 0.5 mg l^{-1} IBA recorded the lowest percentage of callus initiation, even though the time taken for callus initiation was less (21.8 days).

The interaction of auxin and auxin is complex. Rao *et al.* (1988) reported that fresh cotyledons of *A. indica* produced maximum callus when cultured on MS medium supplemented with different concentrations of 2, 4-D and IAA. In the present study, a combination of IAA and IBA at lower concentrations failed to produce callus, whereas, with the increase in concentration of auxins the percentage of callus initiation

also increased. All the treatments producing callus recorded light green colour. Rhizogenesis was a regular occurrence in most of the callus cultures (Plate 13).

Positive effect of 2,4-D with IAA on callus initiation and organogenesis have been reported by many authors in a number of species such as *Casuarina equisitifolia* (Hann Chung and Lo, 1991), *Eucalyptus grandis* x *urophylla* (Zenn Zong *et al.*, 1996). Among the various combinations of 2,4-D and IAA, 1.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} IAA was found to have recorded maximum percentage of callus initiation (83.33%) growth score (3.00) and callus index (249.99). Organogenesis was observed on media containing 1.5 mgl^{-1} 2,4-D + 1.0 mg l^{-1} IAA.

Profuse callusing from the cut ends of leaf explants was obtained in *Madhuca latifolia* when cultured on different concentrations (0.1-10 mg l^{-1}) of 2,4-D and IBA (Chibbar and Bansal, 1998). In the present study, however, the nodal segments did not produce callus at lower concentrations of these auxins. Callus morphology was observed to be light brown in all the treatment combinations. Highest callus initiation occurred on media containing 1.5 mg l^{-1} each of 2,4-D and IBA, which aslo recorded the highest callus index of 212.8.

5.4 ESTIMATION OF AZADIRACHTIN

Alternative pesticides which are ecofriendly and easily degradable are becoming increasing important. One requirement for their utilization in agriculture is constant availability, with standard quality. Various secondary metabolites of plants are known to have insecticidal properties which can act as biopesticides of plant origin. The neem tree, *A. indica*, possesses insecticidally active compounds as secondary metabolites. Among them the most important is the triterpenoid azadirachtin, which has been found to be an antifeedant and a potent inhibitor of growth and development for a large number of pest species (Jacobson, 1986). To date, this compound has not been chemically synthesised. Due to the environmental and genetic factors, the content of azadirachtin in different parts of the trees varies

considerably. Further more, the tree does not grow in moderate climates and is not frost- tolerant. Therefore, in order to obtain constant amounts of standard quality azadirachin, it seems appropriate to employ tissue techniques for its production (Ascher, 1993).

It is well known that *in vitro* cultures are able to produce secondary metabolites, sometimes even in quantities that allow economically feasible production (Fujitha *et al.*, 1981; Hara *et al.*, 1987 and Fujitha, 1988). With this goal in mind in the present study azadirachtin was extracted and estimated from the *in vitro* grown plant samples, by using chromatography and colorimetry method.

The colorimetric method used in the present study allowed direct and rapid measurement of the total azadirachtin in the crude extracts of the plant samples. Commercially available azadiracthin (EID Parry India Ltd. Bangalore) was used to develop a calibration curve (Fig.2) to estimate the content of compounds related to azadirachtin as suggested by Dai *et al.*(1999). The same standard curve was used in both the methods to estimate azadirachtin content.

In TLC the elution and seperation of the compounds depends on the quantity of the extract that is spotted on the plate. Therefore in the present experiment different quantites of the extracts were spotted to find out the optimum amount required for maximum separation and resolution of the constituent compounds. It is already reported that when the concentrations of applied sample is high, the amount of constituent compounds is also high, the quantity of compounds accumulating at any particular Rf value increased proportinately (Hamilton and Hamilton, 1987). However, when higher quantites (> 5 ml)of the *in vitro* plant extracts were spotted on the plate, separation and clarity of the compounds were not clear. All the spots enlarged and merged to form a big smear of compounds on the chromatrogram. It can very well presume that high number of closely related compounds were there in the sample. The maximum separation of the eluted extracts was obtained when 2.5 ml sample was applied as a circular spot on the plate.

