IN VITRO SHOOT REGENERATION AND MICROGRAFTING IN NUTMEG (*Myristica fragrans* Houtt.)

By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

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DECLARATION

I hereby declare that the thesis entitled **"*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, associateship, fellowship or similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis, entitled **"*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)"** is a record of research work done independently by **Liffey Zachariah Antony** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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We, the undersigned members of the Advisory Committee of **Liffey Zachariah Antony**, a candidate for the degree of Master of Science in Agriculture, with major in Plant Biotechnology, agree that the thesis entitled **"*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)"** may be submitted by Liffey Zachariah Antony in partial fulfillment for the requirement for the degree.

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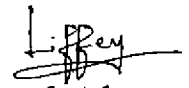

Liffey Zachariah Antony

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ABBEVIATIONS

A.C.	:	Activated charcoal
µM	:	Micro Molar
2,4-D	:	2,4-dichlorophenoxy acetic acid
2ip	:	2-isopentenyl adenine
BAP	:	Benzylamino purine
CH	:	Casein hydrolysate
CW	:	Coconut water
GA	:	Gibberellic acid
hr	:	hour
IAA	:	Indole-3-acetic acid
IBA	:	Indole butyric acid
Kin	:	Kinetin, N^6 – furfuryl acetone
$mg\ l^{-1}$	:	Milligram per litre
min	:	Minute
MS	:	Murashige and Skoog's (1962) medium
NAA	:	α – Naphthalene acetic acid
ppm	:	parts per million
rpm	:	Revolutions per minute
s	:	second
TDZ	:	Thidiazuron
SH	:	Schenk and Hildebrandt (1972) medium
UV	:	Ultraviolet
v/v	:	Volume in volume

Introduction

INTRODUCTION

Nutmeg, (*Myristica fragrans* Houtt.), a member of myristicaceae family originated in the Moluccas islands of Indonesia are now grown in tropical countries of the world. The major nutmeg producing countries are Indonesia, Greneda, India, China and Srilanka. In India, it is grown in Kerala, Karnataka, Tamil Nadu and Maharashtra. This unique spice produces two separate spices namely the nutmeg (kernel of seed) and the mace (the aril covering the seed). In habit, it is a perennial tree and is a suitable intercrop for coconut and arecanut gardens of southern states. This species is dioecious and produces dimorphic branches i.e., erect growing orthotrops and horizontally growing plageotrops. The total area under nutmeg cultivation in India and Kerala are 11984 and 10780 ha respectively. The total production in Kerala is 2746 tonnes in 2005-2006 (Spices Board, 2007).

The export of Nutmeg and Mace from India is 2100 MT during 2006-07. The produce, i.e. nutmeg kernel and aril find varied uses in culinary and indigenous systems of medicines. Mace is used as a spice for savoury dishes. The pericarp is used for pickling. Nutmeg acts as stimulant and carminative. The dioecious nature and long gestation period of the crop causes difficulty in getting quality planting material of known sex. In nutmeg plantations, the ratio of male to female should be 1:10, but in natural seedling population the ratio of male to female is 1:1. So vegetative propagation has relevance in nutmeg cultivation for planting quality seedling of known sex. Raising plantlet from rooted cutting is impossible as regeneration potential under *in vivo* conditions is very limited.

At present, the problems due to dioecious nature of plant are overcome by using grafts, budded plants and *in situ* grafting and budding. For getting erect tree with proper upright architecture, scion material should be from orthotrops. In nutmeg, there is difficulty in getting orthotropic shoots, as the production of orthotropic shoots is confined to single meristematic point of the main stem. Very rarely few orthotropic shoots will be produced in mature trees of 15 or 20 years. So always there is a dearth of orthotropic scion materials for grafting and nutmeg grafts are charged high. So the problem; i.e. "*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)" was selected.

Due to scarce land resources and limited labour force, less labour intensive shade loving cash crops are best suited to Kerala condition. Nutmeg comes in that category and its cultivation is at present profitable. In this context, development of an efficient *in vitro* regeneration system for multiple shoot production and standardizing micrografting with regenerated shoots will be useful for the production of quality planting material of known sex in large numbers. Therefore, the present investigation was undertaken with the following objectives:

i. To identify *in vitro* culture conditions and suitable explants for multiple shoot induction.
ii. To standardize micrografting technique with *in vitro* and *in vivo* shoots as scion.

Review of literature

REVIEW OF LITERATURE

Among the crops used by humankind, the history of spices is perhaps the most adventurous and the most romantic. From the dawn of civilization spices were sought after eagerly. The recorded history of the use of spices goes back to about five thousand years or so from the Indus Valley Civilization. Spices were most valuable among the folk medicinal plants, infact species were valued more as medicinal during the early times and that the use in food became popular probably much later. Spices originating from tree crops are routinely called "Tree Spices". There are 17 tree spices, commonly grown in India, most of them occur naturally in humid tropical forests of South and South East Asia, Pacific Islands and Tropical America. Among them nutmeg, clove, cinnamon, tamarind, garcinia, kokum, curry leaf and allspice are economically important ones. Nutmeg is the only tree that produces two separate spices, namely, nutmeg (kernel of seed) and mace (aril covering seed). Nutmeg belongs to Myristicaceae, one of the most primitive families of dicots.

Nutmeg is a stimulant, carminative, astringent, aphrodisiac and hallucinogenic. Mace is used for flavouring cigarettes and chewing to mask foul breath. Oil of nutmeg or mace is employed for flavouring food products and liquors, soaps, tobacco, dental creams and perfumery products. The volatile oil from the leaf has weedicidal properties. Nutmeg butter is used as a stimulant in ointments, hair lotions and is used in cases of rheumatism, paralysis and sprains. The fleshy pericarp of the fruit is used for making pickles and jelly. Pruthy and Krishnankutty (1984) obtained quality pectin from nutmeg waste (rind).

2.1 Botany of nutmeg

The genus Myristica consists of about 120 species of which five have been described from India. They are *M. fagrans* Houtt., *M. malabarica* Lamk., *M. magnifica* Bedd., *M. beddomei* King. and *M. contorta* Warb. (Santapau and Henry, 1973). Nutmeg is dioecious evergreen aromatic tree usually 10-20 m in height with spreading branches which carry oblong-ovate leaves, acute at apex and base (Varghese, 2000). Nazeem (1979) observed that the shoot growth in nutmeg is cylindrical, a period of growth

followed by quiescence. Six flushes were observed in a year. All the flushes were not seen in all the shoots, which resulted in continuous growth. Two growth peaks were observed, in May-June and September. Joseph (1980) observed that inflorescence is branched raceme in male plant and cyme in female plants. Flowers are drooping, creamy yellow and fragrant.

2.2 Propagation in nutmeg

The general method of propagation is through seeds collected from regular bearing and high yielding trees, yielding more than 10000 fruits per tree per year and having 30g weight per fruit, 1g fresh mace per fruit and 10g fresh weight of nuts per fruit (Bavappa and Ruettimann, 1981). Seeds from healthy well ripe fruits that are naturally split and harvested during June-July are used. Seeds have low viability and hence are to be sown immediately after collection. They can be preserved in moist sand for 3-4 for days (Mathew, 1992). Old seeds and those in which kernels rattle inside the shell will not germinate. Regular watering is necessary for germination (Krishnamoorthy and Rema, 1988).

Nutmeg being a dioecious crop, proper ratio of female and male plants is to be maintained in the plantation. Vegetative propagation is the practical way to achieve this. Various vegetative propagation methods were tested to develop a suitable method for relatively rapid multiplication. Vegetative propagation techniques like epicotyl grafting, approach grafting, budding and top working were tried in nutmeg with varying degree of success. In C.P.C.R.I., Kasargod, Mathew and Joseph (1982) tried epicotyl grafting with 48% survival of grafts. According to them the procedure for epicotyl grafting is as follows. Take twenty days old healthy rootstock. The scion should be of a lead pencil thickness from a high yielding tree. Cut the top and make a vertical slit to a length of 3-4 cm in the root stock. Make a wedge cut of 3-4 cm in the scion and insert the scion into the slit and tie it firmly with a polythene strip. The grafted portion is covered with a polythene bag to ensure high humidity which helps in graft union. Remove the cover bag when new leaf emerges and remove the polythene stripe. Haldankar (1999) observed that

maximum percentage of successful grafts could be obtained if grafting was carried out in August – September (80%).

2.3 General aspects of plant tissue culture

Schleiden and Schwann (1839) postulated the cell theory, which revealed the totipotent nature of plant cell. This forms the basis for plant cell, tissue and organ culture. Haberlandt (1902) reported that isolated cells are capable of resuming uninterrupted growth. Skoog and Miller (1957) with their discovery of auxins and cytokinins made a land mark in the history of plant tissue culture. They formulated the concept of hormonal control of organ formation and showed that root and shoot differentiation was a function of auxin-cytokinin ratio and that it could be regulated by altering the relative concentrations of these growth regulators in the medium. Many pioneer investigators like White (1934), Gautheret (1939) Nobecourt (1939), Miller *et al.* (1956), Reinert (1958), Steward *et al.* (1958), Bergmann (1960) and Vasil and Hildebrandt (1965) have contributed for the successful development of plant tissue culture concepts. A completely defined medium for plant tissue culture was developed by Murashigae and Skoog (1960).

Several aspects of plant tissue culture are being applied in agriculture which include the production of haploid plants, secondary metabolite production, embryo rescue techniques etc. however, the best commercial application of tissue culture is the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981). Murashigae (1974) advocated the possibility of three routes of *in vitro* propagule production, which included enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants.

2.3.1 Factors influencing success of *in vitro* propagation

Success of in vitro propagation depends on several factors directly or indirectly. These factors include genotype of the source plant, age of explant, season of collecting the explant, surface sterilization, systemic contaminants, presence or absence of other media additives, pH, quality and intensity of light and relative humidity (Brown and Thorpe, 1986).

2.3.2 Genotype

Successful application of *in vitro* technology to the production of clone depends upon inducibility of growth and differentiation in tissue of woody plants and the regeneration of true to type viable plants in selected genotypes. Even within a single species, it may vary with varieties. Influence of genotype on the callusogenesis of hypocotyl explants of *Cuphea wrightii* and *C. procumbens* have been reported by Truta *et al.* (2002). However, Mallika *et al.* (1997) observed in nutmeg that there is no genotypic influence on the *in vitro* shoot initiation.

2.3.3 Season for collecting explants

The success of plant tissue culture is influenced to a larger extent by the season of explant collection. Spring season (March-April) is the best time to initiate tissue culture from mature trees. In *Corylus avellana,* Messeguer and Mele (1987) noted that at least 95% aseptic shoot cultures were obtained and buds flushed within 10-12 days in spring season as compared to 5-6 weeks during other seasons. In chestnuts, shoot explants taken during mid May gave rise to plantlets successfully (Chauvin and Salesses, 1988). Yu (1991) reported that in Litchi (*Litchi chinensis*), the explants collected after 10 continuous rainy days was cent percent contaminated and that taken after 15 continuous sunny days had a contamination rate of 20% only. Thakar and Bhargava (1999) reported that in the medicinal tree *Gmelina arborea,* the axillary buds collected during summer responded better to *in vitro* culture than in winter. Mahale *et al.* (2005) reported that in

case of Tamarind, bud break in *in vitro* culture was noticed when the explants were collected during April. In Eagle wood, Nazeem *et al.* (2005) reported that nodal segments collected during March- April from current season shoots responded better to *in vitro* culture.

2.3.4 Surface sterilization

Plant tissue culture media is rich in inorganic salts and organic nutrients including sucrose. This media is a good substrate for the growth of many saprophytic bacteria and fungi. The potential sources of contamination in tissue cultures are culture vessels, nutrient media, plant tissue, instruments and environmental transfer area. The procedure for preparing sterile explants varies with the nature of explant tissues. Sharma *et al.* (2005) surface sterilized the nodal segments of Peach with 70% alcohol for one minute and then in 0.1% mercuric chloride for eight minutes followed by 2-3 washes with sterile distilled water.

2.3.5 Systemic contaminant

The contamination caused by bacteria, fungi or virus present on the surface of bark, glandular hairs at the nodes and internal tissues. It is easy to eliminate the microorganism present on the surface, but contamination due to endogenously present microorganisms is hard to wipe off. It is serious problem with woody plants. Mallika *et al.* (1990) suggested that fungal infection of field explants of cocoa could be substantially controlled by prior fungicidal treatments of mother plants. Freshly prepared chlorine water was found to be an effective sterilant of the explants. Mallika *et al.* (1992) stressed that growing plants under controlled conditions and regularly spraying the plants with systemic fungicides can reduce or avoid contamination problem to certain extent. Dodds and Roberts (1985) reported the use of antibiotics for sterilization should be avoided as they metabolize the plant tissues with unpredictable results.

2.3.6 Culture medium

Selection of culture medium depends on the plant species and purpose of culturing. A wide variety of media have been reported by many researchers. The earliest and widely used basal media were proposed by White (1943) and Heller (1953). Since 1980, most researchers have been using MS (Murashige and Skoog, 1962) medium. Other derivatives of MS medium include B_5 medium developed by Gamborg *et al.* (1968), SH (Schenk and Hildebrandt, 1972) medium and the woody plant medium (WPM) developed by Lloyed and Mc Cown (1980). The MS medium is characterized by high concentration of mineral salts. Skirvin (1980) and Griffins *et al.* (1981) suggested that reducing the strength of MS medium by half was more beneficial for culturing.

2.3.7 Carbon energy source

Cultured plant cells required a source of carbohydrate. Sucrose is the most commonly used carbon energy source for plant tissue culture. Most of the researchers have used 20-30gl^{-1} sucrose in the medium. Carbohydrate not only functions as a carbon source in metabolism but they also play an important role in the regulation of external osmotic potential (Brown and Thorpe, 1980). In apricot, Marino *et al.* (1991) reported that shoot proliferation rate was increased with sorbitol as the carbon source. Nair and Gupta (2003) found that the sucrose concentration of the medium was crucial for the induction of somatic embryos in black pepper and 30g l^{-1} was the optimum quantity for somatic embryogenesis.

2.3.8 Other organic compounds

Van overbek *et al.* (1941) reported that in Datura embryos, coconut water promoted growth and differentiation. Adenine Sulphate can enhance growth and shoot formation in many plants (Skoog and Tsui, 1948). In *Cinhona ledgeriana*, Hunter (1979) found that the addition of Phloroglucinol to the medium promoted the culture growth. Conger (1981) reported the role of complex organic compounds like casein hydrolysate,

coconut water, yeast, malt extract and fruit and vegetable juice for successful growth of tissues and organs. Role of these organic compounds is usually unpredictable and repeatability is also very poor, therefore it has been recommended to avoid their use as far as possible (Gamborg, 1981).

