# TISSUE CULTURE STUDIES ON CINNAMON (*Cinnamomum verum* Bercht. & Presl) AND CASSIA (*Cinnamomum cassia* Blume)

Thesis submitted to the
University of Calicut for the award of the Degree of
**Doctor of Philosophy in Botany**


***By***

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**KERALA, INDIA**


**2009**

# DECLARATION

I hereby declare that the thesis entitled **'Tissue culture studies on Cinnamon (*Cinnamomum verum* Bercht. and Presl) and Cassia (*Cinnamomum cassia* Blume)'** submitted for the award of the Degree of **Doctor of Philosophy in Botany** of the University of Calicut is an original research work done by me and no part of it has been submitted for the award of any other Degree or Diploma.

Place: University of Calicut
Date : 25-09-2009

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## CERTIFICATE

This is to certify that the thesis entitled **'Tissue Culture Studies on Cinnamon (*Cinnamomum verum* Bercht. and Presl) and Cassia (*Cinnamomum cassia* Blume)'** submitted by **Smt. Mini P. Mathai** for the Degree of **Doctor of Philosophy in Botany** of the University of Calicut is a record of bonafide research carried out by her under my guidance. No part of this thesis has been submitted to any other University for the award of any other Degree or Diploma.

**Prof. (Dr.) P. V. MADHUSOODANAN**

# ABBREVIATIONS

| | | |
|---|---|---|
| 2,4-D | - | 2,4 – Dichlorophenoxy Acetic Acid |
| 2iP | - | 2-isopentenyl adenine |
| AA | - | Ascorbic acid |
| ABA | - | Abscisic Acid |
| ANOVA | - | Analysis of Variance |
| B5 | - | Gamborg Medium |
| BAP | - | 6-Benzyl Aminopurine |
| CTAB | - | Cetyl Trimethyl Ammonium Bromide |
| DKW | - | Driver and Kuniyuki Walnut medium |
| $GA_3$ | - | Giberrellic acid |
| ha | - | Hectare |
| HCl | - | Hydrochloric acid |
| $HgCl_2$ | - | Mercuric chloride |
| IAA | - | Indole-3-Acetic Acid |
| IBA | - | Indole-3-Butyric Acid |
| Kn | - | Kinetin |
| mg/l | - | Milligram per litre |
| mm | - | Millimetre |
| NaOH | - | Sodium hydroxide |
| MS | - | Murashige and Skoog medium |
| NAA | - | Naphthalene Acetic Acid |
| PCPA | - | p- Chlorophenoxy acetic acid |
| PCR | - | Polymerase Chain Reaction |
| PVP | - | Polyvinyl Pyrrolidone |
| RAPD | - | Random Amplified Polymorphic DNA |
| SH | - | Schenk and Hildebrandt |
| t | - | ton |
| TDZ | - | 1-Phenyl-3- (1,2,3 – Thiadiazol-5-yl) Urea |
| UV | - | Ultraviolet |
| WPM | - | Woody Plant Medium |
| ZN | - | Zeatin |

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# 1. INTRODUCTION

Cinnamon (*Cinnamomum verum* Bercht. and Presl) and Cassia (*Cinnamomum cassia* (L.) Blume) barks are among the earliest known spices used by the human kind and were among the spices sought after by most of the fifteenth and sixteenth century European explorers (Dao *et al.*, 1999). Frequent references to these spices are available in both pre-biblican and post-biblican writings. The 'true' cinnamon or spice cinnamon is the dried inner bark of *Cinnamomum verum. Cinnamomum cassia* is known as Chinese cassia. They belong to the family *Lauraceae.* The term *Cinnamomum* is derived from the Hebraic and Arabic term 'kinamon' means fragrant spice plant and verum means 'true'.

*Cinnamomum verum* is a native of Sri Lanka and Southern Western Ghats of South India mainly cultivated in Sri Lanka, Seychelles, Madagascar and India. The area under cultivation is estimated to be around 24,000 ha in Sri Lanka and 3,400 ha in the Seychelles producing around 12,000 t and 600 t respectively (Coppen, 1995). Sri Lanka produces more than 90 % genuine cinnamon of the world and is the largest producer of cinnamon accounting for about 65-70 % of the global production with Seychelles, Madagascar, India and other suppliers collectively contributing the balance. The best quality cinnamon is produced in Negambo district of Sri Lanka.

