FINAL REPORT

on

IN VITRO PROPAGATION OF

NUTMEG (Myristica fragrans)

and

CLOVE (Syzygium aromaticum)

by

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Responses to in vitro culture of explants taken from shoot tips from juvenile and adult shoot tips, axillary buds, leaves, ovules and embryo or endosperm of clonal selections of nutmeg and clove were tested. Complete plant regeneration with higher rate of multiplication was achieved with shoot tip culture of juvenile plants of both species. Partial success was achieved in axillary bud culture in clonal trees of both species.

Shoot tip explants taken from juvenile plants of clove and nutmeg were established successfully in Anderson's culture medium supplemented with 2.0 mg/l BA with or without 0.1-0.2 mg/l NAA. Multiplication of juvenile nutmeg (4-5) and clove (6) was obtained within an eight week period in Anderson's solid medium supplemented with 2.0 mg/l BA. Agitated liquid medium with the same nutrient composition gave the highest rate of multiplication (6) with greater shoot growth of nutmeg, whereas, for clove liquid media were found to be not suitable. The best subculture period for nutmeg cultures was about four weeks and the most suitable portion of the shoot to achieve highest multiplication was 0.5-2.5 cm from the apex. Shoot elongation of clove could not be induced by GA₃ or NAA. Nutmeg shoots were successfully rooted in Anderson's medium containing 0.2% activated charcoal with 0.5 mg/l IBA,
whereas for clove rooting took place in 1/3 strength of Anderson's with 0.2% activated charcoal with either 2.0 mg/l IBA or 1.0 mg/l NAA. Plantlets were successfully transferred to soil.

Shoot tips from three physiological stages of clove clonal selections became established in culture only at a low percentage (5%) and produced axillary bud growth in Anderson's medium supplemented with 0.05 mg/l yeast extract, 0.01 mg/l glutamine, 0.01 mg/l biotin 6-10 mg/l BA and mixture of four antibiotics (6.0 mg/l Polymixin B, 6.0 mg/l Rifampicin, 25 mg/l Cephotaxime and 25 mg/l Tetracycline). Higher percentage of culture establishment and low rate of contamination were observed when nodal explants were taken at the end of the major rainy season (December, January). Selected nodal portions with swollen axillary buds took less time to produce axillary bud growth, and with an even higher percentage of success. Although unfertilized ovules were expected to be more suitable for clonal multiplication through somatic embryogenesis, they failed to produce callus.

Shoot tips or nodal explants from plagiotropic or trunk-sprouted orthotropic shoots of nutmeg at different stages gave negative results not only due to a higher rate contamination but also probably to the unsuitability of the
physiological stage of the explants.

Clonal grafted materials maintained in the greenhouse gave a very low rate of contamination but in all the attempts shoot tip cultures failed to regenerate plantlets. Single nodal explants from the grafted plants could be established in 1/3 Anderson’s medium supplemented with 0.05 mg/l yeast extract, 0.01 mg/l glutamine, 0.01 mg/l biotin and 1.5 mg/l BA. Incorporation of even a very little quantity of NAA (0.1 mg/l) was not suitable for nodal culture establishment. Nodal explants at two different physiological stages were tested, and it was found that nodes with emergence of axillary buds (about 5-15 mm long) were more suitable for culture establishment. Initial incubation for three weeks in complete darkness was not effective for axillary bud elongation.

Other explants tested in nutmeg such as leaves, endosperm and embryo gave negative results. Micrografting was also difficult and a failure. Only a limited number of experiments could be done with re-grafted nutmeg plants due to unavailability of explant materials.
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KEY TO ABBREVIATIONS

AC: Activated charcoal
AN: Anderson’s medium (1975)
BA: 6-benzylaminopurine (N’ benzyladenine).
2,4-D: 2,4- dichlorophenoxyacetic acid.
GA₃: Gibberellic acid
B5: Gamborg et al., 85 basal medium.
IAA: Indole-3-acetic acid.
IBA: Indole-3-butyric acid.
2iP: 2-isopentenyl adenine.
KN: Kinetin.
MS: Murashige and Skoog medium.
NAA: α-naphthaleneacetic acid.
PG: Pluroglucinol.
WPM: Lloyd and McCown woody plant medium.
Z: Zeatin.
1.0 INTRODUCTION

The term "plant tissue culture" broadly refers to the cultivation in vitro of any plant part, whether a single cell, a tissue or an organ, under aseptic conditions. Plant tissue culture is a technique with great potential as a method for vegetatively propagating economically important species, which is currently being realized commercially. However, a tissue culture system is also very often a model system which allows one to investigate physiological, biochemical, genetic and structural problems related to plants, and moreover the technique is also being used as an adjunct to more traditional means of plant genetic modification.

The potential of plant tissue culture was demonstrated by the experiments of White (1934, 1939) and Gautheret (1939). The confirmation of Haberlandt's theory (1902) of cellular totipotency was independently demonstrated with carrot cell cultures by Steward et al., (1958) and Reinert (1958). In vitro plant regeneration through somatic embryogenesis, organogenesis and androgenesis has been successfully reported in a large number of taxa. However, successful in vitro propagation of woody fruit species progressed rapidly only after the 1930 Beltsville conference on tissue culture of fruit species (Zimmerman, 1980). The production of apple root stocks has been the first major application of tissue culture to woody fruit species. However, during the last
decade, commercial production of woody horticultural plants by this technique has progressed rapidly. A considerable volume of work has accumulated on tissue culture propagation of temperate fruit and nut crops and a review of such a vast literature is beyond the scope of the present study. Therefore, a concise survey of literature pertaining to in vitro propagation of tropical and subtropical tree species is included to give a background to the work undertaken. In comparison with temperate tree species, relatively little progress has been made on in vitro regeneration of tropical and subtropical trees. The first attempts to regenerate tropical woody fruit species through in vitro techniques were made with Citrus. Nucelli excised from fertilized ovules of C. microcarpa and cultured on medium produced callus which differentiated into globular proembryos, which eventually developed into plantlets. Among dicotyledonous tropical tree species, regeneration of plants from tissues of juvenile origin has been reported in a large number of species e.g., cinnamon, tamarind, avocado and Hevea, but the regenerated plants had only limited usefulness, because the genetic composition of seedling trees was unknown. During the last decade there has been considerable work on regeneration of these recalcitrant trees from shoot tip explants of mature origin, e.g., teak, eucalyptus, guava, Ficus religiosa, mulberry and fig.
Mature tree selections have been very difficult to regenerate from shoot tips and bud or nodal cultures, due to the lack of juvenility of these explants. This has been overcome by selections of other parts (ovules, nucelli) of the mature tree as an initial explant. Extensive studies have been made in citrus, papaya, mango, and to some extent in jaboticaba and Eugenia species. Further, plantlets have been successfully regenerated from cultured pieces of leaves of coffee or a whole leaf of longan. Although these tropical and subtropical tree species have been explored for their in vitro morphogenetic potentialities through somatic embryogenesis, exploitable methods for large-scale clonal propagation of tropical fruit trees have yet to be established except for a few species.

The nutmeg tree Myristica fragrans Houtt., (family Myristicaceae) is a dense evergreen tree, indigenous to the Moluccas and other islands of Indonesia. It produces two different spices, nutmeg and mace. Under cultivation it grows to a height of 40-60 feet and thrives at low elevations in the warm and rainy tropics of both hemispheres. The leading nutmeg producing regions today are Indonesia and Grenada in the West Indies; a comparatively small quantity is produced by Sri Lanka (about 500 metric tones). The area under nutmeg is about 6000 acres and foreign exchange earning is about rupees 65 million
annually. Annual world production of nutmeg and mace is about 7,000 metric tons, 60% of which comes from Indonesia. The principal importing countries are the United States, West Germany, the Netherlands and the United Kingdom.

The fleshy fruit, resembling an apricot, is globose in form, and yellow to light brown in colour. When ripe, it splits in half exposing the net-like membrane or aril known as mace, which encloses a lustrous dark brown brittle shell, inside which is the single glossy brown, oily seed which is the nutmeg of commerce.

The flavour of both nutmeg and mace is sweet and highly spicy, and essential oils are found in the two different structures. Nutmeg and mace are mainly used as milled baking spices. They are also used to flavour sausages, various other meat products, soups, prepared sauces and preserves, and as a medicine for treatment of various ailments of the kidneys and stomach. However, the essential oils of nutmeg and mace also contain about 4% (by dry weight) of a highly toxic substance, myristicin, which, if taken in excessive amounts, can cause degeneration of liver cells.

The dioecious character of nutmeg coupled with a long juvenile phase has prevented expanded cultivation and crop improvement. No method for identifying the sex at the
seedling stage or prior to flowering is known.

Due to continuous cross-pollination within nutmeg plantations, trees are variable for yield, quality and bearing habit. Selected mother trees which yield over 15,000 fruits per year and which possess other desirable characters are available in Sri Lanka. Production increases could be achieved relatively rapidly by distributing to farmers vegetatively propagated plants from superior selections. Vegetative propagation of nutmeg has given limited success and is inadequate to meet the demand.

The cloves of commerce are the dried unopened flower buds of an evergreen tree, *Syzygium aromaticum* (L.) Merr. & Perry belonging to the family Myrtaceae. The plant is indigenous to the Moluccas. The spice, whole or ground, has a number of culinary uses. The world's most important producer of clove is Zanzibar, followed by Madagascar. Sri Lanka exports about 2,000 metric tones of clove and earns about rupees 250 million annually. Indonesia is the world's largest consumer, using some 30,000 tones annually in the "kretek" cigarette industry.

The clove is a small, evergreen tree, reaching 12-15 m in height, conical in shape when young, later becoming roughly cylindrical. The tree can live for about 100 years or
sometimes 150 years. Clove is a normally cross pollinated plant (Tidbury, 1949) but sometimes is referred to as a self-pollinated crop (S. idaran and Bavappa, 1984). The origin of the clove in Sri Lanka was from very limited introductions. Therefore very little variability would be expected within the clove growing areas. The area of clove in Sri Lanka is about 20,000 acres.

The vegetative propagation of cloves has always been difficult and attempts in many regions have failed i.e., rooting of cuttings, inarching and grafting (Purseglove et al., 1981).

The young trees bear a small quantity of cloves when they are 4-5 years old, but trees take 20 years or more to attain full bearing. Therefore, if they remain healthy, they will continue to give crops for another 50 years or so. The mean annual yield of cloves is about 50 kg for a 50-year-old tree and 25 kg for one which is 12-20 years old.

Two main problem in improvement of clove cultivation is the long juvenile phase (about 8 years) and the absence of an acceptable vegetative propagation system. Also variability of yield both seasonal and among the trees exists in the clove plantations although this has not been thoroughly studied. The other problem with this spice tree is that
availability of seeds for propagation is limited to once a year, with no method of seed preservation due to the nature of the seed and harvesting difficulties due to the height of the seedling trees (about 60-75 feet).

The standard approach to fruit tree improvement is selection and vegetative multiplication of superior genotypes already present in natural populations. For this reason an efficient clonal propagation method which ensures the transfer of additive and non-additive gene effects from the parent plant of proven quality to their offspring will be very useful. Horticulturists use the techniques of vegetative propagation to multiply selected plants. However, conventional methods of vegetative propagation such as rooting of cuttings, air layering and grafting are often slow and inadequate. Producing large numbers of clonal plants from one selected genotype can facilitate the yield and quality improvement of tropical perennial tree crops, especially in tree species where breeding cycles are long and vegetative propagation is difficult.

During the past decade the micropropagation of woody plants has moved rapidly from theory to practice at a commercial level. By 1985 at least 50 genera of ornamentals, forest and fruit plants were being produced by tissue culture
(Zimmerman, 1985). In recent years species which were difficult to propagate by vegetative methods such as jak, teak and guava have been successfully propagated through tissue culture techniques. Therefore, tissue culture techniques will be the tool not only to overcome the above mentioned difficulties, but also as an essential prerequisite to the development of new genotypes using techniques such as gene transfer by vector, somatic hybridization or conserving existing germplasm.

No literature is available on successful in vitro propagation of nutmeg or clove except in vitro seed germination of clove by Chandra and Ravishanker in 1986. But closely related genera to clove such as two Eugenia species (Litz, 1984) have produced successful embryogenesis through ovule cultures. Guava (Psidium guajava L.) which is also related to clove has been successfully propagated through tissue culture (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987).

Tissue culture has become an accepted, profitable and established technique for the propagation of ornamentals and some woody temperate tree species. In recent years, large scale production of planting materials through tissue culture has extended to plantation crops e.g., date palm, oil palm, coffee and mulberry and fruit crops or forest
trees e.g., papaya, banana, pineapple, mango, eucalyptus, sandalwood. Therefore, tissue culture propagation may be the most appropriate method for clonal propagation of nutmeg and clove.
2.0 LITERATURE REVIEW

2.1 Methods of \textit{in vitro} clonal propagation

\textit{In vitro} propagation of tree species has progressed significantly during the last decade. There are three different ways of obtaining a large number of plants \textit{in vitro}:

(a) Regeneration via somatic embryos;
(b) Regeneration from adventitious meristems;
(c) Regeneration from existing meristems;

Theoretically, the most efficient method would be to stimulate embryogenesis directly from callus. Via embryogenesis, one could obtain thousands of plants in a very short time from continuous cultures. Several tropical woody fruit crops such as jaboticaba (Litz, 1984a), \textit{Eugenia} species (Litz, 1984b), mango (Litz \textit{et al.}, 1984) and citrus (Manneswari and Rangaswamy, 1958) have been propagated from nucelli or ovule callus and leaf callus of longan (Litz, 1988), cocoa (Litz, 1986) and coffee (Sondahl and Sharp, 1977).

The second method of producing a large numbers of plants is by stimulating adventitious bud formation directly from plant organs such as leaves and stems (organogenesis). It could also be used to produce a large number of plants, but
efficient organogenesis has not been accomplished except with herbaceous plants.

A third method involves the stimulation of axillary bud growth. Using shoot tip cultures, the expansion of dormant axillary buds is stimulated, and each of these buds can become a single shoot that can be rooted and grown into a whole plant. Theoretically, this production system is not as fast as the other two, but the method works well for many fruit crops, and has been utilized widely for proliferation purposes. Plants derived from axillary bud proliferation are generally phenotypically homogenous, thereby indicating genetic stability. The induction of axillary bud proliferation seems applicable in many cases, e.g., rhododendron (Anderson, 1975), apple (Zimmerman, 1983), blueberry (Zimmerman and Broome, 1980b), peach (Hammerschlag, 1982b, 1986), papaya (Litz and Conover, 1975), teak (Gupta et al., 1980), mulberry (Oka and Ohyama, 1986), guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987) jak (Amin, 1986). Although the rate of multiplication by organogenesis and embryogenesis is astonishing, their regeneration capacity usually diminishes rapidly after a number of subcultures, and eventually this morphogenic potential is completely lost (Hu and Wang, 1983). The initial multiplication rate for axillary bud proliferation on the other hand is rather slow, but remain steady during
many subculture cycles. Up to date, plant regeneration from existing meristems is the most common method for clonal propagation of hardwoods.

2.2 Shoot tip cultures

Shoot tip culture is preferred over meristem culture in micropropagation when viral elimination is not part of the objective. Use of larger explants is desirable as they are easier to dissect and have much higher survival and growth rates than smaller explants. Further, shoot tip cultures remain the pre-eminent in vitro method of clonal multiplication for woody plants. The basic reason is that shoot cultures can be manipulated with some success without understanding the basic underlying reasons why the system responds.

Shoot tip and bud culture as a tool for rapid clonal multiplication was initiated as early as 1922 (Robbins, 1922) and the regeneration of transplantable plants using this technique was first achieved by Ball in 1946. The successful cloning of the orchid, Cymbidium (Morel, 1965), resulted in the first practical exploitation of in vitro clonal propagation of an herbaceous horticultural species. However the successful propagation of some tree species,
such as *Populus tremuloides* (Winton, 1968), foreshadowed the rapid expansion in the application of tissue culture methods to propagate woody plants (Anderson, 1975; Jones, 1979, 1982; Mott, 1981; Lane, 1982; Zimmerman, 1983; Amin, 1986; Jaiswal and Amin, 1986; Amin and Jaiswal, 1987). Thorpe (1985) has mentioned some 90 species of hardwood and 18 species of softwood trees from which plants have been regenerated using tissue culture methods.

The greatest success using this technique has been achieved with herbaceous horticultural species. Compared to herbaceous plants, the micropropagation of woody species has lagged far behind. Successful examples of micropropagation of woody and semi-woody plants through *in vitro* axillary bud proliferation include fruit bearing species in the genera *Malus*, *Prunus*, *Pyrus* and *Ribes* and woody ornamentals including *Atriplex*, *Betula*, *Kalmia*, *Rosa*, *Salix*, *Santalum* and *Spirea*. A small number of tropical woody trees has been successfully regenerated from mature origin tissues through axillary bud proliferation i.e., teak (*Tectona grandis*, Gupta *et al.*, 1980), *Eucalyptus* (Gupta *et al.*, 1981), guava (Jaiswal and Amin 1986; Amin and Jaiswal, 1987), jak (Amin, 1986), mulberry (*Norus indica*, Patel *et al.*, 1983), fig (*Ficus carica*, Murithi *et al.*, 1982). There is no report so far on successful shoot tip culture or any tissue culture aspect involving nutmeg or within the family Myristicaceae.
2.3 Stages of micropropagation

Murashige (1974) developed the concept of different stages of micropropagation, which consist of four steps:

1. Stage I or establishment of aseptic cultures
2. Stage II or multiplication of propagules
3. Stage III or rooting of in vitro proliferated shoots
4. Stage IV or hardening and preparation for establishment of regenerates in soil.

Sometimes stages III and IV have been combined as the rooting and acclimatization stage. Some of the cultures, specially dwarf in nature, need a shoot elongation stage prior to the rooting stage. Thereby separation of shoots is easier and also a higher rooting percentage is obtained.

Debergh (1986) has introduced another important stage prior to stage I, stage 0 or preparation of the mother plants. This stage also very important because the condition of the mother-plant can have a profound influence on the subsequent success of a system.
2.3.1 Stage 0: Preparation of the mother-plants

The influence in this stage can be of two kinds, viz., sanitary and physiological effects.

2.3.1.1 Sanitary aspects

For many woody plants it is not easy to find plants that will yield non-contaminated explants after the usual sterilization procedure for tissue culture. As most species are propagated initially from shoot tips or axillary buds, it is necessary to grow the aerial parts of the plant, from which the primary explants will be harvested, under as hygienic conditions as possible. For many plants it is possible to improve the success of stage I considerably by simple irrigation of the mother-plants directly in the container and never overhead (Debergh and Maene, 1981).

