STANDARDISATION OF *in vitro* PROPAGATION TECHNIQUE IN CLOVE *Syzygium aromaticum* (L.)

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled "Standardisation of *in vitro* propagation technique in clove, *Syzygium aromaticum* (L.) is a bonafide record of research work done by me during the course of research and that this has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any University or Society.

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Dedicated to
my loving parents

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LIST OF ABBREVIATIONS

AC	-	Activated charcoal
BAP	-	Benzyl Amino Purine
B_5	-	Gamborg's medium
CH	-	Casein Hydrolysate
cm	-	centimetre
CW	-	Coconut Water
2,4-D	-	2,4-Dichlorophenexy acetic acid
GA_3	-	Gibberellic Acid
IAA	-	Indole Acetic Acid
IBA	-	Indole Butyric Acid
μM	-	micromolar
mm	-	millimetre
MS	-	Murashige and Skoog
NAA	-	Naphthalein Acetic Acid
N	-	Normal
PVP	-	Poly Venyl Pyrrolidone
SH	-	Schenk and Hildebrandt
w/v	-	weight per volume
WPM	-	Woody Plant Medium

Introduction

1. INTRODUCTION

Clove, the dried unopened flower buds of the evergreen tree, Syzygium aromaticum ((L.) Merr. and Perry) is one of the tree spices noted for its flavour and medicinal values. It is indegeneous to Moluccas island (Indonesia). Clove was introduced to India around 1800 A.D. by the East India company in their spice garden in Courtallam, Tamil Nadu. The major producers of this spice today are Indonesia, Zanzibar and Madagascar. World annual production is estimated to be 63,700 tonnes (Kumar et al., 1993).

In India, it is grown in about 600 ha. producing annually 1500 tonnes as against our annual requirement of 6500 tonnes. Kerala is the principal producing area for cloves in India producing nearly 250-300 tonnes annually. India is reported to be the second largest clove consuming country in the world. Since the demand for the spice is much higher than the local production, cloves are being imported from countries like Zanzibar and Madagascar (John et al., 1993).

Clove products may be considered in three major forms: clove buds - used as such or utilized in the preparation of the ground spice, oleoresin, or for the distillation of clove bud oil; clove stem oil and clove leaf oil. Clove stem oil

and leaf oil are principally used as a source of eugenol. The major uses of cloves, both whole and in ground form are for domestic culinary purposes and manufacture of sauces and pickles. In Indonesia which is the world's largest consumer of clove the major use is for making 'kretek' cigarettes. Clove oil is used in perfumeries, pharmaceuticals and flavouring industries. It is also used in histological preparations as a cleaning agent due to its antibacterial property. Clove oleoresin is increasingly used in the food processing industry.

The clove tree is a small, handsome, evergreen tree reaching 12-15 m in height. It belongs to the family Myrtaceae. It is strictly a tropical plant and requires warm humid climate. It thrives well in all situations ranging from mean sea level upto an altitude of 1500 metres. The plants are usually slow growing, and the tree can live for 100 years. There are individual records of trees of 150 years old (Purseglove et al., 1981).

Clove is generally propagated through seed which is called "mother of clove". Eventhough seed propagation is the simplest method, there are certain inherent difficulties in this method. The clove seeds show slow germination and seedling growth as reported by Wit (1969). It has been also found that the clove seeds are recalcitrant, and they loose

viability upto 50 per cent if sowing is delayed by one week after collection of ripe fruits. The availability of seed is also limited since it is a single seeded fruit and as the clove tree sets seed only in high range regions. Moreover, the cost of the seed is as high as Rs. 250-300 per kilogram.

The clove plants are notoriously difficult to propagate vegetatively by conventional methods due to its low meristematic activity (Purseglove, 1981). Works on vegetative propagtion of clove by several workers proved it as a difficult task, with negative results or only a very low percentage of success.

The germplasm collection from India has yielded a few elite plants with high yield potential. Being a single seeded fruit and also due to recalcitrant nature of seeds, the large scale propagation of elite plants is difficult within a stipulated time.

Micropropagation owns a unique distinction as the quick and easy method of deriving plants with identical genetic constitution. This technique has been immensely used by researchers for the commercial exploitation of many taxa. This promises the possibility of the extensive multiplication of elite clove plants through the exploitation of tissue culture techniques. Litz (1984) has also pointed out the

importance of using *in vitro* techniques for the propagation of clove. But there has been no reports on commercial micropropagation of spices except the fact that commercial micropropagation system has been brought through in cardamom (Priyadarshan *et al.*, 1989).

Keeping all these in view, the author has initiated studies with an objective to standardise the *in vitro* propagation technique in clove.

Review of Literature

2. REVIEW OF LITERATURE

2.1 Propagation of clove

The long juvenile phase, difficulty in vegetative propagation, lack of scientific information in nursery practices, methods of culture etc. are the problems faced by the clove growers. The average annual yield after 15th year is only two kg of dried cloves per tree (Anon., 1979). However individual tree yield as high as 15 kg of dried cloves was recorded (Wit, 1969). Exploitation of such high yielding trees has not been possible mainly due to difficulties in vegetative methods of propagation.

2.1.1 Seed propagation

Cloves are usually propagated by seeds. The seeds for sowing are collected from fully ripe 'mother-of-cloves' (Purseglove et al., 1981). The germination of clove seed is fairly low and the viability is very short. Wigg (1940) and Tidbury (1949) gave full and good accounts of nursery techniques. Badami (1938) reported that a rapid 90 per cent germination of clove seeds could be produced by removing the fruit pulp and paper like seed coat and sowing only the embryo with the groove upwards. Easy germination of clove seeds

achieved by a presowing treatment was again described by Nayar et al. (1979). Seedlings are gradually hardened off before lifting when they are about one year old and between 25-50 cm height (Purseglove et al., 1981). While transplanting, the ball of earth must be left on the roots but long main roots may be reduced (Wigg, 1940).

2.1.2 Vegetative propagation

The vegetative propagation of clove has always been difficult and attempts in many regions have failed. Francoise (1936) asserted that young branches of clove can be marcotted fairly easily. However according to Tidbury (1949), this method failed in Zanzibar. Maistre (1964) also suggested the possibility of success through marcottage. Pool et al. (1991) reported the factors affecting marcotting in clove. They showed that for water shoots of 1.0 cm diameter, a mixture of soil and leaf humus as a rooting medium applied immediately following ringing was a simple and effective procedure. This resulted in 64 per cent of marcots producing roots. However, 45 per cent of rooted marcots died during transplanting and hardening.

Terminal leafy soft wood cuttings kept in glazed propagating frames with high humidity, adequate light and bottom heat showed rooting. Success is dependent on the

retention of a proportionate, healthy leaf area (Fernie, 1946). Tidbury (1949) mentioned about one single cutting made from the soft wood portion, which took several months to strike roots.

According to May (1949) one of the grafts of clove on guava made in 1942 was still surviving in 1949. He also succeeded in approach grafting the branch tips of mature clove tree on to young clove seedlings. Experimental grafting between cloves is very difficult with shoots and has so far proved impossible with roots. Volunteer seedlings often grew up closely adpressed to old trees, and produce natural grafts. If the old tree dies from sudden death disease the sapling usually survives but occasionally dies almost simultaneously with the old tree (Sheffield, 1952). Successful inarching has been reported by Yegnanarayanayyar (1960). The compatibility of Syzygium aromaticum with species of Myrtaceae was investigated. No long term compatibility being found between clove and any of the 27 species tried in the case of budding, however, interspecific unions were obtained in more than 50 per cent of seedling approach grafts between Syzygium aromaticum and three other species (S. mulleri, S. pycnanthum and guava Psidium guajava) (Jarvie et al., 1986). According to Menon and Nair (1992) softwood grafting was found successful in clove. A success rate of 33 per cent was

obtained using intact scion shoots with polythene covers with 30 per cent survival after one year.

2.2 General aspects on plant tissue culture

The cell theory postulated by Schleiden (1838) and Schwann (1839), which reveals the totipotent nature of plant cells is the basis of plant cell, tissue and organ culture. Haberlandt (1902) reported that isolated cells are capable of resuming uninterrupted growth. Widespread success with plant tissue culture were reported after the discovery of auxins and cytokinins by Skoog and Miller (1957). They put forth the concept of hormonal control of organ formation and showed that the differentiation of roots and shoots in tobacco pith tissue cultures was a function of the auxin-cytokinin ratio and that organ differentiation could be regulated by changing the relative concentrations of these two substances in the medium. The early development of plant tissue culture technique was based on the efforts of many pioneering investigators including White (1934), Gautheret (1939), Nobecourt (1939), Miller et al. (1956), Reinert (1958), Steward et al. (1958), Bergmann (1960), Vasil and Hildebrandt (1965). It was Murashige and Skoog (1962), who developed a completely defined nutrient medium for plant tissue culture.

Several aspects of plant tissue culture are being applied in agriculture, the best commercial application of tissue culture technique has been in the production of true to type plants at a very rapid rate compared to conventional methods (Levy, 1981). According to Murashige (1974) there are three possible routes available for in vitro propagule multiplication namely, (i) enhanced release of axillary buds, (ii) production of adventitious shoots through organogenesis and (iii) somatic embryogenesis. Such plants are reported to grow faster and to mature earlier than seed propagated plants (Vasil and Vasil, 1980).

2.3 Factors influencing success of in vitro propagation

There are several factors reported to directly or indirectly affect the success of in vitro propagation. These factors include genotype, size, age and type of explant, surface sterilization, presence of systemic microbial contaminants, media used, hormones and their concentrations, presence or absence of other additives, nitrogen source and concentrations, physical condition of the medium, pH, quality and intensity of light, temperature, relative humidity etc. (Brown and Thorpe, 1986).

2.3.1 Genotype

Response of *in vitro* culture vary between plant species, and even in a single species it may vary between varieties. Genotype specific effects have been reported for *Anthurium andraeanum* and *A. schergerianum* (Pierik and Steegman, 1976). Welander (1978) reported that explants from three cultivars of *Begonia* x *Heimalis* differed in survival under *in vitro* culture.

2.3.2 Season of collecting the explants

Season of collecting the explant was also found to influence the success of plant tissue culture. Chauvin and Salesses (1988) reported that successful plantlet production was obtained with shoots taken during mid-May in the case of chestnut. For Litchi (*Litchi chinensis*) the test material taken after 10 continuous rainy days had a contamination rate of cent per cent and that taken after 15 continuous sunny days had a low contamination rate of 20 per cent (Yu, 1991). Season is a very important factor for mature tree tissue culture. Spring (March-April) was the best season to initiate tissue culture from mature trees. At least 95 per cent aseptic shoot cultures were obtained and buds flushed within 10 to 12 days as compared to 5 to 6 weeks during other seasons for *Corylus avellana* (Messeguer and Mele, 1987).

2.3.3 Age of the explant

The capacity to vegetatively propagate a tree, is associated with its juvenility. Generally, the more juvenile the specimen, the easier it is to propagate vegetatively. There is no clearly defined transition from the juvenile to the mature phase in most plants. Often some parts of the tree may be mature, or senescent, while other portions still display juvenile characteristics (Bonga, 1982). Meristematic apices, the centres of growth and organisation in plants, undergo changes when the plant matures. Therefore the tissues derived from these apices behave differently in young and old parts (Bonga, 1980; Hackett, 1980). If we are faced with a recalcitrant in vitro culture from which true to type vegetative propagation does not materialize, it probably is wise to first develop methods for vegetative propagation from highly juvenile material (Bonga, 1982).

Dormant vegetative buds present at the root shoot junction can be induced to grow out, and such tissues will be juvenile in nature. Its cells have low mitotic rate and low number of ribosomes (Bronchart and Nougarede, 1970) both of which may be significant in relation to the morphogenetic capacity of the tissue (Bonga, 1980). In vitro culture of such juvenile sprouts has resulted in clonal propagation of tree species like Sequoia (Ball, 1978). Shoot buds will form

naturally near the apex of roots of some species and have been induced in root cultures of other (Peterson, 1975).

Slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots has been discussed by several workers (Maarri *et al.*, 1987 in pear; Messeguer and Mele, 1987 and Rodriguez *et al.*, 1988 in *Corylus avellana*; and Rajmohan and Kumaran, 1988 in jackfruit). The transfer into soil of rooted plantlets is more critical in the micropropagation process of mature trees than with plantlets regenerated from seedlings in the case of sycamore maple (Hanus and Rohr, 1987).

2.3.4 Explant size and its position on the mother plant

The type of the explant varies with each plant species and the most suitable one should be determined for each species (Skirvin, 1980). Norton and Norton (1986) studied the effects of explant length (2.5 to 20.0 mm), axillary bud number (0 to 6), presence or absence of apex, and explant derivation (top, middle, or base of plant canopy) in the case of prunus and spirea. The number of shoots formed after four weeks increased with the explant length and decreased with the number of buds present. Explants taken from the top of the canopy produced most shoots, but removal of the apex did not affect the shoot number. Rahman and Blake (1988) observed in

jack that nodal explants gave more proliferation than shoot tips, though there was no significant difference between the two types of explants.

2.3.5 Surface sterilization

The explants collected may harbour a lot of micro-organisms which when inoculated onto a nutrient medium, contaminates the entire in vitro system. Hence the objective of surface sterilization is to remove all the micro-organisms present on the explant with minimum damage to the plant part. Sodium hypochlorite (0.5 to 2.0 per cent w/v), calcium hypochlorite (filtered 5 to 10 per cent w/v) and mercuric chloride (0.05 to 0.1 per cent w/v) are the most commonly used surface sterilants. Since the sterilants are toxic to plant cells, it is necessary to wash the tissue twice or thrice with sterile distilled water to ensure dilution of the chemical (Hu and Wang, 1983). Alcohol alone or in combination with other surface sterilants has been used for disinfection (Bonga, 1982).

Hu and Wang (1983) suggested magnetic stirring, ultrasonic vibration or keeping the soaked explants under vacuum to reduce the possibility of trapping air bubbles on the explant surface. Generally a drop of detergent is added

to the surface sterilant to reduce surface tension and to increase the wetability.

2.3.6 Presence of systemic contaminants

Contamination can be caused by bacteria, fungi, or viruses present on the surface of bark, glandular hairs at the nodes and internal tissues (Mathias and Anderson, 1987). Micro organisms present on the outer surface can be eliminated by surface sterilization treatments, but those existing within the internal tissues cannot be removed, and they cause latent contamination, which is a serious problem associated with woody plant tissue culture.

Existence of endophytic or systemic fungi in both reproductive parts, seeds, and older vegetative tissues, that survive surface disinfestation procedures, and produce severe contamination in cultures after explanting were reported by several workers in some woody plant species (Chipley and Heaton, 1971; Hanlin, 1971, Schroeder and Cole, 1977; Knox and Smith, 1980; Wood, 1982). Ten systemic micro organisms (bacteria and yeasts) were isolated from stem sections of ex vitro grown rubber plants (Wilson and Power, 1989).

To avoid latent contamination, we can go in for culturing plant parts free of endogeneous microorganisms. Meristem culture (Eliott, 1972; Galzy, 1972) was reported in

this respect. Growing stock plants under controlled conditions and regularly spraying the plants with systemic and contact fungicides can reduce or avoid the contamination problem to certain extent (Mallika et al., 1992).

Fungicides and antibiotics are also used either as surface sterilant or as medium additives. However, most of the systemic fungicides and some of the antibiotics inhibit growth of the plant cultures. Several workers have reported the use of various fungicides in cultures for reducing fungal contamination (Brown et al., 1982; Sheilds et al., 1984). Dodds and Roberts (1985) suggested to avoid the use of antibiotics for sterilization since they or their degradation products may be metabolized by plant tissues with unpredictable results.

Endophytic fungi associated with mature pecan nuts prevented successful, contaminant free in vitro cultures of embryo explants, even after the rigorous surface disinfection of the nuts and careful aseptic shelling. In this case explanting media with low water availability to suppress fungal growth were developed. A complete medium with 1.5 per cent agar provided control of contamination and encouraged subsequent regeneration from embryo explants (Obeidy and Smith, 1990).

2.3.7 Exudations from the explant

Establishment of *in vitro* cultures of several plant species, especially woody plants, is greatly hampered by the lethal browning of the explant and culture medium. During the course of growth and development *in vitro*, plant tissues not only deplete the nutrients that are furnished in the medium, but also release substances that can accumulate in the cultures. These substances such as phenols, may have profound physiological effects on the cultured tissues (Zaid, 1987). Polyphenols can be oxidized by peroxidases (Mayer and Harel, 1979), or polyphenol oxidases (Mayer and Harel, 1979; Hu and Wang, 1983). Oxidation products are known to be highly reactive and inhibit enzyme activity leading to the death of explant (Hu and Wang, 1983).

Polyphenol interference in culture establishment of different woody plant species was reported by several workers (Anderson, 1975; Lenartowicz and Millikan, 1977; Lloyd and McCown, 1980; Baleriola and Mullins, 1983; Zaid, 1987; Amin and Jaiswal, 1988). In clove, polyphenol interference has been reported by Mathew *et al*. (1987).

2.3.7.1 Methods to overcome polyphenol interference under *in vitro* conditions

2.3.7.1.1 Collection and preparation of explant

Etiolation of branches (Ballester *et al.*, 1989), or growing the stock plants in dark and exposing to one per cent irradiance (Marks and Simpson, 1990) or cold storage of cuttings (Dalal *et al.*, 1992) reduces the endogeneous polyphenol concentration in the plant material.

The degree of wounding during explant preparation can greatly affect the amount of exudate produced during explant establishment. Unwounded tissues as whole leaves, or embryos do not exude phenol and grow well (Reuveni and Kipinis, 1974). Lesser wounding or cutting with a sharp blade reduces the exudation (Ripley and Preece, 1986).

2.3.7.1.2 Pretreatments given to explants for reducing polyphenol exudation

Pretreatments like, soaking explants in water (Gupta *et al.*, 1980) or antioxidant solutions like ascorbic acid or citric acid (Gupta *et al.*, 1980; Zaid and Tisserat, 1983) or adsorbants like polyvenyl pyrrolidone (0.7 per cent) (Gupta *et al.*, 1980) or in a solution of antioxidant and sucrose (Gupta *et al.*, 1981) reduce polyphenol exudation and its

oxidation. Keeping explants under running tap water or agitation of explants with 0.5 per cent PVP in 2 per cent sucrose solution for 30 to 45 minutes at 100 rpm reduced phenolic exudation in guava *in vitro* culture (Amin and Jaiswal, 1988).

Leakage of polyphenols into the medium can be prevented by treating explants with Ca^{++} for apple (Baleriola and Mullins, 1983). Sealing the cut end of the explant with paraffin wax for preventing the polyphenol exudation was reported by Bhat and Chandel (1991) in the case of *Dioscorea alata* L. and banana.

2.3.7.1.3 Selection of basal medium

Reducing the salt concentration of the basal medium was found to be an effective method for reducing polyphenol exudation (Anderson, 1975; Chevre *et al.*, 1983). But Hildebrandt and Harney (1988) observed that increasing the salt concentration of the medium had little effect on the amount of the polyphenols released, until it reached five times that of normal MS medium.

2.3.7.1.4 Rapid transfer of explants into fresh medium

Anderson (1975) observed that the green portion of rhododendron shoot tips had to be transferred to a fresh

medium every three weeks to be kept alive. Similar results were reported by several other workers (Broome and Zimmerman, 1978; Somers et al., 1982).

2.3.7.1.5 Use of liquid medium

Ichihashi and Kako (1977) reported that the browning of cattleya shoot tip was most effectively controlled by addition of antioxidants into stationary liquid medium. However, the same antioxidants were not effective when incorporated into semi solid medium.

2.3.7.1.6 Use of media additives

Incorporation of antioxidants into the culture medium effectively controlled polyphenol interference in different crop plants (Ichihashi and Kako, 1977; Monaco et al., 1977; Hildebrandt and Harney, 1988). Activated charcoal has the ability to adsorb toxic metabolites released into the culture medium (Fridborg and Erikson, 1975). Addition of activated charcoal (1 to 2 per cent) or PVP (0.5 to 1.0 per cent) in the culture medium prevented accumulation of polyphenols in the culture medium and browning of the explants (Bajaj, 1978; Stevenson and Harris, 1980; Zaid and Tisserat, 1983). Adsorbants, along with phenol adsorb hormonal substances also. Inclusion of charcoal reduced the availability of growth hormones and was therefore necessary to apply an abnormally

high concentration of auxin (Tisserat, 1979; Zaid and Tisserat, 1983).

2.3.7.1.7 Selection of growth regulators and carbon source

Plant growth regulators play a major role in darkening the medium by oxidizing the phenols. Cytokinins are known to stimulate the synthesis of polyphenols (Bergman, 1964; Asahira and Nitsch, 1969). Increased secretion of polyphenols was also observed with application of NAA (Zagoskina and Zaprometov, 1979), or abscissic acid (Bagratishviti et al., 1984). Rodriguez (1982) reported that callus induced with 2,4-D and kinetin was incapable of organ formation possibly because of the increased production of polyphenols in the case of walnut.

Rabechault et al. (1976) reported a decreased browning when one per cent sucrose was included in the culture medium. Amorium et al. (1977) reported that increasing the exogeneous glucose level increased phenol synthesis of rose cells.

2.3.7.1.8 Selection of culture conditions

Reduction of light intensity was reported to be an effective method for reducing exudation (Forrest, 1969; Hu and Wang, 1983; Ziv and Halevy, 1983). Activity of enzymes concerned with both biosynthesis and oxidation of polyphenol

was increased by light (Davis, 1972). The release of polyphenols was less at 7°C than at 27°C in Pelargonium x hortum (Hildebrandt and Harney, 1988).

2.3.8 Culture medium

Selection of culture medium depends upon the plant species and purpose of cell, tissue or organ culture, we have resorted to. A wide variety of media have been reported. The earliest and widely used basal media were White's (1943) and Heller (1953). Since 1960, most researchers have been using MS (Murashige and Skoog, 1962) medium. Other derivatives of MS medium include B5 medium developed by Gamborge et al. (1968), Schenk and Hildebrandt (1972) medium and the woody plant medium developed by Lloyd and McCown (1980). The MS medium is characterised by high concentration of mineral salt, so some workers found it beneficial to reduce its strength by half (Skirvin, 1980; Griffis et al., 1981).

2.3.9 Plant growth regulators

Selection and addition of growth regulators at the optimum level is one of the crutial factor for a successful plant tissue culture (Krikorian, 1982). Commonly used growth regulators in tissue culture include three or four groups such as auxins, cytokinins, gibberellins and retardant like abscisic acid. BAP has been the most effective cytokinin for

meristem, shoot tip and bud cultures followed by kinetin (Murashige, 1974).

Cytokinin has been utilized to overcome the apical dominance of shoot to enhance the branching of lateral buds from leaf axils (Murashige, 1974). Lo et al. (1980) reported that a high content of cytokinin was deliterious to the initiation and elongation of roots of both monocotyledonous and dicotyledonous plants.

For axillary bud proliferation exogeneous auxin was not always needed. The young shoot apex has been described as an effective site for auxin biosynthesis (Hu and Wang, 1983). Although exogeneous auxin do not promote axillary shoot proliferation, culture growth has been improved by their presence (Wang and Hu, 1980). One of the possible role of auxin at elongation stage is to nullify the suppressive effect of high cytokinin concentration, there by restoring normal shoot growth (Lundergan and Janick, 1980). Too high a concentration of auxin induce callus formation (Hasegawa, 1980). For rooting of in vitro produced shoots auxins are used.