According to the report of Department of Export Agriculture, Sri Lanka, the main cinnamon producing areas are in the coastal belt, Galte (19647 ha), Matara

(5477 ha), Rathnapura (3620 ha) and Hambantotoa (1985 ha), and the extent of cinnamon plantations at the end of 2005 was 26051 ha. As per the 2005 data the total extent of cinnamon plantations increased by 819 ha during the last 5 years. Leaf and bark oil of cinnamon is obtained by distilling dried cinnamon leaf and bark. The cinnamon oil in world trade is produced from *C. verum, C. Cassia and C. camphora.*

Chinese cassia (*Cinnamomum cassia*, Syn. *C. aromaticum)* is indigenous to Southern China and Vietnam. China is the main producer and the harvested area in 1998 was estimated by FAO at 35,000 ha with a production of 28,000 t. Cassia bark and leaf oil are economically important. Cassia buds, the dried unripe fruits, though rare, are also occasionally used as a spice. The leaf and bark oil of cinnamon finds wide scale application in medicine.

The bark of *C. verum* and *C. cassia* is of great commercial importance due to its aromatic and sweet taste with a spicy fragrance; it also contains a large number of essential oils. A major constituent of cinnamon bark, trans-cinnamaldehyde ($C_9H_8O$) provides the distinctive odour and flavour associated with cinnamon. Oleoresins are the flavour extracts obtained by the solvent extraction of the ground spices.

The bark and leaves of *Cinnamomum verum* and *C. cassia* are commonly used as spice in home kitchens and their distilled essential oils or synthetic analogues are used as flavouring agent in food and beverage industry. Leaf and bark oils are used in the manufacture of perfumes, soaps, tooth pastes, hair oils and face creams and also as an agent for flavouring liquor and dentifrices. They also find application in pharmaceutical industries.

According to Bureau of Economics and Statistics, New Delhi, the area under cinnamon and cassia cultivation is 878 ha during 2008-2009. The import of cinnamon and cassia to India during 2008-2009 was 223 .52 t (worth Rs. 478.55 lakhs) and 8600 t (worth Rs. 3567.40 lakhs) respectively. The export of cinnamon and cassia from India during 2008-2009 was 17.75 t and 137.47 t respectively. The export of cinnamon and cassia in powder form was 647.32 t and 224.97 t respectively. The export of cinnamon and cassia oil was 2.4 t and 0.05 t respectively and the export of cinnamon and cassia oleoresin was 1.12 t and 21.20 respectively during 2008-2009. The import of cinnamon and cassia oil to India was 11.46 t and 1.16 t during 2008-2009.

Crop improvement programmes in cinnamon were initiated in Sri Lanka by the Ceylon Institute of Scientific and Industrial Research and the Department of Export Agriculture. Eight different types of cinnamon were recognized by growers in Sri Lanka based on leaf morphology, bark pungency, grittiness of bark, leaves, etc., (Wijesekera *et al.*, 1975; Anon., 1996).

Department of Export Agriculture of Sri Lanka had identified 19 selections after screening 210 accessions. The Indian Institute of Spices Research (IISR) at Calicut (Kerala, India) has maintained 300 accessions of cinnamon and related taxa. IISR, after evaluating 291 accessions, established five elite lines based on the quality characteristics such as bark oil, oleoresin and leaf oil. One Indian accession IN189 and one Sri Lankan accession SL63 were finally selected based on regeneration capacity, fresh bark yield, dry bark yield, leaf oil, percentage of eugenol in leaf oil and cinnamaldehyde (Krishnamoorthy *et al.*, 1996).

Cinnamon can be propagated either through seeds or clonally by cuttage. When sown immediately after harvest, seeds give 90-94% germination, while on storage for five weeks the viability is completely lost (Kannan and Balakrishnan, 1967).

The major drawback of seed propagation is that, cinnamon being a cross-pollinated plant, exhibits wide variability in yield (Ponnuswamy *et al.*, 1982; Krishnamoorthy *et al.*, 1992), quality of produce and oil content (Paul and Sahoo, 1993) and other morphological characteristics. Hence, seed propagation is not advisable while clonal propagation is recommended in cinnamon (Weiss, 1997). Single node cuttings with leaves can be rooted in a month's time under high humidity conditions (Anon., 1985). Rema and Krishnamurthy (1993) noted much variability in the rooting response of various cinnamon accessions. Variations in rooting during different seasons have been reported (Anon., 1996). Air layering is also a successful method of vegetative propagation. But seasonal variation in rooting pattern has also been observed in air layers (Ranaware *et al.*, 1995).