The solvent system used in a TLC also affects the elution and separation of the compounds in the extract. Touchstone and Dobbins(1978) have stated, that the Rf value of any spot depends on the polarity of the sample itself, the eluting solvent and the adsorbent (silica gel in this case). The theory states that, while moving upwards, the running solvents dissolve the compounds applied in the sample and carry them upward with themselves. A competition arises between the silica gel on the TLC plate and the running solvent for retention of the eluting sample. Though both of them try to retain the sample, only the one having a better ability to adhere the sample retains it by electrostatic force or covalent bonding. Adhesioin of the sample can also occur due to the attraction between the ions on the sample with those on the solvent or silica (polarity).

Among the different solvent systems attempted during the present study methanol: water was found to be suitable for elution and separation of the compunds. The proportion of water present in the mixture was found to directly effect the movement of the samples. It is known that water when added to methanol, increased the polarity value of the solvent mixture. This in turn was found to reduce the Rf value of the sample. On the other hand with lower proportion of water in the solvent mixture the distance travelled by the compound increased. The increased Rf value of the sample was found to be associated with lesser separation of the compounds, while a more discernable separation of the components could be seen at lower Rf values. The ideal solvent mixture as per the observations made in our present study was methanol: water(30:70), which gave an Rf value of 0.60 with a single spot, necessary for quantifications of azadirachtin. Hamilton and Hamilton (1987) have suggested that Rf value between 0.3 and 0.7 are better as they are not disturbed by either the less eluted samples usually lying below Rf 0.3 and due to fluctuations in solvent front, which occur normally above Rf 0.7. It has been already reported by Touchstone and Dobbins (1978) that solvent of low polarity produces low Rf values for highly polar samples and high Rf values for low-polar samples. Azadirachtin is known to be a highly polar compound (Huang and Morgan, 1990). This probably can be attributed as the reason for the slow movement of azadiracthin in the above mixture.

In order to detect the azadirachtin on the plates 5 per cent sulphuric acid in 96 per cent ethanol was used as a spray reagent (Wewetzer,1998). However, during the present investigation sufficient colour development was not obtained with this spray reagent. Acidified methanol solutions of vanillin have been used as spray reagents to visualize terpenes such as limonene (Eweig and Shermer, 1972) and limonoids such as azadirachtin (Yamasaki *et al.*,1986; Allan *et al.*, 1994) on thin-layer chromatography (TLC) plates.In our studies also azadirachtin was found to develop bright brown colour spots when one per cent vanillin in sulphuric acid reagent was sprayed on to the eluted plates.

Dai *et al.* (1999) estimated azadirachtin from the neem seed kernels, by using colorimetry method. They reported that the advantage of using vanillin as a colouring reagent helps in rapid and convenient estimation of relative azadirachtin content directly from crude extracts without the need to perform lengthy HPLC analysis.

A persual of the data obtained from the two methods indicated that the *in vitro* plant parts contain azadirachtin but in much lower concentrations in comparison to the *in vivo* materials. While the quantity of azadirachtin in the various *in vitro* plant parts ranged from 0.11 to 6.81 $\mu g\ g^{-1}$ of the plant sample, it ranged from 9.42 to 12.45 $\mu g\ g^{-1}$ in leaves of *in vivo* plants. In some of the earlier studies by Fu *et al.* (1998) the azadirachtin content in callus obtained from bark, stem, root and leaf explants was 0.40, 0.50, 0.57 and 0.63 $\mu g\ g^{-1}$ DW of the explants respectively. In case of dried seed kernel, azadirachtin content ranged from 0.14 to 1.66 per cent and the kernel oil contained 0.01 to 0.09 per cent (w/w of oil) (Rengasamy *et al.*, 1993). Azadirachtin has been reported in callus derived from explants such as leaf and bark (Wewetzer, 1998), but in extremely low concentrations.