2.3.10 Carbon and energy sources

Sucrose is the most commonly used carbon energy source

for the plant tissue culture. Most of the workers have used 20 to 30 gl^{-1} sucrose in the medium. Glucose and fructose may be substituted in some cases, but most other sugars are reported to be very poor (George and Sherington, 1984). Marino et al. (1991) reported that shoot proliferation rate was increased with sorbitol as the carbon source than with sucrose in the case of apricot.

2.3.11 Other organic compounds

Conger (1981) reported the role of complex organic compounds for successful growth of tissues and organs. These include casein hydrolysate, coconut water, yeast, malt extract and fruit and vegetable juice. Role of these complex organic compounds are usually unpreditable and repeatability is also very poor, therefore it has been recommended to avoid their use as far as possible (Gamborg and Shyluk, 1981). Coconut water was reported to be promoting growth and differentiation in Datura embryos (Vanoverbeak et al., 1941). Adenine sulphate when added to the medium, often can enhance growth and shoot formation (Skoog and Tsui, 1948). The addition of phloroglucinol to the medium promoted the culture growth in Cinchona ledgeriana (Hunter, 1979).

2.3.12 Culture environment

The physical form of the medium whether liquid or

semi-solid, medium pH, other environmental factors like light, temperature, relative humidity and season of culture etc. play an important role in *in vitro* growth and differentiation.

Light requirement for differentiation involve a combination of several components, namely intensity, quality and duration (Murashige, 1974). According to Murashige (1977) the optimum day light period required is 16 hours for a wide range of plants. Yeoman (1986) reported that the environmental temperature of the species at the original habitat should be taken into consideration during the *in vitro* culture also. Relative humidity is rarely a problem except in arid climate, where rapid drying occurs. Hu and Wang (1983) reported that air humidity is infrequently controlled and when it is controlled, 70 per cent has been found to be the most frequent setting.

2.4 Rooting of *in vitro* produced shoots

In vitro produced shoots can be rooted either through *in vitro* methods itself or through *ex vitro* methods. There are three phases involved in rhizogenesis, namely induction, initiation and elongation. All cytokinins inhibit rooting and auxins usually favour induction of rooting. Among the different auxins, NAA has been the most effective one for root induction (Ancora *et al*., 1981). The concentration of hormone

required is often critical to provide sufficient stimulus to initiate roots while preventing the excessive formation of callus (Yeoman, 1986). A combination of auxins may give better response (Gupta et al., 1980; Mathew and Hariharan, 1990).

Regarding the salt concentration of the medium, usually a medium with low salt concentration promotes rooting (Hu and Wang, 1983). Abundant rooting was observed when the salt concentration in the medium was reduced to one half, one third, or one fourth of the standard strength (Kartha et al., 1974; Lane, 1979; Skirvin and Chu, 1979). For rooting stage sugar content of the medium is also lowered in most of the cases (Roy et al., 1990).

Activated charcoal has got profound influence on rooting of shoots in vitro. Activated charcoal may adsorb toxic substances in the medium there by improving root regeneration and development (Jaiswal and Amin, 1987). It may also adsorb residual cytokinin from the shoot and it also shades in vitro roots from light, which in high intensity may inhibit root growth (Hu and Wang, 1983).

Concentration of agar used for rooting varies from 0 (liquid medium) to 0.9 per cent. Liquid media facilitates the free diffusion of toxic plant wastes and when used with filter paper bridge system provides excellent aeration for root

development (Hu and Wang, 1983). Rooting of shoots in medium jelled with agar was also reported by Goh *et al*. (1988); D'Silva and D'Souza (1992).

Yeoman (1986) has advocated *ex vitro* rooting approach which may provide a simple, highly efficient and more economic methodology. *In vitro* produced shoots were transferred to pots containing a mixture of peat, vermiculite and sand in a ratio of 4:2:1. The shoots were maintained in a high humidity environment and watered daily. During the first two weeks, a water solution containing 15 M NAA was administered four times at equally spaced intervals to promote rooting. Twenty per cent of shoots rooted after eight weeks. Hunter (1979) found that cinchona shoots raised in proliferation media rooted best by a non-aseptic implantation into peat blocks rehydrated with one-tenth concentrtion of MS salts following the application of an ethanolic IBA or NAA dip at 75 or 100 mgl^{-1}.

2.5 Hardening and plant out

Acclimatization is crucial to any micropropagation scheme since shoot and plantlets produced *in vitro* must be readapted to the less humid environmental conditions, outside the culture vessels. Plants produced by tissue culture are generally more expensive than conventionally produced

seedlings. The transfer step is time consuming, labour intensive and may vary with the species or even variety. Changes in both structure and physiology of shoots occur during the period of adaptation.

Microcultured leaves are characterised by the absence or reduced amount of epicuticular wax in comparison to leaves of green house or field grown plants (Grout, 1975; Sutter and Langhans, 1979) which affects the rate of water loss from the leaves. As the humidity is gradually lowered during acclimatization, the density of wax on leaves increases (Wardle et al., 1983).

Kyte and Briggs (1979) found that a porous potting mixture of peat : perlite : composted bark (1:1:1) was the best for rooting tissue cultured rhododendrons. A period of humidity acclimatization was suggested for the newly transferred plantlets to make them adapted to the external environment (Hu and Wang, 1983). Barnes (1979) suggested the method of covering the transplanted plantlet with polythene cover to maintain high humidity. Nutrition of the plantlets during the acclimatization phase is species dependant. Success in acclimatization depends upon not only the post-transfer conditions but also the pre-transfer culture conditions (Ziv, 1986).

2.6 In vitro studies on clove

Attempts on clove tissue culture are rather limited mainly because of the problem of polyphenol interference which rapidly arrest the growth of the explants in vitro and also the slow growing nature of the plant. The published literature on tissue culture of clove, and other members of the family Myrtaceae are reviewed here.

Mathew et al. (1987) induced callus from axillary buds by culturing in MS medium supplemented with NAA, at 2 to 3.5 mgl^{-1} and 0.5 to 10 mgl^{-1} BAP. Medium MS supplemented with high NAA and also low BAP concentration promoted callus initiation, while low NAA and high BAP promoted further growth of callus but without differentiation.

Mathew and Hariharan (1990) induced multiple shoots from nodal segments of clove seedling cultured in half-MS medium WPM and B5 medium, supplemented with BAP, IAA, NAA, IBA and coconut water. Among the three auxins tested IAA proved superior to NAA and IBA, in the number of shoots produced. Nodal segments cultured in half-MS medium supplemented with BAP (0.8 to 3.0 mgl^{-1}), IAA (0.3 to 1.0 mgl^{-1}) and 15 per cent coconut water produced 10 to 15 buds. Shoot tips with axillary buds appeared like a rosette and many small buds of sizes ranging from 1.0 to 5.0 mm could be separated from the

cluster. These buds were subcultured to medium with low BAP (0.2 to 0.8 mgl^{-1}) to facilitate shoot development. Shoots of length 1.0 to 1.5 cm were transferred to rooting medium. Of the different auxins tested for rooting, a combination of NAA and IBA (0.5 to 2.0 mgl^{-1}) were found to be more effective. Small thick roots developed on the shoots after five weeks in culture. After the emergence of roots, the plantlets were transferred within one week to fresh White's medium without any hormones to facilitate the elongation of roots. On each shoot, 5 to 8 creamish brown healthy roots developed (Mathew and Hariharan, 1990).

Superman and Blake (1990) reported that nodal explants of clove seedling produced greater shoot proliferation than shoot tips. Woody plant medium was more suitable for shoot proliferation than half-MS medium.

2.7 *In vitro* studies in other members of Myrtaceae

2.7.1 Related species of clove

Choo *et al*. (1978) developed cultures from internodal sections of *Eugenia grandis* seedlings in a culture medium containing 2,4-D. Nodal segments and shoot tips produced roots and adventitious shoots, when cultured in modified MS medium. Litz (1984) developed adventitious embryos from two

polyembryonic species of Eugenia by culturing fruitlets in medium containing 2,4-D.

In vitro micropropagation of the tropical fruit tree Syzygium cumini L. has been reported by Yadav et al. (1990a). Multiple shoots were obtained from nodal and shoot tip segments of 10 to 15 days-old seedlings of Syzygium cumini L. on modified MS medium supplemented with BAP (0.23 to 8.90 μM) singly or in combination with NAA, IAA, or IBA. Nodal explants produced more shoots than shoot tip explants, with the greatest number of shoots from the explants on medium containing 4.5 μM BAP. Although numerous shoots were formed at different concentrations of NAA and BAP, their growth was inhibited, and only 1 to 2 shoots could be elongated. The shoots were first placed on auxin enriched rooting medium for two weeks and then transferred to MS basal medium. A combination of NAA (1.14 to 2.17 μM) and IAA (2.5 to 5.0 μM) showed a significant increase in root number, but roots grow only upto 0.5 cm in length.

2.7.2 Guava (Psidium guajava)

The cv. 'Banaras Local' of guava was propagated successfully by culturing nodal explants from mature trees on MS revised medium supplemented with 4.5 M BAP alone or in combination with either 0.6 M IAA, 0.5 M IBA or 0.3 μM GA_3.

A prior transfer of shoot clumps to a medium containing lower concentrations of BAP (0.5 μM) before harvesting cuttings for rooting allowed rapid extension of growth and increased the number of usable shoots per culture. Adventitious rooting also occurred on subculturing excised shoots to a medium containing half strength MS salts, 1.5 per cent sucrose, 1 μM each of IBA, NAA and 1.0 gl^{-1} activated charcoal. Regenerated plantlets were successfully established in soil (Amin and Jaiswal, 1987).

Amin and Jaiswal (1988) again reported the micro-propagation studies of another guava cultivar Chittidar. They used nodal explants of field-grown adult trees. Agitation of explants in 0.5 per cent polyvenyl pyrrolidone and 2 to 3 changes of medium for the initial 10 to 15 days were essential for establishment of cultures. On Murashige and Skoog revised medium containing 1 mgl^{-1} BAP, axillary buds grow out within 3 to 4 weeks. Shoots were rooted in half-MS medium containing either, 0.2 mgl^{-1} each of IBA and NAA and 1.0 gl^{-1} activated charcoal or 1.0 gl^{-1} activated charcoal alone.

Loh and Rao (1989) obtained multiple shoots from different parts of guava seedling and the nodal segments of grafted plants. In the case of seedling explants, best regeneration in terms of number of cultures forming shoots and the number of shoots per explant occurred in the presence of

0.01 mgl^{-1} BAP. Root differentiation occurred both in MS basal and MS + 2,4-D media (0.01 to 1.0 mgl^{-1}) after 3 to 6 weeks in culture. In the case of nodal segments from grafted plants, highest frequency of explants with shoots was obtained with medium containing 1.0 mgl^{-1} BAP and the highest number of shoots per explant developed in medium with 0.2 mgl^{-1} BAP. At higher concentrations of BAP, shoots were stunted and they elongated upon transferring to a lower BAP concentration. In about 5 per cent of the regenerated shoots derived from seedling explants protuberances appeared spontaneously on intact leaves of elongated shoots growing on media supplemented with BAP (0.1 to 5.0 mgl^{-1}). Nearly all the protuberances grow into shoots by subculturing the leaves. More than 90 per cent of the plants survived transplantation to soil. Papadatou et al. (1990) has reported the micro-propagation of guava using seedling explants.

2.7.3 Eucalyptus

Morphogenesis and plant regeneration from cotyledonary cultures of Eucalyptus was reported by Lakshmisita (1979). Callus cultures were established from hypocotyl and cotyledons derived from young seedlings of Eucalyptus citriodora. Successful plantlet production from cotyledonary callus was achieved within 6 weeks on MS basal medium supplemented with zeatin (1.0 mgl^{-1}) and IAA (0.2 mgl^{-1}). Leaf and shoot callus

obtained from one year old plants did not differentiate. Micropropagation of Eucalyptus gunni and E. staurtiana have been reported by Curir et al. (1986) using seedling explants.

Nodes from field-grown coppice regrowth and mature trees of eucalyptus when cultured on a modified MS medium showed axillary shoot proliferation with BAP (2.0 to 4.0 μM) and NAA (0.5 to 1.0 μM). IBA (10 μM) was more effective than NAA (10 M) in stimulating the formation of adventitious roots (Burger, 1987). According to Damiano et al. (1989) a pre-rooting step involving four weeks in a medium containing kinetin or zeatin was required to avoid the inhibitory effect of BAP on root induction.

A highly embryogenic culture of Eucalyptus citriodora was obtained by repeated embryogenesis from somatic embryos cultured in the dark on a medium containing 500 mgl^{-1} each of glutamine and casein hydrolysate, 30 gl^{-1} sucrose and 5 mgl^{-1} NAA. When incubated in light on a hormone-free medium, 50 per cent of the embryos produced plantlets of which 70 per cent survived transfer to a sand and soil mixture (Muralidharan et al., 1989).

2.7.4 Callistemon and Melaleuca

Species in these genera and also Leptospermum are of interest to ornamental horticulture for their attractive

flowers. Melaleuca has been micropropagated with ease using standard methods of preparation (Shipton and Jackes, 1986).

2.7.5 Verticordia

Micropropagation of verticordia was reported by deFossard and deFossard (1988). McComb et al. (1986) have reported a high level of success in the rooting stage. They subcultured the shoots to the multiplication medium and after one week incubation in constant light at 25°C, the culture tubes were placed in dark for two weeks to etiolate shoots. The etiolated shoots were then placed on their rooting medium (10 μM IBA) in the light. They reported that 58 per cent of cultures rooted as compared with 24 per cent for cultures from non-etiolated cultures.

Materials and Methods

3. MATERIALS AND METHODS

The present study was carried out at the tissue culture laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Kerala Agricultural University, Vellanikkara, Thrissur during March 1992 to February 1994, with an objective to standardise the *in vitro* propagation technique in clove *Syzygium aromaticum* ((L.) Merr. and Perry).

3.1 Materials

3.1.1 Chemicals

Chemicals used for the preparation of various media were procured from British Drug House (BDH), SISCO Research Laboratory (SRL), Merck or Sigma.

3.1.2 Glasswares

Borosilicate glasswares of corning/borosil brand were used for the experiment. They were cleaned by initially boiling for half an hour, after cooling they were thoroughly washed with the detergent solution, then rinsed with potassium dichromate solution in sulphuric acid, washed using tap water and finally rinsed with distilled water. Washed glasswares were dried in hot air oven at 60°C, and were stored away from dust and contaminants.

3.1.3 Culture medium

3.1.3.1 Composition of media

Murashige and Skoog's (MS) medium, (Murashige and Skoog, 1962), Schenk and Hildebrandt (SH) medium (1972), B5 medium (Gamborg et al., 1968), woody plant medium (WPM) (Lloyd and McCown, 1980) and White's medium (Whites, 1943) were used as basal media in the present study. Composition of these media are given in Table 1. The basal media were supplemented with different levels of cytokinins, auxins, vitamins, casein hydrolysate, aminoacids, phloroglucinol, coconut water etc. in different experiments.

3.2 Preparation of medium

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

Specific quantities of the stock solution of chemicals

Table 1. Composition of various basal media tried for *in vitro* culture of clove

Ingredients mgl^{-1}	MS	WPM	B5	SH	White's
Inorganic constituents					
$(NH_4)\ NO_3$	1,650	400			
$(NH_4)_2\ SO_4$			134		
$(NH_4)\ H_2PO_4$				300	
KNO_3	1,900		2,500	2,500	80
K_2SO_4		990			
KH_2PO_4	170	170			
KCl					65
$Ca(NO_3)_2.4H_2O$		556			288
$CaCl_2.2H_2O$	440	96	150	200	
$MgSO_4.7H_2O$	370	370	250	400	737
$Na_2SO_4.10H_2O$					460
$NaH_2PO_4.H_2O$			150		19
$FeSO_4,7H_2O$	27.8	27.8	27.8	15	27.8
Na_2EDTA	37.3	37.3	37.3	20	2.67
$MnSO_4.4H_2O$	22.3	22.3			1.5
$MnSO_4.H_2O$			10	10	0.75
$ZnSO_4.7H_2O$	8.6	8.6	2.0	1.0	
H_3BO_3	6.2	6.2	3.0	5.0	

Contd.

Table 1 (Contd.)

Ingredients mgl^{-1}	MS	WPM	B5	SH	White's
KI	0.83		0.75	1.0	
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.1	
MoO_3					0.0001
$CuSO_4.5H_2O$	0.025	0.25	0.025	0.2	0.001
$CoCl_2.6H_2O$	0.025		0.025	0.1	
Organic constituents					
Myo-inositol	100	100	100	1,000	
Nicotinic acid	0.5	0.5	1.0	5.0	0.5
Pyridoxine HCl	0.5	0.5	1.0	0.5	0.1
Thiamine HCl	0.1	1.0	10.0	5.0	0.1
Glycine	2.0	2.0			3.0
Sucrose	30,000	30,000	20,000	30,000	20,000

were pipetted out into a beaker. Sucrose and inositol were added fresh and dissolved, and the volume was made upto the required level using distilled water. The pH of the solution was adjusted between 5.6 to 5.8 using 1.0 N NaOH or 0.1 N HCl. For preparing semi-solid medium, good quality agar was added at 0.75 per cent level and the medium was boiled till a clean solution was obtained. In case of liquid medium, agar was avoided and filter paper bridges were provided to support the explants. About 15 ml medium was poured into the culture tubes (15x2.5 cm or 20x2.5 cm sizes). The tubes were then plugged with non absorbant cotton and autoclaved at 121°C and 15 psi (1.06 kg/cm^2) for 20 minutes (Dodds and Roberts, 1982). The medium was allowed to cool to room temperature and stored in cool, dry place.

3.3 Source of explants

The explant sources used for the study included field grown mature trees, in vitro germinated seedlings and nursery grown seedlings maintained in a glass house.

3.3.1 Mature field grown tree

Explants were collected from mature trees planted at the Instructional farm, Vellanikkara and also from plants grown in the coconut plantation under the Department of Plantation Crops and Spices, College of Horticulture,

Vellanikkara. In order to reduce the fungal contamination in cultures, prophylactic sprays were given to the mother trees with fungicides like dithane M-45 (0.1 per cent), bavistin (0.1 per cent), and aureofungin (160 mgl^{-1}) at three days interval. Fungicides were selected according to the nature of contamination and response of the fungus.

3.3.2 *In vitro* seedlings

Mature clove seeds ('Mother-of-cloves') were procured from Brindavan estate, Kanyakumari during July-August season. Seeds obtained were soaked in tap water for 24 hours. After this presoaking, the seeds as such were surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) for one hour. They were then dehulled and the exposed seeds were surface sterilized with $HgCl_2$ (0.1 per cent) for 8 minutes in a laminar air flow cabinet. The seeds were washed free of $HgCl_2$ by rinsing with sterile distilled water for three times and were allowed to drain out over blotting paper. These seeds were implanted to half-MS basal medium containing 3 per cent sucrose and vermiculite-sand (1:1) medium wetted with sterile distilled water.

The cultures were incubated at 26 $\pm$ 1°C and kept under both darkness and light condition (16 hour light period) of 1000 lux intensity per day. The cultures kept under dark

condition were transferred to illuminated condition after the full emergence and growth of the radicle. Various explants like nodal segments, internodal segments, and leaf bits were taken from the seedlings, 15 to 20 days after germination. Explants from *in vitro* seedlings raised in vermiculite-sand medium were again surface sterilized with $HgCl_2$ (0.1 per cent) for three minutes before inoculation.

3.3.3 Nursery grown seedlings

Mother of cloves after soaking in water were dehulled and sown in earthen pots and polythene bags filled with river sand. These bags were kept in glass house condition and watered regularly. Explants like nodal, internodal segments, leaf segments and apical shoots were collected 10 to 15 days after germination.

3.4 Preparation of explants

Stem segments of approximately 10 to 15 cm size with 3 to 4 nodes obtained from mature trees and those of length 5.0 to 7.0 cm length from seedlings were carefully excised using surgical blades and brought to the laboratory as quickly as possible. The leaves were removed and the nodal and internodal segments were cut into a length of 2.0 to 2.5 cm. Nodal segments with petiole attached and detached were used. For leaf explants leaves were cut into 4 x 8 mm size. All the

explants were thoroughly washed in tap water with few drops of detergent (Teepol).

Explants collected from mature trees were given a presoaking treatment with different fungicides. Fungicides used, their concentration and the duration of treatments are presented in Table 2. Proceeding to the presoaking treatment they were thoroughly washed with tap water and excess water was allowed to drain off. Explants from seedlings were not treated with the fungicides.

3.5 Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of explants, preparation and inoculation of explants and subsequent subculturing were carried out under the hood of a clean laminar air flow chamber (Thermadyne). The working table of the laminar air flow chamber was first surface sterilized with absolute alcohol and then by putting on the ultraviolet light for 20 to 30 minutes. The petridishes as well as the inoculation aids were first steam sterilized and then flame sterilized before each inoculation. The hands were also scrubbed with absolute alcohol before inoculation.

Table 2. Presoaking treatments given to the explants collected from mature field grown clove trees

Fungicide used	Concentration (per cent)	Duration of treatment (minutes)
Aureofungin Sol	0.125	60
	0.25	60
	0.50	60
Bavistin	0.25	60
	0.50	60
	1.0	30
Aureofungin + Ascorbic acid	0.50 + 100 mgl^{-1}	60
Aureofungin followed by Bavistin	0.50 and 0.25	30 each
Aureofungin followed by Bavistin	0.50 each	30 each

3.6 Culture establishment

3.6.1 Surface sterilization

Surface sterilization was carried out under perfect aseptic conditions in the laminar air flow chamber. The washed explants were put into the sterilant, and kept immersed for the required period and were continuously agitated manually. Explants from glass house grown seedlings were surface sterilized in 0.1 per cent $HgCl_2$ for 5 to 10 minutes. The different sterilization treatments tried for field explants are listed in Table 3. The explants after surface sterilization, were rinsed thrice with sterile distilled water to remove traces of the sterilant from the surface.

3.6.2 Treatments to avoid latent microbial contamination

Despite the prophylactic spray treatments, the field grown explants were found to harbour endogeneous micro-organisms and the organism was identified by microbiological tests. Several treatments were followed for preventing the contamination caused by the endogeneous fungus. Details are given in Table 4. Observations were recorded for the contamination rate, browning of explants, survival and bud break.

Table 3. Different surface sterilization treatments carried out for the nodal explants collected from mature field trees of clove

Treatment	Concentration (per cent)	Duration (minutes)
$HgCl_2$ soaking	0.1	8 10 12 15 20
$HgCl_2$	0.5	4
Double sterilization with $HgCl_2$	0.1	5 each
$HgCl_2$ + Teepol 2 drops	0.1	15
Chlorine water soaking - $HgCl_2$ soaking	3.5,0.5	4, 5
Alcohol soaking $HgCl_2$ soaking	100,0.5 100,0.5 100,0.1 100,0.1 70, 0.1 50, 0.1	2, 4 2, 5 1, 15 2, 12 2, 12 2, 12
Alcohol wiping - Alcohol soaking - $HgCl_2$ soaking	70,100,0.1 resp.	-,1,15
Alcohol soaking - Chlorine water soaking - $HgCl_2$ soaking	100,3.5,0.5	2,4,6 2,3,4 2,2,4 2,1,3

Table 4. Treatments undertaken for preventing the latent microbial contamination of mature clove explants

Treatment	Chemical used and concentration	
Mother plant spraying at three days interval	Dithane M.45	0.1 per cent
	Bavistin	0.1 per cent
	Aureofungin	160 mgl^{-1}
Treatments given to explants	Presoaking treatments (given in Table 2)	
Addition of fungicides as medium supplements	Aureofungin (filter-sterilized)	8 mgl^{-1}
		20 mgl^{-1}
		40 mgl^{-1}
		50 mgl^{-1}
		150 mgl^{-1}
		250 mgl^{-1}
	Bavistin (heat sterilized)	1 per cent
		2 per cent
Providing moisture stress to the explants	Agar	0.75 per cent
		1.50 per cent

3.6.3 Standardisation of pre-treatments to overcome polyphenol interference

Both vegetative and reproductive tissue of clove contain a wide variety of polyphenols. Since they were found to interfere in the *in vitro* cultures of mature field explants, response of explants in the five different basal media such as MS, half-MS, WPM, B5, SH and White's medium were assessed. The best basal medium was selected and various treatments were tried to overcome the interference. Details are given in Table 5. All the treatments were kept both under dark and light conditions, to study the effect of light on polyphenol interference. Intensity of polyphenol exudation was recorded based on the media discolouration 15 days after incubation. Effect of different explants, pretreatments, basal media and media combinations were assessed.