2.3.1.2 Physiological aspects

During the preparation stage, stage 0, physiological condition of the mother plant can be programmed. There are many ways, for example:

(a) For most bulbous species cold storage is required to release dormancy.
(b) Control of the photoperiod under which the plants are grown allows control of the vegetative or generative state.

(c) A weaker sterilization is called for when the plants are grown under hygienic conditions and this minimizes hyper-sensitivity reactions upon introduction into culture.

(d) Appropriate pruning yields healthier, better sized and more active explants.

(e) Mother plants or branches can be pretreated with growth regulating, nutritional and infection controlling products.

2.3.2 Stage I: Establishment of aseptic cultures

The principal purpose of stage I is to establish a microbe free explant culture. It is also desirable that the explant should initiate new growth during this stage. This permits the selection of the strongest pieces for multiplication in stage II. The culture establishment period itself consists of three phases (McCown, 1986) namely, isolation, stabilization and shoot production.
The isolation protocol for tissue from woody plants does not differ greatly from that used for most herbaceous plants. The greatest differences between woody perennial and herbaceous plants are observed during the stabilization phase of culture establishment. Growth characteristics of explants in this phase are unpredictable and often abnormal. In most annuals and herbaceous perennials, this phase takes a relatively short time and can be accomplished in a small number of subcultures after initial isolation. In woody perennials, this phase can require relatively little time (shrubs), may require several years (mature deciduous trees), or may not ever be completed (some forestry species). One of the important factors influencing the stabilization of a shoot culture is the phase-state of the original stock plants. Hence, more juvenile explants are easy to establish in culture. This becomes particularly apparent when isolates are taken from different portions of the same plant such as young shoots originating from the collar region and buds from the apical portions of well-established branches. With the elepidote rhododendrons, collar-shoots established more readily than shoots from flowering stems. Another factor is the type of shoot growth dynamics that the plant normally displays in its seasonal cycle. In general, plants that show a pattern of continuous shoot growth throughout the season are much easier to stabilize in culture than the species denoted by episodic or
flushing growth (McCown, 1986).

A continuing problem in woody plants is bacterial contamination of the cultures at the time of explanting or later during proliferation. In many cases, however, the problem seems to be caused by bacteria growing initially in the tissue. Recently a technique using four antibiotics simultaneously has been reported to control bacterial infections in a number of different plant genera (Young et al., 1984). As an alternative to using antibiotics it may be possible to encourage the rapid growth of shoots with the objective of producing bacteria-free shoot tips (Zimmerman, 1985). Condition favoring rapid shoot elongation, e.g. a low concentration of cytokinin in the medium and dark incubation, may promote suitable shoot tips (5 mm or less in length) for excision and further culture. However, to obtain sterile explants specially from tropical woody plants seems to be very difficult. This may be overcome to some extent by weekly spraying of the buds with a mixture of an antibiotic and a systemic fungicide (streptomycin 0.1% + benlate 0.1%) and maintaining the donor plants in a greenhouse and avoiding overhead watering.
2.3.2.1 Explant

Many variables may influence the suitability of a plant part as a source of explant, and closely related varieties do not necessarily respond similarly in vitro. Three important considerations are the regenerative capacity, physiological stage, and health of the donor plant. Mature trees that are demonstrated as superior genotypes are most attractive for mass propagation. However, it is more difficult to establish shoot cultures from mature trees than from juvenile plants (Bonga, 1987; Hackett, 1987). In spite of advantages of juvenile wood for tissue culture, juvenile tissue may have limited usefulness. The reason is that most of the fruit trees have been selected for mature characteristics such as growth habit, flowers, fruit and bearing habits which cannot be fully assessed until the plant has passed its juvenile phase. Moreover, plantlets originating from mature tissues are expected to require less time for bearing. In order to facilitate establishment of explants from mature trees one can select the most juvenile tissues within the tree or one can pretreat the parts of the donor tree. Explants from the lower branches of the crown are normally easier to establish than explants from mature tissues in the upper part of the crown (Bonga, 1987). Stump sprouts are a favourable juvenile alternative where such exist, for example in Quercus robur and Tilia cordata (Chalupa, 1984), Hevea (Mascarenhas et
Pretreatment techniques include spraying of buds with cytokinin before incubation in vitro and repeated grafting of mature buds on juvenile rootstocks before excision for in vitro culture (Ahuja, 1987). Furthermore, bud break can be stimulated either by culturing newly isolated buds on medium supplemented with cytokinin or by pulse-treating buds with cytokinin (Bergman et al., 1984). The time of the excision is often a key factor for establishment of explants of woody species with their episodic growth. The best season for bud initiation of most trees are spring, coinciding with bud break, and late summer (Bonga, 1987; Welander, 1988). However, consideration has to be taken of the risk of contamination which also is season dependent (Bonga, 1987). Use of small explants, for example meristem tips, facilitate separation of meristematic tissue from surrounding, more differentiated tissues.

2.3.2.2 Culture media

There is no general purpose medium yet available for meristem, shoot tip or bud culture. Among the various media used in shoot tip or bud cultures, Murashige and Skoog medium (1962), with some modifications, is the one used most frequently and with the greatest success as demonstrated for
papaya (Litz and Conover, 1978), Eucalyptus (Gupta et al., 1981), apple (Johnson and O'Ferrell, 1977; Lundergan, 1980; Snir and Erez, 1980), pear (Lane, 1979c), Rosa (Hasegawa, 1979), teak (Gupta et al., 1980), tea (Arulpragasam and Latiff, 1986), cocoa (Passey and Jones, 1983), coffee (Sondahl et al., 1984), sandalwood (Lakshmi, 1986), jak (Amin, 1986) and guava (Jaiswal and Amin, 1986; Amin and Jaiswal 1987).

A woody plant medium developed by Lloyd and McCown (1980) is significantly different from MS with low nitrate, high potassium and low sugar, and has been reported to result in successful regeneration of Kalmia latifolia.

Anderson's rhododendron formula (1975), probably the first report of in vitro propagation of an economically important woody plant, is a modified MS with lower nitrate, higher iron, sodium phosphate, and adenine sulphate. It is specially recommended for acid-loving plants. Linsmaier and Skoog medium (1965), White's medium (1963), and Nitsch and Nitsch medium (1969) are occasionally used for successful shoot tip culture of woody plants.
2.3.2.3 Growth regulators

Although small quantities of cytokinin may be synthesized by shoots grown *in vitro* (Koda and Okazawa, 1980), seeds, embryogenic tissues and roots are the principal sites of cytokinin biosynthesis. It is unlikely that the meristem, shoot tips and bud explants have sufficient endogenous cytokinin to support growth and development. Therefore, cytokinin within the range 0-10 μM is used at this stage. Sometimes higher concentrations of kinetin such as 50mg/l for papaya (Litz and Conover, 1981) and 20 mg/l for Prunus (Skirvin and Rukan, 1979) have been used to establish cultures. Benzyladenine is most effective for meristem, shoot tip and bud cultures, followed by kinetin. 2-isopentyl adenine been used less frequently (Nair et al., 1979; Lloyd and McCown, 1980). Sometimes cytokinin may be species specific such as 2iP for plants of the family Ericaceae (McCown, 1986). Some cultures can be established without cytokinin. It is likely that a sufficient quantity of endogenous hormone is already present in the explant. Furthermore, some of the species which could produce adventitious roots readily may act as a new source of cytokinins.

Auxin is another hormone required for shoot growth. Since the young shoot apex is an active site for auxin
biosynthesis, exogenous auxin is not always needed in the medium, especially when relatively large shoot tip explants from actively growing plants are used. There are cases when exogenous auxin is not essential but is beneficial for growth (Dale, 1975). Resting buds and meristems 0.4 mm or less may not produce or retain enough endogenous auxin for shoot growth. NAA, IBA, IAA, 2, 4-D and zeatin are the most frequently used auxins in plant tissue cultures. NAA is the auxin routinely used by most laboratories for meristem, shoot tip and bud cultures. The usual concentration range used in stage I is between 0.045 and 10.0 μM.

Gibberelins have been included at concentrations of 0.29 μM or less in some of the stage I media, e.g., apple (Zimmerman and Broome, 1980c). Evidently sufficient quantities of this hormone are synthesized by most of the explants. When GA₃ is supplemented, its function is primarily for bud elongation (Schnabdrauch and Si-μ, 1979).

2.3.2.4 Incubation conditions

For meristem, shoot tip and bud cultures constant incubation temperatures ranging between 20 and 28° C are used, with the great majority of cultures requiring temperatures in the middle of the range (24-26° C). A day and night temperature
fluctuation may be desirable for some plants, especially for those adapted to temperate and desert climates.

The duration, intensity and quality of light at incubation are also very important to culture establishment. In order to maximize in vitro growth and prevent induction of dormancy, with few exceptions, long photoperiods (12-24 hrs/day) are used. The most commonly used photoperiod regime is 16 hour light with 8 hour darkness. The light intensity is usually 1-10 klux but low light is beneficial in stage I compared to the other two stages. Since light stimulates tissue browning in explants with high phenols, it is advisable to reduce light intensity below 1 klux or even incubate in darkness for about 1-2 weeks after introduction. In most cases light is produced by fluorescent tubes of the cool white or Gro-lux type.

Air humidity is infrequently controlled during incubation. Those who do control it usually set the humidity between 60 and 80%, with 70% being the most frequent setting. However, closed vessels with controlled aeration provide high humidity (over 90%) within the vessel.
2.3.2.5 Polyphenol oxidation

Many plants are rich in polyphenolic compounds. After tissue injury during dissection such compounds will be oxidized by polyphenoloxidase and the tissues will turn brown or black. The oxidation products are known to inhibit enzyme activity, kill the explant and darken the tissue and culture media. Such phenomena impose a serious hindrance to the establishment of primary cultures, especially in woody plants. Some of the procedures used by various workers to overcome this problem are:

(a) Presoaking or adding of anti-oxidants such as citric acid, ascorbic acid, thiourea or L-cystine. These compounds prevent the oxidation of polyphenols in culture media.

(b) Addition of active charcoal to the medium (concentration of 0.2-3.0% W/V).

(c) Addition of PVP to the medium (concentration of 250-1000 mg/l). PVP is a polymer which adsorbs phenol.

(d) The addition of diethy-dithiocarbonate (DIECA) in the rinses, after sterilization, at a concentration of 2.0 g/l, and its addition as droplets at the time of micro-grafting can also block oxidation.

(e) The addition of three aminoacids (glutamine,
arginine and asparagine).

(f) Frequently transferring explants into a fresh medium whenever browning of the medium is observed.

(g) Incubating during the initial period of primary cultures in reduced light or darkness.

(h) A reduction of wounded tissue can result in a decrease in oxidation.

(i) A reduction of the salt concentration in the culture medium can reduce exudation.

(j) The soaking of explants in water before placing in culture has been an effective method of reducing exudation.

2.3.3 Stage II: Multiplication of propagules

The basal medium used at this stage is mostly the same as for stage I or sometimes with increased concentration.

2.3.3.1 Growth regulators

In "axillary bud proliferation" cytokinin is utilized to overcome the apical dominance of shoots and enhance the branching of lateral buds from leaf axils. Concentration of the cytokinin can be rather high compared to other stages of
**in vitro** cultures. Generally the cytokinin requirement at this stage is about 4.5 $\mu$M, although it can be up to 25 $\mu$M. Occasionally, systems require as high as 90-270 $\mu$M (Schnabdrauch and Sink, 1979; Skirvin and Rukan, 1979). In general it appears that BA is the most effective cytokinin for stimulating axillary bud proliferation, followed by (in decreasing order) kinetin and 2iP (Bojwani, 1980; Papachatzis et al., 1981; Hasegawa, 1980; Kitto and Young, 1981). A quite different order of cytokinin effectiveness may exist in certain species, such as rhododendron (Anderson, 1975). Micropropagated shoots exposed to cytokinins are suppressed in growth and often have a bushy appearance with small undeveloped leaves or needles. A reduction or removal of cytokinins is usually required for shoot elongation and rooting.

Exogenous auxins do not promote axillary shoot proliferation; however, culture growth may be improved by their presence. Wang and Hu (1982) found that decline of multiplication potential of potato shoots after 7-10 subcultures can be eliminated by enriching the medium with 0.5 $\mu$M NAA. Litz and Conover (1978) found that papaya cultures, even in the presence of 0.54 $\mu$M NAA, lost their proliferation ability. One of the possible roles of auxin in stage II medium is to nullify the suppressive effect of high cytokinin concentration on axillary shoot elongation and to
restore normal shoot growth (Lundergan, 1980). Too high a concentration of auxin may not only inhibit axillary bud branching (Hasegawa, 1930) but also induce callus formation, especially when 2,4-D is used. NAA is mostly used as an auxin in this stage followed by IBA and IAA.

Sometimes stage II media have been supplemented with GA₃. It is essential for axillary bud elongation (Schnabdrauch and Sink, 1979).

2.3.3.2 Rate of proliferation

Rate of proliferation is affected by numerous factors. The chemical composition of the medium, especially the type and concentration of the exogenous hormone and the physiological state of the plant material are of major importance. Litz and Conover (1981) found that the rate of proliferation of papaya cultures was dependent on age of plants, time of the year, type of sex and the presence of bacterial contaminants. In carrizo citrange cultures, different rates of proliferation were evident between shoot tips and nodal explants, i.e., 3.0 and 1.7 shoots per explant (Kitto and Young, 1981).

Numerous examples indicated that the recalcitrant state of many species can be gradually modified through serial sub
culturing. McCown and Amos (1979) observed increasing rates of shoot multiplication in bud cultures of *Betula platyphylla* after several subcultures. Litz and Conover (1981) also experienced an increase in shoot multiplication rate with the increase in number of subcultures. They observed that the rate of proliferation was sevenfold after nine subcultures.

2.3.3.3 Incubation condition

Incubating the plant material in a liquid shaking system for a brief period also appears to result in a modification of the physiological state of certain plant species and speed up shoot proliferation. Snir and Erez (1980) found that with bud cultures of "Malling Merton" apple rootstocks, a faster growth rate was observed when in vitro produced shoots were cut and placed in a liquid agitated medium for four days. They also observed intense proliferation when these shoot tips were placed back onto a solid medium. Gupta et al., (1991) induced bud proliferation from explants of mature *Eucalyptus* trees when elongated explants were incubated in a liquid shaking medium for two weeks. Hammerschlag (1982) also found better performance when the cultures were incubated in rollers for only 2-3 weeks. Hammerschlag (1982) also reported that "Sunhigh" and "Compact Red Haven" peach
scions proliferated 20-30 fold when grown in liquid medium, while only an 8-fold proliferation rate was achieved in agar medium. Unfortunately, when shoot tips were grown continuously on agitated cultures they became water soaked and brittle referred as "vetrification". However, Asoken et al., (1984) who worked with Xanthosoma caracay, demonstrated that the number of induced lateral shoots was 4-5 times greater when shoots were kept in a continuous liquid medium than in solid medium. They also found shoots were nearly 2-3 times greater in length.

2.3.4 Stage III: Rooting and acclimatization

During this stage there is de novo regeneration of adventitious roots from shoots obtained in stage II, or in some cases in stage I to form complete plants. Usually in vitro produced shoots of 10 mm or longer are cut and used in stage II cultures. Sometimes elongation of shoots in stage II is inhibited by high cytokinin level; thus, an intermediate shoot elongation medium becomes necessary. Adventitious root formation can be induced quite readily in many herbaceous species. Most woody species can be quite recalcitrant especially with explants taken from mature trees. For these species stage III probably is the most difficult of the three stages to accomplish.
Stage III does not always have to be carried out in vitro. Rooting frequency of 100% was reported by McCown and Amos (1979) when the shoots of Betula platyphylla were placed in 1:1 peat, perlite in a warm (30-35°C), high humidity (above 80%) chamber. Kusey et al. (1980) obtained 60% rooting of Gypsophila paniculata by planting shoots in Jiffy 7 peat moss cylinders in a greenhouse under intermittent mist. Instead of preparing auxin solution, commercial rooting powder, e.g., rootone F, may be used to predip shoot bases before planting in rooting medium (Pyott and Converse, 1981; Wochok and Sluis, 1980). Pyott and Converse (1981) found that rooting in vitro for red raspberry clones was unpredictable. Good rooting was observed in the greenhouse by placing shoots in pasteurized sand under intermittent mist.

2.3.4.1 Culture media - Growth regulators

There are three phases involved in rhizogenesis:

(a) induction
(b) initiation
(c) elongation.

Since it is rather difficult to isolate the induction phase
in most experiments, this phase has usually been combined with the phase of initiation. Many experiments show that de novo root initiation depends on a low cytokinin to auxin ratio. Usually there is sufficient residual cytokinin in shoots from stage II cultures; thus, little or no cytokinin is needed in stage III medium (less than 4.5 μM). Too high an in vitro cytokinin content has been shown to be deleterious to the initiation and the elongation of the roots of both monocotyledonous and dicotyledonous plants (Henny, 1978; Lo et al., 1980; Nemeth, 1979; Pennazio, 1975). Sometimes the residual cytokinin from stage II cultures is high enough to suppress root formation. Transferring these unrooted shoots to a fresh medium of the same composition might result in root formation (Ancora et al., 1981).

Numerous studies have indicated that, among the common auxins, NAA is the most effective auxin for induction of roots (Ancora et al., 1981; Kitto and Young, 1981; Johnson, 1978). Nemth (1981) tested the capacity of several rare synthetic auxins on in vitro root induction of apple rootstocks, and found that 2-chloro-3-(2,3-dichlorophenyl) propionitrile (CDPPN) applied at 5 μM was the most effective and produced up to 90% more roots than IBA which was the most effective common auxin in root induction of apple rootstocks.
Since the developing young shoots are a rich source of auxin, the addition of exogenous auxin to these media becomes unnecessary in many species (Hasegawa, 1980; Lee et al., 1977; Meredith, 1979; Papachatz et al., 1981). When the auxin concentration is too high, callus will form at shoot bases which inhibits normal root development (Lane, 1977a) and elongation (Thimann, 1977). In order to provide hard-to-root apple root stocks with a strong root induction stimulus, as well as to avoid callusing and root growth inhibition, a two-phase procedure has been adopted for stage III cultures (James and Thurbon, 1979; 1981; Snir and Erez, 1980). The shoots were first cultured in an auxin-containing "root induction medium" for 4-8 days, then transferred to an auxin-free "root developing medium". This procedure effectively prevented callus formation, resulted in a 95% rooting, and led to a three-fold increase in root number per rooted culture, compared to those in continuous contact with auxin. To stimulate root induction, Kusey et al., (1980) pre-dipped the base of in vitro-produced Gypsophila shoots in a 0.13 mM NAA or 0.12 mM IBA solution for five seconds and then transferred them into hormone-free White's medium for root initiation and development. A 48 hour soaking in 11.0 mM NAA solution before transferring to White's medium successfully induced root formation in Eucalyptus citriodora (Gupta et al., 1981).
On a few occasions stage III media have been supplemented with GA₃. It has significantly improved the rooting percentage of potato meristem cultures (Pennazio and Vecchiati, 1976). On the other hand, a suppressive effect of GA₃ on the induction phase of \textit{in vitro} peach rooting has also been observed by Mosella \textit{et al.} (1980, quoted by Hu and Wang, in 1983).