Chinese cassia is usually grown from seeds, but can be grown from cuttings also. In cassia, vegetatively propagated plants are not used for commercial planting, as such plants are known to give poor quality stem and bark, less vigorous growth and regeneration (Dao, 2004). Ripe fruits from mother trees producing thick bark of good aroma should be selected for propagation

A few dozens elite cultivars are recognized to possess considerable market relevance. However, conventional breeding methods are cumbersome because they depend on cross pollination, seed germination and selection as well as vegetative

regeneration. Modern plant biotechnology and genetic engineering have adequate potential to reduce the time needed for traditional breeding.

Haldankar *et al.* (1994), Pugalendhi *et al.* (1997) and Joy *et al.* (1998) reported cinnamon selections for crop improvement. Lack of genetic variability for resistance to major diseases and pests and poor availability of quality seeds make conventional breeding programme ineffective and hence micropropagation is needed in the production of large number of elite lines within a short time span.

Tissue culture has emerged as a potent tool for rapid multiplication and propagation of trees (Durzan, 1986) and has been successfully employed for the propagation of various tree species (Mascarenhas and Muralidharan, 1989). The drawbacks of seedlings and cuttages can be overcome to a great extent through biotechnological intervention such as micropropagation which would result in uniform good quality planting material of any elite lines. Successful micropropagation of woody plants is relatively a recent practice (Thorpe, 1990; Bajaj 1997).

Plant tissue culture techniques offer many advantages over conventional methods in crop improvement. Clonal multiplication is a reliable method for plant propagation as it yields true-to-type plants. Thus it helps in maintaining uniformity within the population (Mascarenhas and Muralidharan, 1989). Other important applications of plant tissue culture technique are the production of virus-free plants by meristem culture, production of haploids and thereby homozygous dihaploids for plant breeding purposes, somatic embryo production for the mass propagation of plants, somaclonal variation and cell line selection for crop improvement, ‘embryo

rescue' to overcome the pre- and post-fertilization barriers, slow growth technique and cryopreservation for germplasm conservation, somatic hybridization, production of secondary metabolites, development of novel transgenic plants, etc.

There are only a few reports on micropropagation of C*innamomum* spp. So far there are no reports on *in vitro* shoot multiplication from mature trees of *Cinnamomum verum*. There are no earlier reports on callus regeneration and somatic embryogenesis in *Cinnamomum verum.*

The maintenance of genetic integrity among micropropagated plants vis-a-vis explant source(s) will be one of the most crucial concerns (Larkin and Scowcroft, 1981). In plant propagation, the most crucial aspect is to retain genetic integrity with respect to the mother plant (Jin *et al.*, 2008). This is more important for tree species and other perennial crops where the life span is long and the performance of *in vitro* derived plants can be ascertained only after their long juvenile stage (Brown and Sommer, 1982; Gamborg, 1993). Tissue cultured and woody species take extensive evaluation time to access genetic fidelity (Vendrame *et al.*, 1999). The use of molecular techniques distinctly facilitates and shortens the process of evaluation (Jain 2001).

The use of meristem tip culture is considered least likely to increase genetic instability (Nehra and Kartha, 1994) as demonstrated by RAPD analysis of regenerated plants of *Digitalis obscura* (Gavidia *et al.*, 1996), *Achillea* sp. (Wallner *et al.*, 1996) oak (Barrett *et al.*, 1997) and alfalfa (Piccioni *et al.*, 1997). In spite of this fact, some RAPD differences were found among regenerated plants of poplars (Rani *et al.*, 1995), *Pelargonium* sp. (Cassells *et al.*, 1997) and *Piper longum* (Parani

et al ., 1997) . Occurrence of variability among tissue cultured plants has been investigated extensively and reported in oil palm (Corley *et al.*, 1986), blackberry (Harry *et al.*, 1983), *Coleus* (Marcotrigiano *et al.*, 1990), strawberry (Moore *et al.*, 1991) and plantain (Vuylsteke *et al.*, 1998). Micropropagated plants from the cultures of preformed structures such as shoot tips and axillary buds have been reported to maintain clonal fidelity (Ahuja, 1987; Wang and Charles, 1991; Ostry *et al.*, 1994) but there is still a possibility of generating somaclonal variants employing this method (Rani and Raina, 2000). Because tissue culture system itself act as a mutagenic inducer (Jain 2001), it is necessary to control genetic stability of micropropagated plants. Rani and Raina (2000) showed that some species are inherently more unstable than others during propagation and therefore, genetic diagnostics, especially at the DNA sequence level, should be made an integral component of any micropropagation system aimed at producing true-to-type plants. However, Rani *et al.* (1995) reported variations to the extent of 26% in micropropagated plants of *Populus deltoides*.