Numerical scores were given to quantify the polyphenol exudation. Cultures in which the medium was clear, 15 days after incubation, given zero score. Those cultures in which half the volume of the media turned dark brown within 15 days were scored as 'six'. The others were scored in between 'zero' and 'six' according to the extent of discolouration. Media scoring was difficult in culture medium supplemented with activated charcoal, percentage of survival (after one month) alone was recorded in such cases.

Table 5. Standardisation of treatments for preventing polyphenol exudation and browning of the mature clove explants

I. Pretreatments given to the explant

A. Soaking explants for four hours in

a. tap water alone (control)

b. under running tap water

c. tap water and shaking in an orbital shaker at 100 rpm

d. 100 mgl^{-1} L-ascorbic acid

e. PVP (1.0 per cent) solution

f. 100 mgl^{-1} L-ascorbic acid and 2 per cent sucrose

B. Sealing the cut end of the explant with paraffin wax

II. Use of media additives

g. PVP (0.5 per cent)

h. Activated charcoal (0.5 per cent)

i. Ascorbic acid (100 mgl^{-1})

j. Citric acid (100 mgl^{-1})

k. Citric acid + ascorbic acid (100 mgl^{-1} each)

l. L-cysteine-Hydrochloride (100 mgl^{-1})

III. Combinations of pretreatments and media additives

m. Treatment (d) and (g)

n. Treatment (d) and (h)

o. Treatment (e) and (g)

p. Treatment (e) and (h)

In the case of seedling explants, polyphenol exudation was minimum, and did not interfere with culture establishment.

3.6.4 Culture conditions

The cultures were incubated at 26 ± 1°C in an air conditioned culture room with 16 h photoperiod (1000 lux) supplied by cool day light fluorescent tubes unless, otherwise mentioned in separate experiments. Humidity in the culture room varied between 60 to 80 per cent according to the climate prevailed.

3.7 Direct organogenesis

3.7.1 Shoot induction

3.7.1.1 Standardisation of basal medium

Studies were conducted to determine the most suited basal medium. Different basal media such as MS, half-MS, WPM, SH, B5 and White's medium supplemented with 3.0 mgl^{-1} BAP and 1.0 mgl^{-1} IAA were used for the initial culture, and from the first subculture onwards, growth regulator combination was changed to 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} IAA for all the basal media.

3.7.1.2 Effect of medium supplements

Studies were conducted to determine the effect of

various growth regulators and other medium additives on shoot induction, growth and multiple shoot production from axillary buds, apical shoots and internodal and leaf segments of both seedling and mature clove plant. Details of treatments conducted using modified MS medium and WPM are presented in Table 6 and Table 7. Surviving cultures were subcultured at an interval of 3 to 4 weeks to the very same medium for a total period of four months. The response of cultures in each subculture was observed and recorded. Survival rate in each subculture, number of leaves, number of shoots and shoot length were recorded.

3.7.2 Shoot elongation

Multiple shoots produced in the proliferation medium showed a rosette appearance and so they were subcultured to different combinations of media for shoot elongation. Carbon sources, inorganic nutrients, growth regulators, amino acids, and physical condition of the medium were altered for obtaining shoot elongation. Effect of activated charcoal on shoot elongation was also assessed. Details of treatment combinations are given in Table 8. Observations were recorded on the increase in length of shoots and number of well developed leaves formed, one month after incubation.

Table 6. Standardisation of medium supplements for multiple shoot induction from mature clove explants

Medium	Growth regulators used
MS	BAP 3.0 mgl^{-1} and kenitin 1.0 mgl^{-1}
	3x2 combinations of BAP (1.0, 1.5, 2.0 and mgl^{-1}) and NAA (0.1, 0.5 mgl^{-1})
	10x1 combination of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mgl^{-1}) and NAA (0.2 mgl^{-1})
Half-MS	BAP at 1.0, 2.0, 2.5 mgl^{-1} alone
	5x3 combinations of BAP (1, 2, 3, 4, 5 mgl^{-1}) and kinetin (0.5, 1.0, 2.0 mgl^{-1})
	10x1 combinations of BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 mgl^{-1}) and NAA (0.1 mgl^{-1})
	8x1 combinations of BAP (1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mgl^{-1}) and IAA (0.5 mgl^{-1})
	1x2x1 combinations of BAP (1.0 mgl^{-1}) kinetin (2.0, 2.5 mgl^{-1}) and IAA (0.5 mgl^{-1})
	4x1 combinations of kinetin (1.0, 2.0, 2.5, 3.0 mgl^{-1}) and IAA (0.5 mgl^{-1})
1/4-MS	16x1 combination of BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 mgl^{-1}) and NAA (0.1 mgl^{-1})
WPM	BAP (3.0 mgl^{-1}) and kinetin (1 mgl^{-1})
	6x1 combination of BAP (0.5, 0.8, 1.0, 1.5, 2.0, 2.5 mgl^{-1}) and NAA (0.2 mgl^{-1})

Table 7. Standardisation of medium supplements for multiple shoot induction and proliferation from clove seedling explants

Basal medium	Medium supplements
Half-MS	12x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 mgl^{-1}) and IAA (0.5 mgl^{-1})
	5x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) and NAA (0.5 mgl^{-1})
	7x1x1 combinations of BAP (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 mgl^{-1}, IAA (0.5 mgl^{-1}) and kinetin (1.0 mgl^{-1})
	4x1x1 combinations of kinetin (0.25, 0.5, 1.0, 2.0 mgl^{-1}) BAP (2.0 mgl^{-1}) and IAA (1.0 mgl^{-1})
	5x2x1 combinations of BAP (1.0, 1.5, 2.0, 2.5, 3.0 mgl^{-1}) IAA (0.5, 1.0 mgl^{-1}) and C.W (15 per cent)
	5x2x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) NAA (0.1, 0.5 mgl^{-1}) and C.W. (15 per cent)
WPM	BAP (0.2, 0.8 mgl^{-1}) alone
	5x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) and Kinetin (1.0 mgl^{-1})
	2x1 combinations of BAP (0.4, 0.8 mgl^{-1}) and IAA (1.0 mgl^{-1})
	5x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) IAA (0.5 mgl^{-1})
	7x1 combinations of BAP (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10 mgl^{-1}) and IAA (1.0 mgl^{-1})
	5x1x1 combinations of BAP (0, 0.5, 1.0, 2.0, 3.0 mgl^{-1}) Kinetin (1.0 mgl^{-1}) and IAA (1.0 mgl^{-1})
WPM + BAP and kinetin 1.0 mgl^{-1} each	: Adenine sulphate (25, 50, 100 mgl^{-1}) : Phloroglucinal (50, 100, 200 mgl^{-1})

Table 8. Treatments tried the elongation of multiple shoots induced in clove

Basal medium	Medium supplements
A. Carbon source	
WPM + 0.2 mgl^{-1} BAP	Sucrose (2.0, 5.0 per cent)
	Glucose (2.0, 3.0 per cent)
	Maltose (3.0 per cent)
B. Inorganic nutrients	
WPM + 0.2 mgl^{-1} BAP	NA_4 (NO_3) (410 mgl^{-1})
	$Ca(NO_3)_3$ $4H_2O$ (440 mgl^{-1})
	$MgSO_4.7H_2O$ (370 mgl^{-1})
C. Amino acids	
WPM + 0.2 mgl^{-1} BAP	Leucine (1.0, 2.0 mgl^{-1})
	Lysine (1.0, 2.0 mgl^{-1})
	Arginine (1.0, 2.0 mgl^{-1})
	Tryptophan (1.0 mgl^{-1})
D. Activated charcoal	
WPM + BAP (0.2, 0.3 mgl^{-1})	Activated charcoal (0.1, 0.3 per cent)
Half-MS + BAP (0.2, 0.5 mgl^{-1})	Activated charcoal (0.1 and 0.5 per cent

Contd.

Table 8 (Contd.)

Basal medium	Medium supplements
E. Growth regulators	
WPM	BAP (0.2, 0.3, 0.8 mgl^{-1}) alone
	2x1 combination of BAP (0.4, 0.8 mgl^{-1}) and IAA (1.0 mgl^{-1})
	BAP (0.2 mgl^{-1}) and GA_3 (5.0 mgl^{-1})
	BAP (0.2 mgl^{-1}) GA_3 (10, mgl^{-1}) and IAA (1.0 mgl^{-1})
	GA_3 (10, 16 mgl^{-1}) alone
½MS	BAP (0.2, 0.3 mgl^{-1}) alone
	2x2 combinations of BAP (0.2, 0.8 mgl^{-1}) and IAA (1.0, 2.0 mgl^{-1})
	2x1 combinations of GA_3 (5.0, 10.0 mgl^{-1}) and BAP (0.2 mgl^{-1})
F. Physical condition of the medium	
½MS + BAP (0.2, 0.3 mgl^{-1})	Liquid phase and double phase
½MS+BAP(0.2 mgl^{-1}) and GA_3 (2.0 mgl^{-1})	Liquid phase
a. ½MS + BAP (0.3 mgl^{-1})	Double phase (Solid a + liquid b)
b. ½MS + BAP (0.2 mgl^{-1})	
a. ½MS + BAP (0.3 mgl^{-1})	Double phase (Solid a + liquid b)
b. ½MS + BAP (0.3 mgl^{-1}) and GA_3 (2.0 mgl^{-1})	

3.7.3 Root induction

Shoots of size 1.0 to 1.5 cm excised from the elongated shoot cultures and nodal segments were subjected for in vitro rooting. Shoots were either given a pulse treatment by dipping in auxin solutions of higher concentrations or by culturing in rooting medium containing low levels of auxin. Activated charcoal was also used in the rooting medium. Details are presented in Table 9. Observations on induction of rooting was recorded at monthly intervals.

3.8 Indirect organogenesis

Callus cultures of clove were derived from tissues of seedling origin. The methods applied for the initiation of callus and subsequent induction of morphogenesis, have been more or less the same for all types of seedling explants. Various combinations of growth substances and physical factors were tried to achieve the desired results.

3.8.1 Explant selection and surface sterilization

Explants such as nodal, internodal and leaf segments from both seedling and mature clove plant and cotyledon segments of seeds were used to initiate callus cultures in clove. Surface sterilization of explants was carried out using 0.1 per cent $HgCl_2$ for 10 minutes.

Table 9. Trials on rooting of *in vitro* produced shoots of clove seedling

A. Medium supplements

Basal medium	Medium supplement used (mgl^{-1})
WPM	NAA 0.5 and IBA 0.5
	NAA 1.0 and IBA 1.0
	NAA 2.0 and IBA 2.0
	NAA 6.0
	IBA 3.0
	IAA 0.5
½WPM + 2 per cent sucrose	NAA 1.0 ppm and IBA 0.5
	NAA 1.0 + IBA 0.5 and IAA 0.5
	NAA 2.0 + IBA 0.5 + 0.1 per cent AC
	0 level of growth regulators
	NAA 1.0 + IBA 1.0 + IAA 0.5 + 0.1 per cent AC
+½MS liquid	IBA (0.5) and NAA (0.5)
	IBA (1.0) and NAA (1.0)

B. Pulse treatment given to the *in vitro* produced shoots of *Syzygium aromaticum*

Growth regulator	Duration of treatment (minutes)
NAA 1000 mgl^{-1} (prepared in alcohol)	1
NAA 1000 mgl^{-1} (autoclaved solution)	1½ and 2
IBA 1000 mgl^{-1} (autoclaved solution)	1 and 2

3.8.2 Effect of medium supplements in callus induction

Murashige and Skoog's medium was used at full strength and half strength salt concentration supplemented with different levels of growth regulators, and casein hydrolysate as shown in Table 10. Cultures were kept both under dark and light condition, for the first time. Since darkness was found to favour callus induction, later on all the cultures were incubated in dark condition. The relative performance of different explants for callus induction and proliferation was observed. Observations were recorded for callus induction, growth rate and morphology. Callus index (CI) was worked out as below.

$$CI = P \times G$$

Where P is the percentage of callus initiation and G is the growth score. Scoring was done based on the spread of calli and a maximum score of four was given for those that have occupied the whole surface of the media, 3 months after incubation.

3.8.3 Effect of medium supplements on organogenesis/ embryogenesis from the callus

Calli obtained from leaf segments, and internodal segments were subcultured to the very same medium at 3 to 4

Table 10. Standardisation of medium supplements for the induction of callus/somatic embryoids from clove explants

Medium	Medium supplements
MS	2,4-D (0.5, 1.0, 2.0, 3.0, 4.0, 5.0 mgl^{-1})
½MS	2,4-D, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 mgl^{-1}
	2,4-D 2 mgl^{-1} + Casein hydrolysate 100 mgl^{-1}
	2,4-D 3 mgl^{-1} + Casein hydrolysate 50 mgl^{-1}
	NAA 2, 3, 4, 5, 6, 8 mgl^{-1}
	3x3 combinations of BAP (1.0, 2.0, 3.0 mgl^{-1} and IAA (0.5 mgl^{-1})

Table 11. Standardisation of medium supplements for organogenesis from leaf callus of clove seedling

Medium	Medium supplements
WPM	3x1 combinations of BAP (1.0, 2.0, 3.0 mgl^{-1}) and kinetin (1.0 mgl^{-1})
	2x1 combinations of BAP (1.0, 2.0, mgl^{-1}) and IAA (5.0 mgl^{-1})
	2x1x1 combinations of BAP (2.0, 3.0 mgl^{-1}) and kinetin (1.0 mgl^{-1}) and IAA (1.00 mgl^{-1})
	2x1 combinations of leucine (1.0, 2.0 mgl^{-1}) and BAP (0.2 mgl^{-1})
	2x1 combinations of Lysine (1.0, 2.0 mgl^{-1}) and BAP (0.2 mgl^{-1})
	2x1 combinations of Arginine (1.0, 2.0 mgl^{-1}) and BAP (0.2 mgl^{-1})
	1x1 combinations of Tryptophan (1.0 mgl^{-1}) and BAP (0.2 mgl^{-1})
Half-MS	2x1 combinations of BAP (2.0, 3.0 mgl^{-1}) and NAA (0.5 mgl^{-1})
	8x1 combinations of BAP (0.5, 1.0, 2.0, 3.0, 4.0, 7.0, 8.0, 9.0 mgl^{-1}) and IAA (0.5 mgl^{-1})
	4x1 combinations of kinetin (0.25, 0.5, 1.0, 2.0 mgl^{-1}), BAP (1.0 mgl^{-1}) and IAA (1.0 mgl^{-1})
	1x1 combinations of BAP (2.0 mgl^{-1}) and IAA (2.0 mgl^{-1})
	3x1x1 combinations of $AgNO_3$ (2.5, 5.0, 10.0 mgl^{-1}), BAP (3 mgl^{-1}) and kinetin (1 mgl^{-1})

weeks intervals, and cultured in dark condition for the induction of somatic embryoids from the callus. Treatments were also undertaken for organogenesis from the callus, and were subcultured to different combinations of auxins, cytokinins, and other additives for shoot induction. Details are presented in Table 11. The response of the calli was observed and recorded at fortnightly intervals.

Results

4. RESULTS

The results of various experiments carried out for the standardisation of *in vitro* propagation technique in clove *Syzygium aromaticum* ((L) Merr. and Perry) are presented in detail below.

4.1. Culture establishment

4.1.1 Mature field explants

4.1.1.1 Surface sterilization

The mature field explants harboured a lot of micro-organisms. Hence various surface sterilants were tried, and their effects are presented in Table 12a and b. Internodal and leaf segments were effectively surface sterilized by soaking in $HgCl_2$ (0.1 per cent) for 12 minutes. Whereas none of the treatments were found effective for reducing the microbial interference of nodal explants with their survival. Eventhough higher doses of sterilants greatly reduced the microbial interference, it resulted in browning and death of the nodal explants.

Table 12a. Effect of $HgCl_2$ as surface sterilant on culture establishment of internodal and leaf segments of field grown clove tree

Treatment	Duration of treatment (minutes)	Culture establishment 3 weeks after incubation		
		Contaminated cultures (%)	Uncontaminated cultures (%)	
			Living	Dried
0.1 % $HgCl_2$	8	52	48	Nil
	10	36	64	Nil
	12	10	90	Nil
	15	Nil	82	18

Table 12b. Effect of surface sterilants on culture establishment of nodal explants* from field grown clove tree

Surface sterilant	Concentration (%)	Duration of treatment (minutes)	Culture establishment 3 weeks after incubation			
			Contaminated cultures		Uncontaminated cultures	
			Living (%)	Dried (%)	Living (%)	Dried (%)
$HgCl_2$	0.1	8	100	Nil	Nil	0
		10	100	Nil	Nil	0
		12	100	Nil	Nil	0
		15	100	Nil	Nil	0
		20	88	Nil	Nil	12
Double sterilization with $HgCl_2$	0.1	5 each	100	Nil	Nil	0
$HgCl_2$ + Teepol 2 drops	0.1	15	100	Nil	Nil	0
Chlorine water - $HgCl_2$	3.5,0.5	4,5	0.0	Nil	Nil	100
Alcohol - $HgCl_2$	100,0.1	1,15	100	Nil	Nil	0
	100,0.1	2,12	60	Nil	Nil	40
	70,0.1	2,12	100	Nil	Nil	0
	50,0.1	2,12	99	Nil	1.0	0
	100,0.5	2,4	52	Nil	Nil	48
	100,0.5	2,5	28	Nil	Nil	72
Alcohol wiping - Alcohol - $HgCl_2$	70,100,0.1	-,1,15	100	Nil	Nil	0
Alcohol - Chlorine water - $HgCl_2$	100,3.5,0.5	2,4,6	20	Nil	Nil	80
		2,3,4	68	Nil	Nil	32
		2,2,4	100	Nil	Nil	0
		2,1,3	100	Nil	Nil	0

* Number of explants used was 100

4.1.1.2 Effect of treatments undertaken for reducing systemic microbial contamination

Despite the surface sterilization treatments, fungal mycelia were found extruding from the petiole detached portion of nodal explant and shoot tips (Plate 1). Mycelium was allowed to sporulate and identified as Alternaria sp. by the following characters. Conidia observed were rather large and multicellular with both transverse and longitudinal septa occurring typically. Conidia were borne acropetally in chains. Conidiophores were indistinguishable from the somatic hyphae (Alexopaulos and Mims, 1979).

4.1.1.2.1 Effect of prophylactic spraying given to the source plant

The data on the effect of prophylactic spraying given to the mother plant for reducing systemic microbial contamination is presented in Table 13. All the three fungicides were found to be ineffective in reducing systemic contamination of the explants collected within 8 months after the initiation of spray schedule. Explants collected 8 to 9 months after the cessation of spray schedule gave 56 percentage of uncontaminated cultures.

4.1.1.2.2 Effect of presoaking treatments given to field explants

Presoaking of explants was carried out with different types of fungicides to overcome the systemic microbial contamination. Results of the treatments are tabulated in Table 14. None of the treatments were found effective in reducing systemic fungal contamination. Irrespective of the concentration of the fungicide, or duration of treatment, severe browning of explants was noticed. To avoid this browning ascorbic acid 100 mgl^{-1} was incorporated into the fungicide solution, but showed no favourable results. Petiole detached explants showed more browning than explants having petiole. Certain treatments such as soaking in aureofungin 0.25 and 0.50 per cent resulted only in 12 and 8 percentage of explant browning, but all the survived explants were lost due to contamination. Thus it was clear that if the explants remained alive (green) they showed fungal contamination.

4.1.1.2.3 Effect of fungicides as medium supplements in reducing systemic contamination

Effect of two different fungicides as medium supplements in controlling systemic microbial contamination was studied (Table 13). Incorporation of aureofungin (filter sterilized) was found ineffective in controlling the systemic

Table 13. Effect of treatments undertaken for preventing the systemic microbial contamination of nodal explants of mature clove tree*

Treatment/Chemical	Concentration	Culture establishment 3 weeks after incubation			
		Contaminated cultures		Uncontaminated cultures	
		Living (%)	Dried (%)	Living (%)	Dried (%)
A. Effect of spraying given to mother plant					
1. Control. Explants collected from unsprayed trees	Nil	100	Nil	Nil	Nil
2. Spraying mother plants with fungicides					
a. Explants collected within 8 months after initiating spray schedule					
Dithene M-45	0.1 per cent	100	Nil	Nil	Nil
Bavistin	0.1 per cent	100	Nil	Nil	Nil
Aureofungin	0.016 per cent	100	Nil	Nil	Nil
b. Explants collected 8 months after cessation of spray schedule					
Aureofungin	0.016 per cent	44	Nil	56	Nil
B. Addition of fungicides as medium supplements					
a. Aureofungin (filter sterilized)	10 mgl^{-1}	100	Nil	Nil	0
	20 "	100	Nil	Nil	0
	40 "	92	Nil	Nil	8
	50 "	80	Nil	Nil	20
	150 "	60	Nil	Nil	40
	250 "	52	Nil	Nil	48

Contd.

Table 13 (Contd.)