Srividya *et al.* (1998) reported that nimbin was not detected in *in vitro* callus and root samples but was present in shoots at 0.003 mg g^{-1}. Azadirachtin was detected in roots (0.004 mg g^{-1}) and shoots (0.008 mg g^{-1}). They also observed that callus, had negligible amount of azadirachtin. The results obtained from our study recorded the highest azadirachtin content (6.71 $\mu g\ g^{-1}$) in shoots, followed by callus

(2.24 μg g^{-1}) and roots recorded a negligible amount of 0.32 μg g^{-1} of azadirachtin content in it.

The production of secondary metabolites is influenced by the nutrient medium and the growth regulators supplemented to *in vitro* plants / callus as reported by various authors in species such as *Catharanthus roseus* (Morris, 1986); *Ginkgo biloba* (Carrier *et al.*, 1990) etc. In the present investigation it was observed that MS medium suppplemented with various concentrations of auxins and cytokinins inidividually or in combination recorded different azadirachtin content. Maximum azadirachtin content (6.81μg g^{-1} of shoot) was recorded on medium supplemented with 1.0 mg l^{-1} IAA. Wewetzer(1998) reported that the azadirachtin content was three times higher on MS medium supplemented with 15 g l^{-1} sucrose (<0.5-64 μg g^{-1} DW) as compared with those having 30 g l^{-1} sucrose (<0.5-22 μg g^{-1} DW). Fu *et al.* (1998) reported that high ammonium salt in MS medium resulted in the greatest azadirachtin content in callus. They also observed that White medium, without ammonium salt, resulted in a slow growth rate of the callus, which gave low azadirachtin. But callus growth and azadirachtin formation was found at the second or third sub culture.

Expression of secondary products in plant is dependent on the stage of maturity. Mizrahi (1988) emphasized the necessity of harvesting medicinal crop species at optimum harvest stages, in the context of synthesis of secondary compounds at certain stages of maturity of specialized cells. In cultured materials also such relation between growth stage and metabolite production was observed. It was found that with the increase in the age of cultures the azadirachtin content in the plant sample also increased (Table 30 and 31). Correlation between morpho differentiation and metabolite expression has been observed in *in vitro* cultures of other species such as *Nothapodytes foetida* (Roja and Heble, 1994), *Atropa belladonna* (Eapen *et al.*, 1978) and Opium poppy (Yoshikawa and Furaya, 1982) .

Azadirachtin is a non–volatile and highly polar compound which is unsuited for gas chromatography as well as liquid chromatography (Huang and Morgan, 1990). In the present study a simple and rapid method for the preliminary

estimation of azadirachtin from the crude extracts of neem could be used in the analysis of commercial neem extracts. In both these methods comparable estimates of azadirachtin has been obtained. These results show that *in vitro* cultures are capable of producing azadirachtin in quantities above the limit of detection, depending on nutrient medium, size of explant etc. The quantity of azadirachtin produced has to be increased many fold before large scale production can be considered seriously, so that it becomes economically feasible.

Summary

6. SUMMARY

The Research programme entitled "Micropropagation and evaluation of azadirachtin production in the plantlets of neem (*Azadirachta indica* A. Juss.)" was undertaken in the Tissue Culture Laboratory of the College of Forestry and Biochemistry Laboratory, College of Horticulture, Vellanikkara during the period 2000-2002. The salient findings from the study are highlighted below:

1. The culture contamination was noted to be one of the major problems confronted in neem tissue culture. A fungicidal dip of 0.1 per cent each of Bavistin (Carbendazin) and Indofil M-45 (Mancozeb) for 30 minutes and 15 minutes surface sterilization with 0.10 per cent $HgCl_2$ reduced the percentage of culture contamination to less than 40 per cent. The percentage of culture contamination was found to be high during rainy months.