2.3.4.2 Auxin synergists

The capacity of phenolic compounds to act as auxin synergists in the rooting process is well known, although their mode of action remains obscure. Among the difficult-to-root temperate woody fruit species e.g., apple root stocks (James and Thurbon, 1979) \textit{Fragaria} (James, 1979) and \textit{Prunus insititia} (Jones and Hopgood, 1979) satisfactory results have been obtained using phloroglucinol (PG). Nugeroles and Jones (1979) reported 100\% rooting of peach rootstocks on media with phloroglucinol compared to 60\% rooting without it. They also found that PG improved the average number of roots per culture. Chlorogenic acid also been reported to be useful for rooting \textit{P. cerasifera} in light (Hammerchlag, 1982). On the other hand some researchers have reported absolutely no benefit from these
phenolic substances. Jordan et al. (1980) have reported that chlorogenic acid actually inhibited rooting of P. avium. Great improvement of rooting percentage of P. persica (peach) has been reported by Mosella et al. (1980, quoted by Hu and Wang, 1983), using the phenolics rutin and quercitin. They found improved plant quality and rootability with phloridizin. They also found that phenolics act on the "initiation phase" of rhizogenesis.

It appears that a given phenolic compound will show a synergistic effect only with certain auxins. No synergistic effect between PG and non-indole auxin (NAA) was detected by James (1979). Mosella et al. (1980), quoted by Hu and Wang 1983, found that rutin and quercitin combine best with IAA and NAA.

2.3.4.3 Salt concentration

Sometimes lower salt concentrations of stage III media showed better rooting ability than media with high salt concentrations such as MS, LS, BS (Kartha and Constabel, 1974, Kartha et al., 1981; Lane, 1979a; Skirvin and Chu, 1979).

Although lower salt concentration in a medium may be
beneficial to root induction, it sometimes results in poor top growth. Wang (1978) observed that a 1/9 strength of MS medium stimulated 100% rooting in *Cryptomeria japonica*, but resulted in poor shoot growth. A 1/3 strength MS medium, on the other hand, resulted in 87% rooting with good top growth. Although Gupta *et al.* (1981) successfully rooted *Eucalyptus citriodora* after transferring shoots from MS to WH medium, the rooted shoots failed to survive on this low salt medium. If the plants were transferred within two weeks, just after the emergence of the first roots, back into liquid medium, shoot growth and a well-developed root system were observed.

2.3.4.4 Carrier material

Most of the stage III media use agar as the carrier material to solidify the medium for supporting root growth but sometimes agar may result in poor growth in certain species like *Spirea* and *Prunus* (Lane, 1979a). The reasons may involve growth inhibiting factors, poor aeration and a slow rate of diffusion of toxic metabolic wastes released by growing tissue. Two methods are commonly used to circumvent the inhibitory effect of the agar medium:

(a) Supplementing the agar medium with fine powder of activated charcoal.
(b) Using a liquid medium and filter paper bridge instead of agar.

Activated charcoal may adsorb toxic substances in the medium thereby improving root regeneration and development (Ziv, 1979; Takayama and Misawa, 1980). Activated charcoal may also adsorb residual cytokinin from stage II medium. Takayama and Misawa (1980) reported an inhibition of root formation of Lilium by BA. Such inhibition was completely reversed by addition of activated charcoal. Root formation and growth were even better in BA-free medium containing activated charcoal. Activated charcoal is also capable of shading in vitro roots from light which, in high intensity, may inhibit root growth.

Sometimes liquid media were used at this stage. It facilitates the free diffusion of toxic plant wastes. In combination with the filter paper bridge system, it also provides excellent aeration for root development. Its drawback is the amount of labour involved in preparing the bridge.

Polyurethane foam and vermiculite were used with satisfactory results instead of agar (McComb and Newton, 1981; Barnes, 1979). The use of liquid media with
vermiculite substrate for support and aeration in watermelon culture resulted in a significantly superior root system, with better branches and extensive root hairs, than when grown on 0.4-1.2% agar (Barnes, 1979). Additionally, there was less damage to the roots when removed from the vermiculite during transplanting than from agar, and better explant survival after transplanting.

2.3.4.6 Incubation conditions

In general, high light intensity supports better plant formation (Bojwani, 1980; Ziv, 1979). Increased light intensity results in considerable growth, thus producing plantlets which could be successfully transferred to pots. Direct high light intensity may be harmful to root growth. Improved growth was reported when the culture containers were wrapped with aluminium foil (Hennen and Sheehan, 1978) or when 0.3% activated charcoal was added to the medium (Ziv, 1979). Light intensity used at this stage is usually within a 0.54-10 klux range.

A majority of investigators adopt the same incubating temperature as used in the previous stages. Schnabdrauch and Sink (1979) reported that rooting of shoot tips of Phlox species could be hastened if primordia development was
promoted at 30°C for one week before transferring to 22°C for root elongation.

2.3.5 Stage IV: Acclimatization and planting out

After rooting, the in vitro regenerated plantlets are ready to be transferred from the aseptic containers into pots. Factors that should be considered in transplantation are infection and desiccation. Sterilizing the soil mixture eliminates serious infection problems. Desiccation is the last major block to be conquered in order to reach the goal of micropropagation. Excessively high water loss has been recorded from the leaves of apple immediately after transplanting (Brainerd and Fuchigami, 1981). Such a high rate of water loss is related to:

(a) The reduced quantities of epicuticular wax
(b) High volume of mesophyll intercellular spaces
(c) The slowness of stomatal response to water stress

To compound the problem, the xylem tissue in the regenerated plants forms a closed system across the base of the shoot prior to root formation. The de novo formed roots, arising from callus, have poor connection to the main vascular system of the shoot (Grout and Aston, 1977). Such a
structuring is of no consequence in culture, when plantlets are surrounded by high humidity.

A period of humidity acclimatization is therefore required for newly transferred plantlets to adapt to the outside environment. During acclimatization, humidity is gradually reduced over a period of 2 to 3 weeks. In the mean time, plantlets undergo morphological and physiological adaptations enabling them to develop typical terrestrial plant water control (Grout and Aston, 1977; Brainerd and Fuchigami, 1981).

Various methods have been used to establish in vitro grown plants in soil, i.e., mist beds (Lane, 1979a); plantlets covered by clear plastic cups (Barnes, 1979); plantlets covered by glass jars (Broome and Zimmerman, 1978). Brainerd and Fuchigami (1981) proved that acclimatization can be achieved with direct low humidity exposure. They left apple plantlets in stage III culture jars with lids removed in a room with a 30-40% relative humidity. To prevent drying of the medium, 10 ml of distilled water were added daily. After the lids were open for 5 to 6 days, 80% of the stomata of leaves closed within 15 min. which is the same rate as for greenhouse-grown plants.
3.0 MATERIALS AND METHODS

3.1 GENERAL PROCEDURES

3.1.1 Nutmeg (juvenile plants)

3.1.1.1 Maintenance of nutmeg stock plants.

Approximately 4-year-old nutmeg plants were maintained in a greenhouse at the University of Florida, Tropical Research and Education Center, Homestead, Florida, USA. These plants were regularly pruned to induce new shoots and were sprayed periodically with a solution of 0.1% benlate (Fig. 1). The plants were watered at pot level and vegetable fertilizer mixture applied at the rate of about 10 gm per pot.

Fig.1. Maintenance nutmeg stock plants in greenhouse.
3.1.1.2 Collection of explants.

Newly developed shoot tips about 4-5 cm long, with the characteristic pink colour conferred by anthocyanins were collected from the stock plants. After removing all leaves except for one leaf pair at the apex, the apices were immersed in water, brought to the laboratory and used as experimental material. These were thoroughly washed under running water and then agitated slowly for 15 minutes in 15% Clorox (5.25% sodium hypochlorite solution) with a few drops of Tween 20. Excess sterilant was removed from the explant by three changes of sterile distilled water. Shoot tips were further dissected to produce 2.5-3.5 cm long shoots with two internodes. These explants were placed horizontally on medium and partially embedded. A single explant was introduced into each petri dish or test tube. Establishment of cultures was achieved at the University of Florida, Florida, USA and these cultures were transported to the tissue culture laboratory, Department of Minor Export Crops, Matale, Sri Lanka for further studies.

3.1.1.3 Multiplication and maintenance of shoot cultures of juvenile origin

When the explants produced axillary shoots they were separated from the explant and introduced into test tubes
containing the same medium. At six-week intervals nutmeg shoots were cut into 1-2 cm pieces and each was introduced into separate tubes containing the same medium. This procedure was continued until sufficient numbers of shoot cultures were obtained for further studies.

3.1.2 NUTMEG CLONAL TREES

3.1.2.1 Nutmeg clonal selections

About 25-35 year old, female trees of nutmeg yielding about 10,000-15,000 nuts per year were already selected and used as clonal trees. These trees were located in major nutmeg-growing areas in the mid country wet zone around Kandy such as Kolugala, Hataralivadda and Ambatenna. The trees are owned by farmers and maintained by them with minimum investment and hardly any fertilizer was applied. Various kinds of materials i.e., shoot tips, small branches for nodal cultures, branches for grafting purposes, trunk-sprouted orthotropic branches, immature fruitlets and partially opened leaves were collected for various experiments. However, only a very limited number of orthotropic branches was collected due to the unavailability of such branches in the trees.
Materials were collected in polythene bags maintained at a low temperature in a box and brought to the laboratory for use for culturing or grafting.

3.1.2.2. Grafted plants of nutmeg

Fresh and mature seeds were collected and germinated in polythene bags filled with a mixture of soil and kept in a greenhouse. Scions were collected from the clonal selections and wedge-grafted on to seedlings at the two-leaf stage. Graft-unions were wrapped either with polythene tapes or parafilm and grafted plants were individually covered with large glass jars in order to prevent drying. Plants were watered at pot level. Successful grafted plants were transferred to large cement pots and the recommended fertilizer mixture for nutmeg was added at the rate of 5 gms per pot and plants were sprayed with 0.1% benlate solution once in every two weeks.

3.1.2.3 Nutmeg re-grafted plants

Grafting procedure and maintenance were similar to those for grafted plants except that scions were taken from the grafted plants.
3.1.3 Clove (juvenile plants)

3.1.3.1 Maintenance of clove stock plants

Ripe clove fruits were collected from about 20-year-old selected trees which provide high yields with regular bearing habit in the Hunnasgiriya estate, Matale, Sri Lanka. Seed was extracted from the fruits and was germinated in seed beds in a greenhouse with close spacing. The seed beds were regularly watered. Germination of the seeds occurred after 6-8 weeks. Two months after germination, at the stage of 2 to 3 pairs of leaves, 2.5-3.5 cm long shoot tips were collected. All the leaves were dissected out or the first pair at the apex were left. Then the shoot tips were washed in running water for about half an hour and surface sterilized in 0.1% HgCl₂ with a few drops of Tween 20 with continuous slow agitation (60 rpm) for 10 minutes. The material was then washed with three changes of sterile distilled water and the shoot was further dissected to produce a 2.5 cm long explant with or without a pair of leaves at the apex.

3.1.3.2. Multiplication and maintenance of shoot cultures

After the axillary shoot development from the explants they
were dissected and separated and then individual shoots were introduced into test tubes containing the same medium. Continuous subculture process was done with the shoots separated from the clusters in every 8-10 weeks period until sufficient number of shoots were obtained for further studies.

3.1.4 Clove clonal trees

3.1.4.1 Material collection from clonal trees

Most of the materials were collected from a clove plantation at Hunnasgiriya, mic country wet zone. Plants were about 20-years-old and well-maintained. Shoot tips, buds, leaves, flowers and immature fruits were collected from the trees and immediately brought to the laboratory under low temperature using rigifoam boxes.

3.1.5 Media and environment

3.1.5.1 Media and stock solutions.

Four different media were compared in order to select the optimum basal medium, i.e., Murashige and Skoog (1962) (MS), Woody Plant Medium (1975) (WPM), Anderson's rhododendron
(1975), and BS medium of Gamborg et al., (1968) (Appendix 1).

For all other experiments Anderson's medium was used unless otherwise mentioned, either in the original formulation or modified in order to fulfill the special needs of experiments.

3.1.5.2 Preparation of stock solutions of growth regulators

The basal media were supplemented with various concentrations of different plant growth regulators which were prepared as follows (Table 1).

Table 1. Preparation of growth regulator solutions

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Amount (mg)</th>
<th>Solvent</th>
<th>Final volume (ml)</th>
<th>Final Conc. (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>25</td>
<td>1.0N KOH</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>Kn</td>
<td>25</td>
<td>1.0N HCl</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>Z</td>
<td>10</td>
<td>0.1N HCl</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>2ip</td>
<td>25</td>
<td>1.0N HCl</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>IAA</td>
<td>25</td>
<td>0.1N KOH</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>IBA</td>
<td>25</td>
<td>0.1N KOH</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>NAA</td>
<td>25</td>
<td>1.0N KOH</td>
<td>50</td>
<td>0.5</td>
</tr>
<tr>
<td>2,4-D</td>
<td>25</td>
<td>70% Ethanol</td>
<td>50</td>
<td>0.5</td>
</tr>
</tbody>
</table>
The exact amount of the growth regulator was taken and dissolved in a few drops of either KOH, HCl or ethanol according to Table 1. The final volume of the solution was made to 50 ml by adding double distilled water. All the solutions were stored in a refrigerator at temperature 4°C. Stock solutions stored for more than four weeks were replaced by fresh solutions.

3.1.5.3 Preparation of culture media

Appropriate amounts (mg/l) of all the components prescribed for a particular medium were mixed from freshly prepared stock solutions and the final volume of each medium was made up by adding double distilled water. Sucrose (Sigma or BDH) was added at 30 or 60 g/l and growth regulators were added according to the nature of the experiments and activated charcoal PVP, PG and adenine sulphate etc. were also added to the medium at this time when necessary. After adjusting the pH to 5.6 or 4.8 using 0.1N KOH or 0.1N HCl, 6-8 g/liter Difco-Bacto agar were added as a solidifying agent. Media were autoclaved for 15 minutes at 121°C and 1.1 kg/cm² and about 10 ml each were poured into sterilized petri dishes (60x15 mm). 10 ml of media were poured into test tubes (25x150 mm) and covered with plastic tops before autoclaving.
3.1.5.4 Culture environment

Unless otherwise mentioned all cultures were grown in an air conditioned culture room at 26±2°C and illuminated by white fluorescent tubes of intensity about 3000 lux placed at a distance of 30-40 cm from the culture shelves.

3.1.5.6 Statistical evaluation of data.

The experimental data on counts and percentages were analyzed by non-parametric tests i.e., Kruskal-Wallis test for significance or one way ANOVA (Appendix II), Wilcoxon matched-pairs signed-rank test for comparison of the treatments (Appendix III). The length of the shoots and roots was evaluated by ANOVA and treatment differences were compared by T-test, LSD or DMRT.
3.2 EXPERIMENTAL PROCEDURE

3.2.1 NUTMEG JUVENILE PLANTS

3.2.1.1 Selection of a basal medium.

Four different media were compared in this experiment, i.e., Murashige and Skoog (MS) (1962), Woody Plant Medium (WPM) (1975), Anderson’s rhododendron (1975) and Gamborg et al., (BS) (1958). Appropriate amounts of ingredients (macro, micro, vitamins, organics) and 1.0 mg/liter BA, 0.01 mg/liter NAA and 30 gms of sucrose were added and dissolved. The pH of the media was adjusted to 5.6 for MS, WPM and BS, whereas for Anderson’s medium the pH was adjusted to 4.8. All the media were autoclaved and poured into sterile petri dishes (60x15 mm), each containing about 10 ml of medium. Actively growing shoot tips collected from stock plants were used for this experiment. Sterilized explants were positioned horizontally on culture media. Each treatment was replicated five times. After 14 days data were collected on the number of surviving cultures.
3.2.1.1 The effect of developmental stage of shoot tips—actively growing and dormant—on culture establishment.

The growth of shoot tips originating from pruned stems took place in two clearly distinguishable stages: a fast growing stage which produced pink-coloured stems and leaves followed by a dormant stage when the new leaves and stems subsequently became green. Shoot tips of these two stages were collected separately from the stock plants. Sterilized explants were introduced onto Anderson’s medium with 1.0 mg/l BA with 0.01 mg/l NAA contained in petri dishes (60x15 mm). Explants were placed in the horizontal position. These two treatments were replicated five times. Data were collected on the percentage of surviving cultures and other physical characters after 14 days of culture.

3.2.1.3 The effect of kinetin and IAA on culture establishment.

Actively growing shoot tips collected from stock plants were used for this experiment. Anderson’s medium with all possible combinations of IAA (0, 0.1 and 0.2 mg/liter) and kinetin (0, 0.5, 1.0, 2.0 and 5.0 mg/liter) were used. All treatments were replicated five times. Data were collected after six weeks on number of contaminated cultures, callus
production (according to a scale +, ++ and +++), terminal growth and other morphological characters.

3.2.1.4 The effect of BA and NAA on culture establishment.

Fifteen different hormonal combinations were prepared with Anderson's basal medium. Factorial combinations of five levels of BA (0, 0.5, 1.0, 2.0 and 5.0 mg/liter) with three levels of NAA (0, 0.1 and 0.2 mg/liter) were tested. Actively growing shoot tips were collected from the stock plants, sterilized and introduced onto the medium contained in petri dishes (60x15 mm). Explants were positioned horizontally on the media. There were five uncontaminated cultures for each treatment. Observations were made on rooting, axillary bud break and terminal growth on each of the cultures using a three level scale (+, ++ and ++++) after 8 weeks.