Treatment/Chemical	Concentration	Culture establishment 3 weeks after incubation			
		Contaminated cultures		Uncontaminated cultures	
		Living (%)	Dried (%)	Living (%)	Dried (%)
b. Bavistin (Heat sterilised)	1 per cent	100	Nil	Nil	Nil
	2 per cent	100	Nil	Nil	Nil
c. Control	Nil	100	Nil	Nil	Nil
C. Providing moisture stress by altering the concentration of solidifying agent					
Agar	0.75 per cent	100	Nil	Nil	Nil
	1.50 per cent	100	Nil	Nil	Nil

* Explants (25 nos.) were surface sterilized with alcohol (50 per cent) for 2.0 minutes and $HgCl_2$ (0.1 per cent) for 12 minutes

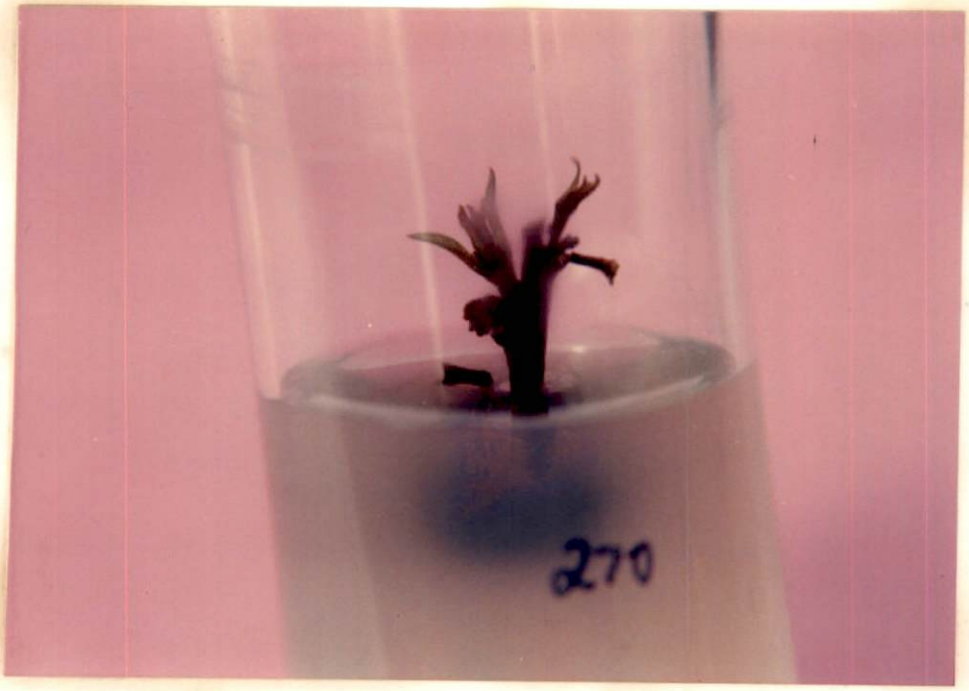
Table 14. Effect of pre soaking treatments given to field explants of clove for preventing systemic microbial contamination*

Chemical	Concentration (%)	Duration of treatment (minutes)	Contaminated cultures (%)	Uncontaminated cultures (%)	
				Dried	Living
Aureofungin sol	0.125	60	68	32	Nil
	0.250	60	88	12	Nil
	0.500	60	92	8	Nil
Aureofungin + Ascorbic acid	0.50,0.01	60	80	20	Nil
Aureofungin followed by Bavistin	0.50,0.25	30 each	16	84	Nil
	0.50,0.50	30 each	60	40	Nil
Bavistin	0.25	60	64	36	Nil
	0.50	60	60	40	Nil
	1.00	30	40	60	Nil

* Explants (25 nos.) were surface sterilized with alcohol (50 per cent) for 2.0 minutes and $HgCl_2$ (0.1 per cent) for 12 minutes

Plate 1. Nodal segment of mature clove tree showing extrusion of fungal mycelia from the petiole detached portion

Plate 2. Polyphenol exudation causing browning and death of the culture


270

fungal contamination at the lower concentrations tried. Higher levels were found to cause browning of the explants. Bavistin (heat sterilized) as medium supplement could not reduce the contamination and resulted in cent per cent loss (Table 13).

4.1.1.2.4 Effect of providing moisture stress to the explant

Moisture stress was provided to the explant and there by to the systemic contaminant, by using 1.5 per cent agar in the culture medium. This treatment was also found ineffective and resulted in cent per cent loss due to contamination (Table 13).

4.1.1.3 Standardisation of treatments to overcome polyphenol interference

When the explants were inoculated it was found that a wide variety of polyphenols were exuding from the cut end of the explant into the medium causing browning and death of the cultures (Plate 2). Several treatments including pretreatment of explants, use of different antioxidants and adsorbants as medium supplements or a combination of two treatments were carried out to alleviate the polyphenol interference.

4.1.1.3.1 Effect of pretreatments in reducing polyphenol interference

Results of the pretreatments are presented in Table 15. Sealing the cut end of the explant with paraffin wax was found to be the best treatment with 'zero' score. Other treatments like soaking the explants in a solution of PVP or ascorbic acid and sucrose were also found effective with relatively low scores. A maximum score of 2.8^{+} was obtained with the control kept under light condition.

Effect of light on polyphenol exudation and browning was also studied for each treatment. It was found that certain treatments such as shaking the explants in an orbital shaker or soaking in a solution of PVP produced greater scores (1.60^{+} and 2.05^{+}) under dark condition, than under light condition. Other treatments showed an equal or slightly lesser scores under dark condition (Table 15).

Survival of explants was found to be negatively associated with the extent of polyphenol exudation. Cent per cent survival was recorded for wax sealing treatment upto a period of 3 to 4 weeks and later on lost due to fungal contamination. The percentage survival recorded was the least (20 per cent) for the control lot kept under light (Table 15).

Table 15. Effect of pretreatments in controlling polyphenol exudation from mature field grown clove explants *

Treatment	Duration of treatment	Intensity of exudation		Cultures surviving (%)	
		Light	Dark	Light	Dark
Keeping explants under running tap water	4 h	1.41+	1.40+	44	44
Shaking explants in an orbital shaker	4 h	1.66+	2.05+	40	32
Soaking explants in a solution of 1 per cent PVP	4 h	0.83+	1.60+	60	40
Soaking in a solution of 100 mgl^{-1} ascorbic acid	4 h	1.66+	1.66+	40	40
Soaking in a solution of 100 mgl^{-1} ascorbic acid + 2 per cent sucrose	4 h	0.83+	0.35+	60	76
Sealing the cut end of the explant with paraffin wax	Nil	0.00+	0.00+	100	100
Control	Nil	2.80+	2.05+	20	32

* Basal medium was half-MS and number of explants used was 25

\+ Intensity of exudation

4.1.1.3.2 Effect of different basal media in reducing polyphenol interference

Six different basal media were tested for finding out their effect on polyphenol exudation and browning. SH medium supported lowest exudation with a score of 2.70^{+} followed by MS, half-MS and WPM. White's medium caused severe exudation, and none of the explants survived (Table 16) in this particular medium.

4.1.1.3.3 Effect of physiological age of explant on polyphenol exudation

Explants of three different positions or physiological ages were tested, and it was found that apical shoots produced more exudate than nodal segments, and among the nodal pieces, mature nodes exuded more polyphenols than tender nodes. Results are presented in Table 16.

4.1.1.3.4 Effect of different medium supplements in reducing polyphenol interference

Among the five different medium supplements tried, PVP and activated charcoal were found to be more effective in nullifying the interference of polyphenol with cent per cent explant survial (Table 17). Among the antioxidants tested, combination of ascorbic acid and citric acid was the best

Table 16. Effect of different basal media and physiological age of explant on polyphenol exudation from field grown explants of clove*

	Intensity of polyphenol exudation	Percentage of cultures surviving
A. Medium		
MS	2.80+	20
Half-MS	2.80+	20
WPM	2.80+	20
B5	3.20+	16
SH	2.70+	20
White's	4.60+	0
B. Explant type		
Apical shoot	2.30+	24
Tender nodal segment	1.40+	44
Mature nodal segment	2.20+	28

* Number of explants used was 25

Table 17. Effect of different medium supplements in controlling polyphenol exudation from mature field grown clove explants*

Chemical used	Intensity of exudation (Mean)		Cultures surviving (%)	
	Light	Dark	Light	Dark
A. Antioxidants				
(i) Ascorbic acid 100 mgl^{-1}	0.31+	0.84+	76	60
(ii) Citric acid 100 mgl^{-1}	0.75+	1.30+	64	48
(iii) Ascorbic acid and citric acid 100 mgl^{-1} each	0.15+	0.16+	92	92
(iv) L-cystein HCl. 100 mgl^{-1}	0.84+	0.83+	60	60
B. Adsorbants				
(i) Activated charcoal 0.5 per cent	0.00	0.00	100	100
(ii) PVP 0.5 per cent	0.00	0.00	100	100

* Basal medium was half-MS and number of explants used was 25

\+ Intensity of exudation

producing 92 per cent explant survival. As in the case of pretreatments, here also the effect of light was found to be varying with different treatments. Phenolic interference was relatively the same or slightly higher with cultures kept under dark (Table 17).

4.1.1.3.5 Effect of combination of treatments in reducing polyphenol interference

All the four treatment combinations tried were found to be equally effective in preventing exudation and oxidation of polyphenols with cent per cent explant survival (Table 18).

4.1.2 Seedling explants

4.1.2.1 Surface sterilization

Both in vitro seedlings and seedlings raised in glass house were used for the study. Surface sterilization of glass house seedling explants was carried out effectively with 0.1 per cent $HgCl_2$ for 10 minutes (Table 19) and percentage of contamination was reduced to 10. Both fungal and surface bacterial contamination (30 per cent) were noticed if the duration was 8 minutes for $HgCl_2$ dip. In vitro seedlings being aseptic, required no surface sterilization procedure, but those raised in vermiculite and sand media, were surface

Table 18. Effect of combination of pretreatment and use of medium additive in controlling polyphenol exudation from field grown explants of clove*

Treatment	Culture condition			
	Intensity of polyphenol exudation+		Percentage of cultures surviving	
	Light	Dark	Light	Dark
A. Soaking in 100 mgl^{-1} ascorbic acid + two per cent sucrose for 4 h. and inoculating to				
(i) ½MS + 0.5 per cent PVP	0	0	100	100
(ii) ½MS + 0.5 per cent AC	0	0	100	100
B. Soaking in 0.5 per cent PVP for 4 h. and inculating to				
(i) ½MS + 0.5 per cent PVP	0	0	100	100
(ii) ½MS + 0.5 per cent AC	0	0	100	100

* Basal medium was half-MS and number of explants used was 25

\+ Intensity of exudation

Table 19. Effect of $HgCl_2$ as surface sterilant for clove seedling explants*

Source of explant	Concentration of $HgCl_2$ (per cent)	Duration of treatment (minute)	Contaminated cultures (per cent)	Uncontaminated cultures (%)	
				Living (%)	Dead
Glasshouse seedling	0.1	8	30	70	Nil
		10	10	90	Nil
In vitro seedling raised in peat: vermiculite medium	0.1	3	0	100	Nil

* Number of explants used was 20

sterilized with $HgCl_2$ (0.1 per cent) for three minutes, which gave 100 per cent uncontaminated cultures.

4.1.3 Effect of different basal media on culture establishment

The effect of six different basal media on the culture establishment of nodal segments of clove are presented in Table 20. The results indicated that for seedling explants cent per cent culture establishment could be obtained in half-MS medium and WPM, while White's medium recorded only 30 per cent of culture establishment. In the case of mature tree explants, due to the high rate of microbial contamination, observations on culture establishment could not be recorded.

4.2 Enhanced release of axillary buds

Detailed experiments were conducted to identify the most ideal growth regulator combination for the multiple shoot induction and proliferation in clove. Different levels of cytokinins (BAP, kinetin) auxins (IAA, NAA) and gibberellins (GA_3) were incorporated in the best suited basal media already identified. Response of different explants like apical shoots, nodal, internodal and leaf segments of both mature tree and seedlings of clove were evaluated. The results are presented here.

Table 20. Effect of different basal media on culture establishment of clove*

Medium	Seedling explants	Mature tree explants	
	Cultures responding to the medium (%)	Contamination (%)	Cultures responding to the medium (%)
MS	70	100	Nil
Half-MS	100	100	Nil
WPM	100	100	Nil
B-5	80	100	Nil
SH	70	100	Nil
White's	30	100	Nil

* Number of explants used was 20

4.2.1 Explants from mature tree

Shoot tips, leaf segments, nodal and internodal segments from mature tree, which survived the sterilization treatments, were utilized to analyse their potential for multiple shoot induction. Survival rate was negligible (1.0 per cent) for the mature tree explants including nodal segments and apical shoots. High rate of microbial contamination was the major limiting factor in drawing out conclusive results in this experiment. Internodal and leaf segments were free of microbial contamination, but showed no response for organogenesis (Table 21).

The cultures of nodal segments that obtained free of microbial contamination, showed multiple shoot induction in WPM supplemented with 3.0 mgl^{-1} BAP and 1.0 mgl^{-1} kinetin (Table 22, Plate 3). For the emergence of two axillary buds it took one month (plate 4a) and within two months period 5 shoots were produced per explant (Plate 4b). The cultures were subcultured to the very same medium at 3 to 4 weeks interval. Subsequent growth of the axillary buds was very slow and the elongation rate was only 1.0 to 2.0 mm per month. Axillary buds were produced from the leaf axils of the initial sprouts and by four months period a total of 12 buds were produced per explant (Plate 4c).

Plate 3. Nodal segment of mature clove tree showing multiple shoot induction in WPM supplemented with BAP 3.0 mgl^{-1} and kinetin 1.0 mgl^{-1}

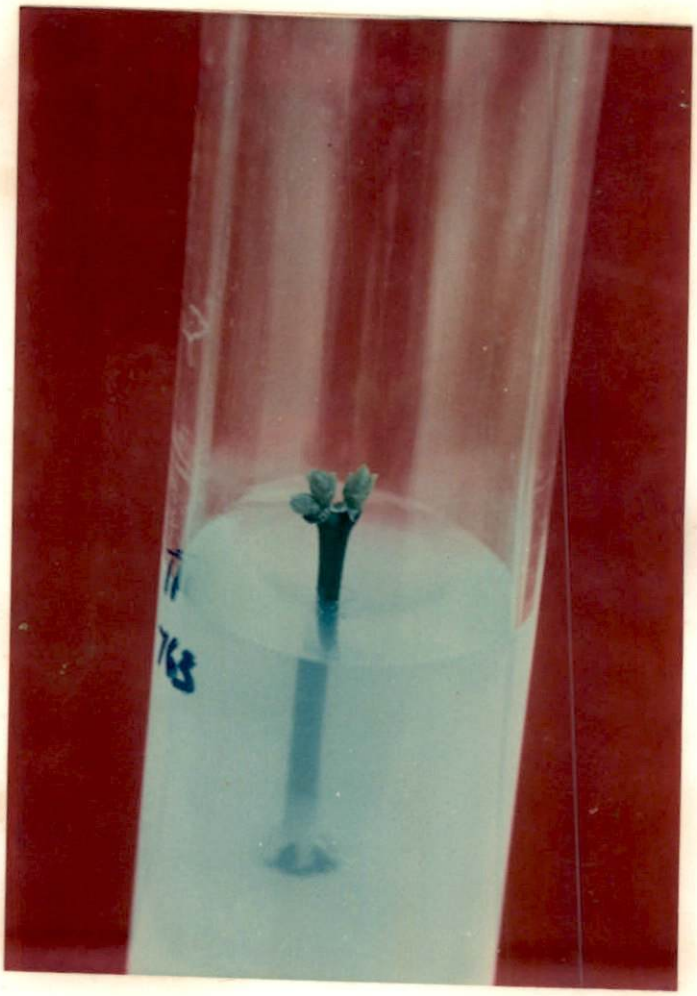


Plate 4a,b,c Growth performance of multiple shoots induced from nodal segment of mature clove tree

Table 21. Effect of different media combinations for direct organogenesis from different explants of mature clove tree

Medium	Type of explant	Cultures surviving one month after incubation	Cultures responding 2 months after incubation (%)
MS + BAP 3.0 mgl^{-1} and and Kinetin 1.0 mgl^{-1}	A*	Nil	Nil
	B*	Nil	Nil
	C*	90	Nil
	D*	90	Nil
MS + BAP (1.0, 1.5, 2.0 mgl^{-1}) and NAA (0.1, 0.5 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
MS + BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mgl^{-1}) and NAA (0.1, 0.5 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
1/2 MS + BAP (1.0, 2.0, 3.0, 4.0, 5.0 mgl^{-1}) and Kinetin (0.5, 1.0, 2.0 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
1/2 MS + BAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 7.0 mgl^{-1}) and NAA (0.1 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil

Contd.

Table 21 (Contd.)

Medium	Type of explant	Cultures surviving one month after incubation	Cultures responding 2 months after incubation (%)
1/2 MS + BAP (1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mgl^{-1}) and IAA (0.5 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
1/2 MS + BAP (1.0mgl^{-1}) Kinetin (2.0, 2.5 mgl^{-1}) and IAA (0.5 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
1/2 MS + Kinetin (1.0, 2.0, 2.5, 3.0 mgl^{-1}) and IAA (0.5 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
1/4 MS + BAP (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 mgl^{-1}) and NAA (0.1 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
WPM + BAP (0.5, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 mgl^{-1}) and NAA (0.2 mgl^{-1})	A	Nil	Nil
	B	Nil	Nil
	C	90	Nil
	D	90	Nil
WPM + BAP 3.0 mgl^{-1} and Kinetin 1.0 mgl^{-1}	A	100	100
	B	Nil	Nil
	C	90	Nil
	D	90	Nil

A - Nodal segment B - Apical shoot C* Internodal segment D* Leaf segment

Table 22. Growth performance of multiple shoots induced from nodal explants of mature clove tree

Medium	Growth performance of multiple shoots					
	Period after incubation					
	1 month		2 months		4 months	
	No.	Size	No.	Size	No.	Size
WPM + BAP 3.0 mgl^{-1} + Kinetin 1.0 mgl^{-1}	2	2 mm	5	3 mm	12	5 mm

4.2.2 Seedling explants

Since the microbial interference was less for seedling explants, more combinations of growth regulators and other media supplements were attempted, so as to induce multiple shoots.

4.2.2.1 Effect of cytokinins

Very low levels of BAP (0.2 and 0.8 mgl^{-1}) were tried for multiple shoot induction using WPM as basal medium. The results are presented in Table 23. Within 75 days incubation, 100 per cent of the cultures produced multiple shoots (2 to 4). Benzyl amino purine incorporated at 0.2 mgl^{-1} was found to give elongated shoots with 4 to 5 well developed leaves (Plate 5). Average length of shoots produced was found to be 6.1 mm and 4.8 mm when BAP was incorporated at 0.2 and 0.8 mgl^{-1} respectively.

Different levels of BAP (0 to 3 mgl^{-1}) were tested with a fixed level of kinetin (1.0 mgl^{-1}) for multiple shoot induction. Nodal segments cultured in WPM supplemented with BAP (0 to 2 mgl^{-1}) and kinetin (1.0 mgl^{-1}) showed 100 per cent response for multiple shoot induction. At zero level of BAP two shoots were produced within 32 days with kinetin at 1.0 mgl^{-1}. But growth of these shoots was very poor and attained only 2.0 mm length. Most obvious effect of BAP was obtained

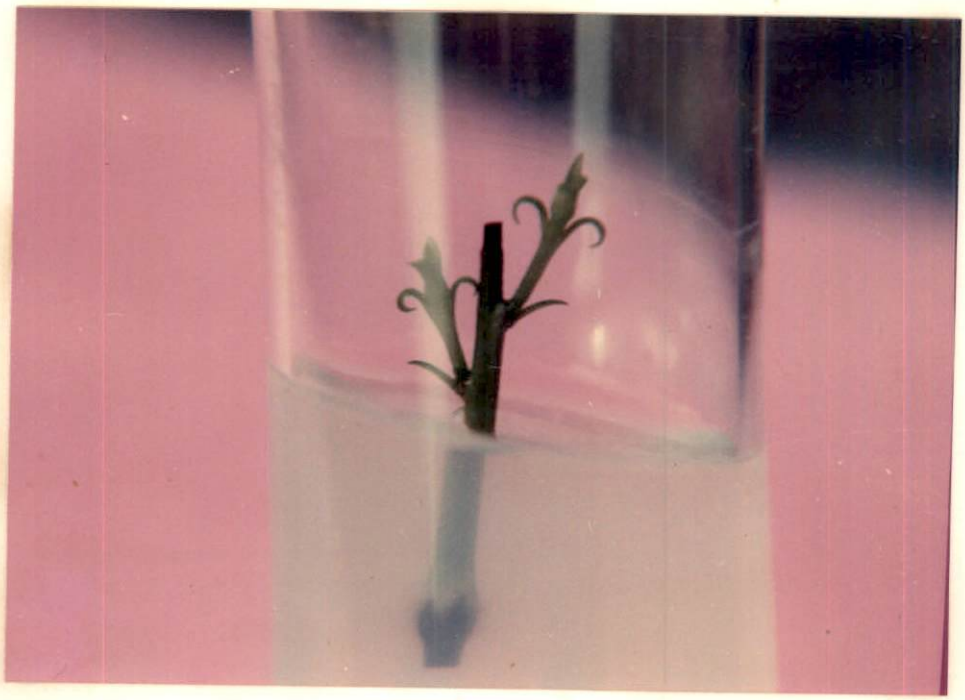
Table 23. Effect of cytokinins on bud proliferation from nodal explants of clove seedling

Basal medium	Concentration of cytokinin used (mgl^{-1})		Cultures showing shoot proliferation	Time taken for shoot proliferation	Average No. of multiple shoots induced per explant	Range of multiple shoots induced	Average length of shoots induced	Range of length of shoots induced	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
	BAP	Kinetin	(%)	(days)		(number)	(mm)	(mm)		
WPM	0.2	0.0	100	75	2.0	1-3	6.1	1-10	2.6	0.0
	0.8	0.0	100	75	2.8	2-4	4.8	1-17	2.5	0.0
	0.0	1.0	100	32	2.0	2	1.1	1-2	0.0	0.0
	0.5	1.0	100	57	2.75	2-4	4.8	1-8	3.3	2.5
	1.0	1.0	100	20	3.4	2-4	4.9	1-15	4.4	4.0
	2.0	1.0	100	20	3.8	2-6	2.7	1-5	0.0	0.0
	3.0	1.0	75	20	2.4	2-3	2.4	1-4	0.0	0.0
½MS	3.0	1.0	80	27	2.0	2	3.3	2-4	0.0	0.0

Plate 5. Nodal segment of clove seedling showing induction of lengthy shoots with leaves in WPM supplemented with BAP 0.2 mgl^{-1}

Plate 6a. Nodal segment of clove seedling showing induction of elongated shoots in WPM supplemented with BAP and kinetin each at 1.0 mgl^{-1}

Plate 6b. Nodal segment of clove seedling showing poor response for multiple shoot induction in WPM supplemented with BAP 3.0 mgl^{-1} and kinetin 1.0 mgl^{-1}



with increased concentration of BAP ranging from 0.5 to 2.0 mgl^{-1}, which initiated a maximum of 4.0 to 6.0 shoots per nodal explants. Maximum elongation of 15 mm was noticed with medium containing 1.0 mgl^{-1} of BAP and kinetin (Plate 6a). With this combination the shoots were of normal growth and produced narrow leaves. At still higher level of BAP (3.0 mgl^{-1}) along with kinetin (1.0 mgl^{-1}) only 75 per cent of the cultures responded. Multiple shoot production and growth of the shoots were also found to be low with this combination (Plate 6b). Benzyl amino purine (3.0 mgl^{-1}) and kinetin (1.0 mgl^{-1}) combination was also tested with half-MS medium. Only 80 per cent of the cultures responded for shoot proliferation, with 2 shoots per nodal explant which attained only 2.0 to 4.0 mm length (Table 23).

4.2.2.2 Effect of combinations of cytokinins and auxins

Different concentrations and combinations of cytokinins (BAP and kinetins) and auxins (IAA and NAA) were attempted for multiple shoot induction in two different basal media namely half-MS and WPM.

4.2.2.2.1 BAP and IAA combinations

The response of seedling explants for multiple shoot induction at different levels of BAP (0 to 10 mgl^{-1}) and IAA (0.5 mgl^{-1}) in half-MS as basal medium is presented in

Table 24. The results indicated that, in the absence of BAP none of the cultures responded for shoot proliferation, even when IAA was incorporated at a level of 0.5 mgl^{-1}. Benzyl amino purine was found to be very much essential for multiple shoot induction. When concentration of BAP was increased from 0.5 to 3.0 mgl^{-1} along with 0.5 mgl^{-1} IAA, 100 per cent cultures showed shoot proliferation with 2 to 8 multiple shoots per explant (Plate 7). By contrast at higher levels of BAP (above 3.0 mgl^{-1}) the response of nodal segments for shoot proliferation was found to be poor. Maximum number of multiple shoots induced at higher levels was only three as compared to eight multiples at lower levels (1.0 to 3 mgl^{-1}) in combination with IAA.