Occurrence of somaclonal variation is a potential drawback when the propagation of an elite tree is intended, where clonal fidelity is required to maintain the advantages of desired elite genotypes (Rahman and Rajora, 2001). On the other hand, stable somaclonal variations of specific type may be advantageous for the improvement of certain traits (Antonetti and Pinon, 1993; Karp, 1995; Jain *et al.*, 1998). Somatic embryogenesis is frequently regarded as the best system for the propagation of superior genotypes mostly because both root and shoot meristems are present simultaneously in somatic embryos (Jin *et al.*, 2008). Before somatic

embryogenesis is used for the above purpose, the genetic fidelity of cultures needs to be determined (Vendrame *et al.*, 1999).

Random amplified polymorphic DNA (RAPD) based detection of genetic polymorphism (Welsh and Mc Clelland, 1990; Williams *et al.*, 1990) has found successful application in describing somaclonal variability in regenerated individuals of several plant species (Isabel *et al.*, 1993; Hashmi *et al.*, 1997). RAPD analysis could also be useful for studying the genetic influence of different hormonal combinations during morphogenesis (Soniya *et al.*, 2001).

The present investigation is an attempt in this direction and undertaken with the following objectives

1. Standardization of micropropagation protocols for *Cinnamomum verum* and *Cinnamomum cassia.*
2. To develop a callus regeneration protocol for *Cinnamomum verum*.
3. To develop a protocol for direct somatic embryogenesis in *Cinnamomum verum.*
4. To standardize the hardening of *in vitro* developed plantlets and field establishment.
5. To compare the genotype of the regenerated plants with that of the mother plant using RAPD markers.

# 2. REVIEW OF LITERATURE

The genus *Cinnamomum* Schaeffer, comprises the evergreen trees and shrubs, found from the Asiatic mainland to Formosa, the Pacific lands, Australia and in Tropical America. According to Mabberley (1997), the genus comprises of 350 species.

## 2.1 *Cinnamomum verum* Bercht. and Presl

[ Syn. *Cinnamomum zeylanicum* Blume ]

Cinnamon (*Cinnamomum verum* Bercht. and Presl) is a tropical evergreen tree, native to South India and Sri Lanka, belonging to the Laurel family (Lauraceae) growing up to 8-17 m in wild state. Bark is smooth, light pinkish brown, up to 10 mm thick with a strong pleasant cinnamon smell and a spicy burning taste. Leaves are opposite or sub-opposite. Petiole 1-2 cm long and stout, lamina 5-18 cm long and 3-10 cm wide, glabrous, thinly to stiffly coreaceous, ovate to elliptic to lanceolate-ovate or narrowly elliptic, shortly or broadly acuminate, upper surface dark green, shining, smooth, lower surface paler, dull, the three main nerves prominent on both surfaces. The young leaves of the flush are reddish in colour, later turning dark green above with paler veins and pale glaucous beneath. Inflorescence is paniculate cymose, the initial branching of the inflorescence paniculate, with alternate or opposite branches, while the flowers are arranged in

cymes. Panicles axillary or terminal up to 20 cm long consisting of a long main peduncle and a few stiff short branches (Kostermans, 1983).

The peduncles are creamy white in colour, softly hairy and 5-7 cm long. The individual flowers are pale yellow, ca. 3 mm in diameter and subtended by a small ovate hairy bract. Calyx is companulate and pubescent with six acutely pointed sepals. Corolla is absent. There are nine stamens, with the six outer stamens being opposite the perianth lobes and an inner whorl of three, which are staminodes. Each perfect stamen has a short, hairy filament with two small glands present at its base and a four celled anther opening by four small flap-shaped valves. The ovary is superior, unilocular, with a single ovule, tapering to a short style.

The flowering time varies from October to February and the fruit ripens during May-June. The trees start putting forth flushes in the monsoon period, around July to September. The flowers when open have a pleasant smell and are visited by a number of insects, especially bees. Flowers exhibit protogynous dichogamy, the male and female phases are separated by almost a day. The fruit is a black fleshy ovoid drupe, 1.52 cm long when ripe, with an enlarged calyx at the base.

Cinnamon is a hardy plant which can grow well in almost all types of soils under semi-dry or wet conditions. The ideal temperature for growing cinnamon is between 20$^{o}$C and 30$^{o}$C. Rainfall should be on the range of 1250-2500 mm. It thrives well as a forest tree at 300-350 m above the sea level. The quality of the bark is greatly influenced by soil and ecological factors. The best quality cinnamon is produced on white silicacious sandy soils like the ‘silver sands’ of the Negombo district. Yield is higher in other soils but the quality is coarser than in sandy soils.

