EFFECT OF PHYSIOLOGICAL PRE-CONDITIONING OF EXPLANTS AND EXPLANT SOURCES OF *Myristica fragrans* Houtt. ON *IN VITRO* CULTURE ESTABLISHMENT AND GROWTH

BY

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THESIS
SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN HORTICULTURE
FACULTY OF AGRICULTURE
KERALA AGRICULTURAL UNIVERSITY

DEPARTMENT OF HORTICULTURE
COLLEGE OF AGRICULTURE
VELLAYANI
THIRUVANANTHAPURAM
1995

DEDICATED TO MY BELOVED FATHER

Declaration

I hereby declare that this thesis entitled "Effect of physiological pre-conditioning of explants and explant sources of *Myristica fragrans* Houtt. on *in vitro* culture establishment and growth", 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, diploma, associateship, fellowship or other similar title of any other University or Society.

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Certificate

Certified that this thesis, entitled "Effect of physiological pre-conditioning of explants and explant sources of *Myristica fragrans* Houtt. on *in vitro* culture establishment and growth", is a record of research work done independently by Ms. Geetha S. (Ad.No. 92-12-10) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Acknowledgement

I hereby express my heartfelt gratitude and indebtedness to Dr. B.R. Reghunath, Associate Professor, Department of Horticulture, Major Advisor and Chairman of the Advisory Committee for suggesting the problem and for his valuable guidance and critical supervision and constructive criticisms throughout the course of investigation and preparation of the thesis.

I also express my deep sense of gratitude to Dr.G.Sreekantan Nair, Professor and Head, Department of Horticulture, Member of the Advisory Committee, for his sincere help and constructive criticisms in the conduct of experiment and preparation of the thesis.

My sincere gratitude are also due to Dr.S.Ramachandran Nair, General Co-ordinator, Kerala Horticultural Development Programme, Member of the Advisory Committee for his keen interest and critical suggestions in the conduct of experiment and preparation of the thesis.

Also, I wish to place on record my indebtedness to Dr.N.Mohanakumaran, Director of Research (i/c) Kerala Agricultural University for his encouragement in taking up this experiment.

Dr. D.S.Radha Devi, Associate Professor, Department of Plant Breeding and Genetics, Member of the Advisory Committee had extended valuable help and critical suggestions in the conduct of the experiment and preparation of the thesis. I thank her very much for this.

Dr. K. Rajmohan, Associate Professor and Project Co-ordinator (Bio-technology), Department of Horticulture had been one of the most helpful person who had also taken keen interest and extended valuable help in the conduct of the experiment. I wish to thank him from the depth of my heart.

Smt. I, Sreelathakumari, Assistant Professor, Department of Horticulture, had always been with me not only in the conduct of experiment but also while preparing the manuscript. Words fail in expressing my gratitude to her.

My sincere thank are also due to Dr.P. Saraswathy, Professor and Head, Department of Agricultural Statistics, College of Agriculture, Vellayani for her valuable help in the analysis of the data.

May I express my heartfelt gratitude to; G.R. Sulekha, Associate Professor, Department of Horticulture, for her valuable suggestions in the conduct of the experiment. Bindu C.P., Ms. Bindu C.S., Ms.Suneetha S., Ms. Sherly Kuriakose, Sri Biju S.V., Sri Ajithkumar, P.V., Sri Nizam S.A. and all my friends for their co-operation and encouragement.

The staff members of the Department of Horticulture, College of Agriculture, Vellayani for their sincere support extended throughout the course of investigation.

Sri C.E. Ajithkumar, Junior Programmer, Department of Agricultural Statistics, College of Agriculture, Vellayani for analysing the experimental data.

Indian Council of Agriculture Research, New Delhi for awarding me the Junior Fellowship during the tenure of the study.

My parents, brother, and sisters for their encouragement and help at every stage of my study.

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List of Abbreviations

AM	Abou-Mandour (1977)
BAP	6-benzylaminopurine
CH	Casein hydrolysate
2,4-D	2,4-dichlorophenoxyacetic acid
GA_3	Gibberellic acid
IAA	3-indoleacetic acid
IBA	3-indolebutyric acid
2iP	N^6-(2-isopentyl) adenine
QL	Quoirin and Lepoivre (1977)
MS	Murashige and Skoog (1962)
NAA	1-naphthylacetic acid
Na_2 EDTA	Sodium salt of ethylene diamenetetra-acetic acid
PG	Phloroglucinol
PVP	Polyvinylpyrrolidone
SH	Schenk and Hildebrandt (1972)
WPM	Woody Plant Medium

Introduction

Introduction

Nutmeg (*Myristica fragrans* Houtt.), a valued tree spice as well as an important medicinal plant, is unique among the spice crops as it produces two separate and distinct products viz., the seed spice and the mace. The former consists of the kernel of mature seed, whereas the latter consists of the aril which covers the seed. The nutmeg seed, mace and even its fleshy pericarp is widely used in Ayurvedic medicines, confectionary and culinary preparations. Nutmeg butter is used as a soothening agent in pain balms and also in treating rheumatism and paralysis. The flavour of nutmeg and mace is distinct due to their fatty acid composition (Johny, 1994).

In India, nutmeg is mainly cultivated in Kerala covering an area of 3Ø5Ø hectares. Its annual production is estimated to be 5537 t (1989-'9Ø). Production of nutmeg in India has always been short of demand necessitating import (Varghese *et al.*, 199Ø). The current market price for a quintal of seed is Rs. 11,ØØØ/- and for mace it is Rs. 32,ØØØ.

Nutmeg is a tall, evergreen tree with a long pre-bearing age. Being dioecious in nature, identification of female plants before the commencement of their flowering, is a problem in nutmeg cultivation. Many of the plants turn out to be unproductive males after 5-7 years of careful nurture (Baby *et al.*, 1993). No reliable methods have been reported yet to identify the sex of the seedlings before flowering. Vegetative propagation through grafting and budding have been attempted but the percentage of success is low (Babu *et al.*, 1993). Standardisation of a viable *in vitro* technique for clonal propagation of female nutmeg plants can solve both the problems of long pre-bearing age and sex to a great extent. However, due to the existence of dimorphism (orthotropism and plagiotropism), it is always difficult to get sufficient number of orthotropic shoots (which only can give erect growing trees) required for *in vitro* culture (Rema and Krishnamoorthy, 1993). Hence, the present study was conducted with the following as the major objectives:

1. To induce sufficient number of orthotropic shoots by various physical and chemical methods.

2. To find out the most suitable culture medium and to standardise the optimum composition of various components of the medium viz., growth regulators, inorganic and organic growth adjuvants and vitamins for achieving better establishment in *in vitro* culture.

3. To study the response of explants (collected from different explant sources viz., mature tree, graft and seedling, subjected to various *in vivo* pre-treatments) on *in vitro* culture establishment and growth.

Review of Literature

Review of Literature

Micropropagation or *in vitro* propagation is widely recognized as an efficient method for propagating plants in large scale. *In vitro* propagation methods include shoot culture for proliferation of axillary or adventitious shoots and callus culture for regeneration of shoots or embryoids. Both these methods have been applied to a wide range of tree species with varying levels of success.

It is evident from the literature that the response of perennial tree species to *in vitro* culture is remarkably low when compared to herbaceous and other annual species. To explain this phenomenon properly the physiological status of each tree species has to be studied in detail. Physiological manipulation of explant sources (parent plants) under *in vivo* condition and *in vitro* manipulation of explants may help in increasing the response of tree species in *in vitro* culture.

2.1. Induction of juvenility under *in vivo* conditions

The physiological age of the plant influences the type and extent of morphogenesis. The youngest and less differentiated tissues are found in plant meristems. Such meristems are successfully cultured *in vitro* in a wide range of species (Hughes, 1981). During the maturation process of

the plant several physiological changes take place that influence the *in vitro* behaviour of the explants (David, 1982). The ability of the explants to produce adventitious and axillary buds, the rate of shoot elongation and their ability to root, all are attributed to such physiological changes.

Explants from mature trees do not usually give any favourable response under *in vitro* conditions (Durzan, 1984). Juvenile tissues and cuttings taken from seedlings or young plants generally exhibit rooting potential which disappears when the plants have reached the adult stage (Bajaj, 1991). Bonga (1982) has emphasised the importance of selecting such tissues for *in vitro* culture. In general, for tissues other than meristems, young tissues have a higher degree of morphogenic competence than older tissues. Because of the decreased morphogenic ability of mature material it has not been generally possible to apply the techniques developed for juvenile material to mature plants.

Pre-treatments such as hormonal sprays, pruning and grafting modify the topophysical pre-disposition of tissues in trees with varying intensity. The growth rate in general, the inhibition on the emergence of lateral buds and shoots, the branch angle and plagiotropic growth, all can be altered by the application of growth regulators. In Alnus and Platanus, application of BAP could change the branch angle and their plagiotropic growth habit. Also the

elongation growth of buds was inhibited. Apparently, this was a consequence of the induction of axillary bud development which consumed a major part of the available energy (Evers, 1987).

Hackett (1987) found that reversion to the juvenile condition does occur naturally during sexual and apomictic reproduction and can be induced by various nutritional, hormonal or environmental treatments. In some plants like *Hedera helix*, the juvenile phase can also be maintained in a stable morphological condition by propogation through cuttage.

Juvenility is of major significance in relation to the vegetative propagation of trees by conventional and *in vitro* methods. However, the transition from the juvenile phase appears to be reversible to some extent and it is common for some tissues of the adult tree to have some of the physiological characteristics of seedlings such as high capacity for adventitious root production. Jones (1986) found that the physiological status of the explant is of prime importance, in the case of adult trees. Extensive studies at the Association Foret-Cellulose (AFOCEL) Research Institute in France indicated that explants from adult forest trees were frequently slow to commence growth *in vitro*, or failed completely unless selected from rejuvenated tissues. Where rejuvenation does not occur naturally, it has been induced by various treatments such as the grafting

of shoots on to seedlings, shoot pruning, the maintenance of high fertiliser levels, vegetative propagation or treatment with cytokinins.

Rema and Krishnamoorthy (1990) obtained 1-3 shoots from each nutmeg graft by pruning orthotropic grafts of known sex above the graft union. When nutmeg trees of 8-9 years old were detopped at 3 m height, on an average 21 shoots were produced and further pruning of the orthotropic shoots produced 1-3 orthotropic shoots from each shoot. They also attempted to convert plagiotropic shoots to orthotropic shoots using hormones such as GA_3 (1000 and 2000 mg/l) and cytokinin (50 and 1000 mg/l); but the response was not encouraging. An attempt to patch bud nutmeg using dormant orthotropes was also not successful.

In cashew, when explants of different age groups were cultured *in vitro*, juvenile explants collected from the seedlings gave best results. Physiological age of the explants exhibited significant influence on their performance *in vitro* (Nair, 1991). She found that nodal segments of partially etiolated shoots induced from cuttings of mature cashew trees, produced multiple shoots under *in vitro* conditions.

Grafting of scions from mature individuals on juvenile rootstock followed by removal of all mature leaves from the scions will lead to the induction of juvenility in mature forest tree species (Franclet *et al.*, 1987).

Graftage has enhanced some juvenile features in mature buds grafted on to juvenile stocks, most notably in ivy and rubber. In ivy, reversion to juvenility is most evident when only juvenile leaves are present on the graft combination and when temperatures are relatively high (Leopold and Kriedemann, 1975; Hartmann and Kester, 1989).

Rajmohan and Mohanakumaran (1988) reported that shoot proliferation was maximum in the case of jack explants taken from seedlings than from 10 and 30 year old trees and six month old grafts. Though the rate of multiplication *in vitro* was very low in all the cases except seedlings, the six month old grafts gave 100 per cent survival of the shoots produced. Philip (1993) also obtained similar results.

In many species GA_3 application extend the juvenile period. This effect of GA_3 was first reported in ivy, in which a temporary reversion from mature to juvenile morphology after GA_3 application was observed. (Robbins, 1957, Hartmann and Kester, 1989). Luckwill (1970) observed that GA_3 application to Ipomoea extended the juvenile period, as evidenced by the extension of time over which juvenile leaf form is retained.

Bassuk and Maynard (1987) reported that stock plant etiolation led to the initiation of new plant growth. Partial etiolation of branches was proposed as a suitable pre-culture treatment for *in vitro* propagation of selected

mature trees, when physiologically juvenile materials such as stump sprouts, epicormic shoots or root suckers are available. Localized etiolation of branches in the crown of a 30 year old chestnut tree gave plant materials that responded much better to establishment and multiplication *in vitro* than unetiolated plant material (Ballester *et al.*, 1989).

Shoot cultures of *Quercus robus* were established by Vieitez *et al.* (1985) from explants taken from new growth of stump sprouts of mature trees. Brand and Lineberger (1988) observed that in *Halesia carolina*, explants taken two weeks after bud break produced the greatest number of shoots in a period of nine weeks.

2.2. Physiological pre-conditioning under *in vitro* conditions

In rubber, pre-culture soaking of shoot explants in 10 mg/l BAP helped in the bud break of 70 per cent of the explants after four weeks of culturing (Enjarlic and Carron, 1982). Pre-treating vegetative buds with cytokinin was also shown to have favourable effect in overcoming apical dominance under *in vitro* culture conditions and also to overcome dormancy (Borkowska and Habdas, 1982).

Rajmohan (1985) found that in jack, a pre-culture treatment given to shoot tip explants by keeping them for 24 hours in a solution containing BAP (10 mg/l) and

GA_3 (1mg/l) under refrigerated condition was effective for culture establishment. In cardamom, cytokinin infusion (200 mg/l kinetin/BAP for 6-24 hours) of juvenile shoot primordia *in vivo* has resulted in enhanced shoot proliferation when they were cultured *in vitro* (Reghunath and Bajaj,1992).