2.3.9 Culture conditions

The culture conditions such as physical form of medium, pH, light, temperature and relative humidity are very important in *in vitro* growth and differentiation. Number of shootlets from inflorescence was increased by growing on a static liquid medium obtained by Blake and Eeuwens (1982). According to Murashige (1977) the optimum day light period required is 16 hours.

Workable protocol for the *in vitro* propagation has been worked out for many broad leaved species. The review of literature pertaining to the micropropagation of some perennial trees has been highlighted below:

Cashew (*Anacardium occidentale*)

In cashew, Keshavachandran (2005) reported that the nodal segments were cultured in MS medium supplemented with kinetin (5 mgl^{-1}), NAA (1 mg l^{-1}), Brassinolide (0.1 mg l^{-1}) and kept in dark for seven days. After that the cultures were kept in light. High per cent of sprouting was observed when kept in the above medium. High rate of multiplication was noticed when the sprouts were subcultured into MS medium supplemented with BA (2 mg l^{-1}), Brassinolide (0.1 mg l^{-1}) and charcoal (0.5g l^{-1}). Rooting was observed when the shoots were treated with IBA (1000 mg l^{-1}) for 2-3 minutes followed by transfer to quarter MS medium supplemented with IBA (1 mg l^{-1}).

Guava (*Psidium guajava*)

HariPrakash *et al.* (1997) reported that guava cultivar Sardar was successfully propagated through *in vitro* culture using nodal buds as explants of mature trees on MS medium supplemented with 3 mgl^{-1} BAP in combination with IBA 0.4 mgl^{-1}. Highest number (15-18) of usable shoots were developed on nodal segments taken from *in vitro* proliferated shoots by enhancement of axillary branching after four subculture on MS medium supplemented with BAP 1 mg l^{-1} and IBA 0.2 mg l^{-1}. GA_3 at 0.5 mg l^{-1} adversely affected shoot proliferation and root induction. *In vitro* grown micro cuttings rooted profusely in half MS medium containing IBA 0.2 mg l^{-1}, NAA 0.2 mg l^{-1} and Activated charcoal 1.5g l^{-1} with 93% rooting efficiency.

Papaya (*Carica papaya* L.)

Babylatha *et al.* (1999) reported that maximum culture establishment of 70% was obtained for seedling shoot tips on MS medium with NAA 0.1 mg l^{-1} and BAP 0.5 mg l^{-1}. This treatment also took minimum time for culture establishment. MS medium supplemented with different levels of kinetin fail to establish the shoot tips. On B_5 medium, culture establishment was obtained with NAA 0.1 mg l^{-1} and BAP 0.5 mg l^{-1}. The lateral buds were found less amenable for *in vitro* culture compared to shoot tips. 20-30% of culture establishment was obtained on MS and B_5 media with NAA and BAP. Maximum number of shoots were produced with IAA 0.25 mgl^{-1} and BAP 0.2 mg l^{-1} and Adenine sulphate 50 mg l^{-1} in MS medium.

Belanites (*Belanites aegyptica)*

Belanites aegyptisea is a semi arid forest tree and Ndoye (2003) reported a protocol for the multiplication of the crop using axillary bud explants from matured trees. Cultures were established in MS medium supplemented with BAP 2.5 mg l^{-1} and NAA 0.1 mg l^{-1}. Shoot multiplication required BAP 2.5 mg l^{-1} and shoot length was

significantly affected by the presence of BAP. Rooting was obtained on MS medium containing IBA 20 mg l^{-1}.

Sandal (*Santalum album*)

Endosperm tissues of *Santalum album* when cultured on MS with 2, 4-D (2mgl^{-1}), Kinetin (0.1 mg l^{-1}) and BA (2 mg l^{-1}) induced callus formation (Sita *et al.*, 1980). Parthiban *et al.* (1998) reported that axilary shoot multiplication was achieved in sandal when nodal segments were inoculated on to MS medium containing BA 2 mgl^{-1}.

Aegle marmelos

Arumughan and Rao (1996) reported production of *in vitro* shoots form the cotyledonary nodes, excised from 15 days old seedlings when cultured on MS medium supplemented with BAP 3 mg l^{-1} . Rooting was obtained on MS medium containing IBA 4 mg l^{-1}. A high frequency of adventitious shoot regeneration was obtained from the radical tissues of same species, when the explants was cultured on MS medium supplemented with BA 1 mg l^{-1} and NAA 0.2 mg l^{-1} (Islam *et al.*, 1996).

Albizia

Ahlawat (1997) reported that in *Albizia procera*, maximum number of shoot per explant was obtained on MS medium containing BA 2 mgl^{-1} and NAA 0.5 mg l^{-1} in 60 days. Rooting of micro shoots was achieved in half MS supplemented with 2 mg l^{-1} IBA and 0.1% activated charcoal.

Majumdar *et al.* (1998) reported that *in vitro* differentiation of adventitious shoots from cotyledonary explants of *Albizia procera* on MS media containing BA 4mg l^{-1} and coconut water 15% $^{v}/_{v}$.

Bamboo (*Bambusia edulis*)

Lin and Chang (1998) reported that the nodal explants form 10 year old *Bambusia edulis* produced multiple shoots on MS medium supplemented with Thiodiazurm (TDZ) 0.1 mg l^{-1}.

Betula

Katayse (1995) successfully obtained plantlets form winter bud explants of 30 year old *Betula maximowicziana* tree by culturing on Woody Plant Medium (WPM) containing NAA 0.2 mg l^{-1} and BA 0.2 mg l^{-1}. The basal portion of the regenerated shoot was dipped in 50ppm IBA solution and roots were regenerated on half strength MS medium.

Garcinia cambogia

Madhusoodhanan and Rao (2003) reported that the shoot tips and small nodal cutting of *Combogia* were cultured on 0.8% agar supplemented with BA (5mg l^{-1}) in combination with NAA 0.5 mg l^{-1} and GA_3 0.5 mg l^{-1} and sucrose at 5%. Best rooting efficiency was achieved by culturing of shoot on MS liquid medium at quarter strength supplemented with 1 mg l^{-1} each of IBA, IAA, and NAA for 15-20 days and subsequently transferring them to auxin free medium with activated charcoal at 50mg l^{-1}.

Jack (*Artocarpus heterophyllus*)

Rajmohan (1986) reported cent percent survival and production of healthy growing cultures of jack shoot apices under dark conditions. The explants were dipped in 95% ethyl alcohol for 10 seconds before thoroughly washing with sterile water. It was then surface sterilized by keeping in two percentage sodium hypochlorite solution for 30 minutes. Following through washing in sterile water, the explants were placed in a solution of 2% sucrose and 7% PVP and agitated for 30-45 minutes. The explants after

surface sterilization were rinsed 3-4 times with sterile water and inoculated. A combination of BAP (18.0 mg l^{-1}) and IBA (0.2 mg l^{-1}) in MS medium was reported to be a suitable medium for culture establishment of jack by Singh and Thivari (1996).

Khirni (*Manilkhara hexandra*)

Hedge *et al.* (1996) reported successful micro propagation of khirni, which is widely used as root stock for sapota. The explants collected from newly sprouted shoots were more ideal than the mature one for the establishment of cultures. The growth from the shoot tips and nodal segments were achieved on MS medium with NAA 5 mg l^{-1} and IBA 5 mg l^{-1}. Shoot tip growth was observed and axillary buds sprouted within one month of inoculation.

Sorbus aucuparia

Chalupa (2002) reported the multiplication and axilary bud proliferation of mature tree of *Sorbus aucuparia* on MS medium supplemented with low concentration of cytokinin (0.2 mg l^{-1}) and IBA (0.1 mg l^{-1}). Nodal explants placed on MS agar medium started to form shoots within 2-3 weeks. Micro shoots excised from multiplying cultures were transferred to WPM supplemented with IBA (0.4 mgl^{-1}) and NAA (0.2 mg l^{-1}) for rooting. Adventitious roots started forming within 2-3 weeks.

Cinnamomum camphora

Huang *et al.* (1998) developed a micro propagation protocol *Cinnamomum camphora* using 3-5mm shoot tips from newly emerged laterals of two year old trees as initial explants. Nine shoots per nodal segments were obtained on MS medium supplemented with BA (4.4μM). Rooting of shoots occurred best on medium supplemented with NAA (0.54μM).

Citrus jambhiri

Singh *et al.* (1999) observed maximum shoot proliferation of *Citrus jambhiri* , when shoot tip explants was cultured on MS medium supplemented with BA (1.0 mgl^{-1}), adenine sulphate (1.0 mg l^{-1}) and NAA (0.25 mg l^{-1}) rooting was best observed on MS supplemented with BA (1.0 mg l^{-1}), Adenine Sulphate (0.1 mg l^{-1}), NAA (0.5 mg l^{-1}) and IBA (1.0 mg l^{-1}).

Casurina equisetifollia

Shoot apices (8-10 mm long) excised from 10 year old tree formed bud like structures within 4-5 weeks, when cultured on MS and Gamborg media with out growth regulators. Supplementation with cytokinin, BA (1.5-2.5 mg l^{-1}) enhanced shoot proliferation. Activated charcoal promoted shoot elongation (Nanda and Gupta, 1991).

2.3.10 Surface sterilization

Different surface sterilization techniques were carried out in explants from perennial crops to remove all the microorganisms present on them. Kesavachandran (2005) reported that the nodal segments of cashew were surface sterilized by first treating with tetracycline 250mg per 100ml along with two drops of exalin for five minutes. After washing with sterile water, it was treated with Bavistin (0.1%) for 15 minutes and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) for 30 seconds. After washing with sterile distilled water for four to five times, the nodal segments were cultured. In nutmeg, Mallika *et al.* (1997) used 70% alcohol for swabbing the nodal segments followed by carbendazim treatment (0.1%) for 10 minutes in orbital shaker. It is followed by $HgCl_2$ wash for 15 minutes and the explants were thoroughly washed in sterile distilled water before inoculation.

In *Gmelia arborea,* George (2007) reported that the most effective surface sterilization was achieved by the combination treatment of soaking the explants in 70% alcohol for 30 seconds followed by soaking them in 0.1% $HgCl_2$ for four times which

resulted in 80% of survival of cultures. Seeds of *Tilia platyphyllos* were washed thoroughly in running tap water and its endosperm including the embryo were later soaked in 3% sodium hypochlorite for 15 minutes followed by sterile distilled water wash (Ucler *et al.*, 2001). Hussain (2007) treated the seeds of *Sterculia ureus* with concentrated Sulphuric acid (H_2SO_4) for one minute, wash thoroughly with running tap water, then rinsed in 5% teepol for five minutes followed by treatment with cetrimide for five minutes. Then they were given 70% ethanol wash for one minute and treated with $HgCl_2$ (0.1%) for 20 minutes followed by five to six rinses with sterile distilled water.

Rashmi *et al.* (2004) washed nodal segments of *Acacia mangium* with 5% ($^{v}/_{v}$) detergent solution, teepol for 10 minutes and surface sterilized with 0.1% ($^{w}/_{v}$) $HgCl_2$ solution for 20 minutes and washed four to five times with sterile distilled water.

2.4 *In vivo* and *in vitro* seed germination

Cardamom seeds have highest germination (39.33%) after acid scarification for 10 minutes and soaking GA_3 for 24 hours combined with sowing in the open site (Raja, 1993). Germination of cardamom seeds improved significantly after acid treatment (Nitric acid 25% for 10 minutes) and continuous washing in water for 24 hours giving 85% germination as observed by Chaudary and Chandel (1995). Korikanthmath and Mulgae (1998) found acid treatment (20% for 10 minutes), GA_3 100 mgl^{-1} and Planofix (NAA 75 mgl^{-1} for 12 hours) increased germination of cardamom seeds. Higher doses of growth regulators decreased germination.

Spiegel *et al.* (1985) reported Nitsch medium with IAA 10^{-5} M and GA_3 as the best for germination of seeds of seedless grape cultivars. Deemir and Gunay (1996) observed increased germination percentage in seeds of cucumber after priming with 3 % KNO_3 for 5 days. *Accacia nilotica* seeds scarified with H_2SO_4 followed by washing and drying and then treated with KNO_3 at 2% for 24 hours showed higher germination percentage as reported by Palani *et al.* (1996). Treatment with KCl and KH_2PO_4 for 24 hours had no germination effect. Shanmughavelu (1977) reported the effects of plant

growth regulators on seed germination. According to him cashew seeds soaked in 100 ppm GA_3 solution recorded about 100% germination. But as the concentration increased the germination percentage decreased.

Thimmappaiah *et al.* (2001) reported germination of cashew seeds 20-25 days after inoculation in absorbent cotton. Bhattacharya and Khuspe (2000) reported germination of papaya seeds *in vitro* when MS medium supplemented with Thidiazurol 20μm l^{-1}. Buyun et al (2004) reported germination of orchid seeds and its proliferation in MS medium supplemented with 1g activate charcoal.

2.5 Micrografting

Micrografting is a relatively new grafting technique and consists of grafting an apex taken from a mother plant on to (a) a young green house or nursery grown plant in accordance with accepted grafting technique (*in vivo* micrografting), (b) a decapitated young plant grown from a seedling under aseptic conditions or microcuttings obtained from *in vitro* vegetative multiplication (*in vitro* micrografting) (Jonard, 1986). This technique is usually used to transfer the meristem tips of virus infected plants to virus free seedlings (Navarro *et al.,* 1975). The success of this technique suggest that it could be adopted for the micropropagation of other tree crops to graft known varieties on to dwarfing root stock (George and Sherrington, 1984), to rejuvenate the mature shoot materials (Francelet, 1979; Hackett, 1985) and to study the histological nature of graft unions (Gebhardt and Goldbach, 1988).

In vitro grafting has been described for peach (Alskieff, 1977), Apple (Lundergan *et al.*, 1978), Plum (Negueroles and Jones, 1979) etc. Micrografting is effected by inserting small shoot apices into inverted T shaped incision immediately below the cut surface of a decapitated rootstock. Alternatively the apices are placed directly on to the cambium layer of the cut surface. The survival of micrografted citrus apices depends on their size. Although very small apices have to be used for virus clones, layer apices could be carried out for propagation purposes (George and Sherrington, 1984).

Yidana *et al.* (1989) reported that cocoa is recalcitrant to micropropagation with only occasional spontaneous rooting. They suggested that the buds produced under *in vitro* condition can successfully be grafted on to aseptically germinated seedlings. This is of greater utility in the gene banks and also as *in vitro* propagation method for recalcitrant material. Novarda (1990) reported shoot tip grafting in vitro of citrus as a means of obtaining virus free plants. Citrus plants produced by shoot tip grafting maintained the same ontogenic age as the infected shoot tip source plant and in many cases showed an increase in vigour presumably due to elimination of the pathogen.