In two to three years after planting, depending on the climatic factors, the plants attain a height of 1.5-2m, with three to four shoots, and then ready for harvesting. Generally, cinnamon can be harvested two to three times per year.

## 2.2 *Cinnamomum cassia* (L.) Blume

[Syn . *C. cassia* Bercht. and Presl ; *C. aromaticum* Nees ]

Cassia (*Cinnamomum cassia)* is an evergreen tree of Lauraceae growing up to 18-20 m height and 40-60 cm in diameter with a straight and cylindrical trunk. Terminal buds are ca. 3 mm long, acuminate and are densely tomentose with grey hairs and broadly oval scales. Leaves are simple, alternate or sub-opposite, oblong-ovate or narrowly elliptic to sublanceolate, thick, shining green, glabrous above, minutely hairy below, tripli-nerved from about 5mm above the base, the apex slightly acute, side veins ascending to the apex and exstipulate. Inflorescence is axillary panicle, sub terminal, 8-16 cm long, exceeding the leaves, triplicate-branched, the end of the branching being 3-flowered, peduncle as long as half of inflorescence. Flowers are white, 4.5 mm long, pedicels 3-6 mm long, perianth present. There are nine fertile stamens. Ovary is ovoid with about 1.7 mm long, glabrous, style slender, as large as ovary, stigma small and conspicuous. Fruit ellipsoid, 10x7 mm, glabrous, pink-violet when mature, with a perianth cup. The root system is deep and strong. When mature the bark is 13-15 mm thick and brown in colour. Whole branches and small trees are harvested for cassia bark, unlike the small shoots used in the production of cinnamon; this gives cassia bark a much thicker and rougher texture than that of true cinnamon. The flowering time is from October to December. Closely related species of cassia are cinnamon

(*Cinnamomum verum*, the true cinnamon), Saigon or Vietnamese cinnamon (*Cinnamomum loureirii)*, camphor (*Cinnamomum camphora*), malabathrum (*Cinnamomum tamala,* the Indian cassia) and *Cinnammomum burmannii* (Indonesian cinnamon).

The main cultivated areas of cassia cinnamon in China are characterized by mean daily temperature of about 22ºC and an annual rainfall of 1250 mm in about 135 wet days. It is grown in Southern China at an altitude up to 300 m. North Vietnam has the same ecological conditions as South China. It is a light demanding tree, preferring cool and wet condition with a mean annual rain fall of 1500 mm (2500-3000 mm) in South Vietnam. The crop needs acidic soil with pH 4.5-5.5 depending on the rainfall and soil fertility. After harvesting, the leaves are separated and the stems are peeled to remove the bark for processing. The economic life span of a cassia plant is about 30-40 years (Senanayake, 1977; Wijesekera *et al*, 1975). Wijesekera *et al.* (1975) reported eight different cultivars of cinnamon grown in Sri Lanka. They are distinguished by pungency of bark and petiole, texture of bark and the structure of leaves. In addition, in Sri Lanka there are 19 high quality, high yielding selections, identified through screening of 210 different accessions by the Department of Export Agriculture of Sri Lanka.

**2.3 Cinnamon products**

Quills are the most valuable cinnamon product, which are obtained from the bark of the cinnamon tree. Mature shoots of cinnamon are coppiced or cut back to a height of about 5-8 cm from the ground. Two or three crops are taken annually depending on the rainfall. The process of bark extraction involves scraping the bark

with a steel scrapper, peeling, filling up and drying. The outer cork from the bark is scrapped prior to peeling. Then the bark is thoroughly rubbed with a wooden or brass rod to loosen it from the woody stem. The longitudinal line is then drawn on the bark from end to end with a sharp pointed knife. A similar line is drawn on the opposite side of the bark. By using the knife skillfully, two equal halves of long barks are peeled off from either side of the stick.

The bark halves are packed one inside the other until cigar like quills are obtained. The hollow inside of the quill is then packed with pieces of thin bark. They are never dried in direct sunlight, but in shade. The dried bark turns pale yellow. Sometimes the bark is fumigated with sulphur dioxide to obtain a uniform yellow colour and to kill any insects and micro organisms in the quills. The moisture content at this stage will be about 10%. They are then packed in polythene lined jute bags.

Cinnamon quills represent the most valuable product originating from the cinnamon bush. The dried quills are tightened into small bundles, each bundle containing about 30-40 quills. The edges are then trimmed, making them ready for marketing. Best quality quills are produced by Sri Lanka, which are specially designated as Ceylon cinnamon.