3.2.1.5 Evaluation of four different cytokinins on axillary bud proliferation.

Anderson's medium solidified with 7 g/l agar with four different cytokinins (BA, kinetin, zeatin and 2iP) each at five concentrations (0, 0.5, 1.0, 1.5 and 2.0 mg/l) was prepared in test tubes (10 ml each) for this experiment.
About 1 cm long in vitro-multiplied shoot pieces were placed horizontally on the medium. There was one shoot piece per test tube. Each treatment was replicated five times. The number of axillary buds produced in each experimental unit was recorded after 8 weeks.

3.2.1.6 The effect of BA concentration on axillary bud proliferation.

Anderson's medium was supplemented with BA in 14 different concentrations (0, 0.5, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25, 2.50, 2.75, 3.0, 3.5, 4.0 and 5.0 mg/l). The shoots from established cultures grown in Anderson's medium with 1.5 mg/liter BA were dissected to about 1 cm lengths and randomly introduced to 10 ml of media in test tubes. Shoot pieces were placed on medium in the horizontal position and were partially imbedded. Each treatment was replicated five times. Observations were made on the production of axillary buds from each of the stem segments.

3.2.1.7 The effect of solid and liquid media (with filter paper bridges) on axillary bud proliferation.

Anderson's liquid and solid medium (agar 7 g/l) supplemented with 1.5 mg/l BA was prepared 10 ml each in test tubes. Stem pieces 1 cm long taken from the in vitro-multiplied nutmeg
cultures were introduced vertically onto the solid media, and inserted into small holes in filter paper bridges in liquid media. Each treatment was replicated five times. Data were collected on the number of new shoots produced after 8 weeks.

3.2.1.8 Comparison of shoot multiplication in liquid and solid medium.

Anderson's liquid and solid medium with 1.5 mg/l BA was prepared and decanted into in 250 ml Erlenmeyer flasks each containing 50 ml of medium. Solid medium was prepared with the addition of 7 g/l of Difco Bacto agar. In vitro multiplied shoots (in 2.0 mg/l BA) were harvested and five 1 cm long shoot pieces without the large leaves were introduced into each of the flasks. The experiment was replicated five times. Observations were made on the number of axillary buds produced and their lengths after 9 weeks.

3.2.1.9 Effect of axillary bud position on regeneration.

Four stem sections about 1 cm long were made from single in vitro grown shoots about 5 cm long which were grown in 2.0 mg/l BA. The terminal portion about 0.5 cm long was
discarded and four sections were made, each about 1 cm long and labelled as follows:

- 0.5 cm - 1.5 cm First section
- 1.6 cm - 2.5 cm Second section
- 2.6 cm - 3.5 cm Third section
- 3.6 cm - 4.5 cm Fourth section

These sections were placed horizontally on Anderson's medium containing 1.5 mg/l BA 10 ml in test tubes. All treatments were replicated five times. Axillary bud proliferation per shoot piece was recorded after 8 weeks.

3.2.1.10 Determination of subculture frequency

Approximately 1 to 2 cm long shoot tips were collected from in vitro-multiplied stocks (BA 2.0 mg/l). These shoot tips were then introduced onto the same medium contained in 10 ml test tubes. Shoot length was measured and recorded at one week intervals for five weeks.

3.2.1.11 Influence of auxins and activated charcoal on adventitious root formation.

From the in vitro-grown shoot clusters individual shoots
about 2.0 cm length were carefully excised, basal leaves were removed and shoots were transferred to rooting medium in order to measure the efficiency of rhizogenesis. The basal medium of Anderson's was supplemented with IBA or NAA (0, 0.2, 0.5, 1.0 and 2.0 mg/l) with or without 0.2% activated charcoal. The number of roots initiated, their length and physical characters were recorded after 8 weeks of culture.

3.2.1.12 Transfer of regenerated plants to soil and acclimatization

Rooted nutmeg shoots were carefully removed from test tubes after 8 weeks and thoroughly washed in running water. They were dipped into a 0.1% benlate solution and transplanted into pots filled with a sterilized mixture consisting of equal parts of sand: top soil: verimposed cowdung. This mixture was sieved through 0.5 cm mesh before sterilization. Plantlets were kept in a growth room for 3-4 weeks with high humidity (over 80%) and then transferred to a greenhouse. After two months plantlets were repotted into non-sterile potting mixture.

3.2.2 Nutmeg clonal trees
3.2.2.1 Shoot tip culture of plagiotropic branches

Newly grown shoots were collected from the clonal selection of nutmeg in a nutmeg growing area. These shoots were brought to the laboratory under low temperature conditions. All the leaves and stem portion were discarded leaving about 1.5 cm long shoot tips. After sterilization with 0.1% HgCl₂ for 10 minutes, shoot tips were thoroughly washed in sterilized distilled water three times. Then one leaf was removed in order to expose the shoot tip and shoot tips were introduced to Anderson's medium containing 1.5 mg/l BA with following hormonal combinations as treatments.

1. Factorial combination of GA, (0, 0.5 and 1.0 mg/l) with NAA (0, 0.5 and 1.0 mg/l).
2. Factorial combination of GA, (0, 0.5 and 1.0 mg/l) with IBA (0, 0.5 and 1.0 mg/l).
3. Factorial combination of GA, (0, 0.5 and 1.0 mg/l) with Kn (0, 0.5 and 1.0 mg/l).
4. BA (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l).
5. Kn (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l).

3.2.2.2 Shoot tip culture of trunk sprouted orthotopic shoots.
Rapidly growing orthotropic shoots were collected from the mother trees and brought to the laboratory under low temperature. About 2.0 cm long shoot tips were prepared and washed 1 hour in running water and sterilized in 0.05% HgCl₂ for three minutes. After three rinses with sterilized distilled water shoot tips were vertically introduced to Anderson’s medium containing 1.5 mg/l BA in test tubes. Cultures were incubated in a 16/8 light and dark period.

3.2.2.3 Effect of cytokinin and its concentration on culture establishment of unselected nodal explants.

About 6” long newly emerged plagiotropic shoots were collected from the mother trees transferred under low temperature. All the leaves were discarded in the laboratory and the following procedure for sterilization was adopted:

I. Washed one hour in running water
II. Brushed axils with liquid soap
III. Agitated 10 minutes in 0.1% HgCl₂
IV. Washed with sterilized distilled water, covered with aluminium foil and kept overnight in the growth room.
V. Re-sterilized with 0.1% HgCl₂ for 10 minutes and thoroughly washed with sterilized distilled water.

Then nodal explants were obtained after dissecting out
unnecessary portions. Nodes were vertically placed in Anderson's medium containing BA or Kn (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l) in test tubes. Each was replicated 10 times and five cultures were kept in total dark for first two weeks and then in 16/8 hour light and dark. The other five cultures were kept in 16/8 hour light and dark photoperiod from the beginning.

3.2.2.4 Culture of selected nodes with developed axillary buds.

Soft or semi-hard wood nodes with developed axillary buds about 1-2 cm long were selected from the mother trees and brought to the laboratory and washed in running water for 1 hour. Then they were sterilized with 0.05% HgCl₂ for three minutes and introduced to Anderson's medium supplemented with 1.5 mg/l BA after three rinses with sterilized distilled water.

3.2.2.5 Etiolation of nutmeg branches.

About 12" long branches were collected from mother trees and all the leaves were discarded leaving 3-4 at the terminal end. Five of them were placed in 250 ml conical flasks containing 50 ml of liquid Anderson's medium. Five conical
flasks were prepared and shoots were weekly sprayed with 100 mg/l solution of BA and kept in a greenhouse.

3.2.2.6 leaf culture

Newly emerged, light green, whole leaves were collected from the clonal selections and brought to the laboratory and sterilized in 0.1% HgCl₂ for 10 minutes. After washing three times with sterilized distilled water these leaves were introduced to B5-2 medium supplemented with 0.4 g/l glutamine, 50 g/l sucrose and 7 g/l agar contained in petri dishes. All possible combinations of 2,4-D (0, 0.5, 1.0, 2.0 and 3.0 mg/l) and Kn (0, 1.0, 2.0 and 3.0 mg/l) were used as treatments. Four number of leaves were placed in an erect position in medium. The treatments were replicated 10 times. All the cultures were incubated in dark.

3.2.2.7 Endosperm cultures

About one month old fruitlets (about 1-2 cm in diameter) were collected from the mother trees and brought to the laboratory under low temperature. These fruitlets were sterilized with 20% clorox for 10 minutes. After washing with sterilized distilled water fruitlets were split into two halves. Since it was to difficult to remove nucellar tissue, split endosperm was introduced to the following
medium with four concentrations of 2,4-D (0, 0.5, 1.0 and 2.0 mg/l).

BS-2 basal medium with
Glutamine 0.4 g/l
Ascorbic acid 0.1 g/l
Sucrose 30 g/l
Agar 7 g/l

Four number of explants were introduced per petri dish with the cut edge in contact with the medium.

3.2.3 Materials from grafted plants maintained in the greenhouse

3.2.3.1 Shoot tip culture.

3.2.3.1.1 Effect of pre-treatment by spraying 50 mg/l GA₃ on culture establishment.

About 6 number of grafted plants were pre-treated by weekly spraying with a solution of 50 mg/l GA₃. About 4 cm long shoot tips were collected, all the leaves were discarded and the shoot tips sterilized with 0.1% HgCl₂ for 10 minutes. Then shoot tips were washed three times with sterilized distilled water and introduced to the following media
contained in test tubes: Anderson's medium supplemented with all possible combinations of BA (0, 0.5, 1.0, 2.0 and 5.0 mg/l) with NAA (0, 0.2 and 0.5 mg/l).

3.2.3.1.2 The effect of GA\textsubscript{3} with NAA or IBA or KN on shoot tip culture establishment and axillary bud elongation.

Newly sprouted shoots were collected from grafted nutmeg plants grown under greenhouse conditions. All the leaves and stem portions were discarded leaving about 4.0 cm long shoot tip. After sterilization with 0.1\% HgCl\textsubscript{2} for 10 minutes shoot tips were thoroughly washed three times in sterilized distilled water. The shoot tips were dissected to three sizes i.e.. 0.5, 1.0 and 1.5 cm, and introduced to Anderson's medium containing 1.5 mg/l BA with following hormonal combinations as treatments.

1. Factorial combination of GA\textsubscript{3} (0, 0.5 and 1.0 mg/l) with NAA (0, 0.5 and 1.0 mg/l).
2. Factorial combination of GA\textsubscript{3} (0, 0.5 and 1.0 mg/l) with IBA (0, 0.5 and 1.0 mg/l).
3. Factorial combination of GA\textsubscript{3} (0, 0.5 and 1.0 mg/l) with KN (0, 0.5 and 1.0 mg/l).
3.2.3.1.3 Comparison of liquid and solid medium on culture establishment of shoot tips.

About 1-1.5 cm long shoot tips were introduced to solid and liquid medium in test tubes containing 10 ml and 125 ml in conical flasks. The basal medium was Anderson's and 1.5 mg/l BA supplemented with factorial combinations of IBA (0, 0.5 and 1.0 mg/l) and GA₃ (0, 0.5 and 1.0 mg/l). A single shoot tip was introduced to each container and liquid cultures were slowly agitated in an orbital shaker.

3.2.3.1.4 Effect of manipulation of various basal media on culture establishment of shoot tips.

About 1-1.5 cm long shoot tips were introduced to the following basal media with factorial combination of IBA (0, 0.5 and 1.0 mg/l) and GA₃ (0, 0.5 and 1.0 mg/l).

1. B5-2 medium
2. MS medium
3. Anderson's medium
4. Modified MS (1/2 strength of macro salts)

The above basal media were supplemented with 20% coconut water, 0.01 g/l yeast extract, 0.05 g/l glutamine, 0.01 g/l
biotin, 30 g/l sucrose and 7 g/l Difco-bacto agar.

3.2.3.2 Nodal cultures from grafted plants

3.2.3.2.1 Nodal cultures from un-selected nodes

Terminal parts of branches with 3-4 nodes were collected from grafted materials in the greenhouse. Single nodal explants were prepared using the second or third node, dissecting out the other parts. These explants were sterilized in 0.05% HgCl₂ for 3 minutes after agitation in 0.1% benlate solution for one hour. After three rinses with sterilized distilled water explants were introduced to Anderson's medium supplemented with following concentrations of growth regulators.

1. BA 0, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l
2. KN 0, 2, 4, 6, 8, 10, 15 and 20 mg/l
3. BA 1.5 mg/l + 0, 1, 2, 4 and 6 mg/l NAA

Five cultures of the above treatment were kept in 16 hr light and 8 hour darkness, whereas the other five cultures were kept in darkness for the first two weeks and then transferred to 16 hr light and 8 hour dark photoperiod.
3.2.3.2.2 Nodal cultures from selected nodes

Nodes with about 5 mm long axillary buds were carefully selected from branches of the grafted plants. These nodes were collected and slowly agitated in 0/1% benlate solution for 1 hour. Then these nodes were sterilized in 0.05% HgCl₂ solution with three minutes dipping period. After three rinses with sterilized distilled water these explants were vertically placed in the following culture medium supplemented with 1.5 mg/l BA, 0.01 g/l yeast extract, 0.05 g/l glutamine, 0.01 g/l biotin, 30 g/l sucrose and 7 g/l agar.

I. 1/3 strength Anderson’s
II. Full strength Anderson’s
III. 1/3 strength blueberry medium.
IV. Full strength blueberry medium.

Twenty five cultures of each medium were kept in complete dark in first three weeks and then exposed to 16/8 light and dark period whereas the other 25 cultures were kept 16/8 light and dark period at the beginning and then transferred to 16/8 photo period. Subculturing period was 6 weeks.
3.2.3.2.3 Requirement of NAA on axillary bud elongation of nodal cultures.

Established nodal cultures in 1/3 Anderson's medium supplemented with 1.5 mg/l BA, 0.01 g/l yeast extract, 0.05 g/l glutamine, 0.01 g/l biotin, 30 g/l sucrose and 7 g/l agar were taken for this study. Established cultures were randomly introduced to the same media with three concentration of NAA (0, 0.1 and 0.2 mg/l). Four replicates were maintained due to unavailability of established cultures.

3.2.3.3 Hard pruning of grafted plants

Grafted plants maintained in pots were decapitated at 15 cm above the union paint was applied at the cut edge and the plants were maintained in the greenhouse with regular watering.

3.2.3.4 In vitro grafting

Mature fruits were harvested from the clonal trees and brought to the laboratory. These fruits were washed in water and sterilized with 20% clorox for 20 minutes. After three rinses with sterilized distilled water fruits were split and seeds were taken and introduced to MS medium contained in
wide-mouth jam jars. These seed cultures were kept until seed germination took place. After seed germination seed cultures were kept in 16/8 light and dark photo-period. The seedlings at two leaf stage were taken for micrografting. Established shoot tips from grafted plants were taken as scions and wedge grafting was adopted within the laminar-flow hood. Micrografted plants were transferred to fresh medium in jam jars.

These micro-grafted plants were incubated in the normal culture environment in the growth room.

3.2.4 Re-grafted plants

An adequate number of materials could not be obtained from the re-grafted plants due to low degree of success and plants being smaller.
3.2.5 CLOVE JUVENILE PLANTS

3.2.5.1 Status of the explant on culture establishment.

About 2-3 cm long shoot tips were collected from greenhouse grown seedlings with two pairs of leaves. The materials were sterilized in 0.1% HgCl₂ solution for 10 minutes and explants were prepared in two ways.

I. All the leaves were discarded leaving only the shoot tip.

II. Other leaves were discarded leaving one pair of leaves at the apex.

These explants were placed in a horizontal position on Anderson's medium with 1.0 mg/l BA and 0.1 mg/l NAA in test tubes. The experiment was replicated five times. Observations were made on death and survival of cultures.

3.2.5.2 The effect of BA and NAA on culture establishment and proliferation.

The medium used for rhododendron micropropagation by Anderson (1975) was used as basal medium. The growth regulators were added as a factorial combination of five levels of BA (0, 0.5, 1.0, 2.0 and 5.0 mg/l) with three
levels of NAA (0, 0.1 and 0.2 mg/l). Sucrose was added at a concentration of 30 g/l. The pH was adjusted to 4.8 before addition of agar (0.7% Difco Bacto). The media were poured into 25×150 mm test tubes, each containing a volume of 10 ml, and autoclaved. The shoot tips were positioned horizontally on the media. Each of the treatments was replicated five times. The number of axillary buds produced from each shoot was recorded.

3.2.5.3 The effect of BA concentration on axillary bud proliferation.

Anderson’s medium was supplemented with BA in 8 different concentrations: 0, 0.25, 0.5, 0.75, 1.25, 1.5, 1.75 and 2.0 mg/l. Regenerated shoots from established shoot tip explants in medium containing 2.0 mg/l BA were separated and about 1 cm long shoots were selected and introduced to the above media. Shoots were introduced to media in a vertical position. The experiment was replicated five times. The number of buds produced from each of the treatments was recorded.

3.2.5.4 Effect of NAA and GA3 on shoot elongation.

About 5-10 mm long shoots were harvested from stock cultures of clove, maintained in Anderson’s medium containing 2.0
mg/1 BA, and introduced to the same medium supplemented with factorial combinations of GA, (0, 0.5 and 1.0 mg/l) and NAA (0, 0.05 and 1.0 mg/l). The experiment was replicated five times. Results were taken on axillary bud production and average shoot length.

3.2.5.5 Evaluation of different media on root formation.

About 1 cm long shoots were harvested from stock cultures grown in Anderson's medium supplemented with 2.0 mg/1 BA. These shoots were introduced to following media contained (10 ml each) in test tubes.

I. Anderson' basal + IBA or NAA (0, 0.2, 0.5, 1.0 and 2.0 mg/l) with or without 0.08% activated charcoal.

II. Anderson's basal + 800 mg/l phloroglucinol + IBA or NAA (0, 0.2, 0.5, 1.0 and 2.0 mg/l).

III. Anderson's medium at 1/3 of salt concentration + IBA or NAA (0, 0.2, 0.5, 1.0 and 2.0 mg/l) with 0.08% activated charcoal.

The experiment was replicated five times and number of roots produced and root length were recorded.
3.2.6. CLONAL SELECTION OF CLOVE

3.2.6.1 Shoot tip cultures.

Attempts were made to culture various stages of shoot tips from clonal selections, i.e., actively growing shoot tips with pink coloured young leaves, shoot tips with two green leaves, rapidly growing shoot tips sprouting from the trunk and shoot tips with dormant buds. Anderson's medium supplemented with BA at three levels (0, 1.0 and 1.5 mg/l), was prepared in test tubes (10 ml each) and explants introduced after sterilization followed by final dissection. Over 3,000 cultures were made with different sterilization procedures after 30 minutes washing in running water:

I. Washing the materials with liquid soap
II. 70% ethanol for 1 minutes
III. 10% clorox for 10 minutes
IV. 0.01% HgCl₂ for 10 minutes.
V. 1% benlure solution for 1 hour

The explants were thoroughly washed in sterilized distilled water.
3.2.6.1.1 Pre-treatment of branches

Selected branches of clone mother trees were sprayed with a mixture of 1% benlate and streptomycin at 10 day intervals and shoot tips were collected after 2-3 spraying cycles.