2. Size of the explant had a significant effect on culture contamination. Microbial contamination was low in large sized (2.00 cm long) explants compared to the small sized (1.00 cm long) ones.

3. Out of the two basal media tried, Murashige and Skoog (MS) medium was found to be better than woody plant medium (WPM).

4. It was observed that lower concentration of Kn in MS had pronounced effect on culture establishment and growth.

5. Among the auxins (IAA, NAA and IBA), IAA was found to be better in terms of culture establishment and growth in MS medium.

6. Medium containing 0.5 mg l^{-1} BA + 1.5 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 0.5 mg l^{-1} IAA, 1.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA, 1.0 mg l^{-1} BA +1.5 mg l^{-1} IAA, 2.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA and 0.5 mg l^{-1} Kn + 1.0 mg l^{-1} NAA were found better than all other treatment combinations in terms of high percentage of bud and leaf initiation.

7. Combination of MS + 1.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA was found better for the induction of multiple shoots (4.00 number).

8. For *in vitro* rooting of shoots, half strength MS media supplemented with 1.5 mg l^{-1} IAA was found to be effective.

9. Out planting in sterile vermiculite or in sand + vermiculite (1:1) at high humidity condition and gradually bringing to ambient condition facilitated acclimatization of *in vitro* plantlets.

10. Callus cultures were successfully initiated from nodal segments, when MS media were supplemented with auxins (IAA, IBA, 2,4-D).

11. Among the several auxins tried 1.5 mg l^{-1} IAA showed maximum percentage of initiation of calli. However, the growth performance of the callus was better on MS + 1.5 mg l^{-1} 2,4-D.

12. Combination of 1.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IAA produced maximum callus followed by that of 1.5 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} IBA.

13. Organogenesis was observed on medium supplemented with 1.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} IAA.

14. Azadirachtin content from *in vitro* and *in vivo* plant sample were estimated by using TLC and colorimetry method.

15. Regarding the standardization of procedure of the TLC method to estimate azadirachtin from *in vitro* cultures, the running solvent system comprising of methanol: water (30: 70) was found to be the best for eluting azadirachtin into a single condensed spot.

16. To detect azadirachtin from *A. indica* on TLC plates, one per cent vanillin in concentrated sulphuric acid was found to be the best spray reagent.

17. *In vitro* plants showed different quantities of azadirachtin at different stages (18^{th} and 30^{th} day for shoot and root whereas, 21^{st} and 50^{th} day for callus) of their growth.

18. Variation in the content of azadirachtin was noticed depending on the growth regulator in the medium. Maximum azadirachtin content (6.81µg $g^{-1}$of shoot) was recorded on medium supplemented with 1.0 mg l^{-1} IAA.

19. Azadirachtin content in the various *in vitro* plant parts ranged from 0.11 to 6.81 µg g^{-1}. It ranged from 9.42 to 12.45 µg g^{-1} in leaves of *in vivo* plants.

20. TLC and colorimetry method can be used for the preliminary estimation of the azadirachtin from the crude extracts of the plant sample.

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* Originals not seen

Plates

Plate 1. Bud sprout in nodal segment of neem

Plate 2. Initiation of leaves from axillary bud of neem

Plate 3. Leaf enlargement and internodal elongation

Plate 4. Formation of globular structure in MS + 2.0 mg l^{-1} BA


1


2


3


4


5


6


7


8


9


10

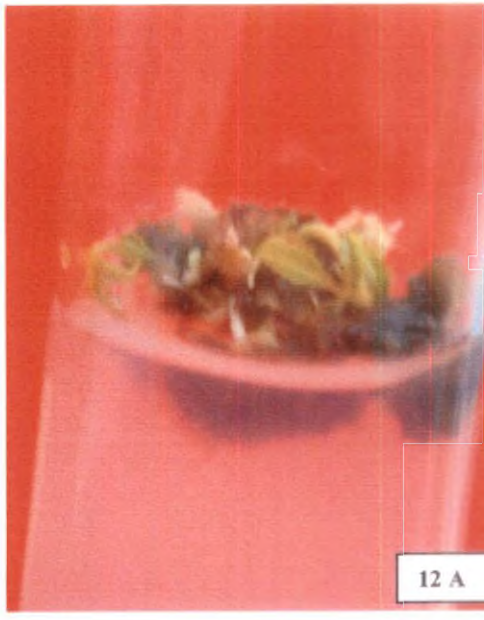
Plate 11. Callus induction in nodal segments of neem in MS + 1.5 mg l^{-1} 2, 4 – D after 22 days