There are several other by-products generated during the processing of quills. They are classified into three major commercial groups as quillings, featherings and chips. Quillings are made from broken pieces and splits of all grades of cinnamon quills. They contain featherings and chips but their quantities should not exceed 3% by mass.

Featherings are feather-like pieces of inner bark consisting of shavings and small pieces of bark left over from the process of making quills. Scraping from the bark or small twigs and stalks of cinnamon shoots including a minimum quantity of chips are also considered as featherings.

Cinnamon chips are obtained from the rough, greenish brown, mature, thick pieces of unpeelable barks scrapped off from thicker stems and which are inferior to quality cinnamon. Cinnamon is also prepared into other forms such as small pieces and powder.

### 2.3.1 Cinnamon bark oil

Cinnamon bark oil is one of the expensive essential oils in the world market. It is produced by hydro-distillation (Wijesekera, 1989). To obtain commercial cinnamon bark oil, broken pieces of quills, quillings, pieces of inner bark from twigs and twisted shoots are distilled.

### 2.3.2 Cinnamon leaf oil

Leaf oil is obtained by distillation of leaves. When the shoots are harvested for bark the leaves are trimmed in the field, which are allowed to remain in the field for three to four days. Oil is obtained by steam distillation of these leaves. Cinnamon leaf oil is graded according to the eugenol content (CS 184).

### 2.3.3 Cinnamon oleoresin

Oleoresins are solvent extracts of spices that contain the volatile oil, non-volatile resinous material and the active ingredient that characterizes the spice as hot

or pungent when such an ingredient is present. After distillation, the oleoresin is stored in suitable containers such as S.S. (stainless steel) drums or epoxy coated HDPE (high density poly ethylene) containers.

The British, Indian and Argentinean pharmacopoeia listed the following pharmaceutical preparations such as compound cinnamon powder, distilled cinnamon powder, concentrated cinnamon water, tincture of cinnamon, aromatic chalk mixture, ammoniated quinine and cinnamon spirit, cinnamon syrup, etc.

## 2.4 Cassia products

In early times, cassia was usually described as a somewhat inferior substitute for cinnamon but now it has its own market. Bark of Chinese cassia resembles that of cinnamon in appearance but it has a stronger aroma. The bark powder of cassia is reddish brown in colour, unlike cinnamon powder, which is tan. As in cinnamon, cinnamaldehyde is the major component (70-95%) of cassia bark and cassia bark is interchangeable in many applications with cinnamon bark. The general composition of Chinese cassia is similar to that of cinnamon, but the mucilage content of various plant parts is higher in cassia. Cassia bark oil and leaf oil contains cinnamaldehyde, while, cinnamon leaf oil contains eugenol and bark oil cinnamaldehyde. Methods of distinguishing cinnamon bark oil from cassia oil are based on the presence/absence of minor components such as orthomethoxy cinnamaldehyde, eugenol and coumarin (Dao *et al.*, 1999)

Dried unripe fruits of *C. cassia* are known as cassia buds. Since ancient times, Chinese cassia has been used as a spice, due to its pleasant aroma and taste.

The major uses of cassia bark, both in whole and ground forms, are for culinary purposes and for processed foods.

### 2.4.1 Cassia oil

Cassia oil is obtained by the distillation of leaves, leaf stalks and twigs. The distillation material consists of partially dried leaves and branchlets from the shoots used for bark production. Depending upon the proportion of leaf and stem (twigs) the quality of oil produced may vary. Usually ¾ part leaves and ¼ part twigs are used. The best quality oil is obtained from material harvested in the summer and autumn from trees that are five to seven years old (Purseglove *et al.*, 1981).

The leaves yield more oil than twigs (0.54% versus 0.2%). The highest oil yield is from vigorous, five to seven year old trees. The quality of the oil depends on the leaf material used and varies according to season. In winter and early spring, the oil quality is said to be inferior to that from leaves harvested in mid-summer and autumn (Guenther, 1950). Cassia oil is used for the purposes similar to that of cinnamon bark oil. It is used mainly for flavoring soft drinks, confectionary and liquors and other beverages. Its use in perfumery is limited because of its skin sensitizing properties (Coppen, 1995).

### 2.4.2 Cassia oleoresins

Cassia oleoresins have similar applications as those of the ground spice in the flavoring of proceeded foods. Cassia oleoresin is stomachic, carminative, mildly astringent and capable of decreasing the secretion of milk. It is used mainly to assist and flavor the other drugs used against diarrhoea, nausea, vomiting, uterine haemorrhage and to relieve flatulence. It is a strong local stimulant and a powerful

germicide. Accidental intake of considerable proportions or overdoses of cassia may result in acute poisoning and inflammation of the gastro-intestinal mucous membrane.