The multiple shoots produced at the best combination of BAP and IAA attained a maximum length of 8.0 mm with the highest mean 3.8 mm when BAP and IAA were incorporated at 1.0 mgl^{-1} and 0.5 mgl^{-1} respectively in the basal medium (Plate 8a). As the concentration of BAP increased, the shoots became more and more rosette in appearance (Plate 8b). Subsequent subcultures did not prove to be beneficial for shoot elongation or further growth of the buds. However more buds started to appear from the axil of the existing buds.

When IAA was used at higher levels (1.0 and 2.0 mgl^{-1}) in combination with BAP (0.2 and 0.8 mgl^{-1}), the cultures

Plate 7. Nodal segment of clove seedling showing multiple shoot induction in medium supplemented with BAP 2.0 mgl^{-1} and IAA 0.5 mgl^{-1}

Plate 8a,b. Nodal segment of clove seedling showing induction of elongated and stunted shoots in medium supplemented with lower and higher concentrations of BAP along with IAA ($0.5\ mgl^{-1}$).

Table 24. Effect of BAP-IAA combinations on multiple shoot induction and proliferation from nodal explants of clove seedling

Medium	Cytokinin concentration (mgl^{-1})	Auxin/ concentration (mgl^{-1})	Cultures showing shoot proliferation (%)	Time taken for shoot proliferation (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
½ MS	BAP	IAA								
	0	0.5	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	0.5		100	23	2	1-3	3.8	1-9	6	Nil
	1.0		100	73	4.2	2-8	3.8	2-8	5	2.7
	2.0		100	59	3.9	2-7	3.3	1-7	4	3
	3.0		100	73	4.25	2-7	3.0	1-7	Nil	3
	4.0		34	47	2.0	1-3	1.5	1-3	Nil	Nil
	5.0		34	47	2.0	1-2	1.5	1-3	Nil	Nil
	6.0		28	47	2.0	1-3	1.5	1-3	Nil	Nil
	7.0		28	47	2.0	1-2	1.5	1-3	Nil	Nil
	8.0		22	47	2.0	1-2	1.5	1-3	Nil	Nil
	9.0		19	47	2.0	1-2	1.5	1-3	Nil	Nil
	10.0		7	47	2.0	1-2	1.5	1-3	Nil	Nil
WPM	0	0.5	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	0.5		100	37	3.5	2-4	4.7	1-9	4	2
	1.0		100	37	3.8	2-6	3.8	1-7	Nil	Nil

Contd.

Table 24 (Contd.)

Medium	Cytokinin concentration (mgl^{-1})	Auxin/ concentration (mgl^{-1})	Cultures showing shoot proliferation (%)	Time taken for shoot proliferation (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
	2.0		100	48	4.0	4	3.8	1-6	Nil	Nil
	3.0		100	56	2.6	2-5	2.75	1-5	Nil	Nil
	4.0	1.0	66	47	2.5	2-4	2.5	1-5	Nil	Nil
	5.0		63	71	2.5	2-4	2.3	1-5	Nil	Nil
	6.0		50	71	2.3	2-4	2.0	1-5	Nil	Nil
	7.0		50	44	2.3	2-4	1.8	1-3	Nil	Nil
	8.0		48	44	2.0	2-3	1.6	1-3	Nil	Nil
	9.0		33	44	2.0	2-3	1.6	1-2	Nil	Nil
	10.0		27	44	2.0	2-3	1.4	1-2	Nil	Nil
½MS	0.2	1.0	66	44	1.5	1-2	1.66	1-2	1	Nil
	0.8		75	71	2.3	1-4	2.14	1-3	Nil	Nil
	0.2	2.0	33	44	2.0	2	2.0	2	Nil	Nil
	0.8		75	71	3.0	2-4	2.6	2-4	Nil	Nil

showed only low response for multiple shoot induction and proliferation. The number of multiple shoots induced and the length of shoots were not considerably altered by change in IAA concentrations (Table 24).

Combinations of BAP (0 to 10 mgl^{-1}) and IAA (0.5 and 1.0 mgl^{-1}) were tested with WPM for multiple shoot induction and later proliferation of shoots. Changing the basal medium did not much influenced the multiple shoot induction and shoot proliferation at different levels of BAP and IAA tried in this study (Table 24).

With lower levels of BAP and IAA tested, shoot tip explants continued to grow very slowly, giving a single shoot (Table 25). With higher concentrations of BAP, 2 shoots developed per explant which had a rosette appearance with small scale like leaves and highly condensed inter nodes (Plate 9).

4.2.2.2.2 BAP-NAA combinations

Different levels of BAP (0 to 3.0 mgl^{-1}) were tried with NAA (0.5 mgl^{-1}). It was observed that the omission of BAP from culture media could become growth limiting factor (Table 26). Addition of BAP (0.5 to 3.0 mgl^{-1}) showed shoot proliferation, with nodal explants. The average number of multiple shoots induced per explant was found to be

Table 25. Response of shoot tip explants of clove seedling for multiple shoot induction and proliferation

Medium	Response (2 months after incubation)
WPM/½MS+3x1 combinations of BAP (0, 0.5, 1.0 mgl^{-1}) and IAA (0.5 mgl^{-1})	Elongation of apical bud to 3.0 to 4.0 mm length
WPM/½MS+2x1 combinations of BAP (2.0, 3.0 mgl^{-1}) and IAA (0.5 mgl^{-1})	Emergence of two lateral buds of 3.0 mm length along with the apical bud
½MS+7x1 combinations of BAP (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 mgl^{-1}) and IAA (0.5 mgl^{-1})	No response, drying after 2nd subculture
WPM+2x1x1 combinations of BAP (0, 0.5 mgl^{-1}), kinetin (1.0 mgl^{-1}) and IAA (1.0 mgl^{-1})	Elongation of apical bud to 3.0 to 4.0 mm length
WPM+2x1x1 combinations of BAP (1.0, 2.0 mgl^{-1}) kinetin (1.0 mgl^{-1}) and IAA (1.0 mgl^{-1})	Emergence of two lateral buds (3.0 mm long) along with the apical bud

Table 26. Effect of BAP-NAA combinations on multiple shoot induction and proliferation from nodal segments of clove seedling

Medium	Cytokinin/ concentration (mgl^{-1})	Auxin/ concentration (mgl^{-1})	Cultures showing shoot proli-feration (%)	Time taken for shoot proli-feration (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
½MS	BAP	NAA								
	0	0.5	0	Nil	Nil	Nil	Nil	Nil	Nil	Nil
	0.5		100	43	2.8	2-4	3.8	2-9	6	2
	1.0		100	43	2.6	2-4	4.0	2-7	10	1
	2.0		100	29	2.8	2-4	3.4	2-5	Nil	Nil
	3.0		100	43	2.6	2-4	1.8	1-5	Nil	Nil

Plate 9. Shoot tip explant of clove seedling showing multiple shoot induction in medium supplemented with BAP (2 to 3.0 mgl^{-1}) and IAA (0.5 mgl^{-1})

Plate 10. Nodal segment of clove seedling showing induction of elongated shoots in medium supplemented with BAP (0.5 mgl^{-1}), kinetin (1.0 mgl^{-1}) and IAA (1.0 mgl^{-1})

more or less similar for these combinations (Table 26). Shoot tip explants cultured in different combinations of BAP and NAA showed similar response as obtained with BAP and IAA combinations. Of the two auxins tested (IAA and NAA) IAA was found to be slightly superior over NAA for multiple shoot induction and proliferation (Tables 24, 26) from nodal explants.

4.2.2.2.3 BAP-kinetin - IAA combinations

Nodal and shoot tip explants were cultured in WPM supplemented with different levels of BAP (0 to 3 mgl^{-1}) and constant level of kinetin and IAA (1.0 mgl^{-1} each). Kinetin and IAA in the absence of BAP failed to induce multiple shoots, but stimulated the existing axillary buds in the node. Subsequent subcultures did not prove to be beneficial for shoot proliferation in the absence of BAP. Increasing the concentration of BAP from 0.5 mgl^{-1} to 3.0 mgl^{-1} induced a maximum of 5.0 to 7.0 multiple shoots per nodal explant. Lowest concentration of BAP tried (0.5 mgl^{-1}) along with kinetin and IAA produced longest shoots (20 mm) of normal morphology with four fully developed leaves (Plate 10). Average number of multiple shoots induced with this combination was only 1.8 (Table 27). Increasing the concentration of BAP to 1.0 mgl^{-1} in combination with kinetin and IAA induced four multiple shoots per nodal explant, with a

maximum length of 10 mm, within 62 days. The multiplication rate remained relatively the same with increase in BAP upto 3.0 mgl^{-1}. Whereas mean shoot length showed a decreasing trend with increase in BAP concentration.

Much higher levels of BAP (4.0 to 10 mgl^{-1}) along with constant level of kinetin (1.0 mgl^{-1}) and IAA (0.5 mgl^{-1}) further reduced the multiplication rate as well as the length of multiple shoots induced (Table 27).

The response of shoot tip explants was found not encouraging when kinetin was supplemented in addition to BAP and IAA in the medium. The effect was the same as described earlier in section 4.2.2.2.1.

Effect of different levels of kinetin (0.to 2.0 mgl^{-1}) was assessed with fixed level of BAP (2.0 mgl^{-1}) and IAA (1.0 mgl^{-1}) using half-MS as basal medium. .All the combinations showed more or less similar response for induction of multiple shoots producing 1 to 4 shoots per culture (Table 27). Few shoots produced with 0.5 mgl^{-1} kinetin showed an elongation upto 8.0 mm with scale like leaves. Whereas shoots induced in other combinations, appeared as rudimentary structures without any leaves.

Table 27. Effect of BAP-Kinetin-IAA combinations on multiple shoot induction and proliferation from nodal explants of clove seedling

Medium	Cytokinin/ concentration (mgl^{-1})		Auxin/ concentration (mgl^{-1})	Cultures showing shoot proli-feration	Time taken for shoot proli-feration	Average No. of multiple shoots induced per explant	Range of multiple shoots induced	Average length of shoots induced	Range of length of shoots induced	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
	BAP	Kinetin	IAA	(%)	(days)		(number)	(mm)	(mm)		
WPM	0	1.0	1.0	100	20	2.0	2.0	1.9	1-3	Nil	Nil
	0.5			100	47	1.8	2-5	6.8	1-20	4	2
	1.0			100	62	4.0	2-7	3.9	1-10	Nil	Nil
	2.0			100	35	4.0	2-6	3.1	1-6	Nil	Nil
	3.0			100	63	4.2	4-5	2.2	1-5	Nil	Nil
	4.0	1.0	0.5	25	47	3.0	2-3	1.5	1-2	Nil	Nil
	5.0			22	86	2.0	2.0	2.5	1-4	Nil	Nil
	6.0			20	59	3.0	2-4	2.3	1-3	Nil	Nil
	7.0			20	59	3.0	2-4	1.5	1-2	Nil	Nil
	8.0			20	47	2.5	2-4	2.2	1-2	Nil	Nil
	9.0			20	47	2.0	2-3	2.2	1-3	Nil	Nil
	10.0			20	47	2.0	1-2	1.5	1-2	Nil	Nil
½MS	2.0	0.25	1.0	100	35	2.8	2-4	2.1	1-5	Nil	Nil
		0.50		100	35	2.6	2-4	3.2	1-8	2.4	Nil
		1.00		100	35	2.3	1-4	2.4	1-4	Nil	Nil
		2.00		100	35	2.2	1-4	2.2	1-5	Nil	Nil

4.2.2.3 Effect of media additives

Coconut water (CW) was incorporated at 15 per cent level along with different combinations of BAP (0.0 to 3.0 mgl^{-1}) IAA (0.5, 1.0 mgl^{-1}) or NAA (0.1, 0.5 mgl^{-1}). From the previous experiments it was clear that, for multiple shoot induction in clove BAP is very much essential. But nodal segments showed 100 per cent response with 2 to 4 multiple shoots in medium supplemented with coconut water (15 per cent) and NAA (0.5 mgl^{-1}) even in the absence of BAP (Table 28). Multiple shoots produced in media with coconut water and growth regulators showed increase in vigour (Plate 11), and they were thicker than those shoots produced in the absence of coconut water (Plate 12). Coconut water induced callusing at the base of the explant, and such explants produced vigorous shoots.

With constant level of BAP, kinetin and IAA (each at 1.0 mgl^{-1}) different levels of adenine sulphate and phloroglucinol were tried, for multiple shoot induction. Percentage of cultures responding for multiple shoot induction was found to vary (66 to 100) in different combinations of these media additives (Table 29).

Both adenine sulphate and phloroglucinol did not favour multiple shoot induction and later proliferation of the

Plate 11. Nodal segment of clove seedling showing induction of vigorous shoots in medium supplemented with CW and growth regulators

Plate 12. Nodal segment of clove seedling showing induction of thin shoots in medium without CW

Table 28. Effect of coconut water and growth regulator combinations on multiple shoot induction and proliferation from nodal explants of clove seedling

Medium	Cytokinin/ concentration (mgl^{-1})	Auxin/ concentration (mgl^{-1})	C.W/ concentration (%)	Cultures showing shoot proliferation (%)	Time taken for shoot proliferation (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
½MS	BAP	NAA									
	0	0.5	15	100	37	2.30	2-4	1.75	1-4	Nil	Nil
	0.5			100	74	2.70	2-4	6.00	3-12	5.0	Nil
	1.0			100	74	4.00	2-6	3.37	2-5	Nil	Nil
	2.0			100	41	3.20	2-4	2.50	1-6	Nil	Nil
	3.0			100	48	4.70	4-6	3.40	1-7	Nil	Nil
	0.5	0.1	15	100	37	3.0	2-4	6.40	2-12	4.0	Nil
	1.0			100	37	3.2	2-4	3.30	1-8	6.0	2.0
	2.0			100	65	4.25	2-6	2.50	1-5	2.0	Nil
	3.0			100	23	2.8	2-4	3.70	1-7	Nil	Nil
		IAA									
	1.0	0.5	15	100	32	2.00	1-3	3.90	1-9	4.0	4.0
	1.5			100	64	3.00	2-5	3.80	1-8	6.0	4.0

Contd.

Table 28 (Contd.)

Medium	Cytokinin/ concentration (mgl^{-1})	Auxin/ concentration (mgl^{-1})	C.W/ concentration (%)	Cultures showing shoot proliferation (%)	Time taken for shoot proliferation (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
	2.0			100	64	2.25	2-3	3.30	1-9	6.0	4.0
	2.5			100	64	2.25	2-3	3.60	2-6	3.0	Nil
	3.0			100	64	2.50	2-4	3.40	2-5	Nil	Nil
	1.0	1.0	15	100	32	3.00	2-3	3.40	1-9	6.0	3.5
	1.5			100	72	3.20	2-5	3.60	1-10	6.5	6.0
	2.0			100	59	4.00	2-6	3.40	2-8	4.0	2.0
	2.5			100	59	5.25	2-6	3.20	1-9	2.8	2.5
	3.0			75	86	4.70	2-7	3.40	2-5	4.0	2.0

Table 29. Effect of adenine sulphate and phloroglucinol on shoot proliferation from nodal explants of clove seedling

Medium	Additive used	Concentration (mgl^{-1})	Cultures showing shoot proliferation (%)	Time taken for shoot proliferation (days)	Average No. of multiple shoots induced per explant	Range of multiple shoots induced (number)	Average length of shoots induced (mm)	Range of length of shoots induced (mm)	Average no. of leaves induced per shoot	Average no. of axillary buds induced per shoot
WPM + BAP 1.0 + kinetin 1.0 + IAA 1.0 mgl^{-1}	Adenine sulphate	25	100	24	2.5	2-3	4.6	3-9	Nil	Nil
		50	83	46	3.0	2-4	2.5	1-6	Nil	Nil
		100	100	88	2.75	2-4	3.5	1-8	Nil	Nil
	Phloroglucinol	50	100	88	3.20	2-4	3.75	1-8	Nil	Nil
		100	66	46	3.30	2-4	2.9	1-5	Nil	Nil
		200	80	88	2.25	2-3	2.4	1-5	Nil	Nil
	Adenine sulphate + Phloroglucinol	50 each	100	46	2.0	2	2.8	1-5	Nil	Nil
	Without additives (control)	Nil	100	62	4.0	2-7	3.9	1-10	Nil	Nil

shoots. Mean number of multiple shoots induced varied between 2.0 to 3.3 per culture and this was relatively lower than the performance obtained in the absence of these media additives (Table 29). Shoot length was also not favoured except in one combination of adenine sulphate (25 mgl^{-1}).

4.3 Elongation of *in vitro* shoots

Shoot elongation was attempted in different media combinations as described in chapter 3. Varying the level of sucrose from 2 to 5 per cent or substituting sucrose with other carbon sources like glucose or maltose did not favour shoot elongation in clove. Increasing the concentration of inorganic nutrients to double the original level did not prove to be beneficial for elongation of multiple shoots (Table 30).

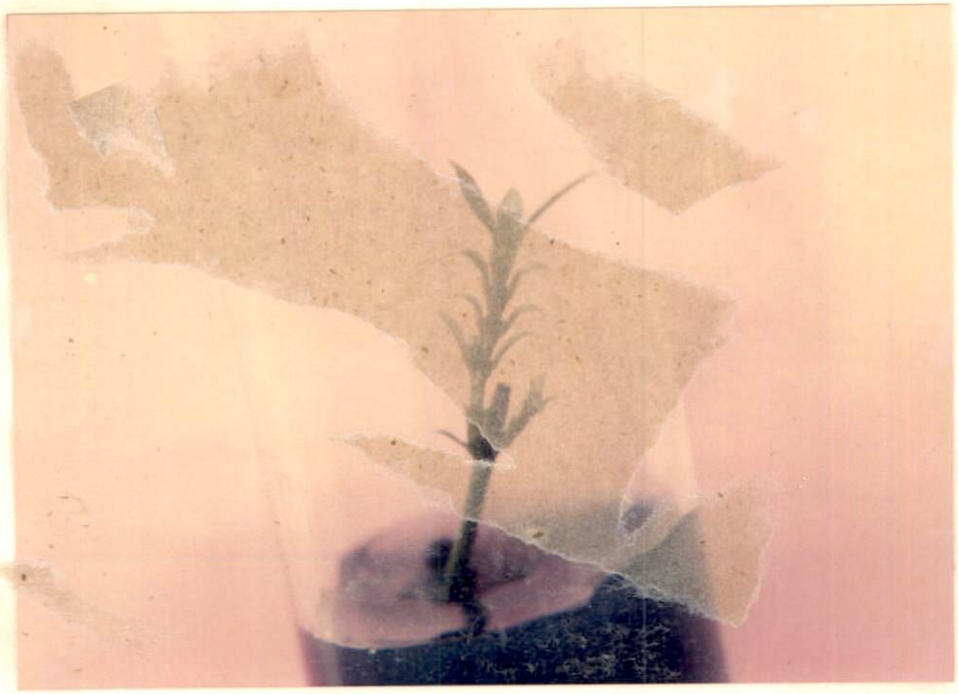
Among the growth regulator combinations tried for shoot elongation, very low level of BAP (0.2 mgl^{-1}) alone showed an increase upto 5.0 to 10 mm. Scale like leaves produced in the multiple shoot induction medium expanded and turned to narrow leaves of normal morphology within 30 days (Plate 13). Incorporation of GA_3 at 5.0 mgl^{-1} showed a slight elongation, but the shoot and leaves appeared lean and narrow. Other growth regulator combinations were found to be ineffective (Table 30).

Table 30. Effect of different media combinations on elongation of *in vitro* multiple shoots of seedling

Treatment tried	Increase in length (mm)	Change in leaf morphology
WPM+0.2 mgl^{-1} BAP+	Nil	Nil
2 per cent sucrose	Nil	Nil
5 per cent sucrose	Nil	Nil
3 per cent glucose	Nil	Nil
5 per cent glucose	Nil	Nil
3 per cent maltose	Nil	Nil
WPM+0.2 mgl^{-1} BAP + (3 per cent sucrose)		
$NH_4 (NO_3)_2$ 410 mgl^{-1}	Nil	Nil
$Ca(NO_3)_2$ $4H_2O$ 440 mgl^{-1}	Nil	Nil
$MgSO_4$ $7H_2O$ 370 mgl^{-1}	Nil	Nil
Leucine 1.0 mgl^{-1}	Nil	Nil
Leucine 2.0 mgl^{-1}	Nil	Nil
Lysine 1-0 mgl^{-1}	Nil	Nil
Lysine 2.0 mgl^{-1}	Nil	Nil
Arginine 1.0 mgl^{-1}	Nil	Nil
Arginine 2.0 mgl^{-1}	Nil	Nil
Tryptophan 1.0 mgl^{-1}	Nil	Nil
Activated charcoal (0.1 and 0.3 per cent)	5.0 to 15	4 to 6 well developed leaves
½MS+BAP 0.2 mgl^{-1}+ activated charcoal (0.1 and 0.5 per cent)	5.0 to 15	4 to 6 well developed leaves
WPM + 0.2 mgl^{-1} BAP	5.0 to 10	3 to 4 narrow leaves
WPM+2x1 combinations of BAP (0.4, 0.8 mgl^{-1}) and IAA (1.0 mgl^{-1})	Nil	Nil
WPM + BAP_1(0.2 mgl^{-1}) and GA_3 (5.0 mgl^{-1})	4.0	3 to 4 narrow leaves
WPM + BAP (0.2 mgl^{-1}) and GA_3 (10 mgl^{-1}) and IAA (1.0 mgl^{-1})	Nil	Nil
WPM + GA_3 (10, 15 mgl^{-1})	Nil	Nil
½MS + BAP (0.2, 0.3 mgl^{-1}) (liquid phase)	5 to 8	3 to 4 narrow, dark green leaves
Double phase media combinations shown in Table 9	Nil	Nil

Plate 13. Multiple shoots showing elongation and leaf expansion WPM supplemented with BAP 0.2 mgl^{-1}

Plate 14. Effect of activated charcoal on multiple shoot elongation and leaf production from nodal segment of clove seedling



Different levels of aminoacids tried did not favour elongation of *in vitro* shoots. Supplementing the basal medium with 0.2 mgl^{-1} BAP and 0.1 per cent level of activated charcoal favoured shoot elongation and leaf production within 45 days (Plate 14). Similar effect was also observed when activated charcoal was increased upto 0.5 per cent level. Multiple shoots transferred to liquid media with BAP (0.2 mgl^{-1}) showed elongation of shoots (5.0 to 8.0 mm) with production of 3 to 4 narrow leaves. Double phase medium tried did not give any response for shoot elongation (Table 30).

4.4 Rooting of *in vitro* shoots

Microcuttings of proliferated shoots having a length of 1 to 1.5 cm as well as explants as such were subjected for rooting. The organic and inorganic components of the basal medium (MS and WPM) as well as the growth regulators were altered for the induction of rooting. Results are presented in Table 31.