The application of shoot tip grafting to some conifers may produce rejuvenation of mature trees. Aguilar *et al.* (1992) attempted micrografting of somatic embryos of cocoa to *in vitro* derived seedling rootstock and found that the complete plant regeneration needed about ten months. Best results were obtained using three week old rootstocks and somatic embryos without cotyledons. A successful micrografting technique was developed for *Pistachia vera* (Abousalim and Montell, 1992). High levels of graft union were achieved when shoots from stage 2 cultures of four year old *P. vera* cv. Mateur were grafted on to *in vitro* raised seedling rootstocks. Light and fluorescent microscopy investigation revealed that vascular continuity was established across grafts by three weeks. Joseph (1994) reported that the plantlets from somatic embryogenesis of cocoa can be successfully micrografted.

Kesavachandran (2005) reported the standardization of *in vitro* micrografting technique in cashew. The nuts after surface sterilization were inoculated into solid MS media supplemented with kinetin (2.5mg l^{-1}) + NAA (0.5 mg l^{-1}) + brassinolide (1 mg l^{-1}) in light conditions. The *in vitro* grown seedlings (8-9 days old) were used as root stocks. 2-3cm shoot tips grown *in vitro* were used as scion. Among the methods tried, side grafting was found to be suitable. A wedge shaped cut was done on scion and was inserted into the slanting cut on the root stock. After 10 days the union was tied with sterilized thread and the grafted plant was transferred to liquid MS medium supplemented with kinetin (5 mg l^{-1}) + NAA (1 mg l^{-1}) + brassinolide (1 mg l^{-1}).

Kadam (2005) attempted *ex vitro* micrografting in wood apple. The seeds were sown in the month of April in poly bags. Micrografting was done on root stocks of varying maturity. The seedling root stocks were decapitated with surgical blade and 1-2cm deep slit was made. The scion microbud (1-2cm) was inserted into the slit. The micropipette cap (0-200µl) was inserted on the grafted portion. After sprouting of the microbud, the pipette cap was removed.

Onay (2004) reported *in vitro* micrografting in pistachio. Excised zygotic embryos that germinated *in vitro* were used as root stocks. Current year shoot tips from mature trees of pistachio micrografted onto *in vitro* juvenile root stocks, resulted in the restoration of shoot bud proliferation. The easiest and most successful method for grafting was slit micrografting. The embryos were cultured in MS media + B_5 vitamins + 3% sucrose + 2µM BA + agar 7g. The cultures were incubated in light. The micrografts were also cultured in the same medium. The scions (4-6mm) were taken from mature plants. The best growth of micro scion was obtained with *in vitro* forced shoot tips rather than with shoot tips excised from tree. Slow growth and lack of axillary shoot development on the micrografts was noticed when they were cultured in the germination medium.

Onay *et al.* (2003) tested success of *in vivo* micrografting in pistachio. The only variable tested was age (1, 5, 10 and 30 year old trees). 10-12 old seedlings grown in pots were used as root stocks. Shoot tips collected from the four age classes of mature trees of pistachio were the sources of scion. Parafilm was used to join scion and root stock. The in vivo micrografting system provided good growth and development for new axillary shoots.

Cortizo *et al.* (2004) describes an *in vitro* micrografting method for *Pinus pienea* trees. Needle fascicles of five selected clones were micrografted onto hypocotyls of two week old germinated embryos. Established micrografts showed a visible intermediate callus within the first week of culture. Callus starts to proliferate in the root stock and then in the scion. Graft was established successfully.

In citrus, Vijayakumari and Shyam (1998) advocated micro budding technique for faster multiplication of virus free planting material produced through 4-6 month old root stocks by taking minute scion bud from current years potted plants. Microbudding enables year round multiplication. They also recommended shoot tip grafting in citrus for producing true to type, healthy and precocious plants. This technique consist of grafting a very small shoot tip excised from an elite mother tree onto to decapitated root stock seedling grown under aseptic conditions. It has been standardized and was first applied with 50% success at NRCC, Nagpur.

Materials and Methods

MATERIALS AND METHODS

The present study entitled "*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)" were carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) of College of Horticulture, Vellanikkara during the period 2005-2007.

3.1 *In vitro* shoot regeneration

3.1.1 Explant Source

Regenerated shoots from coppiced mature trees of twelve year old, two year old grafts and juvenile seedlings of 3 to 4 month old served as the explant source for the study. The grafts and seedlings were maintained at shade net house of 50 per cent shade. The explants were sprayed at weekly intervals with 0.1 per cent carbendazim to reduce the microbial interference in culture establishment. Shoot tips and nodal segments from orthotropic as well as plageotropic shoots were used as explants.

3.1.2 Chemicals and Glass wares

The major and minor elements required for the preparation of the media were of analytical grade. Borosilicate glass wares of Corning/Borosil brand were used for the study. They were cleaned initially by soaking in water overnight, followed by thorough washing with the detergent solution (Teepol 0.1%) and then rinsed with potassium dichromate solution in sulphuric acid. They were then washed free of acid using tap water and finally rinsed with double distilled water. The glasswares were then dried in a hot air oven at 100° C for 24 hours. They were then stored in closed cup boards away from contaminants and dust until used.

3.1.3 Culture Establishment

3.1.3.1 Surface sterilization of explant

Actively growing shoots with axillary buds were excised from parent plant. Nodal cuttings of length 1.5 - 2.5 cm were used as explants. The leaf blades were removed and made into single nodal cuttings. Treatments as detailed in Table 1 were

carried out for removal of microbial flora from the nodal segments. Initially the explants were given detergent wash. Then they were soaked in carbendazim (0.1%) in orbital shaker (100 rpm) for the specified period of the particular treatment. Mercuric chloride (0.1%) treatment was done inside Laminar Air Flow Chamber. It was followed by sterile water wash for four times to remove traces of the sterilant from the surface of the explant. The surface sterilized nodal segments were inoculated in the medium of SH + 3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 0.5% A.C and incubated five days in dark followed by light at an intensity of 1000 lux with a temperature of $26 \pm 2^{\circ}C$. The observation of the cultures was recorded.

Table 1. Surface sterilization treatments in culture establishment of nodal segments of nutmeg

Treatment	Treatment details
T_1	Detergent wash → carbendazim 0.1% (15 min) → $HgCl_2$ 0.1% (5 min) → sterile water wash
T_2	Detergent wash → carbendazim 0.1% (15 min) → 70% alcohol wipe → 0.1% $HgCl_2$ (5 min) → sterile water wash
T_3	Detergent wash → carbendazim (30 min) → 0.1% $HgCl_2$ (10 min) → sterile water wash
T_4	Detergent wash → carbendazim (0.1%) in orbital shaker (100 rpm, 10 min) → 0.1% $HgCl_2$ (6 min) treatment → sterile water wash

Culture medium: SH + 3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 0.5% A.C.
Culture condition: 5 days dark condition followed by light
No. of explants: 15

3.1.3.2 Media for culture establishment

The response of explants in three different media was tested. The culture media were Schenk and Hilderbrant (SH) medium (Schenk and Hilderbrandt, 1972), Murashige and Skoog's (MS) medium (Murashigae and Skoog, 1962) and woody plant medium (WPM) (Llyod and McCown, 1980). Composition of these media is given in Table 1. The basal media were supplemented with 3.0 mg l^{-1} BA+ 1.0 mg l^{-1} IAA and A.C. 0.5% based on report by Mallika *et al.* (1997). The explants used were

nodal segments from orthotrops of mature tree. The percentage of cultures showing bud expansion in each medium was recorded after one month of inoculation.

3.1.3.3 Preparation of the stock solutions

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the medium. Stock solutions of the major and minor nutrients were prepared first by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottles. Stock solutions for the major and minor nutrients were prepared afresh every three months. The vitamin stock solutions were prepared fresh every six to eight weeks and those of growth regulators were prepared fresh every four weeks and stored under refrigerated condition.

3.1.3.4 Preparation of the culture medium

Specific quantities of the stock solution were pipetted out into a beaker. A known quantity of double distilled water was added into the beaker and the equivalent quantity of sucrose was weighed and added as solids and dissolved fully. The pH of the solution was adjusted using an electronic pH meter to 5.8 as required using 0.1N NaOH or 0.1N HCl. The volume was made upto the required quantity. Agar (0.75%) and activated charcoal (0.5%) were added and the medium was boiled till a clear solution was obtained. About 15ml of this molten medium was dispensed into the culture tubes (15 x 2.5 cm or 20 x 3.5 cm) and plugged with non-absorbent cotton.

Table 2. Composition of various basal media tested for *in vitro* culture establishment of nodal segments of nutmeg.

Ingredients	**MS (mg l^{-1})**	**WPM (mg l^{-1})**	**SH (mg l^{-1})**
Inorganic Constituents			
$(NH_4)NO_3$	1650	400	-
$(NH_4)H_2PO_4$	-	-	300
KNO_3	1900	-	2500
K_2SO_4	-	990	-
KH_2PO_4	170	170	-
$Ca(NO_3)_2.4H_2O$	-	556	-
$CaCl_2.2H_2O$	440	96	200
$MgSO_4.7H_2O$	370	370	400
$FeSO_4.7H_2O$	27.8	27.8	15
No_2EDTA	37.3	37.3	20
$MnSO_4.4H_2O$	22.3	22.3	-
$MnSO_4.H_2O$	-	-	10
$ZnSO_4.7H_2O$	8.6	8.6	1
H_3BO_3	6.2	6.2	5
KI	0.83	-	-
$No_2MoO_4.2H_2O$	0.25	0.25	0.1
$CuSO_4.5H_2O$	0.025	0.25	0.2
$CoCl_2.6H_2O$	0.025	0.25	0.1
Organic Constituents	-	-	-
Myoinositol	100	100	1000
Nicotinic Acid	0.5	0.5	5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.1	1	5
Glycine	2	2	-
Sucrose	30000	30000	30000

3.1.3.5 Sterilization of the culture medium

The tubes were plugged with non-absorbent cotton and autoclaved at 121 °C and 15 psi (1.06 kg cm^{-2}) for 20 minutes (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in a cool dry place. The bottles were made airtight by wrapping cling film at the neck of the bottle.

3.1.3.6 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of the explants and subsequent sub culturing were carried out in a clean laminar airflow chamber. The working table of the laminar air flow chamber was initially swabbed with 70 percent alcohol and then exposed to ultraviolet light for 30 minutes. The Petri plates, forceps, knives and other inoculation aids were initially autoclaved and then flame sterilized before inoculation. The hands were washed thoroughly with soap under running tap water, wiped with 70% alcohol before working in the laminar air flow chamber. The explants that were surface sterilized were inoculated into the respective medium under perfect aseptic conditions around the flame using flamed and cooled forceps.

3.1.3.7 Culture conditions

Explants for culture establishment were initially incubated in air conditioned dark culture room for five days followed by light culture room at an intensity of 1000 lux with 18 hour photoperiod supplied by cool white fluorescent light. Relative humidity in the room twintled between 60 and 80 per cent with the prevailing weather conditions.

3.1.4 Standardization of explants for culture establishment

Nodal segments and shoot tips from orthotrophs as well as plageotrophs were tried in culture establishment of nutmeg.

1) Shoot tips 1.5 to 2.5 cm
2) Nodal segments 1.5 to 2.5cm with $1/4^{th}$ leaf blade

3) Nodal segments without leaf blade

The culture medium used was SH + 3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA+ 0.5% A.C Culture conditions for the culture establishment was initial dark followed by light intensity of 26 ± 2° C. The cultures were reinoculated into the same media at an interval of 20 days and the response as bud expansion/elongation were recorded for a period of two months. The effect of season in the culture establishment was also studied.

3.1.4 Effect of Season

The effect of season on culture establishment of nutmeg was studied. The influence of summer and rainy season was studied. Observations were taken at regular intervals.

3.1.5 Treatments to control phenol exudation from the explants.

To control the polyphenol exudation from the explants following treatments were carried out (Table 3). The response of each explant in each media was recorded.

Table 3. Treatments to control phenol exudation from the explants

Sl. No.	Treatment
T_1	Charcoal medium + inoculation in light
T_2	Charcoal medium + inoculation in dark (5 days) followed by light
T_3	Medium without charcoal + inoculation in dark (5 days) followed by light
T_4	Medium without charcoal + inoculation in light.

3.1.6 Effect of Growth regulators in culture establishment

Growth regulator combinations as mentioned in Table 4 were tried to identify the best growth regulator combination for culture establishment. Nodal segments from orthotrops of grafts and mature trees were used as explants. Initially the cultures were maintained in dark for five days and then maintained in light intensity of 1000 lux and temperature of 26±2 °C. The observations on bud expansion and elongation were observed for a period of one month at fifteen days interval.

Table 4. Growth regulator combinations for culture establishment of nodal segments of nutmeg

Treat-ment	Basal medium	Growth regulators (mgl^{-1})
T_1	SH	0.5 BA + 0.5 Kin
T_2	SH	2.0 BA + 1.0 Kin + 1.0 IAA
T_3	SH	3.0 BA + 1.0 IAA
T_4	SH	3.0 BA + 1.0 IBA
T_5	SH	3.0 BA + 2.0 IBA
T_6	SH	0.5 Kin + 1.0 IBA + 3.0 Adenine Sulphate
T_7	SH	1.0 Kin + 1.0 IBA
T_8	SH	1.0 Kin + 1.0 IAA
T_9	SH	2.0 Kin + 1.0 IAA
T_{10}	SH	2.0 Kin + 1.0 IBA
T_{11}	SH	4.0 BA + 1.0 IAA
T_{12}	SH	0.02 TDZ
T_{13}	SH	0.03 TDZ
T_{14}	SH	0.2 TDZ
T_{15}	WPM	2.0 BA + 1.0 NAA
T_{16}	WPM	2.0 BA + 2.0 NAA
T_{17}	WPM	3.0 BA + 1.0 NAA

Culture condition: 5 days dark incubation followed by light
No. of replication: 10
Media supplement: 0.5% A.C.

3.1.7 Effect of carbon source in culture establishment

Studies were conducted to determine the effect of various carbon source concentrations as given in Table 5 on culture establishment using nodal segments. Nodal segments from orthotrops of juvenile seedlings detopped mature trees and mature trees were used as explant source. Surviving cultures were subcultured at an interval of 3-4 weeks to the same media in which they were inoculated. The response of the cultures as bud expansion and shoot elongation was observed and recorded.