Rai and Chandra (1987) could successfully culture cinnamon using shoot tip explants from *in vitro* germinated seeds in MS medium containing 1 mg/l kinetin.

2.2.1. Position and size of explant

D'Amte (1977) observed that apical meristems of buds or the apical part of the growing shoots favoured the production of uniform plantlets, identical to the donor plant. Also shoot proliferation was greatest in the explants derived from the top carrying of the parent plant.

Size of the explant, is also an important factor as that of age while selecting the explant. Monaco *et al.*, (1977) reported that the size of the explant determines the survival of the cultures. When tissues are cut, the exposed cut surfaces turns brown due to the phenolic oxidation to toxic quinones in the damaged cells. If the explant size is small, the cut surface-to-volume ratio will be high and this leads to difficulty in the survival of the explant.

According to Oka (1985) the size of the explant and the level of endogenous growth substances are positively correlated. In mulberry, when 10-15 mm long axillary bud was used to culture, shoots developed in a medium supplemented with NAA alone. But when the stem length was reduced to 3-5 mm, both BAP and NAA were needed for shoot production.

According to Legrand and Mississo (1986), there is a minimum explant size below which buds do not burst particularly in culture medium devoid of growth substances. Norton and Norton (1986) observed that larger explants (10-20 mm) produced greater number of shoots than smaller explants (5-10 mm).

2.2.2. Pre-culture treatments

When explants are cut, phenolic exudation takes place, resulting in the darkening of the tissues as well as the culture medium leading to inhibition of growth. Use of antioxidant solution for pre-treating explants, addition of antioxidants to the culture medium and incubating in reduced light or darkness can lessen the problem. Staritsky (1970) and Mc Comb and Newton (1981) observed that incubation of cultures in the dark initially (for about six weeks) can prevent the production of phenolics in some species including trees.

Jones (1965) and Walkey (1986) employed PVP to prevent polymerization of phenolics.

Sharp *et al.* (1973) added cysteine HCl (1Ø mg/l) to control polyphenol oxidation in *coffea arabica*.

Ascorbic acid enhanced the growth of *Citrus natsudaidai* plantlets by inhibiting action of polyphenols on cut surface of explants (Ohta and Furusato, 1957).

Murashige (1974) recommended ascorbate or citrate dip prior to culture of Eucalyptus to prevent browning of explants, whereas Cresswell and Nitsch (1975) controlled it by rinsing the shoots in water for several hours before excision.

According to Button and Kochba (1981), ascorbic acid enhanced pseudobulbil production in nucellar tissues of *Citrus sinensis*.

Gupta *et al.* (198Ø) found that anti-browning agents such as hydrogen peroxide (5 per cent) ascorbic acid (Ø.28 mg/l), soluble PVP (Ø.7 per cent) and Polyclar AT (Ø.7 per cent) when used along with sucrose was effective for shoot tip cultures of teak.

In nutmeg and bread fruit immersion of explants in a solution containing 2 per cent sucrose and Ø.7 per cent PVP for 3Ø minutes prevented browning (Rajmohan, 1985). In nutmeg, Jayasree (199Ø) also obtained similar results.

Legrand and Mississo (1986) reported that treatment of orthotropic shoots of cocoa with 2% orthodifolatan before collection of explants helped 95 per cent of the cultured explants remain healthy.

Pre-culture treatment of citrus explants with 1N hydrochloric acid for Ø.5 minutes; 7Ø per cent ethanol for 2.5 minutes plus Ø.1 per cent tween for 5 minutes and three rinses with sterile water reduced the phenolic darkening and also contamination of the cultures, without harming the explant (Moore, 1986). Amin and Jaiswal (1987) could eliminate interference of phenolic substances in shoot explants collected from apical portion of 15 year old guava plants by thorough washing in running tap water, followed by treatment with 'Cetavlon' for 5 minutes and agitation for 3Ø to 4Ø minutes in Ø.5 per cent solution of PVP containing 2 per cent sucrose.

In date palm, activated charcoal effectively prevented browning of explants from mature trees (Tisserat, 1982). Hu and Wang (1983) found that treating the explants with PVP, washing them with sterile water and inclusion of activated charcoal in the medium reduced the oxidation of polyphenols because of the absorption of the oxidation products by these chemicals.

Inclusion of charcoal and PVP in the culture media was not found to be helpful in absorbing phenolic substances in clove cultures. Alternatively, quick transfer of explants on to fresh media at the rate of 4 to 6 transfers a day was found useful (Priyadarshan *et al.*, 1989).

In Jack, Rajmohan (1985) reported that treatment of explants in a sterile solution containing BAP (1Ø mg/l) and GA_3 (1 mg/l) for 24h under refrigerated conditon reduced polyphenol activity.

Stafford (1974) observed that heat treatment as well as chilling treatment removed the constraints of maturity in woody species. Nitsch and Norreel (1976) reported that the cold treatments gave a greater number of androgenic anthers of *Datura innoxia*.

Bonga (1982) reported that by starvation, cold treatment and centrifugation treatment, partial damaging of cell organelles and their DNA could be achieved, thereby rejuvenating the mature explants. Fabijanski *et al.* (1991) found that initial incubation of Broccoli (*Brassica oleracea* var. Italica) at elevated temperatures followed by subjecting to low temperature enhanced the somatic embryo formation in anther culture. Philip (1993) observed that cold shock for five minutes was found to be the best among the stress treatments.

2.2.3. Surface sterilization of explants

Contamination of cultures due to micro organisms, particularly moulds and bacteria, is one of the major problems to be tackled during the initial stages of micro-propagation. Sodium hypochlorite or common house hold bleach is largely used for this purpose (Hu and Wang, 1983). However, in humid tropical countries, where there is the presence of microbial spores in the environment throughout the year, milder sterilants such as sodium hypochlorite will not be always effective. In many crops, mercuric chloride is reported to be an effective surface sterilant.

Zhang and Davies (1986) observed in *Lagerstroemia flos-reginae* 95 per cent survival of cultures after surface sterilisation of shoot explants in 7Ø per cent ethanol for 1 minute, followed by Ø.5 per cent sodium hypochlorite with Ø.1 per cent Liquinox, for 1Ø min.

Ball (1987) worked on the surface sterilisation of various explants and observed that the most rapid and efficient method for buds, leaves and stem pieces is the treatment with 1Ø per cent hydrogen peroxide for 1 or 2 minutes, washing in sterile water, followed by 1 or 2 minutes treatment in 2 per cent commercial bleach (5 to 25 per cent sodium hypochlorite).

Frisch and Camper (1987) found that disinfestation of the internodal stem sections of tea with an initial water rinse, Ø.1 per cent hydrochloric acid for 1.5 to 2 minutes; rinsing in autoclaved distilled water for 5 minutes; 3.75 per cent sodium hypo- chlorite for 2Ø minutes; followed by rinsing in autoclaved distilled water for 5 minutes and of 5 per cent calcium chloride for 1Ø minutes and final rinses in sterile water gave the best results.

Gupta *et al.* (1984) used Ø.Ø5 per cent mercuric chloride for 1Ø minutes to surface sterilise the apical regions of stem, leaves and young inflorescence collected from 2Ø year old coconut palms.

In bread fruit, sequential treatment with Ø.5 per cent sodium hypochlorite for 1Ø min and Ø.1 per cent mercuric chloride for 1Ø minutes could effectively surface sterilise the explants from shoot apices (Rajmohan, 1985).

According to Nair *et al.* (1986) Ø.2 per cent solution of mercuric chloride was optimum for disinfecting the endosperm of *Annona squamosa* Linn. In *Lagerstroemia flos-reginae*, surface disinfection was achieved by treating with Ø.Ø1 per cent mercuric chloride and Ø.Ø1 per cent sodium lauryl sulphate for 7-1Ø min (Paily and D'Souza, 1986).

While culturing nodal explants of mature guava trees, Amin and Jaiswal (1987) obtained maximum surface sterilisation by treating them with Ø.Ø5 per cent mercuric chloride for 2 minutes after a brief rinse in 7Ø per cent ethanol.

Surface sterilisation of explants of mulberry shoot tips was done after washing in distilled water for 5 minutes followed by Ø.1 per cent mercuric chloride solution for 5 minutes and finally rinsing with sterile distilled water (Ivanicka, 1987).

In clove, treatment with mercuric chloride (Ø.1 minutes) for 7 minutes could effectively surface sterilise axillary bud explants from mature trees (Mathew *et al.*, 1987; Mathew and Hariharan, 1992).

In nutmeg, treatment with mercuric chloride (Ø.1%) for 15 min could effectively surface sterilise shoot tip explants from mature trees (Jayasree, 199Ø).

Surface sterlisation of cashew explants with mercuric chloride (Ø.1 per cent) for 12 min was found to be effective in eliminating microbial contamination to a great extent. A few drops of Labolene were added to the sterilants (Nair, 1991). D'Silva and D'Souza (1993) reported that contamination of cashew seedling cultures could be controlled by germinating the nuts *in vitro*. The pericarp and testa were removed and nuts were agitated in Bavistin solution for 5 hours, before culturing.

Several workers have reported on the use of various fungicides in cultures for reducing fungal contamination (Brown *et al.*, 1982; Tanaka *et al.*, 1983; Shield *et al.*, 1984). However, only 'Benomyl' (a carbendazim fungicide) had been shown to have broad spectrum effect for use in plant cell and protoplast culture (Forsberg, 1969; Gupta and Hadley, 1977; Hauptmann *et al.*, 1985). Jones *et al.* (1977) had used Ø.1 per cent benomyl in the second stage of sterilisation of Malus (M-26) root stocks.

2.3. Culture establishment

2.3.1. Basal Media

Hussey (1979) pointed out that the exact composition of the medium has to be adjusted according to the requirements of the different groups of plants and that some species require additional supplements.

In Japanese Persimmon Suguira *et al.* (1986) used full strength MS medium supplemented with BAP and 2-ip, MS salts having only half strength nitrate, Lepoivre salts and Woody Plant Medium (WPM). Best results were obtained from MS (1/2 NO_3).

In *Garcinia mangostana* L., Goh *et al.* (1988) obtained proliferating shoots from cotyledon segments cultured on modified MS (1962) medium with BAP. They also obtained adventitious buds from juvenile leaf segments on WPM basal medium.

Berthon *et al.* (1987) used MS medium at half strength mineral salts for transplanting shoot explants of *Sequoiadendron gigantium.*

Rajasekaran and Mohankumar (1992) obtained multiple buds from nodal segments of mature bushes of tea on MS medium supplemented with BAP.

In *Liquidambar styraciflua* L., Brand and Lineberger (1988) obtained prolific shoot production using WPM with relatively high levels of BAP (2.5 mg/l).

The SH medium has been formulated to support the growth of a wide variety of tissue of both monocotyledons and dicotyledons (Narayanaswamy, 1977). When nodal segments of partially etiolated shoots induced from cuttings of mature cashew trees were cultured on SH medium with supplements, multiple shoots were formed. *In vitro* rooting of micro cuttings obtained from these cultures could be achieved on WPM or on half strength MS medium with supplements (Nair, 1991). Abou-Mandour (1977) developed a standard medium for tissue culture of medicinal plants in which a large number of different tissues from different

origin could be successfully grown. On an average 10 fold growth of callus in culture was achieved in 8 to 12 weeks.

Kathryn (1987) observed better culture establishment of mature and juvenile radiata pine on Le-Poivre nutrient media containing 5 mg/l BAP.

The Le-Poivre (Quorin and Le-Poivre, 1977) mineral formula elicited better growth responses than did Knop, when juvenile tea explants were cultured *in vitro* (Samartin, 1989).

2.3.2. Culture conditions

Growth of plants cultured *in vitro* is influenced by temperature, light and humidity. Usually temperature is adjusted to between 24°C and 26°C. However, a temperature of 26°C favoured growth of coffee cultures (Monaco *et al.*, 1977). Although the influence of temperature and light may not be always large, there is need for the use of controlled environment for *in vitro* studies (Hussey, 1979). Incubating the cultures at 26 ± 2°C either under 16 hours photoperiod (supplied by cool white flourescent tubes) or in darkness, gave good results for cashew cultures (Nair, 1991).

2.3.3. Organic compounds

According to Ma and Shii (1972) shoot proliferation in banana was dependant on the presence of adenine sulphate (160 mg/l), kinetin and IAA (2 mg/l each) and

tyrosine and meso-inositol (1ØØ mg/l each). They also reported that high rate of shoot proliferation can be obtained in liquid cultures too, even in the absence of growth regulators.

Rapid shoot proliferation was reported in *Costus speciosus* (Koenig) when shoot tips excised from rhizomes were cultured on modified SH medium containing BAP (Ø.5 mg/l), IAA (1 mg/l) and adenine sulphate (15 mg/l) (Chaturvedi *et al.*, 1984). Supplementation of Adenine sulphate to the medium containing BAP enhanced growth and shoot formation remarkably (Skoog and Tsui, 1948). This type of synergism between adenine sulphate and kinetin, as first reported by Skoog and Miller (1957), was observed by several researchers.

In cases where nutritional requirements have not been established, mixtures of aminoacids such as CH (casein hydrolysate) may be added between Ø.Ø5 and Ø.1 % (Huang and Murashige, 1977).

The discovery of Pollard *et al.* (1961) that myo-inositol was present in coconut water and had growth promoting activity, led to the inclusion of inositol in plant tissue culture media.