Cassia forms one of the components of Japanese herbal medicines such as "TJ 960" which is recommended for hippocampal neuron damage (Sugaya *et al.*, 1991). Chinese herbal medicine formulations contain the bark of cassia and also used to treat blood hyperviscosity, hyperlipemia, hypercoagulability (Toda *et al.*, 1989), and gynecological disorders like hyper menorrhea, dysmenorrhea and infertility (Sakamoto *et al.*, 1988). It is also administrated for gonorrhoea and the leaves are used in rheumatism as a stimulant. The bark was prescribed for the enlargement of spleen, disorders of nerves and for the retention of urine. Cassia was used by the ancients for its stomachic and soporific properties. It is also given as a decoction or powder for the suppression of lochia after childbirth. The bark is also a minor constituent of Unani medicine jawarish jalinoos, a drug prescribed for gastro-enterological complaints (Asolkar *et al.*, 1994). Trans-cinnamaldehyde isolated from the cortex showed antimutagenic activity in *Escherichia coli* (Kakinuma *et al.*, 1984).

Table 1. Composition of Cinnamon and Cassia essential oils

(A and B- Cinnamon bark oil; C- Cinnamon leaf oil; D- Cassia leaf and stem oil)

| **Compound** | **Cinnamon(%)** | | | **Cassia(%)** |
|---|---|---|---|---|
| | **A** | **B** | **C** | **D** |
| α-Pinene | 0.07 | 0.035 | 0.3 | traces |
| Camphene | 0.04 | 0.049 | 0.14 | traces |
| β-Pinene | 0.03 | 0.02 | 0.12 | traces |
| δ-3-Carene | 0.01 | 0.015 | 0.025 | traces |
| A-p-menthene | 0.06 | 0.06 | - | traces |
| Myrcene | 0.08 | 0.07 | traces | traces |
| Limonene | 0.09 | 0.095 | 0.18 | traces |
| β-phellandrene (+1,8-cineole) | 0.4 | 0.64 | 0.28 | Traces |
| p-Cymene | 0.35 | 0.25 | 0.79 | traces |
| α-Copaene | 0.05 | 0.04 | 0.68 | traces |
| Benzaldehyde | 0.05 | traces | 0.17 | 0.94 |
| Linlool | 0.7 | 1.06 | 2.4 | traces |
| β-Caryophyllene | 1 | 2.0 | 3.33 | traces |
| α-Humulene | 0.2 | 0.62 | 0.61 | - |
| Sailicylaldehyde | - | - | - | - |
| α-Terpieol (+ borneol) | 0.35 | 0.62 | 0.35(0.065) | 0.02 (borneol) |
| Hydrocinnamaldehyde | 0.1 | 0.7 | - | 0.06 |
| δ-Cadinene | - | - | 0.2 | - |
| Phenethyl acetate | - | - | traces | 0.24 |
| *cis*-cisnnamaldehyde | 0.12 | 0.98 | - | 0.14 |
| Safrole | - | - | 1.05 | - |
| *o*-Methoxybenzaldehyde | 0.15 | 0.09 | - | 0.7 |
| Caryophyllene oxide | traces | 0.12 | 0.64 | 0.02 |
| *trans*-Cinnamldehyde | 72 | 82.15 | 5.7 | 65.45 |
| *trans*-Cinnamyl acetate | 3.65 | 3.24 | 0.2 | 3.55 |
| Eugenol | 13.3 | 1.07 | 68.5 | traces |
| *trans*-Cinnamyl alcohol | 0.6 | 0.50 | - | - 0.24 |
| Acetyleugenol | - | - | - | 0.15 |
| *o*-Mehoxycinnamaldehyde | 0.8 | 0.3 | traces | 2.65 |
| Isoeugenol | - | - | 1.1 | - |
| Coumarin | - | - | - | 8.73 |
| Benzyl benzoate | 1.0 | 0.4 | 4.06 | 0.63 |
| Phenethyl benzoate | - | - | traces | 0.07 |
| Total | 95.18 | 95.12 | 90.89 | 83.79 |

Source: Vernin *et al.*, 1994

Note: Essential oils A, C, and D are commercial products. Oil B is obtained by steam distillation from commercially available quills (ESPIG). The composition was determined by gas chromatographically.