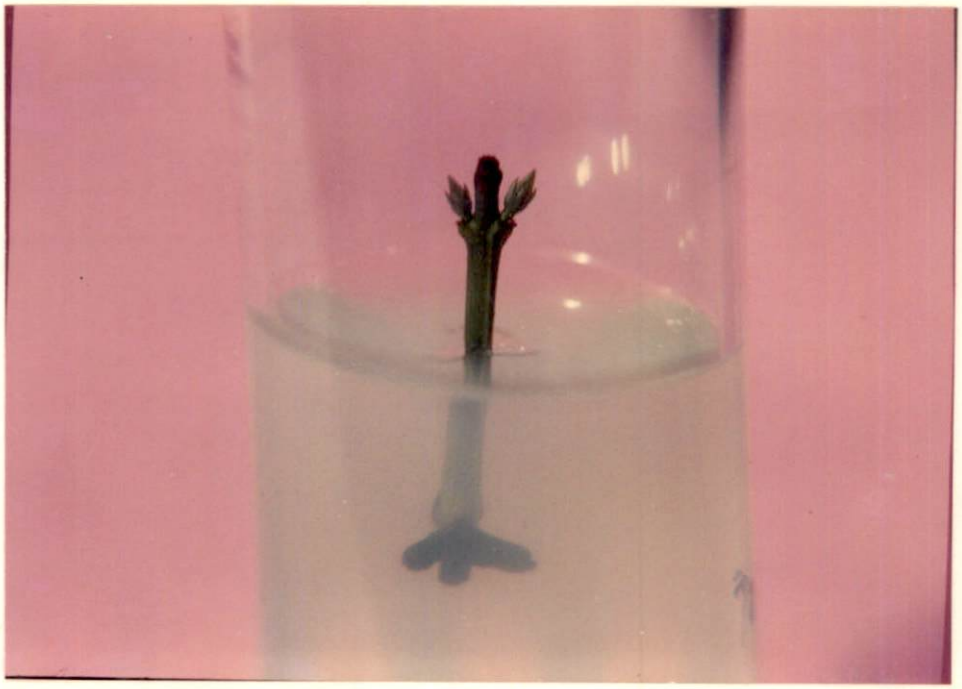
Explants cultured in WPM containing 3 per cent sucrose and 0.5 mgl^{-1} IAA induced 2 to 3 stout root like structures from the base (Plate 15). They were subcultured to the very same medium and thereafter to White's basal medium for further elongation, but showed no elongation. Shoot explants cultured in WPM containing 2 mgl^{-1} each of IBA and NAA produced 2 to 4

Table 31. Effect of different media combinations on *in vitro* rooting of clove seedling

Medium	Number of roots induced	Average length of roots (one month after induction)
WPM+NAA 0.5 mgl^{-1} + IBA 0.5 mgl^{-1}	Nil	Nil
WPM+NAA 1.0 mgl^{-1} + IBA 1.0 mgl^{-1}	Nil	Nil
WPM+NAA 2.0 mgl^{-1} + IBA 2.0 mgl^{-1}	4 roots	5 mm
WPM+NAA 6.0 mgl^{-1}	Nil	Nil
WPM+IBA 3.0 mgl^{-1}	Nil	Nil
WPM+IAA 0.5 mgl^{-1}	3 stout root like structures	3 mm
½WPM basal	Nil	Nil
½WPM+NAA 1.0 mgl^{-1} + IBA 0.5 mgl^{-1}	Nil	Nil
½WPM+NAA 1.0mgl^{-1} + IBA 0.5 + IAA 0.5	Nil	Nil
½WPM+NAA 1.0 mgl^{-1} + IBA 0.5 mgl^{-1} + Activated charcoal 0.1 per cent	Nil	Nil
½WPM+NAA 2.0 mgl^{-1} + IBA 0.5 mgl^{-1} + Activated charcoal 0.1 per cent	Nil	Nil
½WPM+NAA 1.0 mgl^{-1} + IBA 1.0 mgl^{-1} + IAA 0.5 mgl^{-1} + Activated charcoal 0.1 per cent	Nil	Nil
½MS+NAA 0.5 mgl^{-1} + IBA 0.5 mgl^{-1} : liquid phase	Nil	Nil
½MS+NAA 1.0 mgl^{-1} + IBA 1.0 mgl^{-1} : liquid phase	Nil	Nil
½MS + 2 per cent sucrose + IBA 1.0 mgl^{-1}	Nil	Nil

Plate 15. Nodal segment of clove seedling showing induction of root like structures in WPM supplemented with IAA 0.5 mgl^{-1}

Plate 16. Nodal segment of clove seedling showing root induction in WPM supplemented with NAA 2.0 mgl^{-1} and IBA 2.0 mgl^{-1}



roots of 5.0 mm within 45 days (Plate 16). Other combinations tried were unsuccessful in inducing rooting of clove shoots and showed callusing at the base.

Quick dip treatment given to the shoots with 1000 mgl^{-1} IBA prepared in alcohol showed complete browning of shoots. Heat sterilized IBA (1000 mgl^{-1}) used for quick dip treatment showed no browning and rooting.

4.5 Indirect organogenesis

4.5.1 Callus induction and proliferation

Cotyledons of mature clove seeds when inoculated onto MS and half-MS medium containing 2,4-D or NAA at different levels did not exhibit any signs of callusing even after 2 months incubation period.

The response was found to be varying when internodal segments of both seedling and mature clove plants were used. Explants from mature trees showed no response and remained as such for several weeks. Explants from seedlings showed a slow rate of callusing with half-MS medium supplemented with 0.5 mgl^{-1} IAA and 2.0 mgl^{-1} BAP (Plate 17). Callusing was observed from the two cut ends of the internode.

Leaf segments of different developmental stages from 15 to 30 days old seedling and mature clove tree were cultured

in MS and half-MS medium supplemented with different types of growth regulators. Leaf segments at all developmental stages of mature tree failed to develop callus. Mature dark green leaves of seedling also failed to induce callus. Whereas young, pink or flesh coloured leaves of seedling showed callusing. Callus induction was observed from the cut edges of the leaf segments within 8 to 10 weeks in culture. Proliferation was observed mostly at the cut surfaces as well.

Half-MS medium containing 3.0 mgl^{-1} 2,4-D and 50 mgl^{-1} casein hydrolysate showed highest percentage (93) of callus initiation within 64 days, followed by half-MS medium containing 4.0 mgl^{-1} of 2,4-D (62.50 per cent). Leaf segments cultured in MS medium with different levels of 2,4-D showed only lower response compared to half-MS medium. Among the different levels of NAA tested with half-MS medium, NAA at 6.0 mgl^{-1} induced callus in 43.0 per cent of the cultures (Table 32).

Maximum growth of callus (C.I.371.40) was obtained with half-MS medium containing 2,4-D (3.0 mgl^{-1}) and casein hydrolysate (50 mgl^{-1}). Callus index in other media combinations varied between 14.28 to 250.00 (Table 32).

Morphology and texture of callus produced from young leaf explants of clove seedling are shown in Table 32. Callus

Table 32. Effect of different media combinations on callus induction and proliferation from leaf explants of clove seedling

Medium	Cultures showing callus initiation	Time taken for callus initiation	Callus rating (5 months after induction)	Callus index (5 months after induction)	Nature of callus one month after induction
MS+2, 4-D 0.5 mgl^{-1}	33.30	96	2.0	66.60	Cream yellow granular callus
MS+2, 4-D 1.0 mgl^{-1}	27.14	96	2.0	54.28	Cream yellow granular callus
MS+2, 4-D 2.0 mgl^{-1}	16.66	96	2.0	33.32	Cream yellow friable nodular callus
MS+2, 4-D 3.0 mgl^{-1}	33.33	96	2.0	66.66	Cream yellow friable nodular callus
MS+2, 4-D 4.0 mgl^{-1}	42.85	96	2.0	85.70	Cream yellow friable nodular callus
MS+2, 4-D 5.0 mgl^{-1}	35.71	96	2.0	71.92	Cream yellow compact callus
½MS+2, 4-D 0.5 mgl^{-1}	35.60	76	2.0	71.20	Cream friable callus with light green globular structures
½MS+2, 4-D 1.0 mgl^{-1}	34.00	76	2.0	68.00	Cream friable callus
½MS+2, 4-D 1.5 mgl^{-1}	34.00	76	2.0	68.00	Cream friable granular callus
½MS+2, 4-D 2.0 mgl^{-1}	60.00	76	2.0	120.00	Cream friable granular callus
½MS+2, 4-D 2.5 mgl^{-1}	31.10	76	2.0	62.20	Cream friable granular callus
½MS+2, 4-D 3.0 mgl^{-1}	51.50	76	4.0	206.00	Cream friable granular callus
½MS+2, 4-D 3.5 mgl^{-1}	32.20	96	2.0	64.40	Cream friable nodular callus
½MS+2, 4-D 4.0 mgl^{-1}	62.50	76	4.0	250.00	Cream compact callus
½MS+2, 4-D 2.0 mgl^{-1} + CH 100 mgl^{-1}	45.00	76	2.0	90.00	Cream compact callus
½MS+2, 4-D 3.0 mgl^{-1} + CH 50 mgl^{-1}	92.85	64	4.0	371.40	Gray friable nodular callus
½MS+NAA 2.0 mgl^{-1}	36.36	96	2.0	72.72	Cream yellow compact callus
½MS+NAA 3.0 mgl^{-1}	10.00	96	2.0	20.00	Cream yellow compact callus
½MS+NAA 4.0 mgl^{-1}	12.00	96	2.0	24.00	Cream yellow compact callus
½MS+NAA 5.0 mgl^{-1}	20.83	96	2.0	41.66	Cream yellow compact callus
½MS+NAA 6.0 mgl^{-1}	42.85	96	2.0	85.70	Cream yellow compact callus
½MS+NAA 8.0 mgl^{-1}	Nil	Nil	Nil	Nil	Nil

Plate 17. Internodal segment of clove seedling showing callus induction in medium supplemented with BAP 2.0 mgl^{-1} and IAA 0.5 mgl^{-1}

Plate 18. Creamy yellow compact callus induced from leaf explants of clove seedling in medium supplemented with 2,4-D 4.0 mgl^{-1}

Plate 19. Friable granular callus induced from leaf segment of clove seedling in medium supplemented with 2,4-D 1.0 mgl^{-1}

obtained were initially creamy yellow in colour. General appearance and morphology of the callus was found to vary with the growth regulator combinations. At all levels of NAA and at higher levels of 2,4-D tried, calli induced were compact (Plate 18). Friable, granular and nodular calli were induced in the other combinations of 2,4-D tried (Plate 19).

Shining globular structures were produced from those calli induced in half-MS medium containing 0.5 mgl^{-1} of 2,4-D (Table 32). These globular structures on further subculturing, turned to calli and failed to produce embryoids.

4.5.2 Organogenesis/embryogenesis from the callus

Different media combinations as shown in Table 12 were tried for indirect organogenesis/embryogenesis. None of the treatment combinations induced organogenesis/embryogenesis from the callus. Greening of the callus was observed with those cultured in WPM containing 3.0 mgl^{-1} BAP and 1.0 mgl^{-1} kinetin. Incorporation of silver nitrate at 5.0 mgl^{-1} in the same medium did not alter the response.

Discussion

5. DISCUSSION

Clove, Syzygium aromaticum is usually propagated through seeds. Being recalcitrant in nature, germination percentage of the seed reduces to 50, if sowing is delayed by one week. Since it is a single seeded fruit and as the flower bud is the commercial part used, the availability of seed and propagation rate is very low, limiting the large scale multiplication within a stipulated period. Vegetative propagation of clove is rather difficult due to its poor meristematic activity, low rooting of cuttings and layers or its inability in making successful unions by grafting or budding method. In view of these facts, the standardisation of in vitro propagation technique has great relevance in the production of true-to-type propagules at a cheaper rate within a reasonable time.

Attempts were made to standardise the in vitro propagation technique in Syzygium aromaticum at the Plant Tissue Culture Laboratory, Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara during 1992 to 1994. The results obtained are discussed hereunder.

5.1 Mature field grown explants

5.1.1 Culture establishment

Surface sterilization of nodal explants collected from mature field grown trees of clove was rather difficult and none of the surface sterilization treatments were found effective. Incidence of fungal infection in cultures of field explants has been observed as a serious problem in micropropagation. Doublin (1984) observed that the percentage of infection was more than 90 for other field explants regardless of the procedure used for their sterilization.

In clove fungal mycelia were found to be extruding from the stem at the petiole detached portion and not from the outer surface of the explant. Fungal development was observed in the abscission layers of both petiole and upper internode of Piper nigrum (Fitchel-Parnell, 1990).

Existence of endophytic fungi that survive surface disinfestation procedures and producing severe contamination in cultures after explanting has been reported by several workers in other crop plants (Chipley and Heaton, 1971; Hanlin, 1971; Knox and Smith, 1980).

Fungicidal spraying of mother plants has been suggested by Legrand and Missiso (1986) as a method for

reducing culture contamination in cocoa. In the case of clove, explants collected during the spray schedule of eight months gave only one per cent of uncontaminated cultures, after surface sterilization with ethyl alcohol and mercuric chloride. Explants collected eight months after the cessation of spray schedule gave 56 per cent of cultures free of microbial contamination. Clove being a woody plant and having a very low absorption of exogeneously applied chemicals/ nutrients (Nazeem, 1989), would have taken longer period for the absorption and expression of the systemic fungicide.

Presoaking the explants in fungicidal solution for reducing fungal contamination has been suggested by Broome and Zimmerman (1978) in black berry, Manzanera and Pardos (1990) in Quercus suber (Cork oak). Fungicidal soaking given to clove explants gave no reduction in microbial interference and resulted in browning and death of the explants. These findings are in confirmity with the findings of Dodds and Roberts (1985) for other crops plants.

In clove addition of fungicide to the culture medium at lower concentrations was not sufficient to check the microbial interference. Higher levels (150 and 250 mgl^{-1}) of aureofungin tried had remarkable influence in reducing the rate of contamination. The level of fungicide identified for reducing the microbial interference was highly toxic to the

plant material. Similar fungicidal toxicity has been reported by Brown et al. (1982) in orchids and Scheilds et al. (1984) in other crop plants.

Providing moisture stress to the explant for reducing systemic contamination was suggested by Obeidy and Smith (1990) in pecannuts. But for clove, increasing the concentration of agar to 1.5 per cent and thereby providing moisture stress to the explant did not yield any favourable response.

Culture establishment of mature field grown explants of clove faced yet another problem, that is polyphenol interference. Eugenol (a polyphenol) is the major constituent (80 to 95 per cent) of clove bud, stem and leaf oil (Gopalakrishnan et al., 1988). While doing in vitro cultures, these polyphenols ooze out to the medium. Survival of explants reduces to 20 per cent if polyphenol exudation is left unchecked. Polyphenols can be oxidized either by peroxidases (Mayer and Harel, 1979; Vaughn and Duke, 1984), or by polyphenol oxidases (Mayer and Harel, 1979; Hu and Wang, 1983). The oxidized compounds are highly toxic, they form covalent bonds with the plant proteins thus inhibiting the enzyme activity (Hu and Wang, 1983) causing browning and death of the explant. Explant establishment of clove, thus required special procedures to escape or avoid problems that are associated with polyphenol oxidation. Similar problem has

been reported in other woody plant species by Lenartowicz and Millikan (1977); Lloyd and McCown (1980).

Among the pretreatments tried for reducing the polyphenol interference, sealing the cut end of the explant with paraffin wax was found to be the best, with cent per cent culture establishment. Bhat and Chandel (1991) reported that sealing the cut end of the explant with paraffin wax was the best treatment for preventing polyphenol interference in Dioscorea alata and banana.

Since wax sealing acts as a physical barrier, it may reduce the absorption of nutrients and growth regulators. So several other treatments were also carried out for alleviating the problem of polyphenol interference.

Treatments like soaking explants in a solution of PVP or ascorbic acid and sucrose were found to be effective in reducing polyphenol interference in the in vitro cultures of clove. Culture establishment was 60 and 76 per cent respectively. Use of these chemicals for reducing polyphenol interference has been reported by Gupta et al. (1980 and 1981) in teak and eucalyptus.

Use of PVP or activated charcoal as medium supplements completely prevented the polyphenol interference in clove with

cent per cent culture establishment. Similar results were reported by Christiansen and Fonnesbech (1975); Bajaj (1978); Zaid and Tisserat (1983) in other woody plant species.

Combining two treatments together, that is the best pre-soaking treatment and best media additive also completely avoided the polyphenol exudation in clove. Similar result has been previously reported by Ziv and Halevy (1983) in Strelitzia reginae; Amin and Jaiswal (1988) in guava. Apical shoots of clove was found to release more polyphenols than nodal segments. The factors like age of the stock plant and location on the stem from which the explants are removed have been reported to greatly affect the establishment of tissue in vitro (Compton and Preece, 1986). Providing dark culture conditions for clove was found not effective in reducing the polyphenol interference unlike in other crop plants as reported by Forrest (1969) and Hu and Wang (1983).

Among the various basal media tried for culture establishment (MS, 1/2 MS, WPM, B5, SH and White's), cent per cent culture establishment could be obtained in half-MS and WPM. White's medium recorded only 30 per cent of culture establishment. Polyphenol interference was relatively more in White's basal medium. The MS medium is characterised by high concentration of mineral salts, so some workers found it beneficial to reduce its strength by half (Skirvin, 1980;

Griffis et al., 1981). Woody plants usually require a low salt medium, and WPM formulated by Lloyd and McCown (1980) was found to be the best basal media for other woody plants by several workers (Vieitez et al., 1983 in chestnut; Flyinn et al., 1990 in cocoa). Mathew and Hariharan (1990) reported that in vitro cultures of clove produced more or less similar results with half-MS, WPM, and B5 medium. Superman and Blake (1990) reported that for clove, WPM was the best basal medium.

5.1.2 Enhanced release of axillary buds

Nodal segments of mature field grown clove tree showed multiple shoot induction in WPM supplemented with 3.0 mgl^{-1} BAP and 1.0 mgl^{-1} kinetin. Generally cytokinin has been utilized to overcome the apical dominance of shoots to enhance the branching of lateral buds from leaf axils (Murashige, 1974). For the induction of multiple shoots in clove, cytokinin was found to be very much essential. Similar reports have been made in various other crops by Amin and Jaiswal (1987); Manzanera and Pardos (1990) and Yadav et al. (1990a).

Explants from mature field grown trees of clove exhibited dormancy in induction of multiple shoots and was very slow in subsequent growth. Mature field explants induced five shoots within four months period which attained only a

maximum of 5.0 mm. The capacity to vegetatively propagate a tree is associated with its juvenility. Generally the more juvenile the specimen the easier it is to propagate vegetatively (Bonga, 1980), or more easily will organ formation occur *in vitro* (Thorpe and Patel, 1984). Slow growth, low propagation rate and weak *in vitro* performance of mature explants as compared to juvenile shoots has been discussed by several workers in other woody plant species (Maarri *et al.*, 1986; Masseguer and Mele, 1987; Rodriquez *et al.*, 1988; Rajmohan and Kumaran, 1988).

Culture establishment of mature field grown explants of clove faced polyphenol interference and high rate of microbial contamination. In the present study polyphenol interference was completely controlled, but the treatments tried to thrive over microbial contamination were not effective to a satisfactory level. Moreover the performance of the established cultures in subsequent growth stages was very poor. Similar problems have been reported by Chauvin and Sulesses (1988) in chestnut and Yadav *et al.* (1990a) in *Syzygium cumini*. To overcome these problems they utilized juvenile explants. Hence seedlings of clove were raised aseptically and also under controlled glass house condition to get juvenile explants for inoculation.

5.2 Seedling explants

5.2.1 Culture establishment

Surface sterilization of seedling explants was effectively carried out by $HgCl_2$ (0.1 per cent) soaking for 10 minutes. Polyphenol interference was observed only to a very limited extent as compared to mature clove explants. Similar results with juvenile explants have been reported by Yadav *et al*. (1990a) in *Syzygium cumini*.

5.2.2 Enhanced release of axillary buds

Incorporation of BAP even at very low levels (0.2 to 0.8 mgl^{-1}) in the media induced multiple shoots (2 to 4) of 4.8 to 6.1 mm length in clove *in vitro* cultures. Kinetin (1.0 mgl^{-1}) could induce only two shoots of 2.0 mm length. Benzyl amino purine was found superior to kinetin for the induction, proliferation and subsequent growth of the multiple shoots in clove.

The direct effect of cytokinin in tissue culture may vary according to the particular compound used, the type of cultures and the plant species from which it was derived (George and Sherrington, 1984). Superiority of BAP over other cytokinins like kinetin or 2ip has been reported by several other workers like Yadav *et al*. (1990a) in *Syzygium cumini*;

Yadav *et al*. (1990b) in mulberry; Lundergan and Janick in apple (1980).

In clove multiple shoot induction was enhanced (4.0 to 6.0) when concentration of BAP was increased from 0.5 to 2.0 mgl^{-1} along with 1.0 mgl^{-1} of kinetin. Better shoot proliferation in double cytokinin (BAP and kinetin) has been reported by Roy *et al*. (1990) in the case of jack fruit. Increasing the concentration of cytokinin above 3.0 mgl^{-1} was found to have inhibitory effect on multiple shoot induction for clove. Similar inhibitory effect of BAP at higher concentrations has been reported in guava (Jaiswal and Amin, 1987); mangosteen (Goh *et al*., 1988) and in pepper (Philip *et al*., 1992).

Results of BAP-IAA combinations revealed that none of the cultures responded for shoot proliferation even when IAA was incorporated at a level of 0.5 mgl^{-1}. Among the different combinations of BAP and IAA tried, BAP at 0.5 to 2.0 mgl^{-1} along with 0.5 mgl^{-1} IAA was found to be the best.

Normal shoot morphology was observed for the cultures in lower concentration of BAP (0.5 to 1.0 mgl^{-1}) and IAA (0.5 mgl^{-1}). In this case shoots attained a maximum length of 8.0 to 9.0 mm in the multiplication medium. It was also observed that length of the *in vitro* shoots decreased with

increase in BAP concentration. Production of stunted shoots at higher levels of BAP has been reported by Goh et al. (1990) in mangosteen and Yadav et al. (1990b) in mulberry. The treatments revealed that for multiple shoot induction and further growth in clove BAP is very much essential but needed only at low levels (0.5 to 2.0 mgl^{-1}).

Shoot tip explants of clove seedling showed more or less similar response in all the combinations of growth regulators tested. At lower concentrations of cytokinin (0.5 to 1.0 mgl^{-1}) the apical bud continued to grow very slowly giving a single shoot. When higher levels of cytokinin (2.0 to 3.0 mgl^{-1}) were tried, 2 lateral buds emerged from the apex, which had a rosette appearance. Shoot tip explants were proved to be unsuitable for multiple shoot induction and proliferation in clove when compared with nodal segments. Similar results have been reported in clove by Mathew and Hariharan (1990); Superman and Blake (1990) and in other woody plants by Yadav et al. (1990b) and Hutchinson (1982). Endogeneous auxin level was reported to be higher in the apical meristem (Hu and Wang, 1983) and this might be the reason for the poor response of shoot tip explants for multiple shoot induction.

Organic supplements like coconut water, adenine sulphate and phloroglucinol were tried so as to study their

effect on bud break and later growth. In the absence of BAP nodal segments of clove failed to induce multiple shoots with kinetin, and auxins like IAA or NAA. But addition of C.W. at 15 per cent level induced multiple shoots (2 to 4) even in the absence of BAP. Shoots induced in medium containing C.W. and growth regulators showed a vigorous or healthy appearance. The favourable effect of coconut water in the promotion of growth and differentiation of excised tissues and organ have been attributed to the presence of cytokinin and gibberellin like substances in it (Straus and Rodney, 1960). Favourable effect of C.W. in enhancing multiple shoot induction and proliferation were previously reported by Bhaskar (1991) in banana and Lakshmidevi (1992) in orchid, dendrobium.