2.3.4. Phloroglucinol

Phloroglucinol (PG) in the nutrient medium is found to produce a two to three fold increase in the proliferation and rooting of shoots of the root stock M.7

apple cultures (Jones, 1986). Baleriola-Lucas and Mullins (1984) reported the effectiveness of phloroglucinol in overcoming the difficulty in establishing initial explants of woody species. But this effect of phloroglucinol is found to be dependant on both the cultivar tested and on the physiological state of the tissues (Jones, 1986). Hammatt and Grant (1993) reported the effect of this phenolic substance in hastening the process of rejuvenation in *in vitro* culture of mature wild cherry. Phloroglucinol when added to the rooting medium significantly improved the production of roots.

2.3.5. Growth hormones

Popov and Vyosotskii (1978) observed that MS medium supplemented with BAP (Ø.5 mg/l) and kinetin (Ø.5 mg/l) stimulated leaf growth and development in sourcherry, black currant and plum. Dublin (198Ø) in his experiments found that shoots regenerated after six weeks from apical meristems of *Coffea arabica* cv. *caturra x c. canephora* hybrid, when cultured on MS medium with 3% sucrose, 1ØØ mg/l myo-inositol, 1 mg/l thiamine, 3Ø mg/l L-cysteine, Ø.1 to Ø.3 mg/l BAP and Ø.1 to Ø.2 mg/l NAA.

Koblitz *et al.* (1983) obtained shoot regeneration in cinchona on a medium containing BAP or Zeatin. Gibberellic acid (GA_3) which had an initial stimulatory

effect on cotyledonary bud growth, prevented further growth in prolonged culture.

In fig, Pontikis and Melas (1986) initiated aseptic shoot cultures on MS medium containing Ø.5 mg/l BAP and Ø.1 mg each of IBA and GA_3.

Suguira *et al.* (1986) established dormant bud explants of Japanese persimmon on a modified MS medium supplemented with BAP (5 mg/l) and obtained shoot elongation using 2-ip.

Rao and De (1987) used various parts of the plant as explants for generating callus in *Albezia lebbeck*. Callusing could be obtained in hypocotyl segements in MS medium containing 1 mg/l each of NAA and kinetin. For stem explants 1 mg/l each of 2,4-D and kinetin were required whereas for leaf Ø.5 mg/l of 2,4-D and 1 mg/l of kinetin gave good callusing.

In teak (*Tectona grandis*) multiple shoots were induced from terminal buds excised from seedlings of 1ØØ year old trees on MS medium containing Ø.1 mg/l BAP and Ø.1 mg/l kinetin (Gupta *et al.*, 198Ø).

In *Eucalyptus citriodora*, (Gupta *et al.*, 1981) could obtain multiple shoots from terminal buds of 2Ø year old trees on MS medium supplemented with BAP (Ø.3 mg/l) kinetin (Ø.2 mg/l) and calcium pantothenate (Ø.1 mg/l).

Barlass and Skene (1982) found that multiple shoot formation from juvenile and mature nodes of *Alnus* species and hybrids was stimulated on MS medium supplemented with BAP 2.25 mg/l.

Barghchi and Alderson (1985) found that in *Pistacia vera*, multiple shoots were induced from shoot tips and nodal bud explants on MS medium supplemented with BAP (4 mg/l).

Large number of multiple shoots could be produced in apple root stock (M4) cultures using BAP and IBA (Ø.15 or Ø.2Ø mg/l each) (David *et al.*, 1985).

In *Emblica officinalis* morphogenesis and plant regeneration from the endosperm was achieved by Sehgal and Khurana (1985) on MS medium containing BAP (Ø.2 mg/l) and IAA (Ø.1 mg/l).

Yashpal *et al.* (1985) obtained multiple shoot formation using lateral bud explants of subabul on MS medium supplemented with BAP (3 mg/l) and NAA (Ø.Ø5 mg/l) in 4-5 weeks old cultures.

In *Lagerstroemia flos-reginae*, BAP (7.5 to 2Ømg/l) was best suited for shoot tip culture (Paily and D'Souza, 1986).

In guava, BAP (1 mg/l) was optimum for inducing multiple shoots from lateral bud explants (Amin and Jaiswal, 1987).

In the absence of growth substances, hypocotyl segments of seedlings of *Cinnamomum zeylanicum* failed to develop multiple shoots. When BAP and kinetin was given in combination (1 mg/l each) 2 to 4 shoots were developed in 1Ø-15 days (Rai and Chandra, 1987).

Rajmohan and Mohanakumaran (1988) observed maximum number of elongated shoots from shoot apices of fresh stem sprouts of 5 year old jack trees when cultured on MS medium containing BAP (5 mg/l). Growth of the cultures were supported with 3Ø-4Ø g/l of sucrose or 2Ø-3Ø g/l of glucose. They reported no beneficial effect in terms of the rate of shoot multiplication and growth of cultures, when GA_3 was used.

MS medium containing 2 mg/l BAP and Ø.5 mg/l NAA induced the highest number of shoot buds in flood tolerant jack. These shoots continued to proliferate through seven or more subcultures with an average of 1Ø shoots per transfer (Roy *et al.*, 1994).

Multiple buds were obtained from nodal segments of mature bushes of tea (*Camellia* sp.) on MS medium supplemented with BAP (Rajasekaran and Mohankumar, 1992).

Liaw *et al.* (1994) reported that multiple shoots were obtained when *Pyrus serotina* shoot were cultured on half strength MS salts containing Ø.2 mg/l IBA, 1 mg/l BAP Ø.5 mg/l kinetin, 4 mg/l adenine sulphate and 3Ø g/l sucrose, for 3Ø days.

Materials and Methods

Materials and Methods

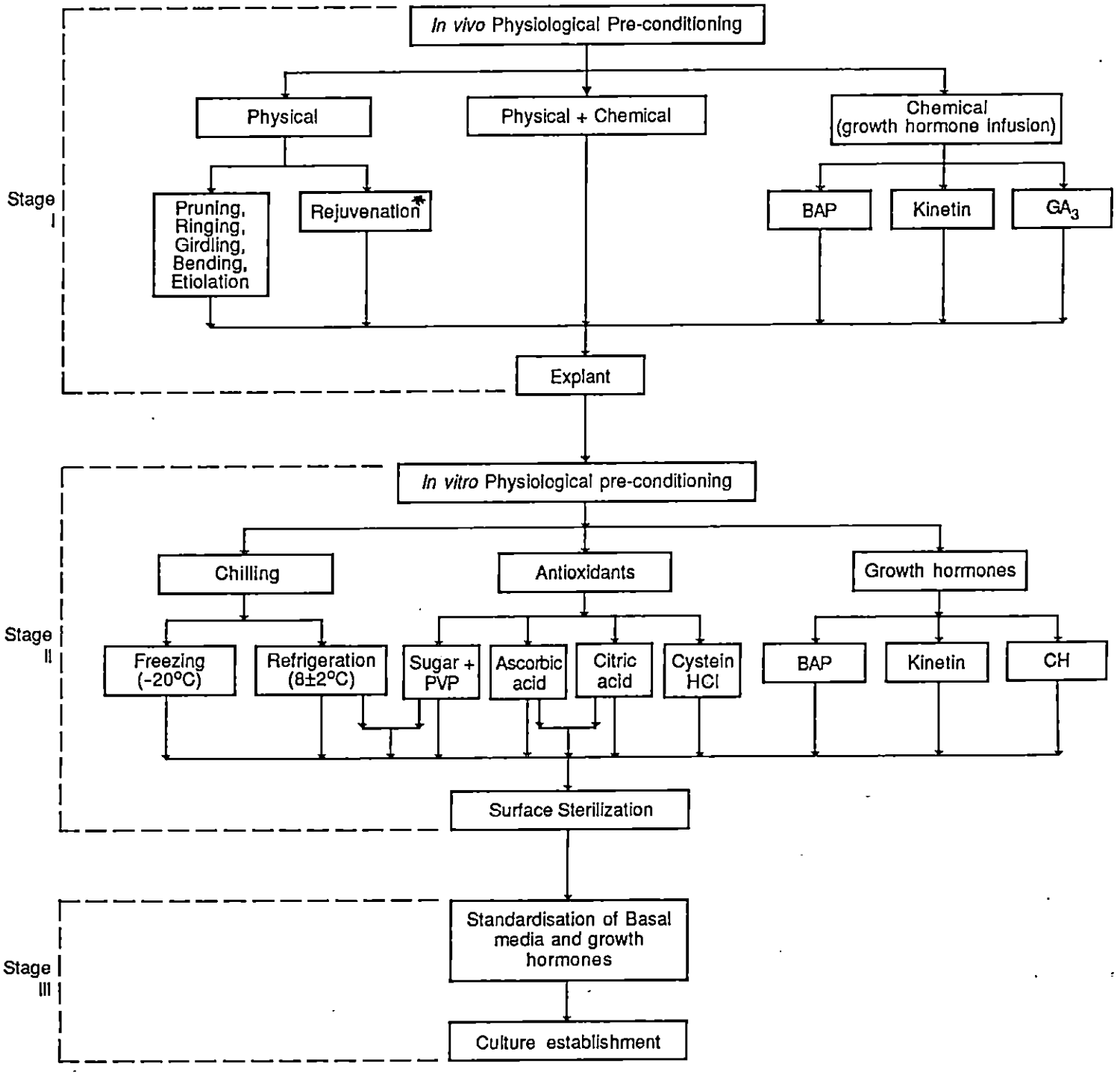
The studies on "The effect of physiological pre-conditioning of explants and explant sources of *Myristica fragrans* Houtt. on *in vitro* culture establishment and growth" were carried out in the Tissue Culture Laboratory attached to the Department of Horticulture, College of Agriculture, Vellayani during the period from 1992 to 1994. The sequential steps of physiological pre-conditioning and *in vitro* culture establishment of shoot tip explants of nutmeg are presented in Fig. 1.

The materials and methods used for the *in vivo* treatments carried out in explant sources (parent plants) in order to induce more number of Orthotropic shoots and to enhance the establishment and growth of explants *in vitro* are detailed below.

3.1. Physiological pre-conditioning of explants under *in vivo* conditions

The following physical and chemical treatments were tried in the field grown female plants.

Fig. 1 Sequential steps of physiological pre-conditioning and *in vitro* culture establishment of shoot tip explants of nutmeg



* By grafting on to one year old seedling root stock.

3.1.1. Physical treatments

3.1.1.1. Pruning

Orthotropic branches were cut off from the second jorquette in order to enhance the emergence of rejuvenated orthotropic shoots, on selected 5 year old grafts.

Three replications were given for this treatment. Observations were made on the number of newly induced orthotropic shoots.

3.1.1.2. Etiolation

Selected orthotropic branches were subjected to shoot tip pruning and these pruned tips were covered with black polythene to give etiolation. Three weeks after etiolation, the cover was removed and observations were made on the number of newly induced orthotropic shoots.

3.1.1.3. Ringing

On selected six year old grafts, a strip of bark having a width of Ø.5 cm was removed 45 cm away from the growing tip. Observations were made on the total number of newly induced orthotropic shoots three weeks after ringing.

3.1.1.4. Half ringing

On selected six year old grafts a strip of bark having a width of Ø.5 cm, was removed partially 45 cm away from the growing tip. Observations were made on the total number of newly induced orthotropic shoots three weeks after half ringing.

3.1.1.5. Bending

The orthotropic branches were bent and staked at ground level. Observations were made on the number of newly induced orthotropic shoots three weeks after bending.

3.1.1.6. Grafting

Grafting (approach grafting) was done in one year old seedlings using scions collected both from lateral branches and orthotropic shoots. The rejuvenated shoot tips collected from the scion part of growing grafts were used as the explant. The graftlings produced using orthotropic branches were erect (Plate 2a), while those produced using plagiotropic branches showed a lateral growing habit (Plate 2b).

3.1.1.7. Girdling

On selected six year old field grown grafts, iron wire of diameter 75 mm was wound tightly around the main growing branch, 45 cm below the growing tip. Observations

Plate 2a.

Nutmeg grafts showing straight growing scions, taken from orthotropic shoots

Plate 2b.

Nutmeg graft showing horizontal growth of scion taken from plagiotropic shoots

were taken on the number of newly induced orthotropic shoots, three weeks after bending. Combined effect of hormone treatment and pruning/etiolation was also studied after evaluating the response of individual treatments.

3.1.2. Chemical treatments

Growth hormone infusion

Solutions of BAP and kinetin 250, 500 and 1000 mg/l each were prepared by dissolving 125, 250 and 500 mg BAP and kinetin in small quantities of 0.1N sodium hydroxide, and then making them upto 500 ml with double glass distilled water. While, solutions of GA_3 50, 100 and 200 mg/l were prepared by dissolving 25, 50 and 100 mg GA_3 in small quantities of ethanol, and then making them upto 500 ml with double glass distilled water. The prepared solutions were sprayed using a hand sprayer on 6 year old mature nutmeg plants, till droplets started dripping from the leaves. Observations on the number of newly induced sprouts were taken and these sprouts were used as explants for *in vitro* culture.

Growth hormones and their concentrations used are given in Table 1. Three replications were given for each treatment. Observations were made on the number of newly induced orthotropic shoots and their response under *in vitro* conditions were studied.

Table 1. Growth hormones and their concentrations used for infusion of explant sources

Growth hormones	Concentration (mg/l)
BAP	250
BAP	500
BAP	1000
Kinetin	250
Kinetin	500
Kinetin	1000
GA_3	50
GA_3	100
GA_3	200

3.2. Pre-culture treatments

Shoot tip explants were subjected to the following pre-culture treatments mainly to eliminate the phenolic interference in *in vitro* culture. The explants were subjected to various treatments immediately after their excision from the plant.

3.2.1. Chilling treatment

3.2.1.1. At 8±2°C (Refrigerated condition) for 6, 12 and 24 hours

Excised shoot apices were rinsed in running tap water and were washed with distilled water containing a few drops of the detergent, `labolene´, and were rinsed in sterile double glass distilled water. These explants of about 3 cm size were kept in a petridish smeared with `Glycerol´. These petridishes were then kept in the refrigerator for 6, 12 and 24 hours.