Table 5. Carbon source combinations for culture establishment of nodal segments of nutmeg

Treatment. No.	Carbon source
T_1	Sucrose 2% + Glucose 1%
T_2	Sucrose 3% + Glucose 1%
T_3	Sucrose 5%
T_4	Sucrose 3%

Culture medium: SH + 0.03 mgl^{-1} TDZ + AC 0.5%
Culture condition: 5 days dark incubation followed by light
No. of explants: 10

3.1.8 Effect of media additives in culture establishment

Media additives like coconut water, casein hydrolysate, brassinolide were tested in varying concentrations using nodal segments as given in Table 6 on culture establishment in bud expansion and bud elongation. The details of the treatments conducted are given in this table. The response of the cultures were observed and recorded. Nodal segments of juvenile seedlings, detopped mature trees and mature trees were used as explant source.

Table 6. Media additives used for culture establishment of nutmeg

Sl. No.	Media additives
T_1	5% v/v Coconut water
T_2	10% v/v Coconut water
T_3	15% v/v Coconut water
T_4	20% v/v Coconut water
T_5	10 mg l^{-1} Casein Hydrolysate
T_6	25 mg l^{-1} Casein Hydrolysate
T_7	50 mg l^{-1} Casein Hydrolysate
T_8	0.05 mg l^{-1} Brassinolide
T_9	0.1 mg l^{-1} Brassinolide
T_{10}	0.2 mg l^{-1} Brassinolide

Culture conditions: 5 days dark incubation followed by light
Culture medium: SH + 0.03 mg l^{-1} TDZ + 0.5% A.C.

3.1.9 Treatments for shoot elongation and proliferation.

Studies were conducted for the induction of multiple shoots. Nodal segments showing bud expansion was transferred to the medium for shoot elongation. The media selected was ½ MS + 1 mg l^{-1} Kinetin + 0.1 mg l^{-1} NAA + 0.1 mg l^{-1} GA_3 + 10 mg l^{-1} Casein hydrolysate and SH + 2 mg l^{-1} BA + 1.0 mg l^{-1} NAA + 50 mg l^{-1} Adenine sulphate. The cultures were sub cultured into the same media at an interval of twenty days and the response of the cultures was recorded for a period of two months.

3.2 Micrografting

Micrografting with *in vitro* and *in vivo* grown scion were attempted under *in vitro* condition. Seeds germinated under *in vitro* as well as *in vivo* were used as rootstock material. *In vivo* grafting with *in vitro* produced shoots was also done.

3.2.1 Seed Germination Studies

3.2.1.1 Surface sterilization

Mature tree burst seeds were used for the study. Treatment details are given below (Table. 7).

Table 7. Surface sterilization treatments in decontaminating seeds for *in vitro* germination culture

Treatment No.	Treatment details
T_1	Detergent wash → wash in 0.25% copper oxy chloride (10 min.) → 0.1% mercuric chloride (5 min.) wash → sterile water wash
T_2	Detergent wash → 70% alcohol wipe → 0.1% carbendazim treatment (20 min.) → 0.1% mercuric chloride (5 min) → sterile water wash
T_3	Detergent wash → 0.1% carbendazim treatment (30 min.) → Streptocyclin treatment 50mg/l (30 min) → 70% alcohol wipe → 0.1% mercuric chloride (7 min) → sterile water wash
T_4	Detergent wash → 0.1% carbendazim treatment (30 min.) →70% alcohol wipe → 0.1% mercuric chloride (7 min) → sterile water wash

Pre-treatments were done outside the Laminar Air Flow Cabinet and the rest were done inside the Cabinet. The sterilant was removed by washing four times with sterile water. Surface sterilized seeds were inoculated in the medium of ½ MS + 2% sucrose + A.C 0.5%. Percentage of cultures showing bacterial and fungal contamination was recorded for a period of four months.

3.2.1.2 *In vitro* seed germination

Attempts were made to germinate nutmeg seeds under *in vitro* condition. The treatment details are given below (Table.8)

Table 8. Treatments for *in vitro* seed germination of nutmeg

Treatment	Medium
T_1	Full MS +A.C 0.5%
T_2	½ MS + 2% sucrose + A.C 0.5%
T_3	¼ MS + 2% sucrose + A.C 0.5%
T_4	¼ MS + 2, 4-D, 2.0 mgl^{-1} + Kin 1.0 mgl^{-1} +2nd stock double +A.C
T_5	½ MS + 2, 4-D, 2.0 mgl^{-1} + Kin 1.0 mgl^{-1} +2nd stock double +A.C
T_6	Water soaked cotton
T_7	Distilled water

Explant: Seeds of 1, 3, 6 and 8 month maturity
Culture condition: dark/light
No. of explants: 5 seeds

Seeds collected from burst nutmeg fruits were used for the study. The response of the cultures / germination in each case was observed and recorded for a period of four months.

3.2.1.3 Plant regeneration through Somatic embryogenesis

Half cut seeds with embryo portion cultured in the medium of ½ MS + 2% sucrose under dark produced somatic embryos. These somatic embryos were transferred to following medium (Table 9) for further proliferation, maturation and germination.

Table 9. Media combinations tested for plant regeneration through somatic embryogenesis

Treatment No.	Media combinations
T_1	½ MS + 2% Sucrose
T_2	½ MS + 3% Sucrose
T_3	B_5 + Kin 0.1mg l^{-1} + NAA 0.01 mg l^{-1} + GA_3 0.01 mg l^{-1} + Casein hydrolysate 10 mg l^{-1} + Agar 0.75% + A.C.0.5%
T_4	B_5 + ABA 0.3mg l^{-1} + Kin 0.5 mg l^{-1} + GA_3 0.05 mg l^{-1} + 2% sucrose +A.C.0.5%
T_5	MS + Kin 1.0 mg l^{-1} + NAA 0.1 mg l^{-1} + GA_3 1.0 mg l^{-1} + Casein hydrolysate 10 mg l^{-1} + 3% sucrose

Culture condition: Dark
No. of explants: 10

3.2.1.4 *In vitro* establishment of *in vivo* germinated seeds.

Seeds germinated under *in vivo* condition were taken out within three days and were surface sterilized by initially giving detergent wash followed by 0.1% Emissan treatment (30 min) and $HgCl_2$ treatment (6 min). The seeds were then given four times sterile water wash. They were then inoculated in ½ MS + 2% Sucrose medium to get establishment and further growth.

3.2.1.5 *In vivo* Seed Germination

Studies were made to determine the different seed treatments on *in vivo* germination of mature nutmeg seeds. Tree burst mature seeds/ fallen seeds within a period of 2 or 3 days were used for the experiment. The details of the treatments were presented in the table 10. The seeds were sown at a depth of 2 cm in sand filled in pots of size 30cm x 30cm. Daily watering was given and the response of each treatment on seed germination was observed for four months.

Table 10. Treatments for *in vivo* seed germination of nutmeg

Sl. No.	Treatment	Duration
T_1	Water soaking	24 hrs
T_2	Chilling (5° C)	24 hrs
T_3	Mechanical scarification	-
T_4	200 mgl^{-1} GA_3	24 hrs
T_5	Mechanical scarification + 200 mgl^{-1} GA_3	24 hrs
T_6	Conc. H_2SO_4 + Water wash	I min
T_7	Conc.H_2SO_4 + 200mgl^{-1} GA_3 + Water wash	1 min + 24 hrs
T_8	Control - without any treatment	-

The germination medium for the treatments was sand filled in pots
Replication - 10 seeds

3.2.2 Micrografting methods

Epicotyl Grafting technique was performed (Plate 12). Grafting was done under *in vitro* and *in vivo* condition

3.2.2.1 Feasibility Study

In order to understand whether juvenile nutmeg seedlings are amenable for grafting, a feasibility study was carried out. Juvenile seedlings of one month old were used as root stocks. *In vitro* grown shoots of length 1.5 to 2.5 cm were used as scion. A wedge shaped cut starting from both sides is made on the lower side of the scion stick. The scion stick is then inserted into the saddle like cut on the rootstock. Epicotyl grafting was performed and the union was firmly tied together with polythene strip and kept in mist chamber for establishment. The observations were taken and the survival percentage was calculated.

3.2.2.2 *In vitro* grafting

3.2.2.2.1 Grafting with Scion and root stock raised *in vitro*

Nutmeg seeds germinated under *in vitro* condition in humid bottles were used as rootstocks. *In vitro* produced elongated shoot tips of 1.5 cm length were used as scion. Stainless steel razor blades mounted on a handle were used for cutting the plant material. Epicotyl grafting technique was followed. The rootstock was decapitated and a vertical slit of 2 cm was made on the stump and the scion base after giving a 'V' shaped cut was fitted in the slit and the union is tied with sterile twine. The micrograft was cultured into bottles with liquid SH + TDZ 0.03mg l^{-1} + Copper oxychloride 50mgl^{-1}. The cultures were observed for scion establishment.

3.2.2.2.2 Grafting with *In vitro* grown scion on *in vivo* raised rootstock

In vivo grown nutmeg seedlings of 10 cm length were used as rootstocks. They were pretreated initially with sterile water, thereafter with 0.1% carbendazim for thirty minutes followed by surface sterilization with 0.1% $HgCl_2$ for seven minutes under aseptic conditions of laminar hood. The scion materials were *in vitro* produced shoot tips from nodal segments. Epicotyl micrografting procedure was carried out by giving a longitudinal transverse cut running 2-2.5 cm centrally down on the beheaded rootstock with the help of a sharp grafting knife and inoculated into presterilized liquid medium of SH + 3.0 mg l^{-1} BA+ 1.0 mg l^{-1} IAA+ 0.5% A.C. in bottles.

3.2.2.3 *In vivo* grafting

Grafting with *in vitro* scion on *in vivo* root stock.

The elongated shoot tips established under *in vitro* condition were used as scion material. The scion was given a hardening for 3 days by keeping the culture tube under room temperature for five hr before grafting. Two month old nutmeg seedlings grown under *in vivo* condition in polybags were used as rootstocks. The scion material was given carbendazim 0.1% treatment for 3 minutes and washed thoroughly. Epicotyl grafting was performed under field condition. It was kept in mist chamber for establishment. The observations on establishment were recorded.

Results

RESULTS

The results of the various experiments carried out for the *in vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.) are described here under.

4.1 Culture Establishment

4.1.1 Surface sterilization of explant

The surface sterilization treatments were carried out with nodal segments (Plate 1) from mature trees, as explants, and the results are presented in Table 11. The percentage of cultures surviving in various treatments ranged from 13% in T_1 to 33 % in T_4 after three weeks of inoculation in culture medium of SH + 3.0 BA+ 1.0 IAA + AC 0.5%. The culture damage was due to fungal contamination and tissue necrosis.

The most effective surface sterilization treatment was T_4 – washing the nodal segments in detergent followed by 0.1% carbendazim treatment in orbital shaker at 100 rpm for 10 min followed by 0.1% $HgCl_2$ treatment for 6 min and four times sterile water wash. The survival percentage was only 33% and the rest of the cultures were destroyed by fungal contamination (34%) and tissue necrosis (33%).

In T_1, which was the least effective treatment, where carbendazim treatment (15 min) and $HgCl_2$ treatment (5 min), the fungal contamination (Plate 2.) was still higher (60%). But tissue necrosis was reduced to 27% and the percentage of cultures survived was 13%. In T_2, besides 0.1% carbendazim treatment (15 min) and $HgCl_2$ wash (5 min), 70% alcohol wipe was also given. But the percentage cultures survived was only 20% and the fungal contamination was more (47%). The percentage of cultures destroyed by tissue damage was 33 %.

a. Juvenile seedling

b. Grafts

c. Mature tree

d. Coppiced tree

Plate 1. Various explant sources

Table 11. Effect of various surface sterilization treatments in the culture establishment of nodal segments of nutmeg

Treat-ment	Surface sterilization	Survival one month after inoculation (%)	Contamin - ation (%)	Cultures dried by tissue damage (%)
T_1	Detergent wash → 0.1% carbendazim (15 min) → 0.1% $HgCl_2$ (5 min)→ sterile water wash	13	60	27
T_2	Detergent wash → 0.1% carbendazim (15 min) → 70% Alcohol wipe → 0.1% $HgCl_2$ (5 min) → sterile water wash	20	47	33
T_3	Detergent wash→ 0.1% carbendazim (0.1%) (30 min) → 0.1% $HgCl_2$ (10 min) → sterile water wash	27	46	27
T_4	Detergent wash → 0.1% carbendazim (0.1%) in orbital shaker (100 rpm, 10 min) → 0.1% $HgCl_2$ (6 min) → sterile water wash	33	34	33

Culture medium – SH + 3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 0.5% AC
Culture condition – 5 days dark incubation followed by light
No. of explants – 15

4.1.2 Effect of different basal media on culture establishment

The effect of three different basal media along with hormones on the culture establishment of the explants of nutmeg is presented in Table 12. In Schenk and Hildebrandt (SH) medium with 3.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA 27% of cultures showed bud expansion. In WPM, only 13% of the cultures showed bud expansion. In ½ MS medium no response was obtained.

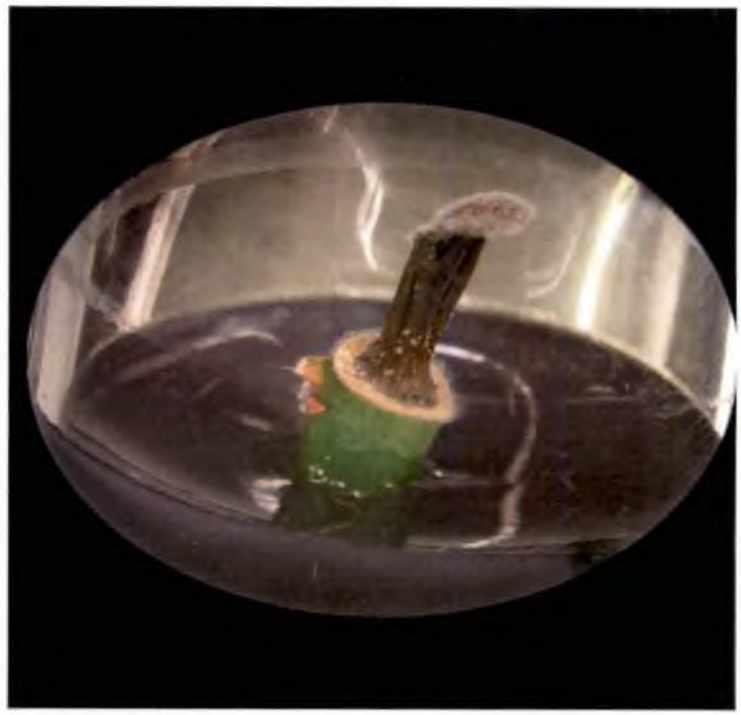

Plate 2. a Fungal contamination in nodal segment explant

Plate 2. b Fungal contamination in nodal segment explant

Table 12. Effect of different basal media on culture establishment

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Nature of response	Cultures responded (%)
T_1	SH	3.0 BA + 1.0 IAA + 0.5% AC	Bud expansion	27
T_2	½ MS	3.0 BA + 1.0 IAA + 0.5% AC	-	-
T_3	WPM	3.0 BA + 1.0 IAA + 0.5% AC	Bud expansion	13

Culture condition – 5 days dark followed by light
No. of explants – 15

4.1.3 Treatments done to overcome polyphenol exudation

Effect of charcoal in the culture medium and culture conditions in controlling polyphenol exudation was studied and the results are presented in the Table 13. The best treatment identified was T_2 in which the explant was green in colour even after two weeks of culturing. The media without charcoal showed browning and the explant later dried up.