3.2.6.1.2. Prevention of browning of the medium

Various stages of shoot tips were sterilized in 0.01% HgCl₂ for 10 minutes and introduced to Anderson's medium with 2.0 mg/l BA with 100 mg/l ascorbic acid, or 800 mg/l PVP or 0.2% activated charcoal.

3.2.6.2 Nodal cultures

3.2.6.2.1 Selection of a cytokinin and the suitable concentration for establishment of nodal cultures.

About 6" long healthy shoot tips were collected from the mother trees and all the leaves and shoot tip portion were discarded to obtain second and third internodal sections. These internodal sections were prepared and introduced to Anderson's medium containing antibiotics (rifampicin 6 mg/l,....
cephotaxime 25 mg/l, tetracycline 25 mg/l and polymyxin B 6 mg/l) with the following cytokinins.

I. BA (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l)
II. Kn (0, 1, 2, 4, 6, 8, 10, 12, 15 and 20 mg/l)

3.2.6.2.2 Evaluation of different physiological stages on culture establishment.

Three different physiological stages of second or third node from the apex were compared for culture establishment and axillary bud enhancing, i.e., without visible axillary bud development at the nodes, swollen axillary buds before bursting and elongated buds.

Anderson's medium was supplemented with 0.01 gm yeast extract, 0.05 gm glutamine, 0.01 gm biotin and 30 gm of sucrose. A mixture of four antibiotics (cephotaxim 25 mg/l + tetracycline 25 mg/l + rifampicin 5 mg/l + polymyxin B 6 mg/l) and 4.0 mg/l BA were also added to the medium. The cultures were introduced to fresh medium after 6-8 weeks.
3.2.6.3 OVULE AND EMBRYO CULTURES

Clove flowers at three different stages, i.e., fully matured but unopened, one week after pollination and 3-4 weeks after pollination were collected from the selected clove plants. These flowers or fruitlets were sterilized in 10% clorox with 10 minute immerse. The flowers were split into two through the ovary and ovules of all three stages and immature embryo at the third stage were carefully removed and cultured in the following medium in petri dishes.

Basal medium BS-2 with
- Ascorbic acid 0.1 mg/l
- Glutamine 0.4 mg/l
- Sucrose 30 gm/l

Four concentrations of 2,4-D were used (0, 0.5, 1.0 and 2.0 mg/l). The ovules contained in a single flower were introduced to one petri dish.

3.2.6.4 Epicotyl, hypocotyl and cotyledons culture of clove

Ripened fruits were collected from the trees and brought to the laboratory, and sterilized with 20% clorox solution for 20 minutes. Then the excess sterilent were removed by transferring the fruits to sterilized distilled water three
times. The pericarp of the fruits were removed within the laminar flow hood and the seeds were introduced to Anderson’s medium with 1.0 mg/l BA in test tubes. About two months after seed germination, at the two leaf stage of seedlings, about 1 cm long epicotyl, hypocotyl segments and the separated cotyledons were introduced to B5-2 medium containing all possible combinations of 2,4-D (0, 0.25, 0.50, 1.0, 2.0 and 5.0 mg/l) with Kn (0, 1.0, 2.0, 5.0 and 10.0 mg/l) in petri dishes. All cultures were kept in the dark.
4.0 RESULTS

4.1 Nutmeg juvenile materials.

4.1.1 Selection of a basal medium.

Percent survival, dead and contaminants were compared for four media MS, B5, WPM and AN (Table 2). All the cultures grown in either MS or B5 became brownish and most of them died within 14 days. Approximately 20 percent of the shoot tips on WPM medium survived. But shoot tips in this medium were buried under the medium and brown polyphenols were spread over the medium due to the semi-solid state of the medium. The shoot tips on Anderson’s medium showed very satisfactory results with 70 percent survival. Browning of the medium was localized at the cut edges of the explants and most of the shoot tips were alive. The amount of contaminated cultures in all the experiments was about 20 percent.

4.1.2 Effect of two different physiological stages of shoot tips on culture establishment.

In this experiment actively growing buds and dormant buds were compared. The shoot tip cultures established from stock plants at a dormant stage showed 90 percent bacterial
Table 2. The effect of four different media on establishment of shoot tips of nutmeg after 14 days.

<table>
<thead>
<tr>
<th>Basal medium</th>
<th>Survival</th>
<th>Dead</th>
<th>Contaminated</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. MS</td>
<td>0</td>
<td>80</td>
<td>20</td>
<td>brown</td>
</tr>
<tr>
<td>2. B5</td>
<td>0</td>
<td>80</td>
<td>20</td>
<td>brown</td>
</tr>
<tr>
<td>3. WPM</td>
<td>20</td>
<td>60</td>
<td>20</td>
<td>buried</td>
</tr>
<tr>
<td>4. AN</td>
<td>70</td>
<td>10</td>
<td>20</td>
<td>green</td>
</tr>
</tbody>
</table>

Table 3. Percentage of culture contamination in actively growing buds and dormant buds after 10 days.

<table>
<thead>
<tr>
<th>Stage of bud</th>
<th>Contaminated</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td>Dormant</td>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>
contamination which originated within the explant, and spread very slowly on the medium after about 8 days of culture. The shoot tips which were obtained from actively growing buds also showed some bacterial contamination but 70 percent of the cultures were healthy and 60 percent survived (Table 3).

4.1.3 Influence of Kinetin and IAA on culture establishment.

Neither culture establishment nor axillary bud break was found in any of the treatments in this experiment (Table 4). In most treatments, two weeks after introduction, shoot tips were separated to 2-3 sections and they formed callus at the separated ends (Fig. 2). Excellent formation of callus (+++) were observed in two treatments i.e., IAA 0.1 mg/l with Kn 0.5 mg/l and IAA 0.2 mg/l with Kn 1.0 mg/l (Table 4). These calluses were light brown in color and never turned green. Terminal growth of 60 percent of the introduced shoot tips was found without any growth regulators.
Table 4. Effect of kinetin and IAA on culture establishment after 6 weeks.

<table>
<thead>
<tr>
<th>regulator mg/l</th>
<th>Callus formation</th>
<th>Terminal growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>IAA</td>
<td>Kn</td>
<td>%</td>
</tr>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>----</td>
</tr>
<tr>
<td>0.5</td>
<td>60(+)</td>
<td>--</td>
</tr>
<tr>
<td>1.0</td>
<td>40(++)</td>
<td>40</td>
</tr>
<tr>
<td>2.0</td>
<td>60(++)</td>
<td>--</td>
</tr>
<tr>
<td>5.0</td>
<td>60(++)</td>
<td>--</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>60(++)</td>
</tr>
<tr>
<td>0.5</td>
<td>100(+++)</td>
<td>--</td>
</tr>
<tr>
<td>1.0</td>
<td>60(++)</td>
<td>--</td>
</tr>
<tr>
<td>2.0</td>
<td>80(++)</td>
<td>--</td>
</tr>
<tr>
<td>5.0</td>
<td>50(++)</td>
<td>40</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0</td>
<td>80(++)</td>
</tr>
<tr>
<td>0.5</td>
<td>40(++)</td>
<td>20</td>
</tr>
<tr>
<td>1.0</td>
<td>100(+++)</td>
<td>--</td>
</tr>
<tr>
<td>2.0</td>
<td>80(++)</td>
<td>--</td>
</tr>
<tr>
<td>5.0</td>
<td>20(++)</td>
<td>60</td>
</tr>
</tbody>
</table>

(+) Poor; (++) Good; (++++) Excellent
4.1.4 Effect of BA and NAA on culture establishment.

The percentage of cultures showing terminal growth, axillary bud break, root formation, contamination and dead are given in Table 5. The average number of axillary bud production per explant from five replicates are also given in Table 5, and Figure 3.
Table 5. The effect of NAA and BA on shoot tip culture after 6 weeks of introduction.

<table>
<thead>
<tr>
<th>NAA</th>
<th>BA</th>
<th>Growth</th>
<th>Terminal Axillary bud</th>
<th>Av of 5 rep.</th>
<th>% Contamination</th>
<th>% Dead</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>80(+)</td>
<td>---</td>
<td>---</td>
<td>20</td>
<td>---</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>100(+)</td>
<td>---</td>
<td>---</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>100(+++)</td>
<td>---</td>
<td>3.75</td>
<td>20</td>
<td>---</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>100(++)</td>
<td>---</td>
<td>---</td>
<td>--</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>80(++)</td>
<td>---</td>
<td>20</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>100(+)</td>
<td>---</td>
<td>80(++)</td>
<td>20</td>
<td>---</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>100(++)</td>
<td>---</td>
<td>3.6</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>0.2</td>
<td>0.0</td>
<td>100(++)</td>
<td>---</td>
<td>---</td>
<td>100(++)</td>
<td>--</td>
</tr>
<tr>
<td>0.5</td>
<td>1.0</td>
<td>80(+)</td>
<td>---</td>
<td>---</td>
<td>20</td>
<td>---</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>80(++)</td>
<td>---</td>
<td>---</td>
<td>--</td>
<td>---</td>
</tr>
<tr>
<td>2.0</td>
<td>5.0</td>
<td>80(++)</td>
<td>---</td>
<td>---</td>
<td>20</td>
<td>---</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td></td>
<td>---</td>
<td>---</td>
<td>--</td>
<td>100</td>
</tr>
</tbody>
</table>

(+++) Poor; (+++) Good; (+++) Excellent
Figure 3. The regulation of axillary bud induction, terminal growth and root formation of shoot tip explants by varying the auxin (NAA): cytokinin (BA) ratio. Note the occurrence of axillary bud induction at 2.0 mg/l BA with or without 0.1 mg/l NAA (ab). Moderate levels of BA (1.0 mg/l), in the absence of auxin, stimulate apical growth. Auxin (NAA 0.1 or 0.2 mg/l) induced root formation in the absence of BA (r). Higher levels of BA (5.0 mg/l) was detrimental to the explant (mag.= 0.33X).

The earliest response of the explants was the change in colour of the shoot tips from pink to green. Axillary bud enlargement and bursting were observed only on media
supplemented with 2.0 mg/l BA with or without 0.1 mg/l NAA after 6-8 weeks of inoculation (Fig. 3). Subsequently these proliferated axillary shoots grew rapidly (Fig. 4). Statistically no difference was found between these two treatments. Root initiation at the bases of the explants was observed after 9-10 weeks in the treatments containing 0.1 or 0.2 mg/liter NAA (Fig. 5). Roots from both treatments were thick and had numerous root hairs but the treatment with 0.2 mg/liter NAA showed a higher frequency of root initiation i.e., about two. Higher concentrations of BA (5.0 mg/l) were detrimental to the explants, inducing browninig.

Figure 4. Axillary bud bursting and growth of the shoot tips in Anderson's basal medium supplemented with 2.0mg/l BA after 6-8 weeks of introduction (magnification=4X).
and ultimate death. Low levels of BA (1.0 mg/l) or treatments without any hormones resulted in only terminal growth of the explant.

Figure 5. Rapid growth and shoot multiplication of axillary shoots in Anderson's basal medium supplemented with 2.0 mg/l BA after 10 weeks of introduction (mag.= 2.5X).

4.1.5 Evaluation of four different cytokinins on axillary bud proliferation.

The average number of axillary buds from five replicates produced from 1 cm shoot pieces in four cytokinins (BA, 2iP, Kn and Z) at five concentrations have been summerized in Table 6 and Figure 7. The average number of axillary bud production was higher in BA at any level (except control)
than 2iP, Z or Kn. The average number of axillary buds increased with increasing BA concentrations from 1.2±0.21 to 5.4±2.19. In the other three cytokinins, no differences were found with increasing concentrations. But zeatin at concentrations of 0.5, 1.0 and 1.5 mg/l produce callus at the bases of the explants and subsequently produced roots in the treatments 1.0 and 1.5mg/l.

Figure 6. Root formation at the base of the shoot tip in Anderson's basal medium supplemented with 0.1mg/l NAA after 10 weeks (mag.= 4X).
Table 6. The average number of axillary buds (five reps) produced from 1 cm shoot pieces in four cytokinins at five concentrations after 6-8 weeks of introduction.

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Concentration mg/liter</th>
<th>Axillary buds</th>
<th>S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA</td>
<td>0.0</td>
<td>1.2</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>3.2</td>
<td>0.84</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4.6</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>4.8</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>5.4</td>
<td>2.19</td>
</tr>
<tr>
<td>Zipt</td>
<td>0.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td>Z</td>
<td>0.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.4</td>
<td>0.25 (C)</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.0 (CR)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.0</td>
<td>0.0 (CR)</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td>Kn</td>
<td>0.0</td>
<td>1.2</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>1.8</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.2</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>1.2</td>
<td>0.24</td>
</tr>
</tbody>
</table>

C = Callus; CR = Callus and Root
Figure 7. Different rates of axillary bud proliferation from 1 cm shoot piece in five concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l) of BA, Kn, 2iP and Z after 6-8 weeks. Also note the occurrence of callus and root formation in Z (—>) at 0.5 and 1.5 mg/l (mag. = 0.75X).
The early response of the explant showed some callus formation at the cut edges of the explants. The callus seemed to be non friable and did not grow further except in some of the treatments in zeatin. The callus in zeatin at concentrations of 0.5, 1.0 and 1.5 mg/l were grown and finally produced roots in 1.0 and 1.5 mg/l. Initiation of axillary buds occurred simultaneously at all levels of four cytokinins after 2-3 weeks of introduction. The treatment which were unable to produced axillary buds by this time were not capable even after 8 weeks.

Figure 8. Maximum rate of axillary bud proliferation (5.4) in Anderson's basal medium supplemented with 2.0 mg/l BA after 6-8 weeks of introduction (mag.= 2X).
More axillary bud development up to 5.4 shoots was observed in media containing 2.0 mg/l BA, (Figure 8). BA at concentrations of 1.0 and 1.5 mg/l also produced 4.6 and 4.8 shoots respectively.

In comparison a very low rate of proliferation was observed in other three cytokinin 2iP, 2 and Kn the highest being 1.8 shoots. Axillary bud proliferation between the control and any concentrations of either 2iP or 2 did not show significant difference. The maximum number of axillary bud about 1.8 produced in Kinetin at the concentration of 0.5 mg/l. However, axillary bud proliferation in other concentrations of Kinetin was not different from the control.

4.1.6 The effect of BA concentrations on axillary bud proliferation.

The rate of axillary bud proliferation (average from 5 replicates) from 1 cm long shoot pieces in response to 14 concentration graders of BA (0.0 to 5.0 mg/l) and percentage of culture contamination are presented in Table 7. The axillary bud production increased with increasing concentration of BA up to 1.5 mg/l and thereafter a reduction was observed at higher concentrations.
Table 7. Average number of axillary buds produced per shoot in media containing BA, six weeks after introduction.

<table>
<thead>
<tr>
<th>Growth regulator BA mg/l</th>
<th>Axillary buds per explant</th>
<th>S.E</th>
<th>Contamination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>20</td>
</tr>
<tr>
<td>0.5</td>
<td>2.2</td>
<td>0.97</td>
<td>--</td>
</tr>
<tr>
<td>1.0</td>
<td>2.0</td>
<td>0.89</td>
<td>--</td>
</tr>
<tr>
<td>1.25</td>
<td>2.2</td>
<td>0.58</td>
<td>--</td>
</tr>
<tr>
<td>1.50</td>
<td>3.0</td>
<td>1.08</td>
<td>20</td>
</tr>
<tr>
<td>1.75</td>
<td>2.75</td>
<td>1.45</td>
<td>20</td>
</tr>
<tr>
<td>2.0</td>
<td>1.2</td>
<td>1.20</td>
<td>--</td>
</tr>
<tr>
<td>2.25</td>
<td>1.2</td>
<td>0.37</td>
<td>--</td>
</tr>
<tr>
<td>2.50</td>
<td>0.2</td>
<td>0.20</td>
<td>--</td>
</tr>
<tr>
<td>2.75</td>
<td>0.6</td>
<td>0.37</td>
<td>--</td>
</tr>
<tr>
<td>3.0</td>
<td>1.0</td>
<td>0.45</td>
<td>--</td>
</tr>
<tr>
<td>3.5</td>
<td>1.0</td>
<td>0.60</td>
<td>20</td>
</tr>
<tr>
<td>4.0</td>
<td>1.0</td>
<td>0.44</td>
<td>20</td>
</tr>
<tr>
<td>5.0</td>
<td>0.4</td>
<td>0.40</td>
<td>--</td>
</tr>
</tbody>
</table>
The cytokinin BA enhanced the production of axillary buds. Higher concentrations of BA over 2.5 mg/l suppressed axillary bud formation, and leaves wrinkled, and turned pink or brown after a few weeks. The medium containing 1.5 mg/l BA resulted in the maximum production of axillary buds (3.0±1.08). However, at the initial stage of axillary bud development in 1.5 mg/l the number of axillary bud production was higher at about 7 per explant. Subsequently the growth of the small axillary buds was continuously suppressed while the large axillary buds were growing (Figure 9). Medium with 1.75 mg/liter BA also produced a considerable number of axillary buds with well elongated shoots. The lower concentrations (0.5, 1.0 and 1.25 mg/l) also produced 2.0 to 2.2 axillary buds. Very low rate of axillary bud proliferation ranging from 0.4 to 1.2 was observed at higher concentrations of BA from 2.0 to 5.0 mg/l.
Figure 9. Initiation of axillary buds (about 7) in 1 cm long shoot piece in basal medium supplemented with 1.5mg/l BA. The growth of the small axillary buds was suppressed while large axillary buds continued to grow (mag. = 6X).

4.1.7 The effect of solid and liquid media (with filter paper bridges) on axillary bud proliferation.

Clear differences were observed in axillary bud proliferation on these media. Explants in liquid medium on filter paper bridges produced an average of 1.4±0.6 axillary shoots (mean of five replicates) whereas explants on solid medium produced a greater number of axillary buds, i.e.,
4.6±1.4 (Fig.10). This confirmed that the filter paper bridge system is ineffective for proliferation of axillary buds of nutmeg.

Figure 10. Rate of axillary bud production of 1 cm long shoot piece in solid medium (left) and liquid medium with filter paper bridge (right).