3.2.1.2. At -20°C (deep freeze condition for 10 and 20 minutes)

Excised shoot apices were rinsed in running tap water and were washed with distilled water containing a few drops of the detergent, `Labolene´, and were rinsed in sterile double glass distilled water. These explants of

about 3 cm size were kept in a petridish smeared with 'Glycerol'. These petridishes were then kept in the freezer (-20°C). Cold shock was given for 5 and 10 minutes.

3.2.2. Chemical treatments

Explants were immersed in the chemical solutions and shaken for 10, 20 and 30 min durations.

Chemicals and their concentrations used are given in Table 2.

3.2.3. Growth hormone treatment

Explants were immersed in the prepared growth hormones and shaken using a gyrotory shaker set at 100 rpm for 10, 20 and 30 minutes.

Growth hormones and their concentrations used are given in Table 3.

3.2.4. Surface sterilization

The explants subjected to pre-culture treatments were surface sterilized inside a Laminar Air Flow Chamber (Klenzoids) and then inoculated to suitable glass containers. Since, Jayasree (1990) has reported mercuric chloride as the suitable surface sterilant, this study was conducted using mercuric chloride at varying durations. The sterilized explants were then trimmed to 25 to 30 mm length and were inoculated in the culture media.

Table 2. Chemicals and their concentrations used for pre-treatment of shoot tip explants to eliminate phenolic interference in *in vitro* culture

Chemicals	Concentration
PVP + Sucrose	Ø.5% + 1.Ø%
PVP + Sucrose	Ø.7% + 2.Ø%
PVP + Sucrose	1.Ø% + 3.Ø%
Ascorbic acid	75 mg/l
Ascorbic acid	15Ø mg/l
Ascorbic acid	3ØØ mg/l
Citric acid	75 mg/l
Citric acid	15Ø mg/l
Citric acid	3ØØ mg/l
Ascorbic acid + Citric acid	15Ø + 15Ø mg/l
Cystein HCl	Ø.1%
Cystein HCl	Ø.5%

Table 3. Growth hormones and their concentration used for pre-treatment of shoot tip explants

Growth hormones	Concentration (mg/l)	Duration of shaking (min)
BAP	200 and 500	10, 20 and 30
Kinetin	200 and 500	10, 20 and 30
CH	500 amd 1000	10, 20 and 30

3.3.1.2. Lloyd and Mc Cown (WPM) (1981)

Further studies were carried out using WPM by incorporating BAP, NAA and 2,4-D at various concentrations. Sucrose, casein hydrolysate, inositol, agar and activated charcoal were also incorporated in the medium at the same rates as shown above. The total number of cultures inoculated, the total number of tubes contaminated and the percentage of survival were recorded at seven, fourteen, thirty and sixty days interval.

3.3.1.3. Quoirin and Lepoivre (QL) (1977)

Studies were conducted using QL medium. Hormones like BAP, NAA and 2,4-D were tried at various hormone combinations. Sucrose, casein hydrolysate, inositol, agar and activated charcoal were incorporated in the medium at the same rates as shown above. The total number of tubes contaminated and the percentage of survival were recorded at seven, fourteen, thirty and sixty days interval.

3.3.1.4. Abou-Mandour (AM) (1977)

By using various hormones like BAP, NAA and 2,4-D at various concentrations and additives like sucrose, inositol, casein hydrolysate, agar and activated charcoal at the rates as shown above, studies were carried out using AM basal medium also. Observations were made as in the above cases.

3.3. Culture establishment

3.3.1. Basal nutrient media

The chemicals used for the study were of the analytic grade from Sisco Research Laboratory (SRL), Qualigens and Merck or Sigma. The basal media used were,

i) Schenk and Hildebrandt (SH) (1972)

ii) Lloyd and Mc Cown (WPM) (1981)

iii) Quoirin and Lepoivre (QL) (1977)

iv) Abou-Mandour (AM) (1977)

The required composition of these media are given in Table 4.

3.3.1.1. Schenk and Hildebrandt (SH) (1972) medium

During the initial stages of study, SH medium was tried and various hormones incorporated were kinetin, 2,4-D, BAP and IBA at various concentrations. 3.Ø% sucrose, Ø.1% casein hydrolysate, Ø.Ø1% inositol and Ø.7% agar and 1.Ø% activated charcoal were also incorporated in the medium.Each treatment was given a code number. The total number of tubes contaminated and the percentage of survival were recorded. The observations were recorded at seven, fourteen, thirty and sixty days interval (sixty days in the case of surviving cultures).

Table 4. Composition of various culture media tried for nutmeg tissue culture

Ingredients	Quantity (mg/l)			
	SH	AM	WPM	QL
Macronutrients				
Ammonium nitrate	-	650.00	400.00	1650.00
Ammonium dihydrogen orthophosphate	300.00	-	-	-
Calcium chloride (anhydrous)	-	45.00	72.50	16.61
Calcium chloride. $2H_2O$	200.00	-	-	-
Calcium nitrate	-	320.00	386.00	-
Disodium orthophosphate	-	150.00	-	-
Magnesium sulphate. $7H_2O$	400.00	265.00	180.70	903.37
Potassium chloride	-	65.00	-	-
Potassium dihydrogen orthophosphate	-	-	170.00	340.00
Potassium nitrate	2500.00	1080.00	-	1900.00
Potassium sulphate	-	-	990.00	-
Sodium dihydrogen orthophosphate. H_2O	-	45.00	-	-
Sodium phosphate	-	900.00	-	-

(contd..)

Ingredients	Quantity (mg/l)			
	SH	AM	WPM	QL
Micro nutrients				
Boric acid	5.ØØ	6.3ØØ	6.2Ø	31.ØØØ
Cobaltous chloride. $6H_2O$	Ø.1Ø	Ø.25Ø	-	Ø.125
Cupric sulphate. H_2O	Ø.2Ø	Ø.Ø75	Ø.25	Ø.5ØØ
Na_2 - EDTA	2Ø.ØØ	745.ØØØ	37.3Ø	37.3ØØ
Ferric sulphate. $7H_2O$	15.ØØ	555.ØØØ	27.8Ø	27.8ØØ
Manganese sulphate. H_2O	1Ø.ØØ	9.ØØØ	22.3Ø	21.ØØØ
Molybdic acid. $2H_2O$	-	-	Ø.25	1.25Ø
Potassium iodide	1.ØØ	Ø.83Ø	-	4.15Ø
Sodium molybdate. $2H_2O$	Ø.1Ø	Ø.25Ø	-	-
Zinc sulphate. $7H_2O$	1.ØØ	7.ØØØ	8.6Ø	43.ØØØ
Vitamins				
Glycine	-	2ØØ.ØØ	-	-
Niacine. HCl	5.ØØ	Ø.5Ø	5.ØØ	5.ØØ
Pyridoxine. HCl	Ø.5Ø	Ø.5Ø	Ø.5Ø	Ø.5Ø
Thiamine. HCl	5.ØØ	Ø.1Ø	5.ØØ	5.ØØ

(Contd.)

Ingredients	Quantity (mg/l)			
	SH	AM	WPM	QL
Others				
Adenine sulphate	4.ØØ	4.ØØ	4.ØØ	4.ØØ
Casein hydrolysate	1ØØØ.ØØ	1ØØØ.ØØ	1ØØØ.ØØ	1ØØØ.ØØ
Folic acid	Ø.3Ø	Ø.3Ø	Ø.3Ø	Ø.3Ø
Myo-inositol	1ØØ.ØØ	1ØØ.ØØ	1ØØ.ØØ	1ØØ.ØØ
Sucrose	3Ø.ØØ*	3Ø.ØØ*	3Ø.ØØ*	3Ø.ØØ*
Agar	7.ØØ*	7.ØØ*	7.ØØ*	7.ØØ*
pH	5.8Ø	5.8Ø	5.8Ø	5.8Ø

* g/l

3.4. Preparation of stock solution

Stock solutions of the different chemicals required for the media were prepared in double glass distilled water and stored under refrigerated condition. Required quantities were pipetted out from the stock solution and made upto 1 or 2 l as needed.

3.5. Preparation of media

The required quantity of stock solutions were pipetted out and sucrose, inositol and casein hydrolysate were added as per the specifications. The media were supplemented with plant growth substances as per requirement. The p^H of the medium was adjusted to 5.8. Agar (7g/l) was added to the solution to solidify the medium. Then, finally, Ø.Ø1% Derosal 5ØWP (Carbendazim, 5Ø%WP Hoechst India Ltd.) was added to the medium to eliminate fungal contamination. Then agar was melted by heating and the final volume was made upto 1 l.

3.6. Glasswares and equipments

Borosil brand test tubes (15Øx2Ø mm) and conical flasks (1ØØ and 15Ø ml) were used. Quantities of 15 ml of the desired media were taken in the test tubes and 4Ø ml in the conical flasks. Sterilization was done at 15 pounds per square inches (p.s.i.) pressure and 12Ø$^{\circ}$C temperature for 2Ø minutes.

3.7. Explants

Explants from mature trees, young seedlings, young grafts and six year old grafts, were used for the present study. Shoot tips from orthotropic and lateral branches, internodal segments and young leaves were used as the explants.

3.7.1. Explant collection

Shoot tips 25-30 mm length were excised from the parent plant (explant source) aseptically. Sterilized razor blades were used for excising the explant from the plant. These explants were immediately transferred to an already sterilized flask containing sterile water.

3.7.2. Explant preparation

Shoot tips of 25-30 mm length were excised, leaves trimmed and washed several times using tap water with few drops of labolene. Later on, these were washed using sterile double glass distilled water containing few drops of labolene and then washed in sterile double glass distilled water.

3.8. Under *in vitro* conditions

3.8.1. Inoculation of explants

Inoculation of the prepared explants was done inside a Laminar Air Flow Chamber (Klenzoids). The explants were transferred to the test tubes or conical flasks with sterile forceps without causing any sort of injury to them. The cultures were incubated at 26±2°c and under a 16 h photo period (1000 lux).

3.9. Statistical analysis

The data generated from the various experiments were subjected to statistical analysis in Completely Randomised Design or Factorial Completely Randomised Design experiments, wherever necessary, as per Panse and Sukhatme (1978).

Results

Results

The greatest limiting factor in the micro-propagation of nutmeg is the lack of sufficient number of orthotropic shoots to be used as explants. Hence the present study was carried out to induce orthotropic shoots by altering the physiological status of the explant source (parent trees) using various physical and chemical treatments in Stage I. The response of these newly induced physiologically pre-conditioned orthotropic shoots in *in vitro* establishment and growth were studied in Stage II.

The results obtained are presented in this chapter.

4.1. Physiological pre-conditioning of explant sources under *in vivo* conditions

4.1.1. Pruning and etiolation

The results of this trial is presented in Table 5. Pruning of shoot tip alone and pruning followed by etiolation were found to be statistically on par in inducing new sprouts. These treatments induced on an average of four orthotropic shoots per plant at 21 days after the operation (Plate 3). Ringing induced three orthotropic shoots. In

Table 5. Effect of physical pre-treatments in the induction of orthotropic shoots+

Physical treatment	No. of induced orthotropes*
Pruning	4 (2.2)
Ringing	3 (2.Ø)
Half ringing	Ø (1.Ø)
Girdling	1 (1.4)
Bending	1 (1.4)
Shoot tip pruning + etiolation	4 (2.2)
Grafting	Ø (1.Ø)
Control	Ø (1.Ø)
CD (1%)	Ø.Ø3

\+ 21 days after treatment in 6 year old female trees.
* Mean of three observations.

Data in parentheses are square root transformed values.

Plate 3.

Orthotropic shoots induced by pruning

girdling and bending, on an average one orthotropic shoot each was induced. Half ringing had no influence in inducing orthotropic shoots.

4.1.2. Growth hormone infusion

The results of this trial are presented in Table 6. Eventhough there is significant variation among different hormones and the control treatment in the induction of orthotropic shoots, there is no significant variation among the various concentration of hormones tried, in inducing orthotropic shoots. Maximum number of orthotropic shoots (12 per plant) was induced by BAP and kinetin both at 500 and 1000 mg/l concentration (Plate 4a), followed by kinetin 250 mg/l and GA_3 200 mg/l, both giving rise to 10 orthotropic shoots per plant. The control (distilled water) induced no shoots after 21 days of hormone infusion (Plate 4b).

The combined effect of pruning and etiolation followed by application of BAP 500 mg/l or kinetin 500 mg/l was also studied and the data (Table 7) revealed that there is no added advantage of combining physical treatments along with BAP or kinetin infusion. In both the cases 12 orthotropic shoots per plant were induced. The control plant (pruning and etiolation alone) induced only four orthotropic shoots.

Table 6. Effect of growth hormone infusion in the induction oforthotropic shoots+

Hormone	Concentration (mg/l)	No. of orthotropes induced*
BAP	250	8 (3.0)
	500	12 (3.6)
	1000	12 (3.6)
Kinetin	250	10 (3.3)
	500	12 (3.6)
	1000	12 (3.6)
GA_3	50	8 (3.0
	100	8 (3.0)
	200	10 (3.3)
Control	(distilled water)	0 (1.0)
CD (1%)		0.19

\+ 21 days after treatment in 6 year old female trees.
* Mean of three observations.

Data in parentheses are square root transformed values.

Plate 4a.

Orthotropic shoots induced by cytokinin infusion (BAP 5ØØ mg/l) of explant source (parent tree)

Plate 4b.