Table 13. Effect of different treatments to control polyphenol exudation from nodal segments of nutmeg

Sl. No.	Treatment	Cultures established (%)
T_1	Charcoal medium + incubation in light	20
T_2	Charcoal medium + incubation in dark (5 days) followed by light	30
T_3	Medium without charcoal + incubation in dark (5 days) followed by light	25
T_4	Medium without charcoal + incubation in light.	10

Average of 10 observations taken two weeks after inoculation
Culture Medium: SH + 3.0 BA + 1.0 IAA + 0.5% AC

4.1.4 Standardization of explants for culture establishment

The effect of different types of explants was tried in culture establishment of nutmeg and the results are presented in Table 14. The percentage of tissue damage (necrosis, Plate 3) was higher with shoot tips. When nodal segments with $1/4^{th}$ leaf blade were used as the explants the rate of contamination was high (53.3%). Nodal segments of 1.5 to 2.5 cm without leaf blade recorded maximum survival percentage (46.7%). So it was selected for further studies (Plate 4 & 5).

Table 14. Effect of different explants in culture establishment

Sl. No	Explant type	% Survival	% Contamination	%Tissue necrosis
1	Shoot tip	13	37	60
2	Nodal segment with $1/4^{th}$ leaf blade	34	52	14
3	Nodal segments without leaf blade	47	34	20

Culture medium: 3.0 BA + 1.0 IAA + 0.5% AC
Culture condition: 5 days dark followed by light
No. of explants: 15

4.1.5 Effect of season

The influence of season on culture establishment was studied and the results are presented in Table 15. The survival of cultures was maximum during summer months (66.72%). Rainy season recorded only 22.24% survival of cultures.

Table 15. Effect of season in culture establishment with nodal segments

Period	% survival	% contamination
April - May	65.71	34.29
June - July	22.85	77.15

Culture medium: 3.0 BA + 1.0 IAA + 0.5% AC
Culture condition: 5 days dark followed by light
No. of explants: 15

Plate 3. a Necrosis affected explants

Plate 3. b Necrosis affected explants

Plate 4. Nodal segments used as explant

Plate 5. Shoot tips used as explant

4.1.6 Effect of carbon source in culture establishment

Carbon sources of various concentrations were tried in the culture establishment of nutmeg and the results were presented in Table 16. Bud expansion was observed with 3% sucrose. 2% Sucrose + 1% glucose or 5% sucrose supported shoot elongation.

Table 16. Effect of carbon source in culture establishment with nodal segments

Treatment	Carbon source	Response	% Cultures showing response
T_1	2% sucrose + 1% glucose	Shoot elongation	40
T_2	3% sucrose + 1% glucose	-	-
T_3	5% sucrose	Shoot elongation	26
T_4	3% sucrose	Bud expansion	20

Culture medium: 3.0 BA + 1.0 IAA + 0.5% AC
Culture condition: 5 days dark followed by light
No. of explants: 15

4.1.7 Effect of various growth regulator combinations in culture establishment

Various auxins and cytokinin combinations were tested for culture establishment in SH and WPM medium and the results were given in the Table 17. The culture condition provided was initial dark for 5 days followed by light. Activated charcoal 0.5% was included in all the treatments.

Among the various combinations tested, bud expansion (Plate 6) was maximum (50%) in T_{13} – SH + 0.03 mg l^{-1} TDZ + 0.5% AC followed by T_3 (25%) and T_{15} (25%). T_3 medium was SH + 3 mg l^{-1} BA + 1.0 mg l^{-1} IAA + AC 0.5% and T_{15} were WPM + 2.0 mg l^{-1} BA +1 mg l^{-1} NAA + AC 0.5%. In T_{13}, mean number of days taken for bud expansion was 9 days (Plate 6). In T_3 and T_{15}, the mean number of days taken for bud expansion was 10 and 11 days respectively.

Lower concentrations of BA and Kin (T_1) did not give any bud expansion. Combinations of BA and Kinetin with Auxin (T_2) also did not give any positive results. Attempts were also made to replace IAA with IBA (1 to 2 mg l^{-1}) but no positive results were observed. Adenine Sulphate was included in T_6 treatment. In WPM, the influence of NAA was studied by replacing IAA. The

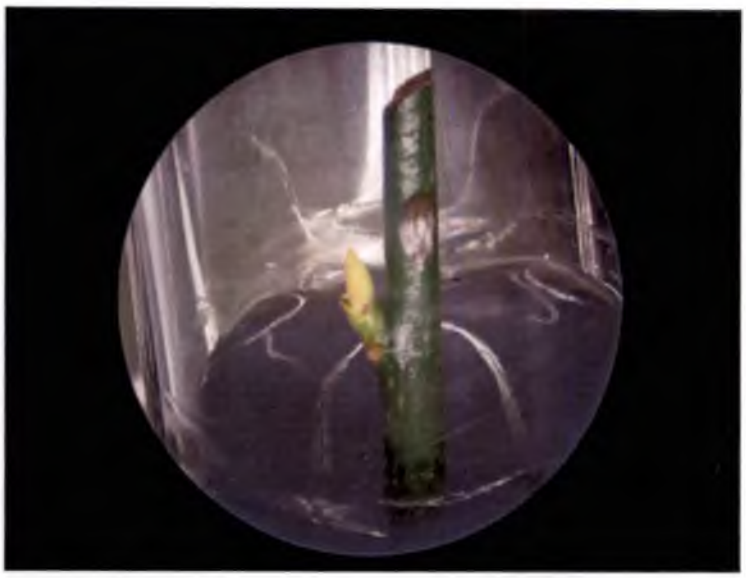

Plate 6. a Bud expansion from nodal segments

Plate 6. b Bud expansion from nodal segments

influence of IBA and Kinetin (T_7 and T_{10}) was also studied. All these treatments did not give any positive results for bud expansion. Among the various concentrations of TDZ (0.2, 0.03 and 0.02 mgl^{-1}) studied, 0.03 mg l^{-1} was found promising for culture establishment.

Table 17. Influence of various growth regulator combinations in culture establishment of nodal segments of nutmeg

Treat-ment	Basal medium	Growth regulators (mgl^{-1})	Response	Cultures Responding	Days taken (mean)
T_1	SH	0.5 BA + 0.5 Kin	-	-	-
T_2	SH	2.0 BA + 1.0 Kin + 1.0 IAA	-	-	-
T_3	SH	3.0 BA + 1.0 IAA	Bud expansion	25%	10
T_4	SH	3.0 BA + 1.0 IBA	-	-	-
T_5	SH	3.0 BA + 2.0 IBA	-	-	-
T_6	SH	0.5 Kin + 1.0 IBA + 3.0 Adenine Sulphate	-	-	-
T_7	SH	1.0 Kin + 1.0 IBA	-	-	-
T_8	SH	1.0 Kin + 1.0 IAA	-	-	-
T_9	SH	2.0 Kin + 1.0 IAA	-	-	-
T_{10}	SH	2.0 Kin + 1.0 IBA	-	-	-
T_{11}	SH	4.0 BA + 1.0 IAA	-	-	-
T_{12}	SH	0.02 TDZ	-	-	-
T_{13}	SH	0.03 TDZ	Bud expansion	50%	9
T_{14}	SH	0.2 TDZ	-	-	-
T_{15}	WPM	2.0 BA + 1.0 NAA	Bud expansion	25%	11
T_{16}	WPM	2.0 BA + 2.0 NAA	-	-	-
T_{17}	WPM	3.0 BA + 1.0 NAA	-	-	-

Culture condition – 5 days dark incubation followed by light
No. of explants – 10

4.1.8 Influence of organic additives in culture establishment

Supplementary effects of organic additives (coconut water, casein hydrolysate, brassinolide) were tested in the medium of SH along with Thidiazuron.

4.1.8.1 Effect of coconut water

Supplementary effects of various levels of coconut water (5, 10, 15, 20% v/v) in culture establishment of nodal segments was studied in the medium of SH + 0.03 mg l^{-1} TDZ + 0.5% A.C and the results are presented in Table 18. Nodal segments from juvenile plants, mature trees and regenerants from coppiced mature trees were used as explants. The explants differed in their response with respect to various levels of coconut water. Explants from seedlings and mature trees gave favorable response for certain concentrations (5 and 15%). Coppiced tree explants did not give any response.

In juvenile explants, coconut water (5, 10, 15% v/v) supported culture establishment and maximum response (50%) was observed with 5% v/v coconut water (Plate 7). It took 8 days for bud expansion in this concentration. In mature trees, at 5 percent v/v, only 25% of cultures showed bud expansion within a period of 10 days.

The percentage cultures showing response was reduced to 25% when coconut water concentration was increased to 10 and 15% v/v. For 10% and 15% concentration, shoot elongation was observed in juvenile explants. Coconut water 20% v/v did not make any favorable response in any of the explants tested.

Table 18. Influence of Coconut water along with Thidiazuron in culture establishment of nodal segments of nutmeg

Sl. No.	Media supplement (% v/v)	Explant	Response	Cultures responded (%)	Number of days taken (Mean)
1	Coconut water 5%	J M.T C.T	Bud expansion Bud expansion -	50 25 -	8 10 -
2	Coconut water 10%	J M.T C.T	Shoot elongation & leaf expansion Nil Nil	25 - -	17 - -
3	Coconut water 15%	J M.T C.T	Shoot elongation Bud expansion Nil	25 25 -	13 10 -
4	Coconut water 20%	J M.T C.T	Nil Nil Nil	- - -	- - -

Basal medium: SH + TDZ 0.03 mg l^{-1} + 0.5% A.C.
Culture condition: 5 days dark incubation followed by light
J- juvenile explants, M.T – mature tree explants, C.T – coppiced tree explants
Average of 8 observations

4.1.8.2 Effect of Casein hydrolysate

Supplementary effect of casein hydrolysate of varying concentrations (10, 25, 50 mg l^{-1}) was studied in the basal medium SH + 0.03 mg l^{-1} TDZ + 0.5% A.C and the results are presented in the Table 19. Nodal segments from juvenile plants, mature trees and regenerants from coppiced trees were used as explants. The explants differed in their response with respect to various concentrations.

In juvenile explants, casein hydrolysate (10 to 50 mg l^{-1}) supported culture establishment. Maximum response (50%) was observed with 10 and 25 mg l^{-1} concentration. At higher level (50 mg l^{-1}), only 37.5% of cultures showed bud expansion. In juvenile explants bud elongation was observed within a period 14 days at 25 mg l^{-1} concentration. In 25% of cultures, shoot elongation to an average length of 1 cm was observed with 25 mg l^{-1} concentration (Plate 8). Regenerants from coppiced mature trees did not give any favorable response to any of the three concentrations.

Table 19. Influence of Casein hydrolysate along with Thidiazuron in culture establishment of nodal segments of nutmeg

Sl. No.	Media supplement (mg l^{-1})	Explant	Response	Cultures responded (%)	Number of days taken (Mean)
1	Casein hydrolysate 10 mg l^{-1}	J M.T C.T	Bud expansion Nil Nil	50 - -	12 - -
2	Casein hydrolysate 25 mgl^{-1}	J M.T C.T	Shoot elongation & leaf expansion Nil Shoot elongation & leaf expansion	25 - 25	14 - 16
3	Casein hydrolysate 50 mg l^{-1}	J M.T C.T	Bud expansion Bud expansion Nil	37.5 25 -	11 10 -

Basal medium: SH + 0.03 mg l^{-1} TDZ + 0.5% A.C.
Culture condition: 5 days dark incubation followed by light
J- juvenile explants, M.T – mature tree explants, C.T – coppiced tree explants
Average of 8 observations

Plate 7. Bud elongation:
SH+0.03 mg l^{-1} TDZ + 10% Coconut water + 0.5% A.C. medium

Plate 8. Leaf expansion:
SH + 0.03 mg l^{-1} TDZ + 25mg l^{-1} Casein hydrolysate + 0.5% A.C. medium

4.1.8.3 Effect of Brassinolide

The effect of Brassinolide in various concentrations (0.05, 0.1, 0.2 mg l^{-1}) was studied in culture establishment. The media SH + TDZ 0.03 mg l^{-1} + A.C 0.5% are given in the Table 20. Nodal segments from juvenile seedlings, mature trees and regenerants from detopped trees were used as explants.

At lower concentrations (0.05 and 0.1 mgl^{-1}), none of the explants showed favorable results. But with 0.2 mg l^{-1} brassinolide, bud expansion was observed in juvenile explants. 25% of cultures gave positive response and the average number of days taken for bud expansion was 11 days. Mature trees and coppiced tree explants did not responded in any of the three concentrations of brassinolide.

Table 20. Influence of Brassinolide along with Thidiazuron in culture establishment of nodal segments of nutmeg

Sl. No.	Media supplement (mgl^{-1})	Explant	Response	Cultures responded (%)	Number of days taken (Mean)
1	Brassinolide (0.05)	J	Nil	-	-
		M.T	Nil	-	-
		C.T	Nil	-	-
2	Brassinolide (0.1)	J	Nil	-	-
		M.T	Nil	-	-
		C.T	Nil	-	-
3	Brassinolide (0.2)	J	Bud expansion	25	11
		M.T	Nil	-	-
		C.T	-	-	-

Basal medium: SH + 0.03 mg l^{-1} TDZ + 0.5% A.C.
Culture condition: 5 days dark incubation followed by light
J- juvenile explants, M.T – mature tree explants, C.T – coppiced tree explants
No. of replication - 8

4.2 Effect of treatments for shoot elongation and proliferation.

Attempts were made for shoot elongation and proliferation in induction of multiple shoots on *in vitro* produced shoots of length 2 to 2.5 cm. The media used were ½ MS + 1mg l^{-1} Kin + 0.1mg l^{-1} NAA + 0.1 mg l^{-1} GA_3 + 10 mg l^{-1} Casein hydrolysate and SH + 2mg l^{-1} BA + 1.0 mg l^{-1} NAA + 50 mg l^{-1} Adenine sulphate. The explants after three weeks were subcultured onto the same media. Multiple shoot induction was not obtained in both the cases. The cultures showed browning and later dried up.