4.1.8 Comparison of shoot multiplication on agitated liquid and solid media.

The number of axillary buds initiated and their average lengths in agitated liquid and stationary solid media indicate that the liquid medium was more suitable than the
solid one (Table 8). However, axillary bud production in stationary liquid medium (Fig.10) in previous experiment showed the low rate of axillary bud proliferation i.e., 1.4 ± 0.6 evident that liquid medium was more suitable only at slow agitation (80 rpm).

Table 8. Axillary bud initiation and growth of regenerated shoots in two types of media after 10 weeks of introduction.

<table>
<thead>
<tr>
<th>Medium</th>
<th>No. of shoots</th>
<th>Shoot length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Av. of 5 reps.</td>
<td>Av. of total shoots</td>
</tr>
<tr>
<td>Liquid</td>
<td>6.2 ± 0.6</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>Solid</td>
<td>2.8 ± 1.2</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

Initiation of axillary bud growth occurred in both liquid and solid media. The number of axillary buds produced in liquid medium (6.2 ± 0.6) shown in Figure 11. was about twice the occurrence in solid medium (2.8 ± 1.2). The growth of the axillary buds was not synchronous. Some of them were suppressed while others grew rapidly. The average shoot length of the axillary buds grown in liquid medium was
greater (3.6±0.6 cm) than the axillary buds which grew in solid medium (1.4±0.2 cm).

Figure 11. Higher number of shoots produced with greater elongation in agitated liquid medium supplemented with 1.5 mg/l BA after 10 weeks of introduction (mag. = 1.5X).

4.1.9 Effect of axillary bud position on regeneration.

Production of axillary buds from four different portions of a shoot clearly indicate that more axillary buds yielded in sections between 0.5 and 2.5 cm from the apex (Table 9).
Table 9. Average number of axillary buds produced (from 5 replicates) from four different positions of axillary buds after 8 weeks.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Length from the apex cm</th>
<th>No. of axillary buds x S.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>0.5 - 1.5</td>
<td>3.4 ± 0.98</td>
</tr>
<tr>
<td>Second</td>
<td>1.6 - 2.5</td>
<td>4.6 ± 0.81</td>
</tr>
<tr>
<td>Third</td>
<td>2.6 - 3.5</td>
<td>1.6 ± 0.78</td>
</tr>
<tr>
<td>Fourth</td>
<td>3.6 - 4.5</td>
<td>1.2 ± 0.71</td>
</tr>
</tbody>
</table>

Axillary bud induction occurred after 3-4 weeks in each treatment. A very low rate of regeneration was observed from the third and fourth sections i.e., 1.6±0.78 and 1.2±0.71 respectively. The explants in these treatments produced a hard black callus at the cut edges. The highest number of axillary buds (4.6±0.81) was found in the second segment (1.6-2.5 cm from apex) followed by the first segment (0.5-2.5 cm from apex) which formed 3.4±0.98 buds.
4.1.10 Determination of subculture frequency.

The average length of ten shoot cultures at one week intervals for a duration of five weeks show a steady increment in growth reaching an increase of 0.612 cm in five weeks (Table 10).

<table>
<thead>
<tr>
<th>Duration (Weeks)</th>
<th>Length (cm)</th>
<th>Incremental growth (cm)</th>
<th>Rate cm/week</th>
</tr>
</thead>
<tbody>
<tr>
<td>After 0</td>
<td>1.561</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>1.594</td>
<td>0.033</td>
<td>0.033</td>
</tr>
<tr>
<td>2</td>
<td>1.720</td>
<td>0.159</td>
<td>0.126</td>
</tr>
<tr>
<td>3</td>
<td>1.905</td>
<td>0.344</td>
<td>0.185</td>
</tr>
<tr>
<td>4</td>
<td>2.106</td>
<td>0.545</td>
<td>0.201</td>
</tr>
<tr>
<td>5</td>
<td>2.173</td>
<td>0.612</td>
<td>0.067</td>
</tr>
</tbody>
</table>

When the explant was first placed in the medium there was a lag period during first week. This was followed by an exponential rise in length between the second and fourth week. After that there was a deceleration. After six weeks, defoliation of culture and media discoloration was observed.
4.1.11 Influence of auxins and activated charcoal on adventitious root formation.

The effect of NAA or IBA with or without 0.2% charcoal on percentage of rooting, average number of roots and length has been summarized in Table 11. The results show that incorporation of activated charcoal assisted root initiation.
Table 11. The effect of NAA and IBA with or without activated charcoal on rooting of shoots, eight weeks after culturing.

<table>
<thead>
<tr>
<th>Growth reg. (mg/l)</th>
<th>Rooting % (5 reps)</th>
<th>No. of roots Av. (5 reps)</th>
<th>Av. root length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC+NAA 0.0</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>20</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>AC+IBA 0.0</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>0.5</td>
<td>60</td>
<td>1.3</td>
<td>0.56</td>
</tr>
<tr>
<td>1.0</td>
<td>25</td>
<td>1.0</td>
<td>0.4</td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NAA 0.0</td>
<td>100</td>
<td>2.2</td>
<td>2.3</td>
</tr>
<tr>
<td>0.2</td>
<td>100</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>1.6</td>
<td>1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>IBA 0.0</td>
<td>80</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>0.2</td>
<td>100</td>
<td>3.0</td>
<td>1.4</td>
</tr>
<tr>
<td>0.5</td>
<td>100</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
<td>1.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>
In most instances emergence of roots in medium started in 2-3 week old cultures. Shoots which failed to form roots during this period did not form roots even after 6 weeks. Anderson’s medium with any concentration of NAA or IBA was ineffective for root initiation, except the treatment with 0.5 mg/liter IBA. The shoots in this treatment produced about two roots but they were very short (0.7 cm). Anderson’s medium with 0.2% activated charcoal markedly improved rooting (80-100%) in all treatments, even in the treatment without any auxins (Fig. 13). The number of roots per shoot varied from 1.2-3.0 per explant and root length ranged from 1-2.3 cm either in IBA or NAA with 0.2% activated charcoal (Figure 14). Roots of microcuttings generally originated within 3 mm of the cutting base and all of the roots had numerous root hairs.

4.1.12 Transfer of regenerated plants to the soil and acclimatization.

About 70% survival was observed following transfer of plantlets to soil. Most of the plantlets which died, rapidly became necrotic, and were overgrown by fungi. Successfully transplanted plantlets initially grew very slowly but following their transfer to greenhouse conditions rapid growth occurred (Fig. 15 and 16).
Figure 13. The development of roots in basal medium supplemented with 0.2% activated charcoal and different concentrations of IBA and NAA after 8 weeks (Mag. = .5X).

Figure 14. Rooted nutmeg shoots in basal medium supplemented with IBA 0.5 and 0.2% activated charcoal after 8 weeks (Mag. = 1X).
Figure 15. A rooted plant transferred to a mixture of sterilized soil after one week (mag. = 0.33X).

Figure 16. A complete plant transferred to a large pot with a mixture of non-sterilized soil after about four months (magnification = 5X).
4.2 Nutmeg clonal materials

4.2.1 Shoot tip culture of plagiotropic branches

Rate of contamination observed was very low (about 25%). Shoot tips in BA survived a longer period, about 6-12 weeks, but failed to induce any axillary buds. The shoot tips in other treatments did not survive beyond four weeks.

4.2.2 Shoot tip culture of trunk-sprouted orthotropic shoots

A small number of orthotropic branches were found in clonal mother trees. Explants turned brown within three days and died. The reason may be due to the soft and tender nature of the shoot tips.

4.2.3 Effect of cytokinin and its concentration on culture establishment of un-selected nodal explants.

Rate of contamination observed was low (about 33%). Cultures were healthy and lived for about 6-8 weeks but no axillary bud-break was observed.

4.2.4 Culture of selected nodes which had developed axillary buds.
The rate of contamination was very high, over 90%. Higher concentrations of sterilent were lethal to the developing axillary buds. Among uncontaminated cultures in about 2% growth and elongation of axillary buds was observed.

4.2.5 Etiolation of the nutmeg branches.

All the shoots died within 14 days.

Figure 17. Unfurling of leaves in presence of 2.0 mg/l 2,4-D after about 12-14 weeks (Mag. = 2X).

4.2.6 Leaf cultures

The rate of contamination was lower (about 20%). At higher concentrations of 2,4-D, 2.0 and 3.0 mg/l, with any
concentration of Kn leaves became wrinkled. No callus was found any of the treatments (Figure 17).

4.2.7 Embryo or endosperm cultures.

Culture media turned dark due to polyphenol-like substances secreted from damaged tissues. Smaller explants (0.5 cm) survived more than 10 weeks and produced a small callus at the cut edges specially with presence of 2,4-D (0.5-2.0 mg/l) (Figure 18). Blackening of culture media was severe in larger and more mature endosperm explants (1 cm) and the explants died after about 4 weeks.

Figure 18. Low frequency callus production in smallest endosperm in 2.0 mg/l 2,4-D after 12 weeks (mag.=1X).
4.3 Materials from grafted plants maintained in the greenhouse.

4.3.1 Shoot tip culture.

4.3.1.1 Effect of pre-treatment by spraying 50 mg/l GA₃ on culture establishment of shoot tips.

Rate of fungal infection observed was very low (about 5%). But about 25%-40% showed bacterial contamination after about 10-14 days of culturing. The best culture establishment was observed in medium containing 2.0 mg/l BA with 0.2 mg/l NAA. Axillary buds developed and very slow growth was observed. Finally all the cultures died without further growth.

4.3.1.2 The effect of GA₃ with NAA, or IBA or Kn on establishment of shoot tip culture and axillary bud elongation.

The smallest shoot tips (0.5 cm) died after about four weeks. Larger shoot tips such as 1.0 and 1.5 cm survived for about 12 weeks. However, the hormonal combination of NAA and GA₃ and Kn with GA₃ failed to induce axillary buds. The shoot tips in IBA with GA₃ combinations survived longer and produced small axillary buds (Figure 19). But these axillary buds did not grow further. The cultures gradually turned
yellow and ultimately died.

Figure 19. Development of small axillary buds of the shoot tip explants in 0.5 mg/l IBA with 1.0 mg/l GA₃ after 12 weeks (magnification = ).

4.3.1.3 Comparison of liquid and solid medium on culture establishment of shoot tips.

Death of the shoot tip was faster in liquid medium. A limited development of axillary buds was observed in shoot tip explants in solid medium. Results were not satisfactory.

4.3.1.4 Manipulation of various basal media on culture establishment of shoot tips.
The cultures in high concentration of salts i.e., BS, MS and 1/2 macro MS gradually turned yellow and finally died within two weeks. The cultures in Anderson's medium showed the development of axillary buds but further growth and elongation of axillary buds were not achieved.

4.3.2 Nodal culture experiments.

4.3.2.1 Nodal cultures from un-selected nodes.

Very low percentage of cultures (about 1%) produced axillary buds regardless of hormone or light and dark treatments.

4.3.2.2 The effect of light and dark and different media on culture establishment of selected nodes.

Nodal cultures were not established either in Anderson's or in Blueberry medium; this may be due to the higher concentration of salts in Anderson's medium and high nitrogen in blueberry medium. Nodal cultures in 1/3 strength of either Anderson's or blueberry medium were comparatively better than their full strength. When carefully examined the cultures in these two media (1/3 of both) were found to have healthy axillary buds and leaves in 1/3 strength of Anderson's medium. The axillary buds were elongated and
leaves were expanded (Figure 20). A single axillary bud was produced in each of the nodal cultures. The average growth of the axillary buds in four media in two types of photoperiods 16/8 hour light and dark, and first 3 weeks in total dark and then exposed to 16/8 photoperiod, was given in Table 12.

The elongation of the axillary buds is significantly more in both media at 1/3 of concentrations. When cultures were exposed to complete darkness for the initial 3 weeks, the growth of the axillary buds was slower than the cultures which were not exposed to the dark.

Table 12. The effect of four different basal media and two photoperiods on axillary bud elongation (mm) after 12 weeks.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Axillary bud growth cm.</th>
<th>16/8 photoperiod</th>
<th>Dark for first 3 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/3 An</td>
<td>2.14</td>
<td>1.55</td>
<td></td>
</tr>
<tr>
<td>Full An</td>
<td>1.01</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td>1/3 BB</td>
<td>2.08</td>
<td>1.35</td>
<td></td>
</tr>
<tr>
<td>Full BB</td>
<td>1.5</td>
<td>0.79</td>
<td></td>
</tr>
</tbody>
</table>
Figure 20. Growth and expansion of the leaves of nodal cultures in 1/3 Anderson's medium after 12 weeks (magnification = 2X).

4.3.2.3 Requirement of NAA for axillary bud elongation of established nodal cultures.

Cultures without NAA showed comparatively better and healthier axillary buds with green leaves (Figure 21), whereas in the presence of NAA, leaves of the axillary buds turned yellow and some of the leaves became detached from the petiole.
4.3.3 Hard pruning of grafted plants.

Haru pruning method for nutmeg grafted plants was failure due to low percentage of survival (about 10%). Therefore, sufficient materials could not obtained to lay out the necessary experiments.

4.3.4 In vitro grafting.

In vitro grafting procedure for nutmeg was laborious and very difficult due to large size of the seed and hardness.
of the rootstock. However, about 10 number of grafted plants were made but all the scion portions dried and fell off after two weeks (Figure 22). Micrografting to hypocotyl of nutmeg seedlings also failure (Figure 23).

Figure 22. Micrografted nutmeg seedling-to the epicotyl.

4.4 Nodal culture establishment in re-grafted materials.

Most of the re-grafted plants in greenhouse produced very attractive axillary bud development in most of the axils (Figure 24). A few cultures could be compared with nodal cultures with grafted materials and no difference was observed. However, this experiment could not be repeated due to unavailability of such nodes in the plants since the
plants were small.

Figure 23. Micrografted nutmeg seedling-to the hypocotyl.

Figure 24. High rate of axillary bud development in re-grafted plants.
4.5 Clove juvenile plants

4.5.1 Status of the shoot tip explant on culture establishment.

Percent survival and contamination with two different physiological stages of the explants are given in Table 13.

Table 13. Percent survival, death and contamination of two different explants after 3 weeks.

<table>
<thead>
<tr>
<th>Explant</th>
<th>Survival</th>
<th>Dead</th>
<th>Contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot tip</td>
<td>10</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>Shoot tip with a pair of leaves</td>
<td>50</td>
<td>10</td>
<td>40</td>
</tr>
</tbody>
</table>

The shoot tip explant with a pair of leaves gave higher survival (50%) than without any leaves (10%). In explants with two leaves the leaves became detached and fell on to the medium after two weeks. Browning was located only at the cut edges. However, in the shoot tips where the leaves were dissected out browning was also observed where the petioles were cut.
4.5.2. The effect of BA and NAA on culture establishment and proliferation of shoot tips.

Shoot tip explants were cultured in Anderson's medium supplemented with a factorial combination of five levels of BA (0, 0.5, 1.0, 2.0 and 5.0 mg/l) and three levels of NAA (0, 0.2 and 0.5 mg/l), and clear differences were observed. The first response was unfurling of the two leaves and subsequently the leaves became detached by basal callus formation of the introduced shoot tip within 2-3 weeks. After transferring shoot tips to fresh medium after removing the leaves, development of axillary buds started after 8-10 weeks in two treatments. Among the growth regulators tested, the treatments 2.0 mg/l BA with 0.2 mg/l NAA showed best shoot proliferation, 5.2 and 6 respectively (Table 14 and Figure 25). Higher concentration of BA (5.0 mg/l) caused death of the shoot tip. A higher concentration of NAA (0.5 mg/l) suppressed the growth of the axillary buds. Absence of BA or a low concentration of BA (0.5 mg/l) was not capable of breaking the apical dormancy, hence growth of the terminal bud was observed. Axillary buds initiated in the explants branched and reproduced axillary shoots at the rate of three per shoot (Figure 26). This branching pattern was similar to the in vivo branching habit of clove.
<table>
<thead>
<tr>
<th>BA</th>
<th>NAA</th>
<th>Axillary buds</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Terminal growth</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>Terminal growth</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>0</td>
<td>3.6</td>
<td>Axillary buds</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>0</td>
<td>6</td>
<td>Axillary buds</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>5.2</td>
<td>Axillary buds</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0</td>
<td>0</td>
<td>Death</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>Death</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>0</td>
<td>Death</td>
</tr>
</tbody>
</table>
Figure 25. Highest rate of axillary bud production (about 6) in Anderson's medium containing 2.0 mg/l BA after 10 weeks (magnification = 1X).

Figure 26. Continuous multiplication at the rate of three from a single shoot tip in Anderson's medium containing 2.0 mg/l BA after 12-14 weeks (magn. = 2X).
4.5.3 The effect of BA concentrations on axillary bud proliferation.

The number of axillary buds produced as an average of five cultures in response to various concentrations of BA are given in Table 15. Generally increasing the concentration of BA also increase the regeneration of axillary buds. The maximum number of axillary buds was produced in medium containing 2.0 mg/l BA (Figure 27).

Table 15. The effect of BA concentration on axillary bud proliferation after 10 weeks.

<table>
<thead>
<tr>
<th>BA mg/l</th>
<th>No. of axillary buds Average of 5 cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>0.25</td>
<td>2.5</td>
</tr>
<tr>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>0.75</td>
<td>2.5</td>
</tr>
<tr>
<td>1.0</td>
<td>2.75</td>
</tr>
<tr>
<td>1.25</td>
<td>4.25</td>
</tr>
<tr>
<td>1.5</td>
<td>2.75</td>
</tr>
<tr>
<td>1.75</td>
<td>5.5</td>
</tr>
<tr>
<td>2.0</td>
<td>5.75</td>
</tr>
</tbody>
</table>
Figure 27. The highest number of axillary bud proliferation in medium containing 2.0 mg/l BA after 10 weeks of introduction (Mag.= 2X).

4.5.4 Effect of NAA and GA₃ on shoot elongation.

The shoot tips introduced to medium with GA₃ and NAA produced axillary buds after 6-8 weeks (Table 16). But the number of axillary buds that elongated in response to GA₃ and NAA did not significantly differ from the control. A higher concentration of both GA₃ and NAA (0.1 and 0.1 mg/l) reduced the production of axillary buds. The average length of axillary shoots also did not differ among the treatments.
Table 16. The number of axillary buds produced and the average length of the shoots in response to GA₃ and NAA after 10 weeks of introduction.