Control plant showing no induction of orthotropic shoots

Table 7. Combined effect of pruning, etiolation and growth hormones in the induction of orthotropic shoots+

Treatment	No. of orthotropes induced
Pruning + Etiolation + BAP 500 mg/l	12
Pruning + Etiolation + Kinetin 500 mg/l	12
Control (Pruning + Etiolation)	4
CD (1%)	0.451

+ Mean of three observations

* 21 days after treatment in 6 year old female trees

4.2. Pre-culture treatments

4.2.1. Chilling treatment

4.2.1.1. Effect of refrigeration pre-treatment

Response of shoot tips kept under refrigerated condition at 8 ± 2°C for 12 h was found to be significantly superior to the other treatments; giving 60 per cent survival of the cultures, while with a treatment time of 6 hours only 40 per cent survival was observed. But, when the treatment time was increased to 24 h, 50 per cent survival was observed. In the control (no refrigeration) only 10 per cent survival was observed. The results are presented in Table 8.

4.2.1.2. Effect of deep freezing the explants

When explants were kept under deep freeze condition of -20°C for varying durations, the results showed that there is significant variation among the various durations tried, in eliminating phenolic interference. Freezing the shoot tips for 5 min followed by shaking them in a solution containing 2 per cent sugar and 0.7 per cent PVP gave significantly superior results than the other treatments tried, giving very slight browning of plant tissue with maximum 50 per cent survival. However, freezing the shoot tips for 5 and 10 minutes gave severe browning of plant tissue and their survival percentage was found to be 40 and 34 respectively (Table 9).

Table 8. Effect of refrigeration pre-treatment at 8 ± 2°C in eliminating phenolic interference in *in vitro* establishment of shoot tip

Nutrient medium : SH

Duration in hours	Survival (%)+	Browning of tissue/media
6	4Ø (38.49)	* * *
12	6Ø (49.98)	* *
24	5Ø (44.98)	* *
Control (no refrigeration)	1Ø (9.99)	* * * *
CD (1%)	3.82	-

\+ Based on 2Ø observations.
* * Slight
* * * Severe
* * * * Very severe

Data in parentheses are angular transformed values.

Table 9. Effect of deep freezing pre-treatment at -2Ø°C in eliminating phenolic interference in *in vitro* establishment of shoot tip explants

Nutrient medium : SH

Duration (min)	Survival (%)+	Browning of tissue/media
5	4Ø (38.49)	* * *
1Ø	34 (32.32)	* * *
Freezing for 5 min + Sugar 2% + PVP Ø.77%	5Ø (44.98)	*
Control (No freezing)	1Ø (9.99)	* * * *
CD (1%)	3.8Ø	-

\+ Based on 2Ø observations.
* Very slight
* * * Severe
* * * * Very severe

Data in parentheses are angular transformed values.

4.2.2. Chemical treatments

4.2.2.1. Sugar + PVP (30 minutes)

Among the various treatments tried to eliminate the problem of phenolic interference, sugar 2 per cent + PVP 0.7 per cent is found to be significantly superior giving 90 per cent survival of cultures. Eventhough, sugar 3 per cent + PVP 1 per cent did not show any browning of the media, only 70 per cent cultures survived. Pre-treating with sugar 1 per cent + PVP 0.5 per cent gave slight browning of the plant tissues with 50 per cent survival of the cultures. While the control (shaking with distilled water for 30 minutes) gave very severe browning of the plant tissues giving 10 per cent survival (Table 10).

4.2.2.2. Ascorbic acid and citric acid

It is evident (Table 11) that eventhough ascorbic acid and citric acid are effective in eliminating phenolic interference, the survival percentage of the treated explants were very poor, compared to sugar and PVP pre-treatment. A combination of ascorbic acid and citric acid each at 150 mg/l concentration, gave very slight browning of plant tissue while their survival percentage was only 40. Ascorbic acid 75 mg/l and citric acid 75 mg/l gave severe browning of plant tissue and their survival percentage was 40. Citric acid at 150 mg/l and 300 mg/l

Table 10. Effect of Sugar + PVP[+] pre-treatment in eliminating phenolic interference in *in vitro* establishment of shoot tip explants

Nutrient medium :SH

Concentration (%)	Survival (%)[x]	Browning of tissue/media
Sugar 1 + PVP 0.5	50 (44.98)	* *
Sugar 2 + PVP 0.7	90 (75.05)	*
Sugar 3 + PVP 1.0	70 (60.19)	Nil
Control (distilled water)	10 (9.99)	* * * *
CD (1%)	3.82	-

\+ Duration of treatment : 30 minutes.
x Based on 20 observations.
* Very slight
* * Slight
* * * * Very severe

Data in parentheses are angular transformed values.

Table 11. Effect of ascorbic acid and citric acid[+] in eliminating phenolic interference in *in vitro* establishment of shoot tip explants

Nutrient medium : SH

Concentration (mg/l)	Survival (%)[x]	Browning of tissue/media
Ascorbic acid 75	40 (38.49)	* * *
Ascorbic acid 150	30 (29.81)	* *
Ascorbic acid 300	30 (29.81)	* *
Citric acid 75	40 (38.49)	* * *
Citric acid 150	40 (38.49)	* *
Citric acid 300	45 (41.74)	* *
Ascorbic acid 150 + Citric acid 150	40 (38.49)	*
Control (distilled water)	10 (9.99)	* * * *
CD (5%)	2.51	-

\+ Duration of treatment: 30 minutes.
x Based on 20 observations.
* Very slight
* * Slight
* * * Severe
* * * * Very severe

Data in parentheses are angular transformed values.

gave slight browning of media and 4Ø and 45 per cent survival of the cultures respectively. When the explants were treated with ascorbic acid 15Ø and 3ØØ mg/l each, the results are found to be on par giving 3Ø per cent survival of the cultures giving slight browning. While, in the control, only 1Ø per cent survival and very severe browning was found.

4.2.2.3. Cystein H Cl

Treating the explants in Ø.1 per cent cystein H Cl for 15 and 3Ø minutes gave 6Ø and 4Ø per cent survival respectively, giving severe browning of the tissues. When explants were treated with Ø.5 per cent cystein H Cl for 15 and 3Ø minutes, the results are found to be on par, giving a survival percentage of 3Ø each; and slight browning of plant tissues. While, in the control (shaking with distilled water for 3Ø minutes), very severe browning was found, giving only 1Ø per cent survival as shown in Table 12.

4.2.3. Growth hormones (BAP and kinetin)

Among the two concentrations of cytokinins (BAP and kinetin) tried for various duration (1Ø, 2Ø and 3Ø minutes), the results revealed that, there was no significant variation in the culture establishment due to the different concentrations or durations tried alone, but their combined effect had significant influence on the culture establishment.

Table 12. Effect of cystein HCl in eliminating phenolic interference in *in vitro* establishment of shoot tip explants

Nutrient medium : SH

Concentration (%)	Duration (min)	Survival (%)+	Browning of tissue/media
Ø.1	15	6Ø (49.98)	* * *
	3Ø	4Ø (38.49)	* * *
Ø.5	15	3Ø (29.81)	* *
	3Ø	3Ø (29.81)	* *
Control (distilled water)	15	1Ø (9.99)	* * * *
CD (1%)		3.24	-

\+ Based on 2Ø observations.
* * Slight
* * * Severe
* * * * Very severe

Data in parantheses are angular transformed values.

Pre-treatment with BAP at 200 and 500 mg/l for 30 minutes gave 40 per cent culture establishment and are found to be significantly superior to the other BAP treatment combinations (Table 13). However, when explants were inoculated without any pre-treatment, much higher rate of culture establishment (55 per cent) was obtained.

Pre-treatment with kinetin 500 mg/l for 20 minutes gave 50 per cent culture establishment and was found to be significantly superior to the other kinetin pre-treatments. Kinetin at 200 mg/l for 30 minutes found to give next best result giving 43 per cent culture establishment. Pre-treatment with kinetin 200 mg/l for 20 minutes and 500 mg/l for 30 minutes are found to be on par giving 33 percent culture establishment (Table 13).

4.2.4. Casein hydrolysate (CH)

Here, the concentration of CH used or the duration of pre-treatment gave no significant variation in their *in vitro* response. But the *in vitro* response of CH pre-treated explants were significnatly superior to the explants taken from control plants (74 per cent against 58 per cent survival).

The results are presented in Table 13 and (Plate 5).

Table 13. Effect of pre-treatment of shoot tip explants with growth hormones and casein hydrolysate (CH) on *in vitro* culture establishment

Nutrient medium : AM (half strength)

Kinetin (mg/l)	Establishment (%)+	BAP (mg/l)	Establishment (%)+	CH (mg/l)	Establishment (%)+
200 (A_1)	34.4 (35.6)	200 (C_1)	23.8 (29.2)	500 (E_1)	71.8
500 (A_2)	35.6 (36.3)	500 (C_2)	24.7 (29.8)	1000 (E_2)	68.6
CD (5%)	NS	-	NS	-	NS
10 min (B_1)	35.0 (36.3)	10 min (D_1)	15.7 (23.3)	10 min (F_1)	69.4
20 min (B_2)	41.7 (40.2)	20 min (D_2)	17.1 (24.4)	20 min (F_2)	69.5
30 min (B_3)	38.3 (38.2)	30 min (D_3)	40.0 (39.2)	30 min (F_3)	71.5
CD (5%)	NS	-	NS	-	NS
A_1 B_1	26.7 (31.1)	C_1 D_1	11.3 (19.7)	E_1 F_1	73.6
A_1 B_2	33.3 (35.1)	C_1 D_2	20.0 (26.6)	E_1 F_2	70.8
A_1 B_2	43.3 (40.9)	C_1 D_3	40.0 (39.2)	E_1 F_3	70.8
A_2 B_1	23.3 (28.7)	C_2 D_1	20.0 (26.6)	E_2 F_1	65.3
A_2 B_2	50.0 (45.0)	C_2 D_2	14.1 (21.9)	E_2 F_2	68.3
A_2 B_3	33.3 (35.1)	C_2 D_3	40.0 (39.2)	E_2 F_3	72.2
CD (5%)	3.2	-	2.7	-	NS
Control (without any pre-treatment)	57.0	-	55.0	-	58.0

+ Mean value of 20 observations.

Data in parentheses are angular transformed values.

Plate 5.

Shoot tip explants pre-treated with CH showing complete unfurling of leaves

4.2.5. Effect of mercuric chloride in eliminating microbial contamination

The results of the trial on surface sterilization of nutmeg explants are presented in Table 14.

The data revealed that significant variation exists among the treatments. Pre-treating the shoot tips with Ø.1per cent mercuric chloride for 1Ø minutes is found to be significantly superior to the other treatments, giving a survival percentage of 72. With a treatment time of 12 minutes, contamination percentage was significantly reduced, but the percentage of live cultures were only 65 since 15 per cent cultures were dead due to the toxic effect of mercuric chloride. The treatment time of 7 and 8 minutes each, gave 6Ø and 65 per cent survival respectively.

In another trial, addition of Ø.Ø1 per cent of Derosal (Carbendazim based fungicide) in the nutrient medium in addition to pre-treatment with Ø.1 per cent mercuric chloride gave 8Ø per cent survival of the cultures. But when the concentration of fungicide was increased to Ø.1 per cent, none of the cultures survived. While in the control treatment, (pre-treatment with Ø.1 per cent mercuric chloride alone) gave 72 per cent survival of the cultures. The results are presented in Table 15.

Table 14. Effect of MC (mercuric chloride Ø.1%) in eliminating microbial contamination and spoilage due to other causes

Nutrient medium : SH

Duration of MC treatment (min)	Spoilage of cultures (%)			Live cultures (%)
	Fungal contamination (%)	Phenolic interference	Toxic effect of MC	
7	3Ø (32.17)	1Ø (14.43)	Ø (Ø.ØØ)	6Ø (49.98)[a]
8	25 (29.99)	1Ø (14.43)	Ø (Ø.ØØ)	65 (53.22)[a]
1Ø	12 (2Ø.13)	8 (1Ø.41)	8(1Ø.41)	72 (58.9Ø)[b]
12	1Ø (14.43)	1Ø (14.43)	15(21.52)	65 (53.22)[a]
CD (1%)	2.7Ø	NS	2.11	2.98

a Based on 2Ø observations.
b Based on 25 observations.

Data in parentheses are angular transformed values.

Table 15 Effect of incorporation of Derosal+ in nutrient medium in eliminating fungal contamination

Nutrient medium - SH

Concentration (%)	Fungal contamination (%)	% Cultures dead due to		% live culture*
		Phenolics interference	Toxic effect of chemical	
Ø.ØI	4 (8.21)	8 (1Ø.41)	8 (1Ø.41)	8Ø (67.Ø)
Ø.1Ø	Ø (Ø.ØØ)	12 (2Ø.13)	88 (62.87)	Ø (Ø.Ø)
Control (.1% HgCl2)	12 (2Ø.13)	8 (1Ø.41)	8 (1Ø.41)	72 (58.9)
CD (5%)	1.32	2.61	1.73	7.77

+ Carbendazim based fungicide (Hoechst India Ltd.)

* Based on 25 observations

Data in parentheses are angular transformed values

4.3. Culture establishment

4.3.1. Basal nutrient media

Among the various basal nutrient media tried, Abou Mandour (AM) medium at half strength of the major nutrient concentration and full strength of minor nutrient concentration (AM1) was found to give maximum culture establishment (67 per cent) (Table 16). Full strength AM medium (AM2) gave only 57 per cent survival. Schenk and Hildebrandt (SH) medium at full strength of major and minor nutrient concentration (SH2) was found to be the second best medium with respect to culture establishment While SH medium at half strength of major nutrient concentration and full strength of minor nutrient concentration (SH1) gave only 33 per cent survival. Lloyd and Mc Cown Medium (WPM) at half strength of the major nutrient concentration and full strength of minor nutrient concentration (WPM1) gave only 50 per cent survival. When full strength of both major and minor nutrient concentration was tried, 56 per cent survival was observed. Similarly, when Quoirin and Le-Poivre (QL) medium at half strength of major nutrient concentration and full strength of minor nutrient concentration (QL1) was used, only 48 per cent survival was obtained; while full strength of both major and minor nutrient concentration gave 53 per cent survival.