Plate 12A . Organogenesis in neem in MS + 1.5 mg l^{-1} 2, 4 – D + 1.0 mg l^{-1} IAA

Plate 12B . Organogenesis in neem in MS + 1.5 mg l^{-1} 2, 4 – D + 1.0 mg l^{-1} IAA

Plate 13. Rhizogenesis of neem in MS + 1.5 mg l^{-1} IAA + 1.5 mg l^{-1} IBA


11


12 A


12 B


13

Plate 14. Thin layer chromatography of azadirachtin from *in vitro* cultures and leaves from field grown plants of *A. indica*

Plate 14 A .Shoot – 1.MS + 1.0 mg l^{-1} Kn (18 day)

2. MS + 1.0 mg l^{-1} Kn (30 day)

3. MS + 1.5 mg l^{-1} Kn (18 day)

4. MS + 1.5 mg l^{-1} Kn (30 day)

5. Standard

Plate 14 B. Shoot –1. MS + 0.5 mg l^{-1} IAA (18 day)

2. MS + 0.5 mg l^{-1} IAA (30 day)

3. MS + 1.0 mg l^{-1} IAA (18 day)

4. MS + 1.0 mg l^{-1} IAA (30 day)

5. Standard

Plate 14 C. 1. Standard

Root -2. MS + 1.5 mg l^{-1} IAA (18 day)

3. MS + 1.5 mg l^{-1} IAA (30 day)

Callus- 4. MS + 1.0 mg l^{-1} IAA + 1.5 mg l^{-1} 2, 4 - D (21 day)

5. MS + 1.0 mg l^{-1} IAA + 1.5 mg l^{-1} 2, 4 - D (50 day)

6. Standard

Plate 14 D. 1. Standard

Shoot- 2. MS + 1.5 mg l^{-1} IAA (18 day)

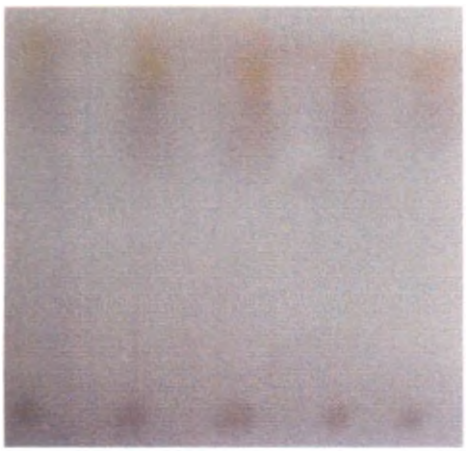
Shoot -3. MS + 1.5 mg l^{-1} IAA (30 day)

4. young leaf from field grown plants

5. mature leaf from field grown plants

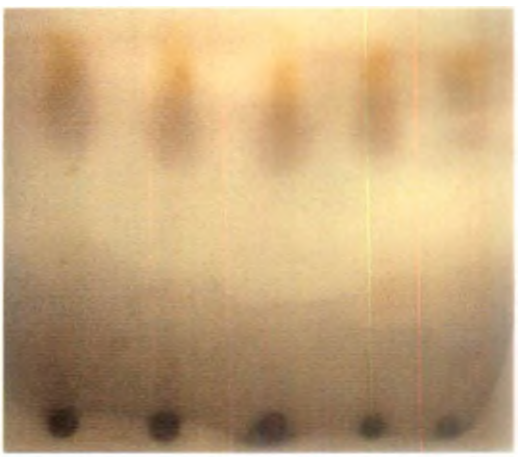
All the samples were eluted in methanol: water (30: 70) and samples were developed by spraying with one per cent vanillin in concentrated sulphuric acid.