<table>
<thead>
<tr>
<th>GA₃ (mg/l)</th>
<th>NAA (mg/l)</th>
<th>No. of axillary buds</th>
<th>Average length of shoots in mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0</td>
<td>3.25</td>
<td>6</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0</td>
<td>3.0</td>
<td>7</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>2.6</td>
<td>5</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0</td>
<td>2.8</td>
<td>6</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0</td>
<td>2.8</td>
<td>4</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>4.2</td>
<td>6</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>2.75</td>
<td>7</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0</td>
<td>2.4</td>
<td>5</td>
</tr>
<tr>
<td>0.1</td>
<td>0.0</td>
<td>2.0</td>
<td>5</td>
</tr>
</tbody>
</table>

4.5.5 Evaluation of different media and growth hormones on root formation.

Full strength Anderson's medium with or without
pluroglucinol and at any concentration of IBA or NAA was not capable of inducing any roots. But introduced shoots were alive and showed growth for a long period. When the level of macro and micro-nutrients was lowered to 1/3, roots were initiated at the bases of the shoots. The rooting % and average root length are given in Table 17.

Figure 28. Formation of roots in 1/3 strength of Anderson's supplemented with 1.0 mg/l NAA after 8 weeks (magnification= 1.5X).
Table 17. The percentage of rooting and average length of the roots in 1/3 Anderson's medium containing five concentrations of IBA or NAA after 8-10 weeks.

<table>
<thead>
<tr>
<th>Conc. (mg/l)</th>
<th>Rooting (%)</th>
<th>Length of roots (in CM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>0.2</td>
<td>60</td>
<td>0.8</td>
</tr>
<tr>
<td>0.5</td>
<td>40</td>
<td>1.0</td>
</tr>
<tr>
<td>1.0</td>
<td>20</td>
<td>1.42</td>
</tr>
<tr>
<td>2.0</td>
<td>100</td>
<td>1.12</td>
</tr>
<tr>
<td>NAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>0.2</td>
<td>60</td>
<td>1.08</td>
</tr>
<tr>
<td>0.5</td>
<td>30</td>
<td>0.84</td>
</tr>
<tr>
<td>1.0</td>
<td>100</td>
<td>1.49</td>
</tr>
<tr>
<td>2.0</td>
<td>60</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Effect of type of auxin and their concentration on rooting of clove microcuttings was clearly observed. 100% rooting
efficiency was observed in 2.0 mg/l IBA and 1.0 mg/l NAA (Figure 29) and also average root length was greater in these two treatments, i.e., 1.42 cm and 1.49 cm. NAA at any concentration gave over 60% rooting. In most of the treatments initiation of a single root was observed and occasionally 2-4 roots were produced from a shoot. Rooted plantlets were successfully transferred to soil (Figure 29).

Figure 29. Regenerated clove plantlets transferred to soil.
4.6 CLONAL SELECTION OF CLOVE.

4.6.1 Shoot tip culture.

Attempts were made to culture various stages of shoot tips from clonal selections, i.e., actively growing shoot tips with pink coloured leaves at the apex, shoot tips with two green leaves, rapidly growing trunk-sprouted shoots and shoot tips with dormant buds. The shoot tips from trunk-sprouted shoots and actively growing shoots turned brown and died after about three days of culture. The shoot tips with dormant buds lived for a longer time, about 14 days. But most of the dormant shoot tip cultures were highly contaminated either by fungus or bacteria. A very high rate of contamination (over 80%) was found in all the sterilization procedures except with 0.01% HgCl₂. The shoot tips from pre-treated branches also had high rate of contamination. All the tested stages of the shoot tips failed to become established in culture.

4.6.2 Nodal cultures

4.6.2.1 Selection of a cytokinin and a suitable concentration for establishment of nodal cultures.

The bacterial contaminants were not completely eliminated
but their growth was suppressed by the use of antibiotics. In a comparison of BA and Kn on culture establishment, BA showed better establishment of cultures whereas the cultures in Kn turned brown and finally died within 8 weeks. After about 10-12 weeks axillary buds had burst in medium containing 6-10 mg/l BA (Figure 30 and 31). Any of the concentrations of Kn was not capable of enhancing axillary bud development. The growth rate of these axillary buds was very slow (0.5-1.0 mm/month). The leaves were very small and these micro leaves did not develop to normal leaves.

Figure 30. Axillary bud break and slow growth of nodal cultures in medium supplemented with antibiotics and 8 mg/l BA after 10-12 weeks (mag. =2X).
BA at the concentration 6-10 mg/l showed the best culture establishment and development of two axillary buds at the two axils. However the percentage of success was very low (less than 5%).

Figure 31. More axillary bud development of nodal cultures in medium supplemented with 0.05 mg/l yeast extract, 0.01 mg/l glutamine, 0.01 mg/l biotin, 8.0 mg/l BA and a mixture of four antibiotics after 20-24 weeks (mag. = 3X).

4.6.2.2 Evaluation of different physiological stages on culture establishment.

Among three different physiological stages of nodes tested
the nodes with presence of axillary buds turned brown and
died within about seven days of culture. At the other two
stages of nodes, remaining portions of leaves became
detached with the formation of callus at the leaf abscission
layer. Nodal cultures with swollen axillary buds were
superior to nodal cultures without visible axillary buds in
terms of emergence of axillary buds. Also nodal cultures
with swollen axillary buds took less time to develop the
axillary buds (about 8 weeks) than the other.

Although bacterial contaminants were visible after two weeks
of culture, the growth of contaminants was very slow and was
suppressed by the mixture of antibiotics. But in most of the
cultures bacterial contaminants were not completely
eliminated. However, a very low percentage of success (about
5%) was obtained.

Repeating the same experiment in dry (February to April) and
wet seasons (November and December) indicated a higher rate
of culture establishment in December (end of the major rainy
season) and the rate of contamination was lower in this
period.
4.6.3 Ovule and embryo culture.

Unfertilized ovules from fruitlets (3-4 weeks after pollination) survived for about 6 months (Figure 32), whereas other stages of ovules did not survive more than 4 months. However, none of the ovules produced any callus.

Immature embryos (4 weeks after pollination) produced large white callus after 8 weeks at the concentration of 2,4-D 1.0 and 2.0 mg/l (Figure 33).

Figure 32. Survival of ovules for longer period from fruitlets 3-4 weeks after pollination in culture medium (magnification = 1X).
Figure 33. Formation of a large white callus from immature embryo (4 weeks after pollination) in B5-2 medium contained 2.0 mg/l 2,4-D after 8 weeks (magnification = 2X).

4.6.4 Epicotyl, hypocotyl and cotyledon culture

Large callus was produced after about 8 weeks in medium containing 2,4-D at the concentration of 0.5, 1.0, 2.0 and 5.0 mg/l without Kn or BA by epicotyl and hypocotyl segments. These calluses turned brown after about 8 weeks and died after a further four weeks (Figure 34).
Figure 34. Callus formation from epicotyl or hypocotyl segments in 85-2 medium containing 2.0 mg/l 2,4-D after 8 weeks (magnification = 1.5X).
5.0 DISCUSSION

The results presented in the previous section on clove and nutmeg tissue culture indicate some similarities specially in juvenile tissues of both plants and some dissimilarities and difficulties in clonal materials. Shoot tip explants from juvenile tissues of both plants are capable of producing multiple shoots. Shoots can be rooted and plantlets have been established in the greenhouse. Regeneration of plantlets from field-grown clonal selections of both trees is much more difficult and it will take some more years to obtain success, specially in nutmeg.

However, with even juvenile materials of both plants some difficulties were encountered during culture initiation. The problems included severe contamination by exogenous fungi at the leaf axils within three days, endogenous bacteria after 10 days and suspected excessive phenolic exudation from the explants which eventually darkened the medium and caused explant death. The rate of polyphenol secretion is comparatively more in clove. Exudation of phenolic compounds from the introduced explants is a basic problem in many woody plants, e.g., teak (Gupta et al., 1980), *Eucalyptus* (Gupta et al., 1981), peach (Hammerchlag, 1985), guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987), apple (Zimmerman, 1983) and tea (Arulpragasam and Latiff, 1986).
However, secretion of phenols into the culture medium could be minimized by adopting one or more steps described by various workers such as initial culture incubation at low light intensity or in the dark (Wang and Huang, 1974; Adams et al., 1979; McComb and Newton, 1981; Monaco et al., 1977), or transferring the explants to fresh medium 1-2 days after initial culturing (Zimmerman, 1980; Lloyd and McCown, 1980; Amin, 1986). The difficulties associated with phenolic compounds could be partially overcome by transferring explants to fresh medium after 24-36 hours and incubating cultures in low light intensity for the first three days. Bacterial contamination of the cultures could not be overcome even with the incorporation of a mixture of four antibiotics to the medium as described by Young et al., (1984). Therefore, a large number of cultures was established initially followed by the selection of clean cultures after about 14 days.

There is no general purpose medium yet available for meristem, shoot tip or nodal cultures, but Murashige and Skoog medium (1962) with modifications, is most frequently used with the greatest success, e.g., guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987), mulberry (Oka and Ohyama, 1986), teak (Gupta et al., 1980), tamarind (Mascaranhas and Nair, 1987), Eucalyptus (Gupta et al., 1981), coffee (Sondahl et al., 1984) and cacao (Dublin,
Anderson's rhododendron formula (1975), a modified MS medium with lowered nitrate, increased iron, sodium phosphate and adenine sulphate, specially recommended for acid-loving plants, has been successfully used for micropropagation of Rhododendron and Kalmia. The pH of this medium is comparatively lower than MS, B5 or WPM media (pH 4.8). Comparative studies with MS, WPM, B5 and Anderson's media for axillary shoot proliferation of nutmeg (juvenile) indicated that the performance of the tissue on the four media differed significantly. The nutmeg shoot tips could be established on Anderson's medium whereas the other media were ineffective, probably due to the higher salt concentration of MS or B5 media than Anderson's. Even though WPM medium is low in salts it was ineffective for establishing nutmeg shoot tips. The same medium used for clove shoot tips (juvenile) gave successful results.

Complete disinfection of many woody species can be extremely difficult, especially when the explants are taken from the field. McCown (1986) has suggested that reduction of the contamination rate in woody species can be achieved by maintaining them in a greenhouse, avoiding overhead watering. Reduction of contaminants was achieved by Litz and Conover (1978) on field-grown papaya by spraying a systemic fungicide (0.1% benlate) at weekly intervals prior to collection of materials for in vitro culture. They also
reported that the rate of contamination depended on climatic season. For the present study the nutmeg and clove juvenile stock plants were maintained in a greenhouse without overhead watering and also sprayed with a solution of fungicide (0.1% benlate) in order to reduce the rate of microbial contamination. However, plants were not grown outside of the greenhouse to compare the rate of contamination.

The selection of the correct physiological stage of the stock plants is one important criterion for successful in vitro culture. Actively growing shoot tips are recommended for meristems, shoot tips and bud cultures because of their low rate of contamination and better culture establishment. Stone (1963) found a better survival rate of carnation meristems excised and cultured during the active season. Zimmerman (1985) also suggested that many apple cultivars could be established with actively growing shoot tips. Many tropical trees have been successfully propagated using actively growing buds, such as in teak (Gupta et al., 1980), tamarind (Mascaranhas et al., 1987), guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987), mulberry (Oka and Ohyama, 1986) and jak (Amin, 1986). In this study, dormant nutmeg shoot tips (juvenile) showed a high rate of contamination (90%). The actively growing nutmeg shoots from the same plants had lower contamination rates (30%) and also
were established readily in culture. Leaving a pair of leaves at the terminal and with clove shoot tips from juvenile plants showed five times higher culture survival than shoot tips alone.

Most (over 85%) culture establishment media have been supplemented with a cytokinin. BA is most effective for meristems, shoot tip and bud cultures e.g., guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987), Pinus lambertiana (Gupta and Durzan, 1985) and mulberry (Oka and Ohyama, 1986), followed by kinetin and 2ip, whereas zeatin has been used less frequently (Nair et al., 1979). Sometimes a mixture of two cytokinins (BA and kinetin) was also used for culture establishment e.g., teak (Gupta et al., 1980), tamarind (Mascaranhas and Nair, 1987) and Eucalyptus (Gupta et al., 1981). The most suitable concentration of cytokinin at this stage varies from 0.5-2.0 mg/l, e.g., with guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987) mulberry (Oka and Ohyama, 1986) tamarind (Mascaranhas and Nair, 1987), coffee (Sondahl et al., 1984) and cocoa (Dublin, 1984). But on a few occasions a high concentration of BA has been used with AC for culture establishment such as 10-20 mg/l for chinese tallow (Krikorian and Kahn, 1987). An auxin is also sometimes required for shoot growth. Since the young shoot apex is an active site for auxin biosynthesis, exogenous auxin is not always needed in stage I medium,
especially when relatively large shoot tip explants from actively growing plants are used, e.g., *Lolium multiflorum* (Dale, 1975), tamarind and teak (Mascaranhas and Nair, 1987), guava (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987), jak (Amin, 1986) and cacao (Dublin, 1984; Passey and Jones, 1983). Sometimes very low concentrations of an auxin have been used with a cytokinin e.g. with coffee (Sondahl et al., 1984). Among other auxins NAA is used most frequently for successful culture establishment in tropical woody plants (Hu and Wang, 1983). For shoot establishment of nutmeg juvenile plants, BA performed best and kinetin was least effective (Figure 2). This study also demonstrated that BA at 2.0 mg/liter with or without 0.1 mg/liter NAA was sufficient for establishing both clove and nutmeg cultures (Figure 3 and 25'). NAA is not required at this stage, probably because of the large size of the initial explant (2.5 cm).

In axillary shoot proliferation a cytokinin is utilized to overcome the apical dominance of shoots and to enhance the branching of lateral buds from each axil. In general, BA is the most effective cytokinin for stimulating axillary shoot proliferation, followed in decreasing order by kinetin and 2ip (Bhojwani, 1980; Hasegawa, 1980; Kitto and Young, 1981; Amin and Jaiswal, 1987; Mascaranhas et al., 1987). It is evident that for nutmeg cultures originating from juvenile
plants BA is superior to other cytokinins tested (kinetin, 2ip or zeatin) for stimulating axillary buds. The cytokinins KN and 2ip produced a maximum number of axillary buds, approximately 1.8 and 1.2/explant, whereas on medium with BA there were 5.4/explant after 8 weeks. The cytokinin zeatin was ineffective and only roots were produced in some of the treatments (0.5, 1.0 and 1.5 mg/l).

The effective concentration of BA to overcome apical dominance varies from plant to plant. Most shoot tips or buds responded to BA at concentrations of 1.0 mg/l or 4.5 μM. Many examples can be given in woody species for successful shoot multiplication in the range of 1.0-3.5 mg/l BA (guava and jak fruit, Amin, 1986; teak, Gupta et al., 1980; willow, Bhojwani, 1980; blackberry, Skirvin et al., 1981; raspberry, James, 1979; rhododendron, Anderson, 1975; Bartlett pear, Lane, 1979c and mulberry, Oka and Ohyama, 1996). The study with nutmeg (juvenile) shoot multiplication in a sequence of BA concentrations also indicates that maximum multiplication (1:3) occurred at a concentration of 1.5 mg/liter BA whereas in clove (juvenile) the maximum rate of multiplication was observed (1:5.75) at the concentration of 2.0 mg/l BA.

During propagule multiplication liquid-agitated cultures have sometimes been used very effectively for shoot
multiplication in comparison with stationary cultures (Walkey and Cooper, 1976; Asokan et al., 1984; Krikorian and Kahn, 1987). Liquid culture has the advantage of exposing explants more uniformly to a given nutrient medium. It obviates the need to remove any remnants of agar during surgical manipulations and it facilitates maintenance of a high humidity environment for the shoots. Asokan et al., (1984) working with *Xanthosoma* cultures in liquid medium, demonstrated that not only higher rate of multiplication but also greater shoot elongation of cultures occurred in liquid medium. This study, with nutmeg juvenile explants, indicated that liquid medium with filter paper supports was ineffective for stimulating axillary bud proliferation (1:1.4) but agitated liquid medium increased axillary bud proliferation about 1:6.2. Moreover, greater shoot elongation occurred in agitated liquid medium (mean 3.6 cm) than in solid medium (mean 1.4 cm). The reason may be poor aeration and very limited contact surface between the explant and medium in the filter paper bridge system. The filter paper bridge preparation requires more labour. In contrast to the advantages of liquid agitated medium for nutmeg cultures, clove cultures in liquid agitated medium have been ineffective. Introduced clove shoot tips died within a few days of culture.
Some workers incorporated GA, to the culture medium in order to achieve shoot elongation of apple (Zimmerman and Broome, 1980c). However, for some plant species GA, was not effective for shoot elongation (Zimmerman and Broome, 1980c). In this study incorporation of NAA and GA, to the culture medium for shoot elongation of clove cultures failed to elongate the shoots. However, keeping cultures a little longer time in the same medium showed shoot elongation of some shoots which were then suitable for rooting.

The buds at early stages of development (proximal to the shoot tip) have often been found to be optimum for shoot regeneration. Roest and Bokelmann (1981, quoted by Hu and Wang, 1983), reported that in carnation nodal segment culture, the percentage of shoots that develop from explants taken from the top and the base of a shoot were 88.6 and 69.8, respectively. Successful methods for most tree crops have been demonstrated using axillary buds between one and four centimeters from the shoot tip (Amin and Jaiswal, 1987; Mascarenhas et al., 1987). Observations of the relationship between axillary bud regeneration and bud position in nutmeg (juvenile) showed distinct differences between nodes proximal and distal to the meristem tip. The highest frequency of axillary bud regeneration (4.5) was observed at the second portion (1.6-2.5 cm from the tip) followed by the first portion (3.4), third portion (1.6) and fourth portion
(1.2) respectively. This finding is an agreement with the generalization that establishment of most tree tissue cultures has been most successful with soft to semi-hard shoot tips (Hu and Wang, 1983).

Maintenance of a stock plant in the rapid multiplication stage is one of the most important steps not only for providing materials for future experiments but also for building up stock plants through recycling. For this reason it is necessary to know the optimum subculture period. The subculture interval mainly depends upon the plant species, the amount of medium and the salt concentration. In general, most of the cultures at the stage of rapid multiplication have been transferred to fresh medium at two to four week intervals. The growth curve of nutmeg shoot elongation (juvenile origin) in relation to time, within a six week period after introduction showed a sigmoidal pattern of growth (Table 10). When the explant material was first placed on medium, there was an initial lag period (0-1 week). This was followed by an exponential rise and linear increase in shoot elongation (2-4 weeks). Finally, there was a gradual decline in growth after 4 weeks. When the cultures were kept more than 6 weeks without transferring to a fresh medium, severe defoliation followed by discoloration of the medium was observed (Fig. 12). Therefore, in order to maintain fast growth of the cultures the shoots should be
subcultured during the stationary phase, i.e., before four weeks.