Table 16. Effect of different basal nutrient media on *in vitro* establishment of shoot tip explants

Nutrient medium	Establishment (%)*
AM^1	67
AM^2	57
SH^1	33
SH^2	61
WPM^1	5Ø
WPM^2	56
QL^1	48
QL^2	53

* Based on 3Ø observations.

AM^1 Half strength of major nutrients + full strength of minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l.

AM^2 Full strength of both major and minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l.

SH^1 Half strength of major nutrients + full strength of minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l.

SH^2 Full strength of both major and minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l.

WPM^1 Half strength of major nutrients + Full strength of minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l.

WPM^2 Full strength of both major and minor nutrient + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l

QL^1 Half strength of major nutrients + full strength of minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l

QL^2 Full strength of both major and minor nutrients + BAP 2 mg/l + NAA 1 mg/l + 2,4-D Ø.5 mg/l

4.3.2. Growth hormones

Results of the effect of incorporating various growth hormones in nutrient medium in culture establishment is given in Table 17. Among the various hormones tried, a combination of BAP, NAA and 2,4-D at 2, 1 and Ø.5 mg/l respectively was found to give maximum culture establishment of 67 per cent and multiple buds were produced in the cultures which survived (Plate 6). A combination of BAP and NAA at 2 mg/l and 1 mg/l respectively gave 5Ø per cent culture establishment followed by BAP, NAA and 2,4-D at 2, 1, and 1 mg/l respectively giving 4Ø per cent culture establishment. Other treatment combination failed to give satisfactory culture establishment.

4.3.3. Phloroglucinol

When phloroglucinol at 2Ø mg/l was added to the basal nutrient media, 2Ø per cent cultures survived. When the concentration of phloroglucinol was increased to 4Ø mg/l, 33 per cent cultures survived. But, when the concentration of phloroglucinol was further increased to 6Ø mg/l the survival percentage was reduced to 16 per cent.

When 4Ø mg/l of PG was added to the nutrient media containing 2 mg/l IBA, 58 per cent survival of the cultures was noticed and significantly superior results were given in their rooting response and roots were produced in 29 per

Table 17. Effect of growth hormones on *in vitro* establishment of shoot tip explants

Basal medium : AM (half strength)

Treatments (mg/l)	% establishment
BAP 2 + NAA 1	50[a] (44.98)
BAP 10 + NAA 2	28[a] (31.51)
BAP 10 + NAA 5	27[b] (30.23)
BAP 2 + NAA 1 + 2,4-D 0.5	67[b] (56.71)
BAP 2 + NAA 1 + 2,4-D 1	40[b] (38.49)
BAP 2 + NAA 1 + 2,4-D 2	0[b] (0.00)
BAP 5 + 2,4-D 1	0[c] (0.00)
BAP 10 + 2,4-D 5	0[b] (0.00)
BAP 10 + 1AA 2	0[a] (0.00)
CD (1%)	7.8

a Based on 18 observations.
b Based on 30 observations.
c Based on 12 observations.

Data in parentheses are angular transformed values.

Plate 6.

Induction of multiple buds in AM medium containing BAP, NAA and 2,4-D at 2, 1 and Ø.5 mg/l respectively

cent of the cultures (Plate 7). Eventhough 57 per cent survival of the cultures were noticed in the control treatment, no roots were produced in any of the cultures (Table 18).

4.4. Subculturing

When the explants were subjected to frequent subculturing at 3 days interval, the cultures responded better by eliminating phenolic interference. The browning of media and the subsequent spoilage of the cultures was higher when the cultures were subjected to subculturing at 14 days interval. When the established cultures were subcultured to fresh media, after giving a cut at the basal region, it was found that cultures were dead due to phenolic exudation.

4.5. Effect of physiological pre-conditioning of explant sources (*in vivo*) in *in vitro* establishment of shoot tip explants

4.5.1 Pruning and grafting

When newly emerged shoot tips taken from graftlings and pruned plants were cultured *in vitro*, they showed no significant variation in culture establishment when compared with that of explants taken from mature plants (Table 19).

Plate 7.

Induction of roots in AM + 2 mg/l
IBA + 40 mg/l PG

Table 18 Effect of phloroglucinol (PG) in the *in vitro* establishment and rooting of shoot tip explants

Nutrient medium : AM (half strength)

Treatments	Establishment (%)	Rooting (%)
T_1 + PG 20 mg/l	20[a] (26.56)	0 (0.00)*
T_1 + PG 40 mg/l	33[a] (35.06)	0 (0.00)
T_1 + PG 60 mg/l	16[b] (23.57)	0 (0.00)
T_2 + PG 40 mg/l	58[b] (49.6)	29 (32.58)
Control (T_1 alone)	57[a] (49.02)	0 (0.00)
CD (1%)	2.32	8.03

T_1 AM (half strength) + BAP 2 mg/l + NAA 1 mg/l + 2,4-D 0.5 mg/l.

T_2 AM (half strength) + IBA 2 mg/l.

a Based on 30 observations.

b Based on 24 observations.

Data in parentheses are angular transformed values.

Table 19. Effect of *in vivo* pre-treatment of explant sources on *in vitro* establishment of shoot tip explants

Nutrient medium : AM (half strength)

In vivo treatment	Hormone concentration in culture medium (mg/l)	Culture establishment (%)*
Pruning	BAP 2 + NAA 1	53
	BAP 2 + NAA 1 + 2,4-D Ø.5	58
Grafting	BAP 2 + NAA 1	59
	BAP 2 + NAA 1 + 2,4-D Ø.5	57
Control (mature tree)	BAP 2 + NAA 1 + 2,4-D Ø.5	5Ø
SEm ±		3.3

* Based on 2Ø observations.

When newly emerged shoot tips taken from pruned plants were subjected to *in vitro* culture, by incorporating BAP, NAA and 2,4-D at a concentration of 2, 1 and Ø.5 mg/l respectively to the AM nutrient medium, 58 per cent survival was obtained. Fifty seven per cent survival was obtained when newly emerged shoot tips from graftlings were used as explants; while only 5Ø per cet survival was obtained in the case of shoot tips taken from mature plants.

When newly emerged shoot tips taken from pruned plants were subjected to *in vitro* culture by incorporating BAP and NAA at a concentration of 2 and 1 mg/l respectively, 53 per cent survival of cultures was obtained whereas 59 per cent survival was resulted when newly emerged shoot tips from graftlings were used.

4.5.2. Growth hormone infusion

When shoot tips collected from GA_3 infused plants were subjected to *in vitro* culture, there was significant variation in the rate of culture establishment among the various concentrations tried and no significant variation in the frequency of the explant collection. Explants collected from 5Ø and 1ØØ mg/l of GA_3 infused plants gave significantly superior results in their *in vitro* response

(Plate 8), giving 7Ø and 68 per cent culture establishment respectively, which are found to be statistically on par. The explants taken from control plant (sprayed with distilled water) gave only 58 per cent culture establishment (Table 2Ø).

Shoot tip explants collected from cytokinins (BAP and kinetin) infused plants (*in vivo*) were *in vitro* cultured, there was no significant variation in the rate of culture establishment among the various concentrations (25Ø, 5ØØ and 1ØØØ mg/l) of the hormones tried and also in the frequency of the explant collection (Table 2Ø).

4.6. Effect of physiological age of shoot tips (from mature and seedling plants) on *in vitro* culture establishment

Significant variation was shown by explants taken from mature and seedling plants under *in vitro* conditions. Ninety eight per cent of the cultures survived when shoot tips were taken from 6Ø days old seedlings while only 67 per cent cultures survived when explants were taken from 6Ø year old trees; while their culture establishment was 6Ø and 5Ø per cent respectively (Table 21).

Plate 8.

Shoot tip explants from GA_9 infused plants showing fully developed leaves

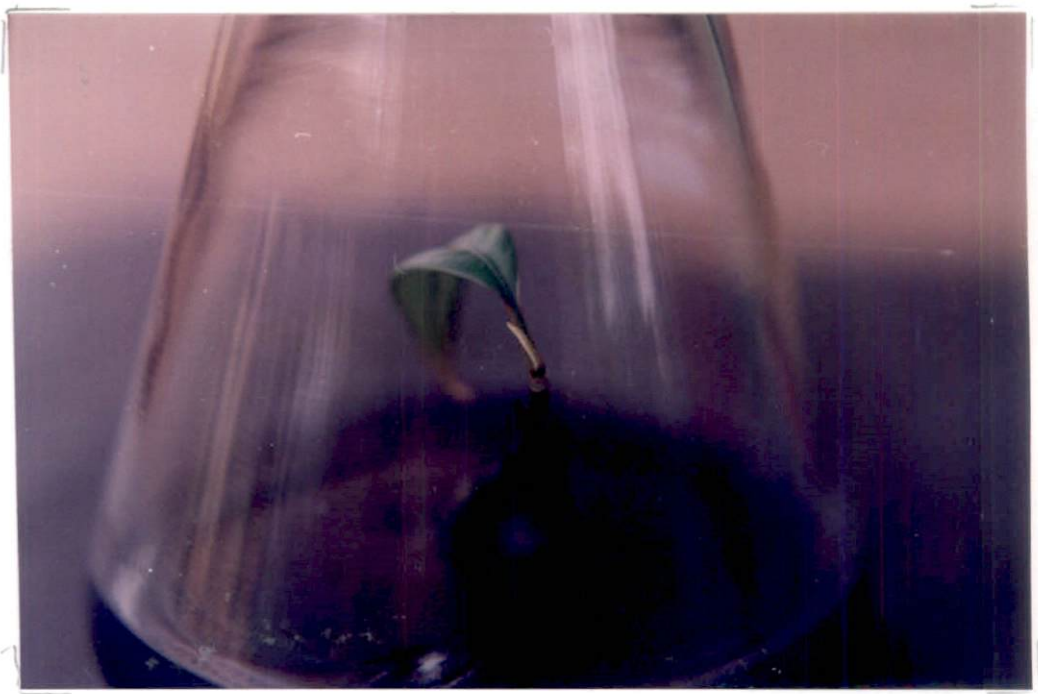


Table 20. Effect of *in vivo* growth hormone pre-treatment of explant sources on *in vitro* establishment of shoot tip explants

Nutrient medium : AM (half strength)

GA_3 (mg/l)	Establishment (%)+	BAP (mg/l)	Establishment (%)+	Kinetin (mg/l)	Establishment (%)+
50 (A_1)	70.0	250 (C_1)	52.9	250 (E_1)	48.9
100 (A_2)	67.5	500 (C_2)	45.8	500 (E_2)	46.3
200 (A_3)	45.7	1000 (C_3)	45.5	1000 (E_3)	43.3
CD (5%)	9.8	-	NS	-	NS
3 days (B_1) 7 days (B_2)	55.2 51.4	3 days (D_1) 7 days (D_2)	50.8 45.3	3 days (F_1) 7 days (F^2)	47.4 45.0
CD (5%)	NS	-	NS	-	NS
A_1 B_1	66.0	C_1 D_1	56.7	E_1 F_1	51.1
A_1 B_2	53.3	C_1 D_2	49.2	E_1 F_2	46.7
A_2 B_1	57.5	C_2 D_1	48.2	E_2 F_1	44.4
A_2 B_2	57.5	C_2 D_2	43.5	E_2 F_2	48.2
A_3 B_1	48.2	C_3 D_1	47.7	E_3 F_1	46.7
A_3 B_2	43.3	C_3 D_2	43.3	E_3 F_2	40.0
CD (5%)	NS	-	NS	-	NS
Control(distilled water)	58.0	-	55.0	-	57.0

+ Mean value of 20 observations.

Table 21 Effect of physiological age* on *in vitro* culture establishment

Explant source	Survival (%)**	Culture establishment (%)**
Mature tree	67	5Ø
Seedlings	98	6Ø
CD (5%)	15.75	2.45

* 6Ø year old mature tree and 6Ø days old seedling.

** Based on 3Ø observations.

Discussion

Discussion

Nutmeg, a valuable tree spice of Kerala, due to its dioecious nature and the long pre-bearing age poses a great problem in its sexual propagation. Also, the success of vegetative propagation methods such as grafting and budding is low. In this context, the standardisation of a viable *in vitro* technique for clonal propagation of female plants assumes great importance. In the Kerala Agricultural University, work in this line was initiated in 1990. Jayasree (1990) observed distinct difference in the *in vitro* response of orthotropic and plagiotropic shoot explants of nutmeg trees.

Lack of availability of sufficient number of orthotropic shoots to be used as explants, is a major hurdle in the *in vitro* propagation of nutmeg trees. In the present study, in Stage I, various physical and chemical methods were tested so as to induce explant sources (parent trees) to produce sufficient number of orthotropic shoots to be used as explants in *in vitro* culture. The response of these explants in *in vitro* culture establishment and growth were studied in Stage II.

The differnt pre-treatments given to the explant sources (parent trees) included pruning, grafting, etiolation, girdling, bending, half ringing, ringing and growth hormone infusion.

The salient findings are discussed below.

5.1. Physiological pre-conditioning of explant sources

5.1.1. Pruning, grafting, etiolation, girdling, bending, half ringing and ringing

In the present study, among the various treatments tried, pruning and pruning followed by etiolation were found to be statistically on par in inducing new sprouts, producing four orthotropic shoots each. Ringing gave rise to three orthotropic shoots while girdling and half ringing was found ineffective in inducing new sprouts. The graftlings obtained by using orthotropic branches as scions were erect growing, while those produced using plagiotropic branches as scions, produced only lateral growing branches.