Plate 14. Thin layer chromatography of azadirachtin from *in vitro* cultures and leaves from field grown plants of *A.indica*



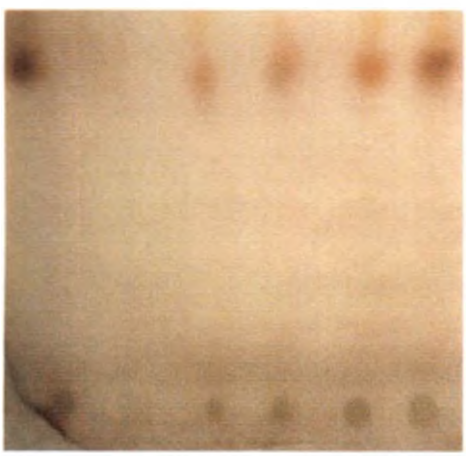
1 2 3 4 5

14 A



1 2 3 4 5

14 B



1 2 3 4 5 6

14 C

1 2 3 4 5

14 D

MICROPROPAGATION AND EVALUATION OF AZADIRACHTIN PRODUCTION IN THE PLANTLETS OF NEEM

(Azadirachta indica A. Juss.)

By

ROSHNI, A. J.

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

The study under the title "Micropropagation and evaluation of azadirachtin production in the plantlets of neem (*Azadirachta indica* A. Juss.)", was carried out at the Tissue Culture Laboratory of College of Forestry and Biochemistry laboratory, College of Horticulture, Vellanikkara during the period 2000-2002. The objective of the programme was to standardize the micropropagation protocol and also to evaluate the secondary metabolite production potential of *in vitro* produced plantlets and callus in neem (*Azadirachta indica* A. Juss.).

Culture contamination mainly due to fungus was prominent in the rainy season. To get contamination free cultures, dipping of explants in a fungicidal mixture of 0.1 per cent each of Bavistin (Carbendazim) and Indofil M-45 (Mancozeb) for 30 min and their sterilization with mercuric chloride (0.10%) for 15 min was found effective in controlling the contamination. Larger sized explants (2.00 cm long) with significantly low culture contamination was found to be better than 1.00 cm long explants.

Murashige and Skoog (MS) medium was found to be better than WPM for culture establishment and growth individual supplementation of Kn to MS medium was found more effective than BA. MS medium supplemented with 1.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA was found to be the best media for shoot proliferation.

Maximum *in vitro* rooting (93.33%) of microshoots was obtained on ½ MS + 1.5 mg l^{-1} IAA. Vermiculite and vermiculite + sand (1:1) were found to be the best media for hardening of *in vitro* raised plantlets.

The auxins evaluated for stimulating callus production were, IAA, IBA and 2,4-D among them IAA was the most potent in callusing followed by 2,4-D. The combination of 1.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} IAA and 1.5 mg l^{-1} 2,4-D + 1.5 mg l^{-1} IBA produced maximum callus.

Azadirachtin content was estimated by using TLC and colorimetry techniques. In the case of TLC for eluting azadirachtin into a single condensed

spot, the running solvent system comprising of methanol: water (30:70) was found to be the best. One per cent vanillin in concentrated sulphuric acid was used as a spray reagent to detect the azadirachtin on TLC plates. Amount of azadirachtin varied depending on the plant part used which was estimated at various growth stages and different concentration of growth regulators supplemented to the medium. It ranged from 0.11 to 6.81 $\mu g\ g^{-1}$ in *in vitro* plants sample and 9.42 to 12.45 $\mu g\ g^{-1}$ in leaves of *in vivo* plants. Both these methods can be followed for the preliminary estimation of azadirachtin.