4.3 Micrografting

4.3.1 Surface sterilization of seeds for *in vitro* seed germination

Surface sterilization treatments were carried out with mature tree burst seeds and the results are presented in the Table 21. The culture damage was mainly due to fungal contamination. The percentage cultures survived in various treatments ranged from 15% to 30%.

T_1 was the least effective treatment which involved copper oxychloride (0.25%) treatment for ten minutes with seed coat followed by mercuric chloride (0.1%) treatment for 5 minutes without seed coat in the laminar air flow cabinet. Survival percentage after one month of inoculation was 15 percent.

In T_2 copper oxychloride (0.25%) for ten minutes was replaced by Carbendazim (0.1%) for twenty minutes. The percentage of cultures survived increased to 20%, but bacterial contamination was noticed. So in T_3 Streptocyclin (500 mg l^{-1}) treatment was included for 30 minutes.

The effective surface sterilization treatment was T_4 i.e., washing the seeds with seed coat in detergent followed by carbendazim (0.1%) treatment for 30 min. 70% alcohol wipe and Mercuric chloride (0.1%) wash for seven minutes. inside laminar air flow cabinet after the removal of seed coat. Before inoculation the

explants were given four times sterile water wash. The survival percentage was 30% and the rest of the cultures were destroyed by fungal contamination.

Table 21. Effect of various surface sterilization treatments in decontaminating seeds for *in vitro* seed germination

Treat-ment	Surface sterilization	% Survival after one month	Nature of contamination
T_1	Detergent wash → wash in 0.25% copper oxychloride (10 min.) → 0.1% $HgCl_2$ (5 min.) wash → sterile water wash	15	F.C
T_2	Detergent wash → 70% alcohol wipe → 0.1% carbendazim (20 min.) → 0.1% $HgCl_2$ (5 min) →sterile water wash	20	F.C, B.C
T_3	Detergent wash → 0.1% carbendazim (30 min.) →streptocyclin 50mg l^{-1} (30 min) → 70% alcohol wipe → 0.1% $HgCl_2$ (6 min) → sterile water wash	25	F.C
T_4	Detergent wash → 0.1% carbendazim (30 min.) →70% alcohol wipe → 0.1% $HgCl_2$ (7 min) → sterile water wash	30	F.C

Culture condition – dark
No. of explants – 20

4.3.2 Effect of media combinations *in vitro* seed germination

Seeds of 1, 3, 6 and 8 month old maturity were tested for germination in vitro and the results are presented in Table 22. Germination of mature seeds was observed only in pre-sterilized bottles with distilled water/water soaked cotton after a period of sixty eight days (Plate 9). Basal media of ¼, ½, and full MS did not support germination of whole seeds of 1, 3, 6 and 8 month maturity. Fortification of $1/4^{th}$ and ½ MS with hormone combination of 2 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} Kin + 2^{nd} stock double also did not support germination of whole seed. Cut seeds with embryo also did not germinate either in $¼^{th}$, ½ MS or full MS alone or fortified with hormones, 2 mg l^{-1} 2, 4-D + 1mg l^{-1} Kin + 2^{nd} stock double. Somatic embryos were formed from the cut end of the endospermous tissue of seeds of six month maturity

Table 22. Effect of media combinations for *in vitro* seed germination in nutmeg

Treatment	Media Combination	Seed Germination/ Response							
		Maturity of Seed							
		1 month		3 months		6 months		8 months	
		Whole	Half	Whole	Half	Whole	Half	Whole	Half
T_1	¼ MS	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
T_2	½ MS	Nil	Nil	Nil	Nil	Nil	Somatic embryos	Nil	Nil
T_3	Full MS	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
T_4	¼ MS + 2,4-D 2mgl^{-1} + Kin 1mgl^{-1} + II Stock double	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
T_5	½ MS + 2,4-D 2mgl^{-1} + Kin 1mgl^{-1} + II Stock double	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
T_6	Distilled water	Nil	Nil	Nil	Nil	Nil	Nil	Germi nated	Nil
T_7	water soaked cotton	Nil	Nil	Nil	Nil	Nil	Nil	Germi nated	Nil

when cultured in ½ MS + 2 % Sucrose. The response was obtained two and a half months after inoculation. The contamination rate was high when *in vivo* germinated seedlings were transferred to *in vitro* condition.

4.3.3 Plant regeneration through somatic embryogenesis

Plant regeneration through somatic embryogenesis could not be obtained even though various treatments were tried (Table 7), but callus proliferation and somatic embryos was observed with the medium B_5 + 0.1 mg l^{-1} Kin + 0.01 mg l^{-1} NAA + 0.01 mg l^{-1} GA_3 + 10.0 mg l^{-1} Casein hydrolysate + . 0.5% A.C. (Plate 11). Further maturation was attempted, but the calli turned dark brown colour.

4.3.4 *In vitro* establishment of *in vivo* germinated seeds

Seeds germinated under *in vivo* condition were taken out within three days and were surface sterilized by giving detergent wash followed by 0.1% Emissan treatment (30 min) and $HgCl_2$ treatment (6 min). They were then given four times sterile water wash. They were inoculated in ½ MS + 2% Sucrose medium to get establishment and further growth. The cultures were destroyed by fungal contamination.

4.3.5 *In vivo* seed germination

The effect of *in vivo* seed germination studies with transformed values were presented in Table 23. Seeds of various treatments took 67 to 77 days for germination. The percentage seed germination ranged from 20 to 50%.

Table 23. Effect of various treatments for *in vivo* seed germination with transformed values

Sl. No.	Treatment	Duration	Number of days for germination (mean)	Seed germination (%)
T_1	Water Soaking	24 hrs	76 (8.745)C	50 (76)AB
T_2	Chilling (5° C)	24 hrs	67 (8.215)A	40 67D
T_3	Mechanical scarification	-	72 (8.514)BC	40 (72)BC
T_4	200 mg l^{-1} GA_3	24 hrs	76 (8.746)CD	20 (76)AB
T_5	Mechanical scarification + 200 mg l^{-1} GA_3	24 hrs	74 (8.63)CD	30 (74)AB
T_6	Conc. H_2SO_4 + Water wash	I min	68 (8.275)A	30 (68)D
T_7	Conc.H_2SO_4 + 200mg l^{-1} GA_3 + waterwash	1 min + 24 hrs	77 (8.802)D	30 (77)A
T_8	Control - without any treatment	-	70 (8.394)AB	50 (70)CD

Germination medium - Sand
No. of replication – 10 seeds
Values in parenthesis indicate angular transformed values

Table 24. ANOVA table for *in vivo* seed germination studies

ANOVA Table						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
VAR00001	Between Groups	365.310	7	52.187	9.613	0.000
	Within Groups	114.000	21	5.429		
	Total	479.310	28			
VAR00002	Between Groups	1.262	7	0.180	9.566	0.000
	Within Groups	0.396	21	0.019		
	Total	1.657	28			

Statistical analysis of *in vivo* seed germination studies was done using CRD and the results are presented in Table 24, 25 and 26. The result showed that treatments T_2, T_6 and T_8 were better treatments compared to other treatments with respect to number of days taken for germination. The days required for germination

in these treatments ranged from 67 to 70 days. Considering the germination percentage, the treatments T_1 (water soaking) and T_8 (control – without any treatment) are superior. In T_1 and T_8 the germination was 50%. T_8 (control) can be considered as the best treatment as it recorded 50% germination within a period of 70 days. ANOVA table showed there is significant difference between the treatments and Duncan's Multiple range test confirmed the same.

Table 25. Duncan's Multiple Range Test for No. of days taken for seed germination

VAR00003	N	Subset for alpha =0 .05			
		1	2	3	4
2.00	4	8.2153 (A)			
6.00	3	8.2759 (A)			
8.00	5	8.3947 (AB)	8.3947 (AB)		
3.00	4		8.5140 (BC)	8.5140 (BC)	
5.00	3			8.6308 (CD)	8.6308 (CD)
1.00	5			8.7454 (CD)	8.7454 (CD)
4.00	2			8.7464 (CD)	8.7464 (CD)
7.00	3				8.8029 (D)
Sig.		0.124	0.275	0.056	0.152
Means for groups in homogeneous subsets are displayed.					
a Uses Harmonic Mean Sample Size = 3.333.					
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.					

Table 26. Duncan's Multiple Range Test for seed germination (%)

VAR00003	N	Subset for alpha =0 .05			
		1	2	3	4
2.00	4	67.0000 (D)			
6.00	3	68.0000 (D)			
8.00	5	70.000 (CD)	70.0000 (CD)		
3.00	4		72.0000 (BC)	72.000 (BC)	
5.00	3			74.000 (AB)	74.0000 (AB)
1.00	5			76.000 (AB)	76.0000 (AB)
4.00	2			76.000 (AB)	76.0000 (AB)
7.00	3				77.0000 (A)
Sig.		0.130	0.280	0.053	0.142
Means for groups in homogeneous subsets are displayed.					
a Uses Harmonic Mean Sample Size = 3.333.					
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.					

Plate 9. ***In vitro*** **seed germination in humid bottle**

Plate 10. ***In vitro*** **seed germination in water soaked cotton**

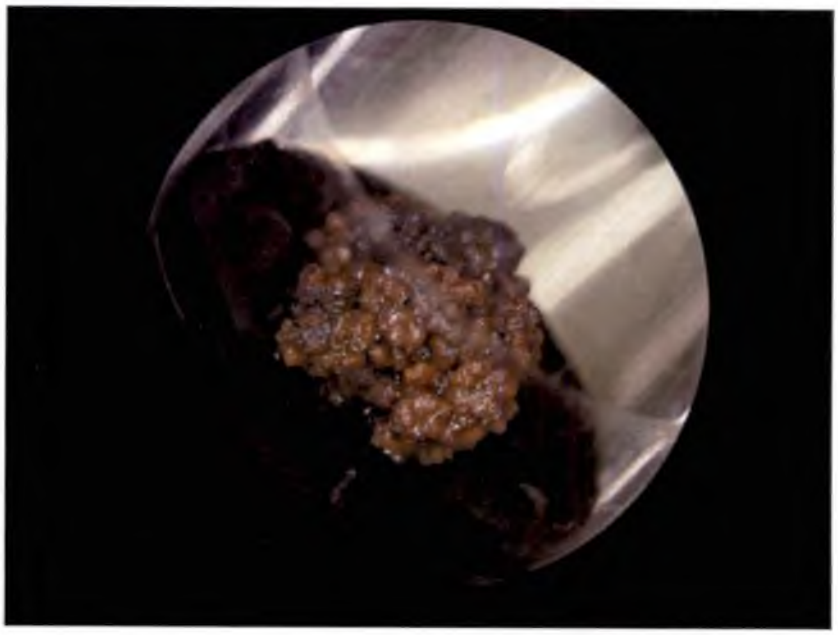

Plate 11. a Somatic Embryogenesis from endosperm (10 X)

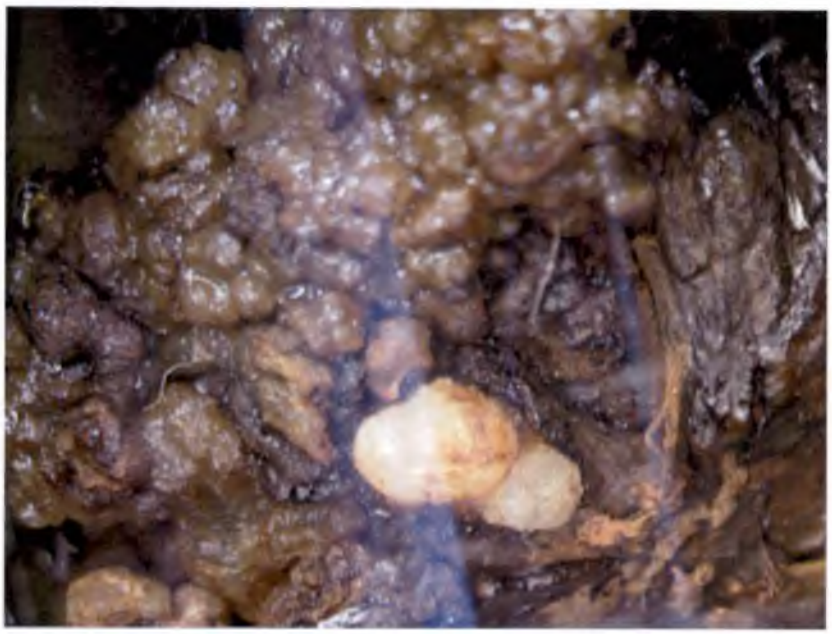

Plate 11. b Somatic Embryogenesis from endosperm (100 X)

4.3.6 Feasibility study of juvenile grafting

In order to understand whether juvenile nutmeg seedlings are amenable for grafting, a feasibility study was carried out. Juvenile seedlings of one month old were used as root stocks. *In vitro* grown shoots of length 1.5 to 2.5 cm were used as scion. Epicotyl grafting was performed and the union was firmly tied together and kept in shade net with humidity 60 to 70%, 50% shade and temperature 30 C for establishment (Plate 12). The graft union was successful and new sprouts were formed within one month. 80% of the grafts survived.

4.3.7 Grafting with scion and rootstock raised *in vitro*

Micrografting was done as described in section 3.2.2.2 of materials and methods. It was inoculated into bottles containing liquid media and cultured in dark at 26 ± 2^0C. The scion was green for two weeks, later fungal contamination occurred in the culture from rootstock. It was again surface sterilized by dipping rootstock portion in $HgCl_2$ (0.1%) for 5 minutes and washed with sterile water. It was sub cultured into fresh media containing Copper oxychloride 50mg l^{-1}. But fungal contamination was not controlled.

4.3.8 Grafting with *in vitro* grown scion and *in vivo* grown rootstock

In vivo grown nutmeg seedlings of 10 cm length were used as rootstocks. They were pretreated initially with sterile water, thereafter with 0.1% carbendazim for thirty minutes followed by surface sterilization with 0.1% $HgCl_2$ for seven minutes under aseptic conditions of laminar hood. Epicotyl grafting was done and the graft was intact and green for one week, but fungal contamination was noticed from rootstock. It was again surface sterilized by dipping the rootstock portion in $HgCl_2$ (0.1%) for six minutes and inoculated in fresh liquid media. Again fungal contamination was noticed and cultures were destroyed.

a. Beheaded root stock with longitudinal incision

b. Wedge shaped scion

c. Scion inserted into the root stock

Plate 12. Micrografting procedure: Epicotyl grafting

Plate 13. Feasibility of epicotyl grafting

Plate 14. *In vitro* micrografting

4.3.9 *In vivo* grafting

The elongated shoot tips established under *in vitro* condition were used as scion material. The scion was given a hardening for three days by keeping the culture tube under *in vivo* for five hr before grafting. Two month old nutmeg seedlings grown under *in vivo* condition in polybags were used as rootstocks. Epicotyl grafting was done on *in vivo* produced seedlings with *in vitro* regenerated shoots of length 2.5 cm. It was kept in mist chamber with 70% humidity for establishment. The graft union was not successful due to the drying up of scion after one week.