To induce adventitious roots of nutmeg microcuttings, the basal medium was supplemented with IBA or NAA at five concentrations with or without 0.2% activated charcoal. Among the concentrations tested, neither IBA nor NAA induced roots in nutmeg microcuttings (juvenile origin). Incorporation of activated charcoal in the medium rapidly produced roots in all of the treatments even without auxin. The reason for poor rooting in agar may be due to lack of aeration and a slow rate of diffusion of the toxic metabolic wastes released by growing tissues. Activated charcoal can adsorb metabolic inhibitors, improve aeration, adsorb toxic substances and adsorb residual cytokinin and auxin from the previous medium (Hu and Wang, 1983). Activated charcoal is also capable of shading in vitro roots from light which, in high intensity, may inhibit root growth. Our results are in accord with those of Snir and Erez (1980), and Cheema and Sharma (1983), who found that the percentage of rooting is increased by the influence of activated charcoal. Anderson (1978) and Damiano (1980) have also reported beneficial effects of activated charcoal on rooting of raspberries and strawberry, respectively. Further, recent studies on rooting of two guava cultivars (Jaiswal and Amin, 1986; Amin and Jaiswal, 1987) and jak fruit culture (Amin, 1986) also
indicated that activated charcoal is capable of favouring early emergence and faster growth of roots even without auxin.

Root induction of clove microcuttings was more difficult compared to nutmeg. The reason may be the same as in in vivo rooting of cuttings. Auxins, activated charcoal and pluroglucinol were ineffective for root initiation of clove with the full concentration of Anderson's medium. When the concentration was reduced to \( \frac{1}{3} \) of basal medium with presence of activated charcoal, roots were successfully produced in clove. These findings are also in accord with Anderson's (1975), who used low concentrations of basal medium to obtain a higher percentage of rooting in rhododendron.

Culturing of leaf discs from juvenile nutmeg plants has given partial organogenesis i.e. only roots were produced from the globular callus. Obtaining a root system from callus is easy, but efficient shoot organogenesis has been a failure most of the time. Epicotyl, hypocotyl and cotyledon cultures of clove seeds produced only callus. But in many forest trees (eucalyptus, sandalwood) efficient organogenesis was observed from the calluses from similar explants (Laksmi, 1995).
Even though some of the tree species can be micropropagated from tissues collected from mature trees, many others can presently be propagated only from tissues from juvenile specimens, i.e., embryo or young seedlings (Bonga, 1987). This study also proved that shoot tips or buds of juvenile plants of clove and nutmeg were cultured with more success and regeneration of plantlets from mature clonal selections is very difficult. The problem areas encountered in clonal materials were high rate of contaminants either with fungus or bacteria, blackening of tissues and media, flushing growth of the shoots, very slow growth of regenerated shoots and impossibility of culture establishment most likely due to phase change.

Two of the woody plants successfully propagated by shoot tip culture where nodal cultures failed are jak (Amin and Jaiswal, 1986) and mulberry (Ohyama and Oka, 1987). With shoot tips from actively growing plagiotropic shoots of nutmeg (clonal) very low rates of contamination and blackening were observed. Manipulation of various media with growth regulators gave negative results on establishment of cultures.

The nodal cultures which were made from the same branches as the shoot tips had higher rates of contamination. But the rate of contamination could be reduced to a considerable
extent by re-sterilizing the shoots after 24 hours of the first sterilization. The two-step sterilization procedure was utilized by Sondahl et al. (1984) to sterilize the leaves from field-grown coffee plants. Most of the contaminants were accumulated at the axils and therefore, brushing the axils with a liquid soap reduced the contamination. Nodal explants were more resistant to re-sterilization due to less hypersensitivity of the nodes to the sterilant than shoot tips.

Obtaining materials from grafted plants has several advantages over field-grown trees in tissue culture (Bonga, 1987; McCown, 1986). Major advantages encountered were low rate of contamination, uniform and controlled environment, ease of pretreatment procedures and reversing the juvenility. Our observation on nutmeg also accords with those on clove. The rate of contamination was lower and low concentration of sterilant could be used on these materials. Avoiding of overhead watering and also spraying a fungicide at regular intervals were extremely beneficial for nutmeg grafted plants.

Shoot tips or nodal cultures from plagiotropic branches of clonal trees of nutmeg always failed to regenerate shoots. McCown (1986) reported that when the shoots reached reproductive maturity their regeneration capacity is
diminished. These shoots will not contain any juvenile characters once flower initiation was observed. The shoot tips of clonal materials always produced flowers in these branches. The ability of rooting of cuttings is one of the indication of the maturity of the tissues. Vegetative propagation studies done by Sultanbawa (1984) in Sri Lanka demonstrated that rooting was very difficult with fruit-bearing plagiotropic branches. The reason may be the absence of juvenility of the shoot tips or nodal cultures of plagiotropic branches of field grown clonal selections.

Various workers has described several rejuvenation procedures for physiologically matured tissues (Hackett, 1987; Franclet et al., 1987; Bonga, 1987). One of the procedures described by them is repeating re-grafting until the plant become juvenile. Shoot tip or nodal cultures from grafted plants of nutmeg (grafting just after the seed germination) showed more success on culture establishment than the shoot tips or nodals directly from mature clonal selections. Considerable amounts of re-grafted plants could not be raised within the duration of this project for proper comparison of the materials from field grown, grafted and re-grafted plants but most of the re-grafted materials in the greenhouse produced high rate of axillary buds (Figure 24). However, the limited number of explants taken from re-grafted plants when grown in vitro did not show results
superior to grafted plants.

Another way of changing the phase from mature to juvenile is by regular spraying of a cytokinin. BA is used mostly in this respect. Our observation in this respect also gave negative results. But grafted plants produced more lateral shoots with this treatment.

Collar pruning and allowing the tree to grow new shoots is also described by Bonga (1987) as a rejuvenation process. In nutmeg grafted plants this treatment was lethal. Collar pruning could be adapted to some plants but plants not having enough reserve food will not tolerate this treatment. But pruning of branches while retaining a few branches was acceptable for the nutmeg plants grown in pots.

Another alternative will be to select the most juvenile tissues within the tree (Bonga, 1987). In several hard wood species, orthotropic shoots that originate from the base of the trunk, or shoots that develop from the bases of the main branches, or shoots that develop from sparooblasts are more juvenile than branches from other parts of the tree. These shoots arise from arrested as well as adventitious buds. They often carry juvenile type foliage and their cuttings are often easier to root than those of other branches of the tree. A recent study done in Sri Lanka on rooting of nutmeg
in potting medium accords with these results and about 70% rooting was found in trunk-sprouted orthotropic branches whereas a very low percentage of rooting was observed with plagiotropic branches. Existence of orthotopic branches on clonal selections of nutmeg was very limited. It is not possible to decapitate the clonal trees and induce orthotropic branches because to owners of these clonal trees are private farmers. However, shoot tips from the orthotropic branches could not be effectively sterilized due to their high hypersensitivity to clorox or HgCl₂.

Successful nodal culture establishment was found with selected nodes from grafted plants with axillary buds about to emerge. Among four media tested, 1/3 strength of Anderson's medium with 0.01 mg/l yeast extract, 0.05 mg/l glutamine, 0.01 mg/l biotin and 1.5 mg/l BA has given successful culture establishment. Incubation of cultures for about three weeks in the dark decreased the elongation of the shoot. Also incorporation of NAA at the rate of 0.1 or 0.2 mg/l was harmful to the growth of nodal cultures.

The maintenance of grafted plants or the repeated grafting procedure could not be utilized with clove because clove plants could not be grafted.
Selection of other juvenile materials from clonal trees and culturing in appropriate medium of somatic embryos were successfully carried out in various subtropical fruit trees (Litz, 1984a; 1984b). An attempt was made to use the same procedure as used by Litz (1988) for culturing of newly emerged whole leaf of longan, on nutmeg clonal selections. But only unfurling of leaves was observed in response to 2,4-D. The other suitable material to obtain somatic embryos were nucellar tissue and ovules as demonstrated with citrus, mango (Litz, et al., 1982), jaboticaba (Litz, 1984a) and Eugenia species (Litz, 1984b). In this respect immature embryo or endosperm was cultured in appropriate medium. To separate nucellar tissues in nutmeg was very difficult. Therefore, whole endosperm was cultured but nothing was observed other than callus formation.

When compared to the results with nutmeg, clonal material of clove showed several drawbacks. Grafting procedure or rooting of cuttings seemed to be very difficult or impossible; they contained a very high level of polyphenol-like substances, eradication of fungal or bacterial contaminants was very difficult due to high hypersensitivity of the tissue to the any sterilant. However, a few cultures could be cultured with a mixture of antibiotics. The use of a mixture of four antibiotics (Young et al., 1984) with culture medium could suppress the growth of bacteria but not
eradicate them completely. Blackening of culture media was overcome by frequent transfer of cultures to fresh medium and incubation for an initial period in low light intensity. However, a few cultures developed axillary buds from nodal cultures with minimum growth of bacteria. The rate of proliferation and growth of the shoots were lower than in nutmeg. The best results were given on nodal explants selected on the basis of swollen axils.

All attempts with various stages of shoot tips e.g. from actively growing, dormant and trunk-sprouted shoots failed to establish in culture medium. Shoot tips either died or were contaminated within a few days.

In flowers of clove a large number of ovules, about 50-75 per flower, is available. After pollination only one ovule gets a chance to become fertilized and eventually this ovule develops an embryo. Other unfertilized ovules remain for another 6-8 weeks. Unfertilized ovules are preferred over other parts as explants due to a number of reasons. Important reasons are that they are the most juvenile parts, free of contaminants and excellent material for clonal multiplication due to the existence of only mother characters of the tissues. This was clearly demonstrated with papaya (Litz and Conover, 1982). The ovules of clove survived a longer period in culture medium, about 4-6 months.
but did not produce even callus. But over 95% cultures were free of contaminants. Immature embryos provided very attractive growth of callus but embryogenesis or organogenesis was not achieved.
6.0 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH.

On the basis of the results following conclusions can be drawn:

(A) NUTMEG JUVENILE

1. Shoot tips from the juvenile plants of nutmeg are amenable to in vitro culture and theoretically, about 3000 plants can be regenerated within a year from a single shoot culture.

2. Actively growing shoot tips of nutmeg are found to be reliable material for culture initiation.

3. Anderson’s rhododendron medium is the best basal medium for all the stages of in vitro propagation of nutmeg.

4. A suitable growth regulator for culture establishment is BA at the concentration of 2.0 mg/l with or without 0.1 mg/l NAA.

5. The rate of proliferation is optimal with BA at a concentration of 1.5 mg/l.

6. An agitated liquid medium is superior to solid or
stationary liquid medium for proliferation and shoot elongation.

7. For continuous subculture the maximum number of axillary buds is regenerated by sections between 0.5 and 2.5 cm from the apex of a shoot whereas a very low rate of regeneration occurred more than 2.6 cm from the apex.

8. The appropriate subculture period is about four weeks.

9. Incorporation of activated charcoal is most favorable for rooting of shoots.

(B) CLOVE JUVENILE

1. Shoot tip of clove juvenile plants with a pair of leaves at the terminal end can be established in Anderson's medium supplemented with 2.0 mg/l BA.

2. Maximum rate of multiplication of clove culture occurred in Anderson's medium supplemented with BA at the concentration of 2.0 mg/l.
3. The best rooting medium (100% rooting) was 1/3 concentration of Anderson's supplemented with 0.08% activated charcoal and IBA 2.0 mg/l or NAA 1.0 mg/l.

(C) NUTMEG CLONAL SELECTION

1. At the first phase clonal materials should be grafted onto very early stages of nutmeg seedlings and maintained in a greenhouse without overhead watering.

2. Selected single nodal explants where axillary buds are about to emerge are suitable explants for culture establishment.

3. The suitable culture establishment medium is 1/3 concentration of Anderson's with 0.01 g/l yeast extract, 0.05 g/l glutamine, 0.01 g/l biotin, 30 g/l sucrose and 7 g/l agar.

(D) CLOVE CLONAL MATERIALS

1. Selected nodal explants just prior to bursting of the axillary buds are suitable material for culture establishment.
2. Cleaning the axils with liquid soap using a soft brush and resterilization after 12-24 hours gave higher percentage of fungus-free explants.

3. Anderson's medium supplemented with 0.01 g/l yeast extract, 0.05 g/l glutamine, 0.01 g/l biotin, 30 g/l sucrose and 7 g/l agar is the suitable medium for culture establishment.

4. Frequent transferring and incubating of cultures in reduced light minimizes the blackening of culture media.

5. Incorporation of a mixture of four antibiotics (cefotaxim 25 mg/l + tetracycline 25 mg/l + rifampicin 5 mg/l + polymyxin B 6 mg/l) was found to be always needed to suppress the bacterial contaminants in culture.

SUGGESTIONS FOR FUTURE RESEARCH

From these studies a protocol for in vitro mass propagation of nutmeg and clove and a technique for nodal culture establishment of clonal selections of both species are available. Future research should be focussed towards clonal materials of both species. Various rejuvenation procedures
and culture establishment should be thoroughly studied for nutmeg clonal selections whereas for clove elimination of bacteria would be the major problem to be solved.
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PROBLEMS AND CONSTRAINTS

Experienced a long delay of supplying instruments and chemicals from foreign countries.
APPENDICES

Appendix 1. Composition of different plant growth media

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount in mg/liter</th>
<th>MS</th>
<th>WPM</th>
<th>AN</th>
<th>B5</th>
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</thead>
<tbody>
<tr>
<td><strong>Major elements</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>400</td>
<td>400</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>---</td>
<td>480</td>
<td>2500</td>
<td></td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>440</td>
<td>96</td>
<td>440</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370</td>
<td>370</td>
<td>370</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>170</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>NaH₂PO₄.H₂O</td>
<td>---</td>
<td>---</td>
<td>380</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂.4H₂O</td>
<td>---</td>
<td>556</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>---</td>
<td>990</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td><strong>Minor elements</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>KI</td>
<td>0.83</td>
<td>---</td>
<td></td>
<td>0.75</td>
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<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>6.2</td>
<td>6.2</td>
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<tr>
<td>MgSO₄.4H₂O</td>
<td>22.3</td>
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<td>---</td>
<td></td>
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<tr>
<td>MnSO₄.H₂O</td>
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<td>22.3</td>
<td>1'9</td>
<td>10.0</td>
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<tr>
<td>ZnSO₄.7H₂O</td>
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<td>8.6</td>
<td>8.6</td>
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<tr>
<td>Na₂MoO₄.2H₂O</td>
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<td>0.25</td>
<td>0.26</td>
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<tr>
<td>CuSO₄.5H₂O</td>
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<td>0.25</td>
<td>0.025</td>
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<tr>
<td>CoCl₂.6H₂O</td>
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<td>---</td>
<td>0.025</td>
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Continued
<table>
<thead>
<tr>
<th></th>
<th>10X</th>
<th>100X</th>
<th>200X</th>
<th>20X</th>
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<tbody>
<tr>
<td>Na₂EDTA</td>
<td>37.3</td>
<td>37.3</td>
<td>74.5</td>
<td>37.3</td>
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<tr>
<td>FeSO₄·7H₂O</td>
<td>27.8</td>
<td>27.8</td>
<td>55.7</td>
<td>27.8</td>
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<tr>
<td><strong>Organic constituents</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Inositol</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
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<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>0.5</td>
<td>---</td>
<td>1.0</td>
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<tr>
<td>Pyridoxin HCl</td>
<td>0.5</td>
<td>0.5</td>
<td>---</td>
<td>1.0</td>
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<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
<td>1.0</td>
<td>0.4</td>
<td>10.0</td>
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<tr>
<td>Glycine</td>
<td>2.0</td>
<td>2.0</td>
<td>---</td>
<td>---</td>
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<tr>
<td>Adenine sulphate</td>
<td>---</td>
<td>---</td>
<td>0.80</td>
<td>---</td>
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<tr>
<td><strong>Carbon sources</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose ('000)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar ('000)</td>
<td>8</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>pH</td>
<td>5.6</td>
<td>5.2</td>
<td>4.8</td>
<td>5.5</td>
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</table>

Separate stock solutions were prepared for major elements, minor elements and organic constituents at the concentration of 10X, 100X and 20X respectively.
Appendix 2. ANOVA table of Kruskal-Wallis test

NP AR 1 W A Y P R O C E D U R E

Analysis of Variance for Variable Y
Classified by Variable DOSE

<table>
<thead>
<tr>
<th>Dose (mg/l)</th>
<th>N</th>
<th>Mean</th>
<th>Among MS</th>
<th>Within MS</th>
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<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>1.20000000</td>
<td>14.0000</td>
<td>7.74000000</td>
</tr>
<tr>
<td>0.5</td>
<td>5</td>
<td>3.20000000</td>
<td>F Value</td>
<td>Prob &gt; F</td>
</tr>
<tr>
<td>1.0</td>
<td>5</td>
<td>4.60000000</td>
<td>1.827</td>
<td>0.1633</td>
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<tr>
<td>1.5</td>
<td>5</td>
<td>4.80000000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>5</td>
<td>5.40000000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average Scores were used for Ties
Appendix 3. Wilcoxon matched-pairs signed-rank test for treatment comparison.

Wilcoxon Scores (Rank Sums) for Variable Y
Classified by Variable DOSE

<table>
<thead>
<tr>
<th>Dose</th>
<th>N</th>
<th>Sum of Scores</th>
<th>Expected Under H0</th>
<th>Std Dev Under H0</th>
<th>Mean Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>20.0</td>
<td>65.0</td>
<td>14.5143607</td>
<td>4.00000000</td>
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<tr>
<td>0.5</td>
<td>5</td>
<td>67.0</td>
<td>65.0</td>
<td>14.5143607</td>
<td>13.40000000</td>
</tr>
<tr>
<td>1</td>
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<td>65.0</td>
<td>14.5143607</td>
<td>17.60000000</td>
</tr>
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<td>1.5</td>
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<td>79.0</td>
<td>65.0</td>
<td>14.5143607</td>
<td>15.80000000</td>
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<td>2</td>
<td>5</td>
<td>71.0</td>
<td>65.0</td>
<td>14.5143607</td>
<td>16.20000000</td>
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</tbody>
</table>

Average Scores were used for Ties

Kruskal-Wallis Test (Chi-square Approximation)

$CHISQ = 10.595$, $DF = 4$, $Prob > CHISQ = 0.0375$