Rema and Krishnamoorthy (199Ø) obtained 1-3 shoots from a nutmeg graft by pruning the orthotropic shoot above the graft union. They also observed that detopping nutmeg trees of 8-9 years old at 3 m height produced, on an average, 21 shoots and further pruning of the orthotropic shoots produced another 1-3 orthotropic shoots from each shoot pruned.

Franclet *et al.* (1987) reported that juvenile rootstocks when grafted with scions from mature individuals, followed by removal of all mature leaves from the scions lead to the induction of juvenility in mature forest tree species. Graftage has induced some juvenile features in mature buds grafted on to juvenile stocks most notably in ivy and rubber (Leopold and Kriedemann,1975).

Bassuk and Maynard (1987) reported that stock plant etiolation led to the initiation of new plant growth. Partial etiolation of branches is proposed as a suitable pre-culture treatment for *in vitro* propagation of selected mature trees, when physiologically juvenile material such as stump sprouts, epicormic shoots or root suckers are used. (Ballester *et al.*, 1989).

Cooper (1935) noted that in citrus, girdling interrupts the flow of growth regulators in the phloem. As a result, the growth regulator accumulates in the girdled region induces new sprouts/roots depending on the plant species and other treatment conditions.

Pre-treatments such as pruning and grafting modify the topophysical pre-disposition of plant tissue in alnus and platanus trees with varying intensity (Evers, 1987). But in the present study, such responses were not obtained by grafting. A combination of physical and chemical

treatments (pruning and etiolation + growth hormone infusion) was also tried and there was no added advantage in such combined treatments (Table 7).

5.1.2. Growth hormone infusion

In order to study the effect of growth hormones in the induction of orthotropic shoots, BAP and kinetin each at 250, 500 and 1000 mg/l and GA_3 at 50,100 and 200 mg/l and a control were tried in the present investigation. Among the treatments tried, BAP and kinetin each at 500 and 1000 mg/l were found to be on par in inducing orthotropic shoots, giving 12 orthotropic shoots each. Similarly, GA_3 at a higher concentration of 200 mg/l gave 10 orthotropic shoots, while the control plant failed to induce any orthotropic shoots during the period.

Rema and Krishnamoorthy (1990), in their experiments to convert plagiotropic shoots to orthotropic shoots using cytokinin (50 and 1000 mg/l) and GA_3 (1000 and 2000 mg/l) did not find any encouraging results. But, the effectiveness of BAP in rejuvenation was suggested by David *et al.*, (1978) in *Pinus pinaster*: Abo-El Nil (1982) in conifers; and Bouriquet *et al.*, (1984) in woody species. Philip (1993) also reported that BAP at a lower concentration produced high rate of rejuvenation of mature jack cultures.

Reports show that in many species, GA_3 and cytokinin application help in extending the juvenile period. Such effects of GA_3 was first reported for ivy, which shows a temporary reversion from mature to juvenile morphology (Robbins, 1957; Hartmann and Kester, 1989). Present study revealed that similar physiological phenomenon is expressed in nutmeg as well.

5.2. Pre-culture treatments

5.2.1. Chilling treatments

Subjecting the explants to cold treatments such as refrigeration, chilling and/or freezing has been reported to stimulate rejuvenation by breaking the physiological relations of the mature phase of the explant (Norreel, 1976). In order to explore the response of shoot tip explants of mature nutmeg trees, chilling pre-treatment such as refrigeration at 8±2°C for 6, 12 and 24 h and deep freezing at -2Ø°C for 5 and 1Ø min were tried.

There was significant variation in the rate of survival of the cultures when the explants were kept under refrigerated condition for varying duration before culturing. After 12 hours of refrigeration when the explants were cultured, 6Ø per cent of the cultures survived. When the duration was increased to 24 hours, the survival percentage was reduced to 5Ø.

Similarly, there was significant variation in the rate of survival of the cultures, when shoot tip explants were kept under deep freeze condition for varying duration. Forty per cent survial was obtained when the explants were kept under deep freeze condition for 5 minutes. When the duration was increased to 1Ø min, there was a reduction in the rate of survival (34 per cent). Freezing the explants for 5 min followed by pre-treating the explants with 2 percent sugar and Ø.7 per cent PVP gave 5Ø per cent survival of the cultures (Table 9).

Many researchers tried to explore the effect of temperature (heat or cold shock) in stimulating juvenility in plants. Fabijanski *et al.*, (1991) observed that by manipulating temperature at elevated and lower levels in anther cultures of broccoli, formation of certain heat shock proteins occurs which enhanced somatic embryo formation. Bonga (1982) reported that by cold treatment, partial damaging of cell organelles and DNA occurs, thereby rejuvenating the mature explants.

5.2.2. Chemical treatments

In the present study, pre-treatment of the explants was tried using sugar, PVP, ascorbic acid, citric acid and cystein HCl for eliminating phenolic oxidation.

Pre-treatment with 2per cent sugar and Ø.7 per cent PVP for 3Ø minutes was found to be significantly

superior than the other treatments tried, which gave 90 per cent survival of the cultures. Jayasree (1990) also obtained similar results with sugar, PVP combination in eliminating phenolic oxidation in nutmeg explants.

5.2.3. Growth hormones

Cytokinins (BAP and kinetin) were used to pre-treat the explants for obtaining better culture establishment and growth. Among the two concentrations tried (200 mg/l and 500 mg/l each) under various durations (10, 20 and 30 minutes), there was no significant variation in the response of cultures due to different concentrations, or different durations tried. However, but their combined effect had significant influence on the culture establishment.

Culture establishment obtained (40 per cent) by pre-treating the explants with BAP both at 200 mg/l and 500 mg/l for 30 minutes was significantly superior to BAP pre-treatment at 20 and 10 minutes (Table 13). However, when explants were inoculated without any pre-treatment, much higher rate of culture establishment (55 per cent) was obtained.

Pre-treating the explants with kinetin at 500 mg/l for 20 minutes gave 50 per cent culture establishment and was found to be significantly superior to the other kinetin pre- treatments (200 mg/l for 10, 20 and 30 minutes and 500 mg/l for 10 and 30 minutes).

The effect of pre-treating shoot tip explants with growth hormones, particularly cytokinins has been reported as both favourable as well as unfavourable for culture establishment in different plant species.

Philip (1993) found that the *in vitro* response of BAP pre-treated explants were not beneficial in the case of jack shoot tips. However, Enjarlic and Carron (1982) in rubber and Rajmohan (1985) in jack, reported that pre-culture infusion of shoot tip explants of these two tree species in 10 mg/l of BAP or kinetin for four hours helped in the culture establishment.

In the present investigation, however, no such favourable response could be obtained. This may be due to the comparatively faster rate of senescence of nutmeg shoot tip explants that occurs during the pre-inoculation period when compared to other plant species.

5.2.4. Casein hydrolysate

Casein hydrolysate (CH) is a complex mixture of amino acids which when incorporated in the culture medium at the rate of 0.05 per cent to 0.1 per cent may enhance the rate of culture establishment and growth (Huang and Murashige, 1977).

In the present study, CH at 500 and 1000 mg/l were tried for pre-treating the explants for 10, 20 and 30 minutes. Results revealed that neither the different levels

of CH nor the duration of treatment had any significant effect in the culture establishment and growth. However, when compared to control, better culture establishment (74 against 58 per cent) was obtained when the explants were pre-treated with 500 mg/l CH for 10 min. Increasing the level to 1000 mg/l was found to have adverse effect with respect to culture establishment and growth.

5.2.5. Surface sterilization of explants

Microbial contamination has long been a major problem in the culture establishment of explants. Since plant parts are exposed in the field for a long time, they harbour various micro-organisms and many of which penetrate into the plant tissue resulting in systemic infection. Cultures from such tissues are hence easily contaminated (Chen and Evans, 1990).

In the present study also, high rate of fungal contamination was observed, since the explants were collected from field grown plants. In order to minimise the rate of contamination, the explants were subjected to various surface sterilization treatments.

Significantly superior results were obtained when nutmeg explants were surface sterilized with 0.1 per cent mercuric chloride for 10 min, giving 72 per cent contamination free cultures. But, with a treatment time of 12 minutes, the rate of contamination was significantly

reduced, but the percentage of live cultures were only 65, rendering 15 per cent of the cultures dead due to the toxic effect of mercuric chloride.

Further, to eliminate microbial contamination, trials were carried out by incorporating Derosal 5Ø WP (Carbendazim fungicide; Hoechst India Ltd.) in the culture medium. This fungicide at Ø.Ø1 per cent concentration gave 8Ø per cent contamination free cultures which was significantly higher when compared to Ø.1 per cent concentration, in which none of the cultures survived.

D'Silva and D'Souza (1993) have reported similar beneficial effects of carbendazim (Bavistin 5Ø WP) in eliminating contamination of cashew seedling cultures by pre-treating nuts devoid of testa and pericarp before germinating them *in vitro*.

Successful use of mercuric chloride (Ø.1 per cent) in surface sterilization of cashew and clove explants for 6-12 minutes have been reported (Nair, 1991; Mathew and Harihanan, 1992).

5.3. Culture establishment

5.3.1. Basal nutrient media

In the present study, explants of nutmeg were inoculated to various nutrient media namely WPM (Lloyd and McCown, 198Ø) SH (Schenk and Hildebrandt, 1972) QL (Quoirin

and Le-Poivre, 1977) and AM (Abou-Mandour, 1977) to assess their *in vitro* response. The nutrient media were supplemented with BAP 2 mg/l and NAA 1 mg/l.

Trials were carried out separately using full strength and half strength of major nutrients, in all the four cases. Among the four basal media tried, best results were obtained in the case of AM medium, using half strength of major nutrients + full strength of minor nutrients, giving 67 per cent culture establishment. The above results are in agreement with those of Abou-Mandour (1977) who standardised AM medium for tissue culture of medicinal plants.

Woody plant medium, generally used for tree species gave only 56% culture establishment in the present study. According to Narayanaswamy (1977) SH medium has been formulated to support the growth of a wide variety of plants. In the case of nutmeg, it gave 61 per cent culture establishment. But with QL, only 53 per cent culture establishment was obtained, though it is reported to give such higher rate in the establishment and growth of tea (Samartin, 1989) and radiata pine explants (Kathryn, 1987).

The macro and micro nutrients contained in different basal culture media are in different proportions. In combination with other organic supplements in the medium

their varied responses in supporting or not supporting explant survival and growth is much sophisticated and needs elaborate studies. Such an attempt is not envisaged in the present experiments.

5.3.2. Growth hormones

According to Hussey (1979), composition of the basal medium has to be adjusted to the requirements of the different groups of plants. He also reported that some species may require additional supplements.

The nature and concentration of growth hormones in the culture medium are critical for *in vitro* growth and morphogenesis of plants (Skoog and Miller, 1957).

In the present investigation, in order to standardise a suitable hormone combination for better culture establishment in nutmeg, studies were carried out using BAP, NAA and 2,4-D at various concentrations. When a combination of BAP, NAA and 2,4-D at 2 mg/l, 1 mg/l and Ø.5 mg/l respectively were added to the basal medium, significantly superior results were obtained in the rate of survival, giving 67 per cent culture establishment, compared to other combinations of BAP and NAA. A combination of BAP and NAA at 2 mg/l and 1 mg/l respectively gave 5Ø per cent culture establishment and a combination of BAP, NAA and 2, 4-D at 2 mg/l, 1mg/l and 1 mg/l respectively gave 4Ø per

cent culture establishment, while the other hormone combinations give less than 3Ø per cent culture establishment (Table 16).

The favourable effects of cytokinins in axillary bud breaking and multiple shoot production has been demonstrated by Murashige (1974). But at higher levels, cytokinions were proved to have deleterious effect on shoot growth of many plants. Auxin added to the medium helps to nullify the suppressive effect of high cytokinin concentrations on axillary shoot growth in these plants (Lundergan and Janick, 198Ø). Auxins, especially NAA, at a concentration ranging from Ø.1 mg/l to 1Ø mg/l were found to support the *in vitro* establishment of many woody plants (Hartmann and Kester, 1989). This effect of hormones may be related to the variation in the endogenous levels of auxins and cytokinins in plant tissues that occurs as a consequence of exogenous application of growth hormones.

5.3.3. Phloroglucinol (PG)

When 4Ø mg/l of PG was added to the basal nutrient media containing 2 mg/l IBA, 58 per cent survival of the cultures was noticed. The most favourable effect of adding PG to culture medium along with 2 mg/l IBA was noticed in the rooting response. Phloroglucinol at 4Ø mg/l induced rooting in 29 per cent of the cultures, while other

levels of PG (2Ø mg/l and 6Ø mg/l) induced no rooting. Similar results were obtained by Jones (1986), who reported that PG could induce two to three fold increase in the proliferation and rooting of shoots in apple root stock (M7) cultures. In wild cherry, Hammatt and Grant (1993) also reported favourable response in the induction of rooting in cultures. Hartmann and Kester (1989) observed that phenols act as auxin co-factors or synergists in root initiation. Being a phenol, phloroglucinol thus helps in the rooting of nutmeg explants.

5.4. Subculturing

Subculturing of nutmeg shoot tip explants at 3 days interval, resulted in better culture survival and growth by eliminating phenolic interference to some extent. But, when the established cultures were subcultured to fresh media, after giving a cut at the basal region, the cultures were dead due to phenolic exudation. Hence, the cultures are to be transferred intact due to the presence of excess of phenols in the shoot tip explants. The favourable effect of frequent subculturing has also been reported in clove by Priyadarshan, *et al.*, (1989).