Discussion

5. DISCUSSION

Nutmeg (*Myristica fragrans* Houtt.), a perennial spice, is dioecious and dimorphic in plant growth and development. In profitable commercial cultivation, male and female plant ratio should be 1:10. In seed propagation, seedlings segregate into male and female at the ratio of 1:1. The sex of the plant can be identified only after flowering i.e. five to six years after planting under good management. So vegetative propagation has relevance in this crop. Vegetative propagation is possible through epicotyl grafting, *in situ* budding etc. The growth habit of the tree is characterized by orthotropic growing main shoot and plageotropic growing side shoots. Grafting/budding with orthotropic scion materials produces erect growing plant architecture, while grafting/budding with plageotropic scion material produces plants with hanging shoots with less topographical growth.

Grafting with orthotropic shoots is limited due to the scarcity of available orthotropic shoots. So the selected study, "*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans Houtt.*)" has relevance. The results obtained are discussed hereunder.

5.1 Culture Establishment

5.1.1 Surface sterilization

The surface sterilization of nodal segments were carried out by soaking the explants in carbendazim (0.1%) for ten minutes (100 rpm) in orbital shaker followed by mercuric chloride (0.1%) wash for six minutes and four times sterile water wash. Thirty three percent of cultures survived. The damage of cultures was due to fungal contamination and tissue necrosis. Exposure of explants to mercuric chloride (0.1%) beyond ten minutes was found to be deleterious. This is in confirmation with the findings of Krishnan and Seeni (1994) in *Woodfordia fruticosa*.

In plant tissue culture of perennial spices, latent contamination in tissue system is the most important problem. In this experiment explants were taken from plants grown in controlled condition which were given prophylactic spray systemic fungicide at an interval of five days. Mallika *et al.* (1997) has advocated the use of

carbendazim (0.1%) for ten minutes in combination with mercuric chloride (0.1%) for fifteen minutes for disinfecting the nodal segments of nutmeg.

The identified surface sterilization treatment reported only 33% of survival of cultures in the present study. So other methods which do not cause tissue damage and reduction in microbial contamination should be explored to improve the survival percentage.

5.1.2 Polyphenol interference

Nutmeg is reported to contain polyphenols that ooze into the medium during culture. Survival of the explants reduced if polyphenol interference is left unchecked. This has been reported by Mathew (1995) in clove. Polyphenols can be oxidized either by peroxidases (Mayer and Harel, 1979) or polyphenol oxidases (Mayer and Harel, 1979; Hu and Wang 1983). The oxidized compounds are highly toxic and form covalent bonds with the plant proteins thus inhibiting the enzyme activity (Hu and Wang 1983) causing browning and death of the explant. Explant establishment of nutmeg thus required special treatment to escape or avoid problems that are associated with polyphenol exudation.

In the present study, culturing the explant initially for five days under dark in charcoal (0.5%) containing medium followed by light recorded maximum culture establishment (30%). The polyphenol exudation was more when cultured in light alone avoiding initial dark incubation. So the cultures were initially kept in dark for five days followed by light.

Chawla (2002) has reported that charcoal in the culture medium 0.2 to 3.0 percent (w/v) will reduce the polyphenol interference in culture establishment.

5.1.3 Standardization of explants for culture establishment

Different types of explants were tried in culture establishment of nutmeg. When nodal segments with $\frac{1}{4}^{th}$ leaf blade were used as the explants, the rate of contamination was high. In shoot tip explants survival was only 13%. Nodal segments

of 1.5 to 2.5 cm without leaf blade recorded maximum survival percentage i.e. 47%. So it was selected for further studies. Roy *et al.* (1996) reported use of nodal segments devoid of leaf blade for multiple shoot induction. Mathew (1995) reported the superiority of nodal segments over shoot tips in the micropropagation of clove.

5.1.4 Effect of season

In the present study, the culture establishment was best in the summer season (April – May) (65.71%) than in rainy season (June – July) (22.85%). During rainy season microbial contamination was very high (77.15%). Mahale *et al.* (2005) reported that in case of Tamarind, bud break in *in vitro* culture was noticed when the explants were collected during April. In Eagle wood, Nazeem *et al.* (2005) reported that nodal segments collected during March- April from current season shoots responded better to *in vitro* culture.

In the in vitro culture of perennial trees the most important problems that has to be tackled are endophytic microbial flora and recalcitrancy in bud break. Growth habit of nutmeg is characterized by periodical flushing. So collecting the explants just prior to flushing avoiding rainy season i.e. June -July will enhance the culture establishment.

5.1.5 Influence of carbon source in culture establishment

Sucrose 2% + glucose 1%, and sucrose 5% supported shoot elongation (40 and 26% respectively). Sucrose at 3% concentration supported only bud expansion. Sucrose is the most commonly used carbon energy source for plant tissue culture. George and Sherrington (1984) reported that glucose and fructose may be substituted in some cases. Marino *et al.* (1991) observed that shoot proliferation rate in Apricot increased with sorbitol as carbon source than with sucrose.

In future studies culture establishment in nutmeg could be done in medium contaiing sucrose 2% + glucose 1%.

50 mg l^{-1}) and Brassinolide (0.05, 0.1 and 0.2 mg l^{-1}) with nodal segments from juvenile seedlings, regenerants from coppiced trees and mature trees

5.1.7.1 Effect of coconut water

Supplementary effects of coconut water (5, 10, 15 and 20% v/v) in culture establishment of nodal segments were studied in the medium of SH + 0.03 mg l^{-1} TDZ + 0.5 % A.C and the results were presented in Table 18.

Juvenile explants responded better to coconut water supplementation than explants from mature trees. In juvenile explants at 5% concentration, 50% of cultures showed bud expansion. At 10 to 15% level, bud expansion and shoot elongation was observed in 25% cultures. Since 10% coconut water produced shoot elongation and leaf expansion, it could be taken as somewhat optimum supplement of coconut water for juvenile explants. Blake and Eeuwens (1982) reported that the addition of coconut water is useful for culture establishment in coconut. Babylatha *et al.* (1996) reported that coconut water enhanced shoot proliferation in papaya.

Mature tree explants showed bud expansion at 5 and 15% coconut water in 25% of cultures. So culture establishment media of mature explants could be supplemented with 5% coconut water. But experiment should be repeated for conclusive results. In regenerants from detopped trees, no response was observed.

5.1.7.2 Effect of Casein Hydrolysate

Supplementary effects of various levels of casein hydrolysate (10, 25 and 50 mg l^{-1}) in culture establishment of nodal segments was studied in the medium of SH + TDZ 0.03 mg l^{-1} + 0.5 % A.C in culture establishment of nutmeg explant. Nodal segments from juvenile plants, mature trees and regenerants from coppiced trees were used as explants. The explants deferred in their response with respect to various concentrations.

The juvenile explants showed bud expansion in all the three combinations. Casein hydrolysate at 25 mg l^{-1} concentration showed bud elongation and leaf

expansion. Maximum bud expansion percentage was in 10 mg l^{-1} (50%). In explants from mature trees, 50 mg l^{-1} Casein Hydrolysate showed bud expansion. The regenerants from coppiced tree explants also produced bud expansion and elongation at 25 mg l^{-1} concentration in 25% of cultures. So Casein hydrolysate concentration of 25 mg l^{-1} is recommended for further experiments.

Incorporation of Casein Hydrolysate, a complex mixture of amino acids, has been attempted by several workers. Enhancement in multiple shoot production by adding Casein Hydrolysate has been reported by Mascarenhas *et al.* (1993) in *Hevea brazelienssis* and Mahato (1992) in *Dalbergia latifolia*. In the present study Casein Hydrolysate (25 mg l^{-1}) supported bud elongation and leaf expansion in explants from juvenile and coppiced trees. So, in the culture establishment of explants from juvenile seedlings and coppiced trees Casein Hydrolysate (50 mg l^{-1}) could be incorporated. For mature tree explant, Casein Hydrolysate could be 50 mg l^{-1}

5.1.7.3 Effect of Brassinolide

The Steroid growth regulator, Brassinolide (0.05, 0.1, 0.2 mgl^{-1}) were also tried in the culture establishment of nutmeg. At 0.2 mgl^{-1} concentration, bud expansion was observed. At lower concentrations (0.05 and 0.1 mgl^{-1}) none of the explant showed favourable results. Brassinosteroids are natural plant growth promoting products widely distributed in the plant kingdom. Physiological responses of Brassinosteroids include effects on elongation, cell division and vascular development (Wada *et al.*, 1981, Fujioka *et al.*, 1995). Kesavachandran (2005) reported the use of Brassinolide for *in vitro* raising of cashew root stocks. Brassinolide shows most stimulator effect at a concentration of 0.1 mg l^{-1} in conifers (Pullman *et al.*, 2003). In the present study, Brassinolide at 0.2 mgl^{-1} showed favourable response in juvenile explants and so it could be included in the culture establishment of nutmeg in future studies.

The suggested media combinations incorporating organic supplements are the following:

1. Juvenile explant - SH + 0.03 mg l^{-1} TDZ + 0.5 % A.C + casein hydrolysate 25 mg l^{-1} or brassinolide 0.2 mg l^{-1} or 10% v/v coconut water

2. Coppiced tree explant - SH+0.03 mg l^{-1} TDZ + 0.5 % A.C + 25 mg l^{-1} casein hydrolysate
3. Mature explants - SH + 0.03 mg l^{-1} TDZ + 0.5 % A.C + 50 mg l^{-1} casein hydrolysate

These organic supplements can also be tried with other two media combinations also

1. SH + 3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 0.5% A.C
2. WPM 2.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA + 0.5 % A.C

Based on the previous experiments carbon source and content in culture establishment media could be 2%sucrose + 1% glucose.

5.2 Shoot elongation and proliferation

Nodal segments of nutmeg did not show multiple shoot induction in the two media tried. Generally cytokinins has been utilized to overcome the apical dominance of shoots to enhance formation of lateral buds (Murashige, 1974). The direct effect of cytokinins in tissue culture may vary according to the particular compound used, the type of cultures and plant species from which it was derived (George and Sherrington, 1984). For multiple shoot production cytokinin in the form of BA was found to be highly essential. Similar observations have been made in guava by Amin and Jaiswal (1987) and in cumin by Yadav *et al.* (1990).

Shoot multiplication is a function of cytokinin activity but sustained growth of shoots depends on a synergistic balance between BA and NAA. The combination of BA and NAA for shoot initiation and multiplication was earlier reported by Razdan (1990) were NAA was considered more effective than IAA due to its better stability. The combination of BA and NAA is reported to be the best for multiple shoot induction in several crops like cashew (Kesavachandran *et al.*, 1998) and jute (Nandy *et al.*, 2005). In the present study multiple shoots were not induced, with combination of cytokinin, Gibberilic acid and NAA as well as with BA and NAA. It appears that to get positive results elaborate work is needed.

5.3 Micrografting

5.3.1 *In vitro* seed germination

Seeds of 1, 3, 6 and 8 months old maturity were tested for *in vitro* germination. Germination of mature seeds was observed only in presterilized bottles with water soaked cotton/distilled water. Basal media of ¼, ½ and Full MS did not support germination of whole/ cut seed. Fortification of ¼ th and ½ MS with hormones 2, 4-D (2.0 mg l^{-1}) + Kinetin (1.0 mg l^{-1}) + 2nd stock double also did not support germination.

Thimmapaiah *et al.* (2001) reported germination of cashew seeds 20 to25 days after inoculation in absorbent cotton. Bhattacharya and Khuspe (2000) reported *in vitro* germination of papaya seeds when MS medium supplemented with Thidiazuron (20µ ml^{-1}). Buyun *et al.* (2004) reported germination of orchid seeds and its proliferation in MS medium supplemented with 1.0 g activated charcoal.

Seedlngs for *in vitro* micrografting could be raised by incubating mature seeds in presterilized bottles with water soaked cotton/ little distilled water. After initial establishment of the graft, it could be transplanted outside to potting mixture medium.

5.3.2 Plant regeneration through somatic embryogenesis

Plant regeneration through somatic embryogenesis could not be obtained even though various treatments were tried (Table 7), but callus and somatic embryo proliferation was observed with the medium B_5 + 0.1 mg l^{-1} Kin + 0.01 mg l^{-1} NAA + 0.01 mg l^{-1} GA_3 + 10.0 mg l^{-1} Casein hydrolysate + . 0.5% A.C. Further maturation was attempted, but the calli turned dark brown colour. Radjojevic *et al.*, (1987) obtained somatic embryogenesis and plant regeneration from zygotic embryo derived callus cultures of *Iris pumila*. But in the present case plant regeneration was not possible even though various auxins and cytokinins were tried. So further studies may be recommended towards this direction. Evans *et al.* (1986) reported that somatic embryogenesis is a more rapid mode of plant regeneration but the positive results are limited to a few species.

Regenerating plants via. Somatic embryogenesis will reveal the tissue response of nutmeg to hormones. This knowledge could be utilized for breaking the recalcitrancy associated with multiple shoot induction.

5.3.3 *In vitro* establishment of *in vivo* germinated seeds

Attempts were made to establish in vivo germinated seeds three days after germination under in vitro condition in ½ MS + 2% sucrose. Even though the surface sterilization treatment involved thirty minutes dip in 0.1% Emissan followed by 0.1% $HgCl_2$ treatment for six minutes, the fungal contamination was not controlled. The culture medium containing major and minor nutrients favoured fungal growth very much. So effective surface sterilization treatment should be developed, guarding that, it is not necrotic to the seedling.

5.3.4 *In vivo* seed germination

The effect of seed treatments on seed germination was studied in detail. Seeds sown without any treatment germinated within 70 days. Chilling of nutmeg seeds for 24 hrs and sand sowing was found to be the best treatment with respect to number of days taken for germination as it germinated within 67 days, but the germination percentage was only 40%. The highest percentage of germination (50%) were obtained with treatments T_1 (water soaking for 24 hr followed by sand sowing) and T_8 (control – without any treatment). Other treatments such as mechanical scarification, GA_3 treatment etc reduced the germination percentage (10 to 20%).