In clove addition of adenine sulphate, in the culture medium did not favour multiple shoot induction and proliferation. Whereas it was even found to inhibit, or reduce the response. Indole acetic acid or adenine sulphate ($20\ mgl^{-1}$) added alone or in combination had no significant effect on either multiplication rate or shoot length in Camellia japonica (Samartin, 1989). Phloroglucinol, a phenolic auxin synergist commonly used for tree species, gave no favourable results in clove, instead it reduced the percentage of cultures responding for multiple shoot induction, and the

number of multiples produced. Similar results were reported by Anu (1993) in Gymnema sylevestre.

Though multiple shoots to the extent of 6 to 9 could be induced by manipulating the growth regulator combinations in the culture medium, further growth of the multiple shoots induced were found not satisfactory.

5.2.3 Elongation of in vitro shoots

Rugini and Verma (1982), recommended the use of a specific shoot elongation medium for almond cultures, wherein the shoots were transferred from the proliferation medium onto a different culture medium. Among the different treatment combinations tried subculturing in vitro multiple shoots of clove to a medium containing very low level of BAP (0.2 mgl^{-1}) showed a maximum elongation upto 10 to 12 mm. Scale like leaves produced in multiplication medium tend to expand and exhibited normal morphology with this low concentration of BAP. Lowering BAP concentration between 0.1 to 0.5 mgl^{-1} permitted shoot elongation in guava (Amin and Jaiswal, 1987); in chestnut (Chauvin and Salesses, 1987); in walnut (Pennela, 1988) and in mulberry (Yadav et al., 1990b). Incorporation of activated charcoal along with 0.2 mgl^{-1} BAP gave better results for elongation of shoots and leaf production. With charcoal, leaves produced were wider than those with BAP

(0.2 mgl^{-1}) alone. Similar results with activated charcoal have been reported in the case of douglas fir by Gupta and Durzan (1987) and in guava by Jaiswal and Amin (1987). Activated charcoal has got the capacity to adsorb nutrients or growth regulators to some extent (Preil and Engelhardt, 1977; Tisserat, 1979). Thus the inhibitory action of BAP on shoot elongation and leaf production was reduced by the addition of activated charcoal into the culture medium. This might be the reason for the development of well-developed shoots and leaves in culture medium containing activated charcoal.

5.2.4 Rooting of *in vitro* shoots

Shoot explants of clove cultured in WPM containing 2 mgl^{-1} each of IBA and NAA produced 2 to 4 roots of 0.5 cm (within 45 days) length. Combination of auxins found better than single auxin for rooting in guava (Amin and Jaiswal, 1987); in jack (Roy *et al.*, 1990a) and in *Syzygium cumini* (Yadav *et al.*, 1990a). Pulse treatment with the rooting hormone IBA did not favour root initiation. Similar result has been reported by Arrillaga *et al.* (1991) in the case of *Sorbus domestica* L.

Rooting of *in vitro* shoots of clove was found to be a difficult process. A serious problem preventing successful micropropagation of many woody species is their reluctance to

form adventitious roots either *in vitro* or *in vivo* (Nemeth, 1986).

5.3 Indirect organogenesis/embryogenesis

5.3.1 Induction of callus

Plants regenerated from excised shoot apices and axillary buds *in vitro* appear to preserve the integrity of the parental genotype, whereas, those regenerated from callus often demonstrate considerable instability (d'Amato, 1978). But as far as the rate of multiplication and induction of genetic variation are concerned, callus mediated somatic organogenesis or embryogenesis are reported to have greater potentialities than axillary bud release method.

In the present study callus mediated organogenesis/ embryogenesis was tried using different explants. Explants from mature clove tree failed to respond for callus induction in the media combinations tried. However callus could be induced with the juvenile leaf explants by manipulating the growth regulator combinations. Among the seedling explants tried, segments from young pink coloured leaves were found to be the ideal one for induction of calli in clove. In general, the more juvenile the material, the more easily will it respond under *in vitro* cultures (Thorpe and Patel, 1984). Takayama and Misawa (1980) reported that, within any plant,

tissues differ in their degree of determination and their ability to undergo morphogenesis. Evans et al. (1981) suggested that within a given plant, success of callus initiation is dependent on the explant source and that variation in different explants may reflect the difference in the phenotypic physiological expression of cells in the original explant.

Callusing was observed from the cut edges of leaf segments. The initiation of callus from the cut surfaces may be due to more accumulation of the endogeneous auxin, level favouring an active cell division producing a mass of cells. This can also be attributed to the exposure of cells at the cut surfaces to an excessive supply of nutritive substances as compared with cells adjoining intact regions of the explant (Mitra et al., 1965).

Among the different growth regulators tried, 2,4-D was found to be the best for callus induction in clove. Use of 2,4-D for callus induction has been reported in related species of clove by Choo et al. (1978) and Litz (1984).

Formation of callus was a very slow process in clove. The obtention of substantial amount of calli was possible only after 2 to 3 months of culture. Similar slow response was observed in coffee (Sondahl and Sharp, 1977). Woody plants

generally show a slower response to culture conditions than herbaceous angiosperms. This can be related to the less effectiveness for penetration of growth regulators to the explants, the cells of which may also be in a physiologically quiescent state.

Callus obtained were initially creamy yellow in colour. Friable granular calli were induced in lower concentrations of 2,4-D tried. Calli induced with 0.5 mgl^{-1} 2,4-D produced shining light-green globular structures resembling proembryoids. Similar result has been reported by Mathew (1992) in clove.

5.3.2 Organogenesis/embryogenesis

None of the treatments tried gave favourable response on callus differentiation through organogenesis or embryogenesis in clove. Callus produced from leaves of both seedlings and mature trees did not produce any shoots, and were not found to be organogenic in mangosteen (Goh *et al.*, 1988) and *Gymnema sylvestre* (Anu, 1993).

Being a woody plant, *in vitro* cultures of clove faced problems such as polyphenol interference, systemic fungal contamination and poor response at all stages of *in vitro* culture. The explants were quiescent in nature for axillary bud break, further proliferation and also for the callus

mediated organogenesis. During the present study it was possible to overcome the polyphenol interference and partly thrive over microbial interference caused by systemic fungus. However microbial interference is at present the serious constraint for the *in vitro* culture of clove with the field explants. By culturing several thousands of explants only a few cultures were obtained as uncontaminated. The survived cultures, eventhough produced 10 to 12 multiples, further studies for their elongation could not be undertaken due to the limited number of cultures available. Due to the very low absorption rate of clove, field explants showed delayed response for the prophylactic spraying. Thus the period of study available for imposing different treatments was limited. Clove is reported to be the slowest growing tree and it showed similar growth pattern in the *in vitro* culture system also.

With clove seedling explants, microbial contamination and polyphenol interference were not observed during the culture establishment stage. Eventhough multiplication rate was found good, further growth of this multiple shoots was very slow, again due to the slow growing nature of the crop. Purseglove (1981) has reported that, due to low meristematic activity, the clove plants are difficult to propagate vegetatively. From the foregoing discussions it can be concluded that the same property is being reflected in the

in vitro cultures as well. In order to break the recalcitrant nature of clove, further treatment combinations are to be tried utilizing, rejuvenated explant source maintained in controlled partly aseptic conditions.

Summary

SUMMARY

The present investigation was carried out during the period 1992 to 1994 in the Tissue Culture Laboratory of the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara with the objective of standardising the *in vitro* propagation technique in clove, *Syzygium aromaticum*. Mature clove trees and seedlings were used as the source of explants. The salient findings of the investigation are presented below.

1. Culture establishment of mature clove explants faced two major problems such as systemic microbial contamination and polyphenol interference.

2. The systemic microbe causing contamination was identified as *Alternaria sp*.

3. Explants collected eight months after the cessation of a spray schedule of eight months gave 56 per cent of uncontaminated cultures, with a surface sterilization treatment including alcohol soaking (50 per cent) for two minutes followed by $Hgcl_2$ soaking (0.1 per cent) for 12 minutes.

4. Other treatments like soaking the explants in fungicidal solutions, addition of fungicides in the culture medium or providing moisture stress to the explant did not give any reduction in the growth of the systemic fungus.

5. Sealing the cut end of the explant with molten paraffin wax found to be the best pretreatment for preventing the polyphenol interference during culture establishment.

6. Among the media additives tried, polyvenyl pyrrolidone or activated charcoal at 0.5 per cent level gave complete control to the polyphenol interference.

7. White's medium caused severe polyphenol exudation

8. Mature nodal segments and apical shoots produced more phenolic exudalion than tender nodal segments.

9. Surface sterilization of seedling explants was carried out effectively with 0.1 per cent $HgCl_2$ for 10 minutes.

10. Among the six different basal media tried, cent per cent culture establishment was obtained in WPM and half-MS medium. White's medium was found to be unsuitable for *in vitro* culture of clove.

11. Nodal segments from mature clove tree induced 10 to 12 buds in WPM containing 3.0 mgl^{-1} BAP and 1.0 mgl^{-1} kinetin. Explants such as apical shoots, internodal segments and leaf segments were found to be nonorganogenic.

12. Age of the explant was found to influence the proliferation of multiple shoots. Multiple shoots induced from nodal segments of mature clove tree attained only 5.0 mm by 120 days.

13. Benzyl amino purine (0.5 to 2.0 mgl^{-1}) was found to be very much essential for multiple shoot induction from nodal explants of clove seedling.

14. Lower concentration of BAP (0.2 mgl^{-1}) gave elongated shoots.

15. Higher concentration of BAP (above 3.0 mgl^{-1}) showed inhibitory action on multiple shoot induction and proliferation.

16. It was observed that among the cytokinins tried, BAP was superior to kinetin and among the auxins tried, IAA was superior to NAA for multiple shoot induction.

17. Apical shoots were proved to be unsuitable for multiple shoot induction in clove.

18. Coconut water induced multiple shoots even in the absence of BAP and shoots induced in medium containing CW and growth regulators showed a vigorous growth appearance.

19. Adenine sulphate and phloroglucinol did not favour multiple shoot induction and later proliferation.

20. Elongation of in vitro shoots along with the production of 4 to 6 well developed leaves was obtained in WPM supplemented with 0.2 mgl^{-1} BAP and 0.1 to 0.5 per cent activated charcoal.

21. Rooting was obtained with shoot explants cultured in WPM supplemented with 2.0 mgl^{-1} each of NAA and IBA.

22. Young leaf segments of seedlings responded better than nodal, and internodal segments, for callus induction and proliferation.

23. Explants from mature clove tree failed to induce callus in any of the media combinations tried.

24. Half-MS medium supplemented with 3.0 mgl^{-1} 2,4-D and 50 mgl^{-1} casein hydrolysate induced maximum callusing with the leaf explants of clove seedling.

25. Leaf segments produced cream friable callus with light green globular structures in half-MS medium supplemented with 0.5 mgl^{-1} 2,4-D.

26. Indirect organogenesis could not be obtained with various treatment combinations tried.

References

REFERENCES

Alexopaulos, C.J. and Mims, C.W. 1979. Introductory Mycology 3rd Ed. Wiley Eastern Ltd., New Delhi. p. 566.

Amato, F.D. 1978. Chromosome number variation in cultured cells and regenerated plants. In: Thorpe, T.A. (Ed.). Frontiers of Plant Tissue Culture. University of Calgary. p. 289-295.

Amin, M.N. and Jaiswal, V.S. 1987. Rapid clonal propagation of guava through in vitro shoot proliferation on nodal explants of mature trees. Pl. Cell Tissue Organ Cult. 9 (3): 235-243.

Amin, M.N. and Jaiswal, V.S. 1988. Micropropagation as an aid to rapid cloning of a guava cultivar. Scientia Horticulturae 36 (1-2): 89-95.

Amorium, H.V., Dargall, D.K. and Sharp, W.R. 1977. The effect of carbohydrate and nitrogen concentration on phenol synthesis in Paris scarlet rose cells grown in tissue culture. Physiologia Plantarum 39 (1): 91-95.

Anderson, W.C. 1975. Propagation of Rhododendrons by tissue culture: Part I, development of a culture medium for multiplication of shoots. Comb. Proc. Inter. Pl. Prop. Soc. 25: 129-135.

Ancora, G., Belli-Donini, M.L. and Cuozzo, L. 1981. Globe artichoke plants obtained from shoot apices through rapid in vitro micropropagation. Scientia Horticulturae 14: 207-213.

Anon. 1979. Clove. Directorate of Cocoa Areacanut and Spices Development, Calicut.

Anu, K.I. 1993. Standardisation if in vitro propagation technique in Gymnema sylvestre R. Br. M.Sc. thesis, Kerala Agricultural University, Vellanikkara.

Arrillaga, I., Marzo, T. and Segura, J. 1991. Micropropagation of juvenile and adult Sorbus domestica L. Pl. Cell Tissue Organ Cult. 27: 341-348.

Asahira, T. and Nitsch, J.P. 1969. Effect of polarity and kinetin on the browning reaction of Dioscorea batatas and D. japonila. Planta 84: 292-294.

Badami, V.K. 1938. Germination of clove seeds. A publication of Department of Agriculture, Mysore State. pp.5.

Bagratishvity, D.G., Zaprometov, M.N. and Butenko, R.G. 1984. Effect of ABA on tea cell culture growth and synthesis of phenolics. Fiziologiya Rastenii 31 (5): 980-982.

Bajaj, Y.P.S. 1978. Regeneration of haploid tobacco plants from isolated pollen, grown in drop culture. Indian J. Exp. Biol. 16: 407-409.

Baleriola-Lucas, C. and Mullins, M.G. 1983. Phloridzin in tissue cultures of apples (Abstract). Aust. hort. Res. Lett. (55): 74.

Ball, E.A. 1978. Cloning in vitro of Sequoia sempervirens. In. Hughes, K.W., Henke, R. and Constatin, M. (Eds.). Propagation of Higher Plants through Tissue Culture. Tech. Inf. Center USD Energy Conf. 7804111, p. 259.

Ballester, A., Sanchez, M.C. and Vietez, A.M. 1989. Etiolation as a pretreatment for in vitro establishment and multiplication of chestnut. Physiologia Plantarum 77 (3): 395-400.

Barnes, L.R. 1979. In vitro propagation of walnut. Scientia Horticulturae 11: 223-227.

Bergmann, L. 1960. Growth and division of single cells of higher plants in vitro. J. Gen. Physiol. 43: 841-851.

*Bergmann, L. 1964. Dar Einflub Von Kinetin auf die Lignibildung und differenzierung in Gewebekulturen Von Nicotiana tabaccum. Planta 62: 221-254.

Bhaskar, J. 1991. Standardisation of in vitro propagation techniques in banana. M.Sc. (Hort.) thesis, Kerala Agricultural University, Vellanikkara.

Bhat, S.R. and Chandel, K.P.S. 1991. A novel technique to overcome browning in tissue culture. Pl. Cell Rep. 10: 358-361.

Bonga, J.M. 1980. Plant propagation through tissue culture, emphasizing woody species. In. Sala, F., Parisi, B., Cella, R. and Ciferri, O. (Eds.). Plant Cell Cultures: Results and Perspectives. Elsevier/North-Holland Biomedical Press, Amsterdam, p. 253-264.

Bonga, J.M. 1982. Vegetative propagation in relation to juvenility, maturity and rejuvenation. In. Bonga, J.M. and Durzan, D.J. (Eds.). Tissue Culture in Forestry. Martinus Nijhoff. The Hague, Netherlands. p. 387-412.

*Bronchart, R. and Nougarede, A. 1970. Evenements metaboliques et structuraux au niveau du meristeme du Perilla nankinensis lors de la phase preflorale. In. Bernier (Ed.) Cellular and Molecular Aspects of Floral Induction. Longman, London. p. 27-36.

Broome, C. and Zimmerman, R.H. 1978. In vitro propagation of blackberry. HortScience 13 (2): 151-153.

Brown, D.C.W. and Thorpe, T.A. 1986. Plant regeneration by organogenesis. In: Vasil, I.K. (Ed.). Cell Culture and Somatic Cell Genetics of Plants. Plant Regeneration and Genetic Variability. Vol.3, Acad. Press, New York. p. 49-66.

Brown, D.M., Groom, C.L., Cyitanik, M., Brown, M., Cooper, J.L. and Arditti, J. 1982. Effects of fungicides and bacteriocides on orchid seed germination and shoot tip cultures in vitro. Pl. Cell Tissue Organ cult. 1: 165-180.

Burger, D.W. 1987. In. vitro micropropagation of Eucalyptus sideroxylon. HortScience 22 (3): 496-497.

Chauvin, J.E. and Salesses, G. 1988. Advances in chestnut micropropagation (Castanea sp.). Acta Horticulturae (227): 339-342.

Chevre, A.M., Gill, S.S., Mouras, A. and Salesses, G. 1983. In vitro vegetative multiplication of chestnut. J. hort. Sci. 58: 23-29.

Chipley, J.R. and Heaton, E.K. 1971. Appl. Microbiol. 22: 252-253.

Choo, V.S., Ho, S.H., Fu, F.M.L. 1978. Preliminary investigations on the propagation of Eugenia grandis through tissue culture. Gardens Bulletin 31 (2): 253-254.

Christiansen, J. and Fonnesbech, M. 1975. Prevention by polyvenylpyrrolidone of growth inhibition of Hamamelis shoot tips grown in vitro and browning of the agar medium. Acta Horticulturae 54: 101-104.

McComb, J.A., Newell, C. and Arthur, N. 1986. Micropropagation of Verticordia grandis and other Verticordia spp. Pl. Propagator 32 (1): 12-14.

Compton, M.E. and Preece, J.E. 1988. Exudation and explant establishment. Newslett. IAPTC 50: 9-18.

Conger, B.W. 1981. (Ed.) Cloning Agricultural Plants via in vitro Techniques. CRC Press, Florida. p. 1-74.

*Curir, P., Damiano, C., Esposito, P. and Ruffoni, B. 1986. The in vitro propagation of Eucalyptus gunni Hook and Eucalyptus staurtiana F.Y.M. Annali dell' Istituto Sperimentale perla floriculturae 17 (1): 73-84.

Dalal, M.A., Sharma, B.B. and Srinivasa Rao, M. 1992. Studies on stock plant treatment and initiation culture mode on control of oxidative browning of in vitro cultures of grape vine. Scientia Horticulturae 51 (1-2): 35-41.

Damiano, C., Curir, P., Esposito, P. and Ruffoni, B. 1989. Present micropropagation research programs at ISF in sanremo. Acta Horticulturae (251): 129-133.

Davis, J.E. 1972. Effects of auxin in polyphenol accumulation and the development of phenylalanine Ammonia-Lyase activity in dark-grown suspension cultures of Paul's Scarlet Rose. Planta 104: 66-77.

Dodds, J.H. and Roberts, L.W. 1985. Experiments in Plant Tissue Culture. Cambridge University Press, London, p.28.

Doublin, P. 1984. Extractable production section IX. In: Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.). Handbook of Plant Cell Culture, MacMillan Pub. Co., New York. p. 541-564.

*Eliott, R.F. 1972. Axenic cultures of shoot tips of apple. Newzealand J. Bot. 10: 254-258.

Evans, D.A., Sharp, W.R. and Flick, C.E. 1981. Plant regeneration from cell cultures. Hort. Rev. 3: 214-314.

*Fernie, L.M. 1946. Preliminary trials on the rooting of clove. East Afr. agric. J. 12 (135): 6.

*Fitchel-Parnell, M. 1990. Successful establishment of Piper nigrum in tissue culture. Inligtingsbulletin - Navorsing - Sinstituut Vir Sitrus en Subtropiese Urugte (212): 4-5.

Flynn, W.P., Glicenstein, L.G. and Fritz, P.J. 1990. Theobroma cacao L. an axillary bud in vitro propagation procedure. Pl. Cell Tissue Organ Cult. 20: 111-117.

Forrest, G.J. 1969. Studies on the polyphenol metabolism of tissue cultures derived from the tea plant (Camellia sinensis L.). Biochem. J. 113: 765-772.

D'Fossard, R.A. and D'Fossard, H. 1988. Micropropagation of some members of the Myrtaceae. Acta Horticulturae (227): 346-351.

*Francoise, E. 1936. Giroflier el girofla. Rev. Bot. Appl. agric. Trop. 589-608.

Fridborg, G. and Erikson, T. 1975. Effects of activated charcoal on growth and morphogenesis in cell cultures. Physiologia Plantarum 34: 306-308.

*Galzy, R. 1972. La Culture in vitro des apex de Vitis rupestris Compt. Rend. D. 274: 210-213.

Gamborg, O.L., Miller, R.A. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Res. 50: 151-158.

Gamborg, O.L. and Shyluk, J.P. 1981. Nutrition, media and characteristics of plant cell and tissue cultures. In: Thorpe, T.A. (Ed.). Plant Tissue Culture: Methods and Applications in Agriculture. Academic Press, New York. p. 21-44.

*Gautheret, R.J. 1939. Sur la possibilite de realiser la culture indefenne des tissus de tubercules de carotte. C. R. Acad. Sci. Paris 208: 118-120.

George, E.F. and Sherrington, P.O. 1984. Plant Propagation by Tissue Culture. Exegetics Limited, Basing Stoke. p. 690.

Goh, H.K.L., Rao, A.N. and Loh, C.S. 1988. In vitro plantlet formation in mangosteen (Garcinia mangostana L.). Annals Bot. 62: 87-93.

Goh, H.K.L., Rao, A.N. and Loh, C.S. 1990. Direct shoot bud formation from leaf explants of seedlings and mature mangosteen (Garcinia mangostana L.) trees. Pl. Sci. Lett. 68 (1): 113-121.

Gopalakrishnan, M., Narayanan, C.S. and Mathew, A.G. 1988. Chemical composition of Indian clove bud, stem and leaf oils. Indian Perfumer 32 (3): 229-235.

Griffis, J.L.Jr., Sheehan, T.J. and Dennis, B. 1981. Excised floral tissues of coconut palm (Cocos nucifera L.) cultured in vitro. HortScience 16: 460.

Grout, B.W.W. 1975. Wax development on leaf surfaces of Brassica oleraceae var. currawong regenerated from meristem culture. Pl. Sci. Lett. 5: 401-405.

Gupta, P.K. and Durzan, D.J. 1987. In vitro establishment and multiplication of juvenile and mature Douglas fir and sugar pine. Acta Horticulturae (212):483-485.

Gupta, P.K., Mascarenhas, A.F. and Jaganathan, V. 1981. Tissue culture of forest trees - clonal propagation of mature trees of Eucalyptus citriodora Hook. by tissue culture. Pl. Sci. Lett. 20: 195-201.

Gupta, P.K., Nadgir, A.L., Mascarenhas, A.F. and Jagannathan, V. 1980. Tissue culture of forest trees: Clonal multiplication of Tectona grandis L. (teak) by tissue culture. Pl. Sci. Lett. 17: 259-268.

*Haberlandt, G. 1902. Kultinver suche mit isolierten pfanzellen. Sber. Akad. Wiss. Wien III: 69-92.

Hackett, W.P. 1980. Control of phase change in woody plants. In. Little, C.H.A. (Ed.) Control of Shoot Growth in Trees. Proc. Joint Workshop IUFRO Working Parties on Xylem and Shoot Growth Physiology, Fredericton, NB, Canada. p. 257-272.

*Hanlin, R.T. 1971. Proc. Ga. Pecan Grow. Assoc. 2: 20-26.

Hanus, D. and Rohr, R. 1987. In vitro plantlet regeneration from juvenile and mature sycamore maple. Acta Horticulturae (212): 77-82.

Hasegawa, P.M. 1980. Factors affecting shoot and root initiation from cultured rose shoot tips. J. Am. Soc. Hort. Sci. 105: 216-220.

*Heller, R. 1953. Recherches sur la nurition minerale de tissus vegetaux cultives in vitro. Ann. Sci. Nat. Bot. Biol. Veg. 14: 1-223.