5.5. Effect of physiological pre-conditioning of explant sources (*in vivo*) in *in vitro* establishment of shoot tip explants

5.5.1. Pruning and grafting

Extensive studies at the Association Foret Cellulose (AFOCEL) Research Institute in France indicated that explants from adult forest trees were frequently slow to commence growth *in vitro* or failed completely, unless selected from rejuvenated tissues. Rejuvenation, when does not occur naturally, it has to be induced by various treatments such as grafting of shoots on to seedlings, shoot pruning or spraying with cytokinins.

In the present study in nutmeg, when newly emerged shoot tips taken from graftlings and pruned plants were cultured *in vitro*, they gave no significant variation in culture establishment when compared with that of explants taken from mature plants. Similar results were obtained in the case of cashew, where poor survival was observed when the explants were collected from grafted plants (Nair, 1991). However, in the case of jack, Philip (1993) obtained maximum culture establishment when the explants were taken from grafted plants.

Janick (1972) reported that if the mature branch of *Hedera helix* is grafted on a juvenile stock, juvenile shoots develop at first on the mature branch. After a few week's growth, the juvenility gradually disappears and the shoot again becomes mature. This may also be the reason for the poor response of shoot tips taken from pruned and grafted nutmeg plants.

5.5.2. Growth hormone infusion

When shoot tips collected from GA_3 infused plants (*in vivo*) were *in vitro* cultured, significant variation was observed in the rate of culture establishment among the various concentrations tried (5Ø, 1ØØ and 2ØØ mg/l) and no such significant response was observed in the frequency of explant collection.. Explants collected from 5Ø mg/l and 1ØØ mg/l of GA_3 infused plants gave significantly superior results in their *in vitro* response giving 7Ø and 68 per cent culture establishment respectively which were found to be statistically on par. The explants taken from control plant (distilled water spray) gave 57 per cent culture establishment. Rajmohan (1985) found that in jack, a pre-culture treatment using GA_3 (1 mg/l) was effective for culture establishment. Similar results were observed by Robbins (1957) who found that GA application can induce juvenility in *Hedera helix* and in Citrus (Luckwill, 197Ø).

When shoot tips collected from various concentrations of Cytokinin infused (BAP and kinetin) plants *in vivo*) were *in vitro* cultured, there was no significant variation in the rate of *in vitro* culture establishment. Also, considerable variation was not observed in the frequency of explant collection (3 and 7 days after shoot induction) (Table 19).

Philip (1993) observed that BAP at higher concentrations became inhibitory to rejuvenation in jack shoot tip cultures. According to Bajaj (1991) the cytokinin loses its organogenetic capacity when the duration of exposure of the plant to cytokinin is increased.

5.6. Effect of physiological age of shoot tips (from mature and seedling plants) on *in vitro* culture establishment

One of the major problems in the *in vitro* propagation of tree crops is its maturity barrier. It is important to select the most juvenile explant. In the case of nutmeg, shoot tips from seedlings exhibited significantly superior results in the rate of culture establishment compared to the explants taken from mature/bearing trees.

According to Rajmohan and Mohanakumaran (1988), physiological age of the explants exhibited significant influence on the *in vitro* shoot proliferation and rooting of jack. In the present investigation, explants taken from

seedlings gave 60 per cent culture establishment while it was only 50 per cent in the case of mature tree explants.

Seedling explants gave continued growth under *in vitro* conditions giving multiple buds and exhibited complete unfurling of leaves. Rajmohan (1985) reported that the rate of multiplication was very poor when explants were taken from mature jack trees where as explant from six month old grafts gave 100 per cent survival of shoots. Durzan (1984) reported that the feeble response observed by explants taken from mature trees is because of the "residual memory" of the explants.

It can be inferred from the above results that the culture establishment of nutmeg explants are highly influenced by the plant growth factors. Juvenile tissues responded better than the mature ones. But the major problem faced in its culture was that, the phenol content in the shoot tip explants was very high and on culture it gets exuded leading to destruction of the explants. Making a cut at the base of the explants during subculturing aggrevated the problem inspite of pre-treatment with various antioxidants. A viable protocol for *in vitro* multiplication of nutmeg through enhanced release of axillary buds should invariably include methods to overcome phenolic problem completely.

Summary

Summary

Investigations were carried out during 1992-'94 at the Department of Horticulture, College of Agriculture, Vellayani, to standardise pre-treatments to induce large number of orthotropic shoots in nutmeg (*Myristica fragrans* Houtt.). Subsequently, at the Plant Tissue Culture Laboratory attached to the Department of Horticulture studies were conducted to reveal the response of nutmeg shoot tip explants to *in vitro* culture conditions.

The pre-treatments tried include explant source (parent trees) treatments *in vivo* and explant treatments *in vitro*. The treatments tried on parent trees were pruning, grafting, etiolation, bending, girdling, half ringing, ringing and growth hormone infusion (kinetin, BAP and GA_3 each at different concentrations). Freezing, refrigeration, antioxidants, plant growth substances and casein hydrolysate (CH) were the different treatments tested on explants as pre-culture treatments. The antioxidants used for reducing phenolic interference were sugar, PVP, ascorbic acid, citric acid and cystein HCl. Growth hormones like BAP and kinetin and CH at different concentrations were used for pre-treating the explants for varying durations.

SH (Schenk and Hildebrandt, 1972), WPM (Lloyd and Mc Cown, 1980), QL (Quoirin and Le-Poivre, 1977) and AM (Abou-Mandour, 1977) were the different basal media tried. They were incorporated with growth hormones like BAP, NAA and 2, 4-D at various concentrations. Pre-treating explant sources (parent trees) with BAP and kinetin each at a higher concentration of 500 mg/l and 1000 mg/l was found to be effective in inducing orthotropic shoots (12 orthotropic shoots/tree), while pruning and etiolation gave only four orthotropic shoots per tree. Also, there was no added advantage in combining pruning + etiolation + growth hormone infusion in inducing orthotropic shoots. The *in vitro* response of all the newly induced orthotropic shoots were more or less similar. Parent tree infusion with GA_3 at 50 mg/l and 100 mg/l induced only eight orthotropic shoots each, but their *in vitro* response was significantly superior compared to explants collected from other treatments and control.

Among the pre-culture treatments tried, surface sterilization with 0.1 per cent mercuric chloride for 10 minutes gave significantly superior results in reducing fungal contamination, giving 72 per cent survival of the cultures. Incorporation of a carbendazim fungicide (Derosal 50 WP) in the basal media at 0.01% concentration further

reduced the microbial contamination, giving 8Ø per cent contamination free cultures. Pre-treating the shoot tips with 2 per cent sugar and Ø.7 per cent PVP was found to be significantly superior in eliminating phenolic interference. However, while subculturing, they had to be transferred intact, otherwise the excess phenols may exude out resulting in the damage of the cultures. Pre-treating the shoot tip explants with growth hormones like BAP and kinetin did not give any significant response under *in vitro* conditions. However, when the explants were pre-treated with 5ØØ mg/l CH for 1Ø minute, it resulted in significantly higher culture establishment rate. The study also revealed that seedling explants establish much better *in vitro* (6Ø per cent) than mature tree explants (5Ø per cent). Among the various basal media tried, AM at half strength of major nutrients and full strength of minor nutrients was found to be the best. A combination of BAP, NAA and 2,4-D at 2 mg/l, 1 mg/l and Ø.5 mg/l respectively gave significantly superior results in the rate of culture establishment (67 per cent). Phloroglucinol (4Ø mg/l) in combination with IBA (2 mg/l) gave significantly superior results in the induction of roots.

Protocol for achieving maximum culture establishment using nutmeg shoot tip explants

1. Best physiological pre-treatment to induce orthotropic shoots.

   Infuse explant source (mature female trees, preferably dwarf statured) with 50 mg/l GA_3, using a hand sprayer with fine mist nozzle, till whole plant surface is wetted.

2. Best explant

   Newly induced orthotropic shoots 25-30 mm length, collected from GA_3 infused plants.

3. Best pre-culture treatment to eliminate phenolic interference.

   Pre-treat the explants with 2% sucrose + 0.7% PVP solution for 30 minutes and wash the explants thrice using sterile double-glass distilled water.

4. Best pre-culture treatment to obtain better culture establishment.

   Pre-treat the explants using 500 mg/l casein hydrolysate for 10 minutes and wash the explants using sterile double glass distilled water, thrice.

5. Best surface sterilization method

Surface sterilize the explants using Ø.1% mercuric chloride solution for 1Ø minutes and then wash the explants three or four times using sterile double glass distilled water.

6. Best culture medium

Basal medium : AM (1977) (half strength of major nutrients + full strength of minor nutrients) + 2 mg/l BAP + 1 mg/l NAA + Ø.5 mg/l 2, 4-D and Ø.7% agar + Ø.2% activated charcoal. Carbendazim (Ø.Ø1%) is useful in controlling fungal contamination if incorporated into the culture medium.

7. Best rooting medium

Basal medium : AM (1977) (half strength of major nutrients + full strength of minor nutrients) + 2 mg/l IBA + 4Ø mg/l phloroglucinol and Ø.7% agar + Ø.2% activated charcoal.

Future trials are to be oriented with the following main objectives:

1. Phenolic interference should be tackled completely by means of waxing or by incorporating chemicals like silver thiosulphate.

2. Effect of various organic additives such as adenine sulphate, myo-inositol and casein hydrolysate should be studied in detail.

3. Other routes of *in vitro* propagation for inducing somatic organogenesis or somatic embryogenesis are to be standardised.

4. Rooting should be further increased by using growth hormones such as IBA and phloroglucinol in combination at varying strength of macro and micro nutrients in the culture medium.

5. Hardening and planting out should be attempted.

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* Originals not seen.

Appendix

Appendix I

Treatments	Mean sum of squares	
	Treatment	Error
A. Effect of physiological pre-treatments in the induction of orthotropic shoots	Ø.ØØ2**	Ø.ØØØ3
B. Effect of growth hormones in the induction orthotropic shoots	Ø.2ØØ5**	Ø.Ø12
C. Combined effect of pruning, etiolation and growth hormones in the induction of orthotropic shoots	61.43**	Ø.56
D. Elimination of phenolics interference		
a. Refrigeration	1Ø68.35**	143.98
b. Freezing	532.19**	56.78
c. Sugar + PVP	2332.8Ø**	125.37
d. AA + CA	236.57*	65.78
e. Cystein HCl.	1Ø6.6Ø**	17.7Ø
E. Elimination of fungal contamination		
a. Mercuric chloride	3989.1Ø**	179.4Ø
b. Mercuric chloride + Derosal	55.67*	15.13
F. Effect of Phloroglucinol on rooting of nutmeg shoot tip cultures		
a. Percentage survival	3.94**	Ø.15Ø
b. Percentage rooting	Ø.265**	Ø.ØØ2

(contd...)

Treatment	Mean sum of squares		
	Treatment	Interaction	Error
G. Effect of *in vivo* pre-treatment of shoot tip on *in vitro* culture establishment			
a. Pruning and grafting	2.31^{NS}	4.12^{NS}	3.17
b. GA_3 infusion	3.32^{NS}	2.59^{NS}	21.27
c. Kinetin infusion	3.Ø4^{NS}	2.85^{NS}	41.12
d. BAP infusion	3.3^{NS}	2.4^{NS}	8.5
H. Effect of pre-treatment of shoot tip explant with growth hormones and CH on *in vitro* culture establishment			
a. Kinetin	115.Ø9**	126.Ø7**	17.93
b. BAP	3Ø.43^{NS}	9.78^{NS}	1Ø.31
c. CH	13.49^{NS}	78.78^{NS}	46.55
I. Effect of mature and seedling shoot tips on *in vitro* culture establishment	8Ø1.6**	2.67^{NS}	78.38

* Significant at 5% level.
** Significant at 1% level.
NS Not significant.

EFFECT OF PHYSIOLOGICAL PRE-CONDITIONING OF EXPLANTS AND EXPLANT SOURCES OF *Myristica fragrans* Houtt. ON *IN VITRO* CULTURE ESTABLISHMENT AND GROWTH

BY

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ABSTRACT OF A THESIS
SUBMITTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN HORTICULTURE
FACULTY OF AGRICULTURE
KERALA AGRICULTURAL UNIVERSITY

DEPARTMENT OF HORTICULTURE
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VELLAYANI
THIRUVANANTHAPURAM
1995

Abstract

The present study was conducted to standardise pre-treatments to induce large number of orthotropic shoots and to enhance the *in vitro* response of explants taken from nutmeg trees by standardising suitable basal media and by incorporating suitable growth hormones.

Among the various physical methods of mother tree pre-treatments tried, pruning and pruning followed by etiolation induced four orthotropic shoots each. Pre-treating explant sources (parent trees) with BAP and kinetin each at 500 mg/l and 1000 mg/l induced 12 orthotropic shoots per tree. However, the *in vitro* response of all these newly induced shoots were more or less similar, except those taken from 50 mg/l and 100 mg/l of GA_3 infused parent trees. Surface sterilisation of the explants with - 0.1 per cent mercuric chloride for 10 minutes and addition of 0.01 per cent of a Carbendazim based fungicide in the culture medium helped to increase the rate of contamination free cultures. Pre-treating the shoot tips with 2 per cent sugar and 0.7 per cent PVP gave significantly superior results in eliminating phenolic interference. Pre-treating the shoot tip explants with CH at 500 mg/l for 10 minutes gave significantly superior results in the culture establishment

of nutmeg explants. The results also revealed the significantly superior influence of seedling explants over mature tree explants. Among the various basal media tried, AM at half strength of major nutrients and full strength of minor nutrients was found to be the best with a hormonal combination of BAP, NAA and 2,4-D at 2 mg/l, 1 mg/l and Ø.5 mg/l respectively, giving 67 per cent culture establishment. Phloroglucinol (4Ø mg/l) in combination with IBA (2 mg/l) gave significantly superior results in the induction of roots in established shoot tip cultures.