It is reported by Flach (1966) that the germination of nutmeg seeds varied from 35% to 70%. Peril (1938) has also reported that the germination of the nutmeg seeds vary widely depending upon the collection of seeds from trees of different yield groups and from female trees which are situated at different distances from the male trees. The possible reasons for these differences have not been explained by him. Hume and Cobin (1943) reported that smaller nutmeg seeds gave lower percentage of germination as compared to the heavier seeds. The stimulatory effect of gibberellic acid treatment on the germination as well as the subsequent growth of the seedlings had been reported by several workers in fruit crops (Elson 1954, Kahn *et al.*, 1957,

Fogle and Mc Crory 1960). However in the present study effect of gibberellic acid was not promising. Statistical analysis with CRD showed that there is significant difference among various treatments and T_2, T_6, and T_8 were found to be the best treatments.

Cardamom seeds have highest germination after acid scarification for 10 minutes and soaking in GA_3 for 24 hours (Raja 1993). *Acacia nilotica* seeds scarified with Sulphuric acid (H_2SO_4) showed higher germination percentage as reported by Palani *et al.* (1996).

Since the treatment control, i.e., sowing seeds without any treatment registered 50% germination within a period of 70 days; it could be treated as the best treatment for germination. Seedlings for *in vivo* grafting could be germinated under *in vivo* by sowing in sand without any treatment.

5.3.5 Micrografting methods

Attempts were made in grafting of *in vitro* grown scion with *in vitro* and *in vivo* grown rootstock as given in section 3.2.5. The scion was *in vitro* produced elongated shoot tips of 1.5 cm length. Epicotyl grafting was done by inserting prepared wedge shaped scion to the rootstock slit of 2 cm length. Micrografted cultures were inoculated into pre-sterilized bottles containing liquid SH media. The grafts survived two weeks only due to bacterial and fungal contamination. Although a second time surface sterilization was given, contamination could not be controlled.

In vitro micrografting could be done successfully if microbial contamination arising from root stocks could be controlled. The possibility of grafting on *in vitro* germinated seedlings in humid bottles and initial establishment with nutrients supplied from seed endosperm could be explored. After initial establishment, the graft could be planted out in potting mixture. This avoids culturing in nutrient medium which is very much favourable for microbial growth. Grafting could be done under *in vivo* also on *in vivo* germinated seedlings with in vitro regenerated scion. Since feasibility studies in juvenile grafing have recorded 80% success this will be feasible

(section 3.2.2.1). The environmental conditions i.e., humidity and temperature level for the perfect graft union has to be identified.

Kesavachandran (2005) reported successful micrografting *in vitro* in cashew. He could obtain high levels of graft union when 2-3 cm long shoots were grafted on to 8-9 month old *in vitro* raised rootstocks. Both *in vitro* and *in vivo* micrografting were successuful in Pistachio (Onay *et al.*, 2003). Braghdin (1986) reported that growth of scion was very slow and elongation did not occurred where *in vitro* micrografting in *Pistachia vera* was attempted. Abou Salim and Mantell (1992) also could not rejuvenate where micrografting was done in the Pistachia crop.

Standardization of micrografting in nutmeg with stage II cultures of orthotrops under *in vitro* or *in vivo* condition has relevance as the nutmeg grafts are costly.

5.4 Future line of work suggested

1. In the present study suitable explant, culture medium and culture conditions for culture establishment was identified. The growth habit of nutmeg is characterized by periodical flushing and dormancy. Initiating culture establishment just before flushing, avoiding rainy season will reduce the contamination and will improve the success rate.

2. Nutmeg is recalcitrant in multiple shoot induction. The scientific reason for that should be explored and treatments to nullify that should be developed.

3. *In vitro* micrografting could be attempted on seedlings germinated in humid bottles with epicotyl grafting techniques. Initial establishment of the graft could be made from the stored food of the seed. Later they could be transplanted to soil and performance should be evaluated.

4. Micrografting could be attempted on *in vivo* germinated seedlings with *in vitro* regenerated scion.

Summary

SUMMARY

The present investigation was carried out during the period 2005-2007 in the Centre for Plant Biotechnology and Molecular Biology Laboratory of College of Horticulture, Vellanikkara with the objective of *In vitro* shoot regeneration and micrografting in Nutmeg (*Myristica fragrans* Houtt.). Juvenile plants, mature trees and regenerants from coppiced trees were used as source of explants. The salient findings of the investigation are presented below:

1. Surface sterilization of nodal segments with soaking in carbendazim (0.1%) for 10 minutes followed by mercuric chloride (0.1%) treatment for six minutes and sterile water wash, recorded 33% survival of cultures. Loss of cultures were due to fungal contamination and necrosis of tissues.

2. Different basal media along with hormones were tested for culture establishment with nodal segments. SH medium was found to be the best for culture establishment (26%) followed by WPM (13%). The response of explants to ½ MS medium was nil. Compared to shoot tips, nodal segments were found to be the best explant for culture establishment.

3. The influence of summer (April - May) and rainy season (June – July) on culture establishment was studied with nodal segments. The survival of cultures during summer season was 66.72% and during rainy season was 22.24%. During rainy season contamination due to fungus was high.

4. The media combination (T_{13}), SH + TDZ 0.03 mg l^{-1} + 0.5% A.C. recorded bud expansion in 50% of the cultures with nodal segments, within a period of 9 days. In (T_3), SH +3.0 mg l^{-1} BA + 1.0 mg l^{-1} IAA + 0.5% A.C. bud expansion were observed with 25% of cultures. In (T_{15}), WPM + 2.0 BA + 1.0 NAA + 0.5% A.C., 25% cultures showed bud expansion.

5. Refinement of culture establishment media was attempted with organic supplements; Coconut water (5, 10, 15 and 20% v/v), Casein hydrolysate (10, 25 and 50 mg l^{-1}) and Brassinolide (0.05, 0.1 and 0.2 mg l^{-1}) with nodal segments from juvenile seedlings, regenerants from coppiced trees and mature trees.

6. In juvenile explants, coconut water (5, 10, 15% v/v) supported culture establishment and maximum response (50%) was observed with 5% v/v coconut water. Shoot elongation was obtained with 10% v/v coconut water. In explants from mature trees, coconut water 5% and 15% v/v showed bud expansion in 25% of cultures.

7. The juvenile explants showed bud expansion in all the three combinations (10, 25 and 50 mg l^{-1}) of casein hydrolysate. At 25 mg l^{-1} concentration shoot elongation and leaf expansion was observed. The maximum bud expansion percentage was in 10 mg l^{-1} (50%). In explants from mature trees, casein hydrolysate 50 mg l^{-1} showed bud expansion. The regenerants from coppiced trees produced shoot elongation and leaf expansion at 25 mg l^{-1} concentration in 25% of cultures.

8. Brassinolide at 0.2 mg l^{-1} supported bud expansion in juvenile explants.

9. The carbon source; 2% Sucrose + 1% glucose or 5% sucrose supported bud elongation and leaf expansion. The suggested media for juvenile as well as mature explants for culture establishment of nodal segments of nutmeg is SH + 2% sucrose + 1% glucose + 0.03mg l^{-1} TDZ + 25 mg l^{-1} Casein hydrolysate + 0.5% A.C.

10. Shoot elongation and proliferation were tried with the media ½ MS + 1.0 mg l^{-1} Kin + 0.1mg l^{-1} NAA + 0.1 mg l^{-1} GA_3 + 10 mg l^{-1} Casein hydrolysate and SH + 2.0 mg l^{-1} BA + 1.0 mg l^{-1} NAA + 50 mg l^{-1} Adenine sulphate. The explants after three weeks were sub-cultured onto the same media. Multiple shoot induction was not obtained in both the cases.

11. Surface sterilization of seeds was carried out by soaking in carbendazim (0.1%) for thirty minutes followed by 70% alcohol wipe and mercuric chloride (0.1%) wash for seven minutes. Microbial contamination greatly hindered the *in vitro* culture establishment of seeds and maximum survival was 30%.

12. *In vitro* seed germination was attempted in various media. Germination of mature seeds was observed only in pre-sterilized bottles with distilled water / water soaked cotton. The basal medium of ¼, ½ and full MS did not support germination of whole seeds/cut seeds of 1, 3, 6 and 8 months maturity. Fortification of $¼^{th}$ and ½ MS media with hormonal combinations of 2, 4 - D (2.0 mg l^{-1}) + Kinetin (1.0 mg l^{-1}) + 2^{nd} stock double also did not support germination of whole or cut seeds.

13. Somatic embryos were formed from the cut end of the endospermous tissue of seeds of six month maturity when cultured in ½ MS + 2 % Sucrose. Proliferation of callus and somatic embryos was observed with the medium B_5 + 0.1 mg l^{-1} Kin + 0.01 mg l^{-1} NAA + 0.01 mg l^{-1} GA_3 + 10.0 mg l^{-1} Casein hydrolysate + 0.5% A.C. The response was obtained two and a half months after inoculation.

14. Attempts to establish *in vivo* germinated seedling under *in vitro* condition after surface sterilization by soaking in 0.1% Emissan for thirty minutes followed by 0.1% $HgCl_2$ for six minutes did not succeed. Seeds were damaged due to fungal contamination.

15. In *in vivo* seed germination, the highest percentage (50%) of germination were obtained with treatments T_1 (water soaking, 76 days) and T_8 (control, 70 days). With respect to the time taken for the germination, it was quicker (67 days) with T_2 (chilling treatment at 5°C for 24 hrs followed by sand sowing).

16. Feasibility of juvenile grafting in nutmeg was studied on one month old seedlings as rootstock and *in vitro* produced shoot tips as scion. The grafts survived with 80% success.

17. For *in vitro* micrografting, elongated shoot tips produced under *in vitro* condition of length 1.5 cm were used as scion. *In vivo* and *in vitro* germinated seedlings were used as rootstock. Epicotyl grafting procedure was followed and the cultures were inoculated into liquid medium of SH+ 0.03 mg l^{-1} TDZ + 0.5% A.C. Fungal contamination severely hindered the establishment of grafts.

18. For *in vivo* micrografting, elongated shoot tips produced under *in vitro* condition were used as scion and two month old nutmeg seedlings grown under *in vivo* condition were used as rootstock. The culture condition for graft was 60 to 70% humidity with 50% shade. The grafts survived one week and later dried up.

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IN VITRO SHOOT REGENERATION AND MICROGRAFTING IN NUTMEG (*Myristica fragrans* Houtt.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Nutmeg (*Myristica fragrans* Houtt.), is dioecious and dimorphic in branching habit with erect growing orthotropic and horizontally growing plagiotropic shoots. The long gestation period and dioecious nature of the crop causes difficulty in the production of quality planting materials of known sex. Vegetative propagation, budding and grafting with orthotropic scion material produces erect growing tree with upright tree architecture. In vegetative propagation, scarcity of orthotropic scion material is a limiting factor in large scale production of planting materials. So the programme "*In vitro* shoot regeneration and micrografting in nutmeg (*Myristica fragrans* Houtt.)" was taken up.

The objectives of the study were: (1) To identify the culture conditions for multiple shoot production from orthotrops of gynoecious plants of nutmeg through enhanced release of axillary buds and shoot tip culture and (2) To standardize micrografting technique with *in vitro* and *in vivo* shoots as scion. The work was done at CPBMB, College of Horticulture, Vellanikkara.

SH medium (Schenk and Hildebrandt, 1972) was found to be the best basal medium for *in vitro* culture establishment of nodal segments of nutmeg compared to ½ MS (Murashige and Skoog, 1962) and WPM (Lloyd and Mc Cown, 1980). Surface sterilization of nodal segments by soaking in (0.1%) carbendazim for 10 minutes followed by (0.1%) $HgCl_2$ treatment for six minutes and sterile water wash, recorded 33% survival of cultures. The best explant for culture initiation was nodal segments. The best season for culture establishment was summer months (April-May) compared to rainy season (June- July). Loss of cultures was due to fungal contamination and necrosis of tissues. The media combination SH + Thidiazuron (TDZ) 0.03mg l^{-1} + Activated Charcoal (A.C.) 0.5% recorded bud expansion in 50% of the cultures within a period of nine days. Nodal segments are superior to shoot tips in culture establishment. Culture condition for culture establishment was 26 ± 2^0C at a light intensity of 1000 lux. The carbon source; 2% Sucrose + 1% glucose or 5% sucrose supported bud elongation and leaf expansion

Refinement of culture establishment media was attempted with organic supplements; Coconut water (5, 10, 15 and 20% v/v), Casein hydrolysate (10, 25 and

50mg l^{-1}) and Brassinolide (0.05, 0.1 and 0.2mg l^{-1}) with nodal segments from juvenile seedlings, regenerants from coppiced trees and mature trees. In explants from juvenile seedlings and mature trees, 5 to 15%coconut water supported culture establishment. In juvenile explants, shoot elongation was also observed at 10% coconut water. Casein hydrolysate supported bud expansion in juvenile and mature tree explants at 10 to 50 mg l^{-1}. Bud elongation and leaf expansion was observed at 25 mg l^{-1} concentration. Brassinolide (0.2 mg l^{-1}) supported bud expansion in juvenile explants.

The suggested media for explants from juvenile as well as coppiced trees for culture establishment of nodal segments of nutmeg is SH + 0.03 mg l^{-1} TDZ + 25 mg l^{-1} Casein hydrolysate + 2% sucrose + 1% glucose + 0.5% A.C. Casein hydrolysate concentration for explants from mature trees could be 50 mg l^{-1}.

In vitro seed germination was observed in mature seeds in presterilized bottles with water soaked cotton/ little water. Somatic embryos were formed at the cut portion of six month old seeds in the medium of ½ MS + 2% Sucrose + A.C. 0.5%. Proliferation of callus and somatic embryos was observed with the medium B_5 + 0.1 mg l^{-1} Kin + 0.01 mg l^{-1} NAA + 0.01 mg l^{-1} GA_3 + 10.0 mg l^{-1} Casein hydrolysate + . 0.5% A.C. The response was obtained two and a half months after inoculation. Three days old *in vivo* germinated seedlings did not established under *in vitro* condition even though surface sterilization treatment with 0.1% Emissan for thirty minutes followed by 0.1% $HgCl_2$ for six minutes was given.

Feasibility of grafting in juvenile plants was studied with epicotyl grafting and got 80% success. Grafting was done on twenty days old seedling with scion material from different seedling.

In vitro epicotyl micrografting was done with *in vitro* raised scion and root stocks. It was also done on *in vivo* germinated seedlings after surface sterilization. Scion shoot of 2.5 cm length was grafted on twenty days old root stock. Graft was cultured in liquid nutrient medium and survived for two weeks. Later fungal contamination destroyed the cultures. Grafting with *in vitro* shoots on *in vivo* raised root stocks did not succeed.