Hildebrandt, V. and Harney, P.M. 1988. Factors affecting the release of phenolic exudate from explants of Pelargonium x hortum, Bailey Sprinter Scarlet. J. hort. Sci. 63 (4): 651-675.

Hu, C.Y. and Wang, P.J. 1983. Meristem, shoot tip and bud cultures. Evans, D.A., Sharp, W.R., Ammirato, P.V. and Yamada, Y. (Eds.).Handbook of Plant Cell Culture Vol.I. Techniques for propagation and Breeding. MacMillan Pub. Co., New York. p. 177-227.

Hunter, C.S. 1979. In vitro culture of Cinchona ledgeriana L. J. hort. Sci. 54 (2): 111-114.

Hutchinson, J.F. 1982. In vitro propagation of apple using organ culture. In. Fugiwara, A. (Ed.). Plant Tissue Culture. Maruzuen, Tokyo. p. 729-730.

Ichihashi, S. and Kako, S. 1977. Studies on clonal propagation of Cattleya through tissue culture method. II. Browning of cattleya. J. Jap. Soc. Hort. Sci. 46: 325-330.

Jaiswal, V.S. and Amin, M.N. 1987. In vitro propagation of guava from shoot cultures of mature trees. J. Pl. Physiol. 130 (1): 7-12.

*Jarvie, J.K., Koerniati, S. and Pool, P.A. 1986. Screening for compatibility between clove (Syzygium aromaticum) and related myrtaceous species. Pemberitaan Penelitian Tanaman Industry 11 (3-4): 56-59.

John, M.D., Gurupreet Singh and Jaiswal, P.K. 1993. Clove. Physico-chemical characteristics of cloves whole and powder. Spices India 6 (6): 2-9.

Kartha, K.K., Gamborg, O.L., Constable, F. and Shyluk, J.P. 1974. Regeneration of cassava plants from shoot apical meristem. Pl. Sci. Lett. 2: 107-113.

*Knox, C.A.P. and Smith, R.H. 1980. In vitro. 16: 651-654.

Krikorian, A.D. 1982. Cloning higher plants from aseptically cultured tissues and cells. Biol. Rev. 57: 151-218.

Kumar, N., Khader, A., Rangaswami, P. and Irulappan, I. 1993. Introduction to Spices, Plantation Crops, Medicinal and Aromatic Plants. 1st Ed. Rajalakshmi Pub., Nagercoil. p. 4.01-4.03.

Kyte, L. and Briggs, B. 1979. A simplified entry into tissue culture production. Proc. Int. Pl. Prop. Soc. p. 290.

Lakshmidevi, S. 1992. Standardisation of explant for in vitro propagation in Dendrobium spp. M.Sc. thesis, Kerala Agricultural University, Vellanikkara.

Lakshmisita, G. 1979. Morphogenesis and plant regeneration from cotyledonary culture of Eucalyptus. Pl. Sci. Lett. 14 (1): 63-68.

Lane, W.D. 1979. The influence of growth regulators on root and shoot initiation from flax meristem tips and hypocotyls in vitro. Physiologia Plantarum **45**: 260-264.

*Legrand, B. and Missiso, E. 1986. Effect of size of the explants and growth regulators on the development of tissues of Theobroma cacao L. var. Amelonado cultivated in vitro. Cafe Cacao The' **30** (4): 239-246.

*Lenartowicz, A. and Millikan. 1977. Meristem culture of Juglans nigra L. Proc. II State Acad. Sci. **70** (2): 193 (Abstr.).

Levy, L.W. 1981. A large scale application of tissue culture - The mass propagation of pyrethrum clones in Ecaudor. Environ. Exp. Bot. **21**: 389-395.

Litz, R.E. 1984. In vitro responses of adventitious embryos of two polyembryonic Eugenia sp. HortScience **19** (5): 720-722.

Lloyd, B. and McCown, B. 1980. Commercially feasible micropropagation of mountain Laurel Kalmia latifolia by use of shoot tip culture. Comb. Proc. Int. Plant Prop. Soc. **30**: 421-427.

Lo, O.F., Chen, C.J. and Ross, J.G. 1980. Vegetative propagation of temperate foliage grasses through callus culture. Crop Sci. **20**: 363-367.

Loh, C.S. and Rao, A.N. 1989. clonal propagation of guava (Psidium guajava L.) from seedling and grafted plants and adventitious shoot formation in vitro. Scientia Horticulturae 39 (1): 31-39.

Lundergan, C.A., Janick, J. 1980. Regulation of apple shoot proliferation and growth in vitro. Hort. Res. 20 (1): 19-24.

*Maarri, K. AL., Duron, M., Arnaud, Y. and Miginaic, E. 1986. Comparative study on the response to micropropagation of adult Passe Crassane pears and seedlings in meristem culture. Comptes Rendus Academie Agriculture France 72 (5): 413-421.

*Maistre, J. 1964. Les plantes a epices. Techniques agricoles el production tropicales G-P. Maison-neuve el Larose Peris.

Mallika, V.K., Sankar, A.M., Sindhu, K., Rekha, C., Vijayakumar, N.K. and Nair, R.V. 1992. Plantlet regeneration in vitro from nodal segments of cocoa (Theobroma cacao L.) and field planting. J. Plantation Crops 20: 114-122.

Manzanera, J.A. and Pardos, J.A. 1990. Pl. Cell Tissue Organ Cult. 21: 1-8.

Marino, G., Magananini, L., Battistini, S. and Righetti, B. 1991. Effect of hormones and main carbon energy sources on in vitro propagation of apricot (Prunus americana L.) cvs. Sancastrese and Portici. Acta Horticulturae (293): 355-362.

Marks, T.R. and Simpson, S.E. 1990. Reduced phenolic oxidation at culture initiation *in vitro* following the exposure of field grown stock plants to darkness or low level of irradiance. *J. hort. Sci.* 65 (2): 103-111.

Mathew, M.K. 1992. Developmental studies on certain spice plants. Ph.D. thesis, Calicut University, Kerala.

Mathew, M.K., Francis, M.S. and Hariharan, M. 1987. Development of callus in cloves (*Syzygium aromaticum* (L.) Merr. and Perry). *J. Plantation Crops* 15 (2): 123-125.

Mathew, M.K. and Hariharan, M. 1990. Micropropagation of cloves (*Syzygium aromaticum* (L.) Merr. and Perry). *J. Plantation Crops* 20 (Supplement): 308-309.

Mathias, P.J. and Anderson, P.G. 1987. Bacterial contamination in tropical hard wood cultures. *Acta Horticulturae* 212: 43-48.

*May, W.B. 1949. Horticultural work with clove tree in Zanzibar. *Gard. Chron.* 125: 178-179.

Mayer, A.M. and Harel, E. 1979. Polyphenol oxidases in plants. *Phytochem.* 18: 193-215.

Menon, R. and Nair, S. 1992. Soft wood grafting of clove (*Syzygium aromaticum* L.) plants. *South Indian Hort.* 40 (1): 62-63.

Messeguer, J. and Mele, E. 1987. In vitro propagation of adult material and seedlings of Corylus avellana. Acta Horticulturae 212: 499-501.

Miller, C.D., Okumara, F.S., Saltzer Von, M.H. and Strong, F.M. 1956. Isolation structure and synthesis of kinetin, a substance promoting cell division. J. Am. Chem. Soc. 78: 1375-1380.

Mitra, G.C., Prabha, C. and Chaturvedi, H.C. 1965. Histogenesis of callus tissues from different organs of Rauwolfia serpentina Benth. in tissue culture. Indian J. Exp. Biol. 216: 222.

Monaco, L.L., Sondahl, M.R., Carvalho, A., Crocomo, D.J. and Sharp, W.R. 1977. Application of tissue culture in the improvement of coffee. In. Reinert, J. and Bajaj, Y.P.S. (Eds.) Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Springer Verlag, Bernin. p. 109-129.

Muralidharan, E.M., Gupta, P.K. and Mascarenhas, A.F. 1989. Plantlet production through high frequency somatic embryogenesis in long term cultures of Eucalyptus citriodora. Pl. Cell Rep. 8 (1): 41-43.

Murashige, T. 1974. Plant propagation by tissue culture. Ann. Rev. Pl. Physiol. 25: 135-166.

Murashige, T. 1977. Clonal propagation of horticultural crops through tissue culture. In: Barz, W., Reinhard, E. and Zenk, M.H. (Eds.). Plant Tissue Culture and Its Biotechnological Applications. Springer-Verlag, New York, p. 392-403.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Nayar, M.N.C., Hariharan, R. and Kosi, M.P.K. 1979. Effect of presowing treatment on the sprouting of clove (Eugenia caryophyllata Thumb.) seeds. Indian Cocoa Arecanut Spices J. 3 (2): 33-34.

Nazeem, P.A. 1989. Deficiency symptoms of mineral nutrients in clove, Syzygium aromaticum (L.) Merr. and Perry. Ph.D. thesis, Kerala Agricultural University, Vellanikkara.

Nemeth, G. 1986. Induction of rooting. In: Bajaj, Y.P.S. (Ed.). Biotechnology in Agriculture and Forestry, Springer Verlag, Berlin. p. 49-64.

*Nobecourt, P. 1939. Sur la Perenvite del' arrgmentation de volume des cultures de tissues vegetaux. C.R. Soc. Biol. Paris 130: 1270-1271.

Norton, M.E. and Norton, C.R. 1986. In vitro shoot proliferation of Prunus and Spirea in relation to explant type. Pl. Propagator 32 (3): 5.

Obeidy, A.A. and Smith, M.A.L. 1990. Establishing axenic cultures from mature pecan embryo explants on media with low water availability. Pl. Cell. Rep. 9: 463-465.

Papadatou, P., Pontikis, C.A., Ephtimiadou, E. and Lydaki, M. 1990. Rapid multiplication of guava seedlings by in vitro shoot tip culture. Scientia Horticulturae 45 (1-2): 99-103.

Pennela, R., Garavito, C., Sanchez-James, R. and Rodriguez, R. 1988. Multiple shoot bud stimulation and rhizogenesis induction of embryogenic and juvenile explants of walnut. Acta Horticulturae (227): 457-459.

*Peterson, R.L. 1975. The initiation and development of root buds. In: Torrey, J.D., Clarkson, D.T. (Eds.). The Development and Function of Roots. Academic Press, London. p. 125-162.

Pierik, R.L.M. and Steegman, H.H.M. 1976. Vegetative propagation of Anthurium schezerianum shoot through callus cultures. Scientia Horticulturae 4: 291.

Philip, V.J., Joseph, D., Triggs, G.S. and Dickinson, N.M. 1992. Micropropagation of black pepper (Piper nigrum L.) through shoot tip cultures. Pl. Cell Rep. 12: 41-44.

*Pool, P.A., Bermawie, N. and Usman. 1991. Factors affecting marcotting in clove (Syzygium aromaticum). Ind. Crops Res. J. 4 (1): 12-16.

Preil, W. and Englehardt, M. 1977. Meristem culture of azaleas (Rhododendron simsii). Acta Horticulturae 78: 203-208.

Priyadarshan, P.M., Radhakrishnan, V.V. and Madhusoodanan, K.J. 1989. Spice India 11 (4): 5-10.

Purseglove, J.W., Brown, E.G., Green, C.L. and Robbins, S.R.J. 1981. Spices Vol. 1. Longman, London. p. 241.

*Rabechault, T.H., Martin, J. and Gas, S. 1976. XII. Effects de substan ces de croissance a des doses superoptimales. Relation avec le brunissement des tissus. Oleag 31 (4): 159-163.

Rahman, M.A. and Blake, J. 1988. Factors affecting in vitro proliferation and rooting of shoots of jack fruit (Artocarpus heterophyllus Lam.). Pl. Cell Tissue Organ Cult. 13: 179-187.

Rajmohan, K., Kumaran, M.N. 1988. Influence of explant source on the in vitro propagation of jack (Artocarpus heterophyllus Lam). agric. Res. J. Kerala 26 (2): 169-174.

*Reinert, J. 1958. Untersuchugen uber die morphogenese an geneelse bulteven. Ber. Vient Botanges 71: 15.

*Reuveni, O. and Kipnis, H.L. 1974. Studies of the in vitro culture of date palm (Phoenix dactylifera L.) tissues and organs. Pamphlet (145): pp. 40.

Ripley, K.P. and Preece, J.E. 1986. Micropropagation of Euphorbia lathyris L. Pl. Cell Tissue Organ Cult. 5: 213-218.

Rodriguez, R. 1982. Callus initiation and root formation from in vitro cultures of walnut cotyledons. HortScience 17: 195-196.

Rodriguez, R., Diaz-Sala, C. and Ancora, G. 1988. Sequential cultures of explants taken from adult Corylus avellana L. Acta Horticulturae 227: 45-49.

Roy, S.K., Rahman, S.L. and Majumdar, R. 1990. In vitro propagation of jack fruit (Artocarpus heterophyllus Lam.). J. hort. Sci. 65 (3): 355-358.

*Rugini, E. and Verma, D.C. 1982. Micropropagation and cell suspensions of a difficult to propagate almond (Prunus amygdalus Batsch) cultivar. In: Fujiwara, A. (Ed.) Plant Tissue Culture. Maruzen, Tokyo. p.741-742.

Samartin, A. 1989. A comparative study of effects of nutrient media and cultural conditions on shoot multiplication of in vitro cultures of Camellia japonica explants. J. hort. Sci. 64 (1): 73-79.

Schenk, R.V. and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.

*Schleiden, M.J. 1838. Beitrage zur phytogenesis, Muller. Arch. Anat. Physiol. Wiss. Med. p. 137-176.

*Schroeder, H.W. and Cole, R.J. 1977. J. Agric. Food Chem. 25: 204-206.

*Schwann, T. 1839. Mikroskopische untersuchungen uber die ubereinstimmung in der struktur und dem wachstume der Tiere und Pfanzan. ('Ostwalds Klassiller der exakten Wissenschaften' No. 176. Englemann, Leipzig, 1910).

Sheffield, F.M.L. 1952. Studies of the clove tree. IV. Natural grafting and its bearing on sudden death disease. Ann. appl. Biol. 39: 103-110.

Sheilds, R., Robinson, S.J. and Anslow, P.A. 1984. Use of fungicides in plant tissue culture. Pl. Cell Rep. 3: 33-36.

Shipton, W.A. and Jackes, B.R. 1986. Clonal propagation of Leptospermum sp. by tissue culture. Pl. Cell Rep. 5: 5-8.

D'Silva and D'Souza, L. 1992. In vitro propagation of anacardium occidentale L. Pl. Cell Tissue Organ Cult. 29: 1-6.

Skirvin, R.M. 1980. Fruit Crops. In. Conger, B.V. (Ed.) Cloning Agricultural Plants via in vitro Technique. CRS Press, Boca, Raton, Florida. p. 51-139.

Skirvin, R.M. and Chu, M.C. 1979. In vitro propagation of 'Foreever Yours' rose. HortScience 14: 608-610.

Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tisues cultured in vitro. Symp. Soc. Exp. Biol. 11: 118-131.

Skoog, F. and Tsui, C. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Am. J. Bot. 35: 782.

*Somers, P.W., Vansambeek, J.W., Preece, J.E., Gaffney, G. and Myeca, O. 1982. In vitro micropropagation of black walnut (Juglans nigra L.). Proc. Seventh N. Am. For. Bio. Workshop. July: 26-28.

Sondahl, M.R. and Sharp, W.R. 1977. High frequency induction of somatic embryos in cultured leaf explants of Coffea arabica L. Z. Pflanzenphysiol. 81: 395-408.

Spices Board. 1993. Business: Indian spices making rapid strides. Indian Spices 30 (2&3): 6-7.

Stevenson, J.H. and Harris, R.E. 1980. In vitro plantlet formation from shoot tip explants of Fuchsia hybrida cv. Swing time. Can. J. Bot. 58: 2190-2199.

Steward, F.C., Mapes, O.M. and Moars, K. 1958. Growth and organised development of cultured cells. Organization in culture growth from freely suspended cells. Am. J. Bot. 45: 705-708.

Straus, J. and Rodney, R.E. 1960. Response of Cupressus funebris tissue culture to gibberellins. Sci. 131: 1806-1807.

*Superman, U. and Blake, J. 1990. Studies on tissue culture of clove tree plant. Indonesian J. Crop Sci. 5 (2): 67-75.

Sutter, E. and Langhans, R.W. 1982. Formation of epicuticular wax and its effect on water loss in cabbage plants regenerated from shoot-tip culture. Can. J. Bot. 60: 2896-2902.

Takayamma, S. and Miswa, W. 1980. Differentiation in Lilium bulbscales grown in vitro. Effects of activated charcoal, physiological age of bulbs and sucrose concentration on differentiation and scale leaf formation in vitro. Physiologia Plantarum 48: 121-125.

Thorpe, T.A. and Patel, K.R. 1984. Clonal propagation: adventitious buds. In: Vasil, I.K. (Ed.). Cell Culture and Somatic Cell Genetics. I. Laboratory Procedure and their Applications, Academic Press, Orlando. p. 49-57.

*Tidbury, G.E. 1949. The clove tree. Crossby Lockwood and Son, Ltd. London.

Tisserat, B.H. 1979. Propagation of date palm (Phoenix dactylifera L.) in vitro. Exp. Bot. 30: 1275-1283.

Vanoverbeek, T., Conklin, M.E. and Blakeslee, A. 1941. Factors in coconut milk essential for growth and development of very young Datura embryos. Sci. 94: 350.

Vasil, V. and Hildebrandt, A.C. 1965. Differentiation of tobacco plants from single isolated cells in micro culture. Sci. 150: 889-892.

Vasil, I.K. and Vasil, V. 1980. Clonal propagation. Int. Rev. Cytol. Suppl. II (A): 145-173.

Vaughn, K.C. and Duke, S.O. 1984. Function of polyphenol oxidase in higher plants. Physiologia Plantarum 60: 106-112.

Vieitez, A.M., Ballester, M.L. and Vieitez, E. 1983. In vitro plantlet regeneration of mature chestnut. J. hort. Sci. 58 (4): 457-463.

Wang, P.J. and Hu, C.Y. 1980. Regeneration of virus-free plants through in vitro culture. In: Fiechter, A. (Ed.) Advances in Biochemical Engineering. Vol. 18, Springer-Verlag, New York, 1st Ed. p. 61-99.

Wardle, K.E., Dobbs, E.B. and Short, K.C. 1983. In vitro acclimatization of aseptically-cultured plants to humidity. J. Am. Soc. Hort. Sci. 108: 386-389.

Welander, T. 1978. In vitro propagation of clones from different cultivars of Begonia x hiemalis. Swed. J. agric. Res. 8: 181-183.

White, P.R. 1934. Potentially unlimited growth of excised auxin and kinetin in apical dominance. Physiologia Plantarum 11: 62-74.

White, P.R. 1943. Nutrient deficiency studies and an improved inorganic nutrient for cultivation of excised tomato roots. Growth 7: 53-65.

*Wigg, L.G.T. 1940. The production of field plants of the clove tree. E. Afr. agric. J. 5: 268-278.

Wilson, Z.A. and Power, J.B. 1989. Elimination of systemic contamination in explant and protoplast cultures of rubber (Hevea brasiliensis Mull. Arg.). Pl. Cell Rep. 7 (8): 622-625.

Wit, F. 1969. The clove tree Eugenia caryophyllus (Sprengel) Bullock Harrison. In: Ferwerda, F.P. and Wit, F. (Eds.) Outline of Perennial Crop Breeding in the Tropics. Veenman and Zoner, N.V. Wageningen. p.163-175.

Wood, B.W. 1982. HortScience 17: 890-891.

Yadav, U., Lal, M., Jaiswal, V.S. 1990a. In vitro micro-propagation of the tropical fruit tree Syzygium cumini L. Pl. Cell Tissue Organ Cult. 21 (1): 87-92.

Yadav, U., Lal, M. and Jaiswal, V.S. 1990b. Micropropagation of Morus nigra L. from shoot tip and nodal explants of mature trees. Scientia Horticulturae 44: 61-67.

Yegnanarayanayyar, A.K. 1960. Cultivation of cloves in India. Indian Council of Agricultural Research, New Delhi.

Yeoman, M.M. 1986. Plant Cell Culture Technology. Blackwell Scientific Pub., Malabourne, p. 33.

Yu, Y.B. 1991. Study on some factors in tissue culture of lychee (Litchi chinensis). Fujian agric. Sci. Tech. (5): 17-18.

Zagoskina, N.V. and Zaprometov, M.N. 1979. The effect of l-NAA on the growth and production of phenolic compounds by tea tissue culture. Fiziologiya Rastenii 26 (4): 681-687.

Zaid, A. 1987. In vitro browning of tissues and media with special emphasis to datepalm cultures - A review. Acta Horticulturae (212):561-565.

Zaid, A. and Tisserat, B.H. 1983. In vitro shoot tip differentiation in Phoenix dactylifera L. Datepalm J. 2 (2): 163-182.

Ziv, M. 1986. In vitro hardening and acclimatization of tissue culture plants. In: Winther, L.A. and Alderson, P.G. (Eds.). Plant Tissue Culture and its Agricultural Application, Butterworths, London. p. 187-196.

Ziv, M. and Halevy, A.H. 1983. Control of oxidative browning and in vitro propagation of Strelitzia reginae. HortScience 18 (4): 434-436.

* Originals not seen

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ABSTRACT OF A THESIS

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ABSTRACT

A study was taken up in the Department of Plantation Crops and Spices, College of Horticulture, Vellanikkara, during 1992 to 1994 to standardise the *in vitro* propagation technique in clove *Syzygium aromaticum* (L.). Mature field grown trees and seedlings of clove were used as sources of explants in the present study. Different routes like enhanced release of axillary buds, and callus mediated organogenesis/ embryogenesis were attempted for the crop.

Culture establishment of mature field grown clove explants faced two serious problems viz. fungal contamination caused by *Alternaria sp.* and polyphenol interference. Prophylactic spraying given to the mother plants with aureofunginsol gave 44 per cent reduction in the contamination rate. Polyphenol interference was completely controlled with wax sealing treatment given to the explants, or by supplementing the culture medium with polyvenyl pyrrolidone or activated charcoal. Multiple shoots were induced from nodal explants cultured in WPM supplemented with BAP (3.0 mgl^{-1}) and kinetin (1.0 mgl^{-1}).

Seedling explants of clove showed very good culture establishment. Microbial contamination and polyphenol interference were observed only to a very limited extent

Multiple shoots were induced from nodal explants cultured in half-MS and WPM supplemented with lower levels and combinations of growth regulators like BAP, kinetin, IAA and NAA. Additives like CW induced vigorous shoots whereas adenine sulphate and phloroglucinol did not give any favourable response for multiple shoot induction. Incorporation of activated charcoal and very low level of BAP (0.2 mgl^{-1}) favoured elongation of shoots and leaf production. Shoot explants showed rooting in WPM containing IBA and NAA each at 2.0 mgl^{-1}.

Callus could be induced from leaf and internodal segments of clove seedling cultured in half-MS medium supplemented with 2,4-D, NAA or BAP + IAA. Addition of casein hydrolysate favoured callus induction and proliferation. Calli induced with 0.5 mgl^{-1} of 2,4-D produced shining globular structures resembling proembryoids. Calli obtained were failed to induce organogenesis with the treatments tried.

Due to low meristematic activity clove plants are difficult to propagate vegetatively. Moreover clove is reported to be the slowest growing tree. The same property is being reflected in the *in vitro* cultures as well, hence much more concerted efforts are required to develop a viable protocol for the micropropagation of clove *Syzygium aromaticum* (L.).