Table 2. Composition of oil from Cinnamomum leaves from Calicut, India.

| | |
|---|---|
| (E)-2-hexenol (0.1%) | Borneol (0.1%) |
| (Z)-3-hexenol (0.1%) | Terinen-4-ol (0.3%) |
| 1-Hexen-3-ol (0.1%) | α-Terpineol (0.1%) |
| Hexanol (0.1%) | Dihydrocarveol (t) |
| α-Pinene (t) | Linalyl acetate (0.1%) |
| (Z)-3-hexenyl acetate (0.1%) | €-cinnamyl acetate |
| (E)-2-hexenyl acetate (0.1%) | Safrole (t) |
| p-cymene (t) | €-cinnamyl alcohol (0.1%) |
| β-phellandrence (t) | Eugenol (3.1%) |
| €-β-ocimene (t) | (E)-cinnamyl acetate (0.9%) |
| 1,8-cineole (0.1%) | β-Caryophyllene (2.4%) |
| Limonene (0.2%) | α-Humulene (2%) |
| *cis*-Linalool oxide (0.1%) | Eugynyl acetate (0.1%) |
| Terpinolene (0.1%) | Caryophyllene oxide (0.1%) |
| *trans*-Linalool oxide (0.1%) | Spathulenol (0.2%) |
| Linalool (85.7%) | Nonanol (0.3%) |

Source: Jirovertz *et al.*, 2001.

Note: t - trace (<0.01%)

Table 3. Comparative percentages, composition of the leaf and bark oils of *Cinnamomum cassia*

| **Compound** | **Leaf oil** | **Bark oil** |
|---|---|---|
| α-Pinene | 0.05-0.36 | 0.10-0.25 |
| Camphene | 0.04-0.05 | 0.05-0.10 |
| β-Pinene | 0.04-0.15 | 0.14-0.22 |
| Myrcene | 0.02-0.03 | t-0.10 |
| α-Phellandrene | 0.01-0.06 | t-0.13 |
| Limonene | 0.13-0.24 | 0.14-0.29 |
| 1,8-Cineole | 0.05-0.08 | 0.06-1.07 |
| δ-3-Carene | 0.03-0.05 | t-0.07 |
| $_{p}$-Cymene | 0.11-0.19 | 0.04-0.18 |
| Camphor | 0.07-0.15 | 0-0.08 |
| Benzaldehyde | 1.42-1.48 | 0.50-1.10 |
| Linalool | 0.11-0.23 | 0.08-0.16 |
| Teepinolene | T | 0-0.04 |
| β-Caryophyllene | 0.16-0.20 | t-0.27 |
| α-Terpineol | t-0.10 | 0.07-2.05 |
| Geraniol | T | 0-0.08 |
| Carvone | 0.57-0.64 | 0-0.34 |
| 2-Methoxybenzaldehyde | 0.08 | 0-0.12 |
| safrole | - | t-0.20 |
| γ-Elemene | 0-t | 0-0.41 |
| δ-Cadinene | T | t-0.13 |
| β- Cadinene | - | t-0.10 |
| Hydrocinnamaldehyde | 0.88-0.89 | 0-0.24 |
| Phenylacetaldehyde | 0.07-0.16 | t-0.27 |
| Methyl eugenol | 0.14-0.15 | t-0.05 |
| (E)cinnamaldehyde | 64.10-68.30 | 80.40-88.50 |
| α-Copaene | 0.41-0.49 | 0.23-0.68 |

| Compound | Leaf oil | Bark oil |
|---|---|---|
| vanillin | T | t-0.10 |
| Salicylaldehyde | 0.05-0.42 | 0.04-0.85 |
| 2-Phenethyl alcohol | 0.11-0.27 | t-0.16 |
| Benzyl alcohol | t-0.05 | - |
| Acetophenone | t-0.1 | 0-0.06 |
| Eugenol | 0.04-0.06 | 0.03-1.08 |
| (Z)-isoeugenol | 0.14-0.28 | 0.12-0.66 |
| (E)-cinnamyl acetate | 4.50-12.50 | 0.60-5.10 |
| γ-Muurolene | T | t-0.50 |
| Anisaldehyde | 0.58-1.02 | t |
| 2-Phenethyl acetate | t-1.55 | - |
| β-Bisabolene | t-0.06 | t-0.18 |
| β-Bisabolol | T | t-0.35 |
| α-Muurolol | 0-0.08 | 0-0.24 |
| Coumarin | 0.03-0.08 | 0.12-3.10 |
| (E)-cinnamic acid | t-0.45 | 0.80-2.48 |
| (E)-2-methoxy cinnamaldehyde | 8.40-10.50 | t-2.50 |
| Hydrocinnamic acid | 0.18-0.51 | 0-0.24 |
| 4-Hydroxy-2-phenethyl alcohol | 0-0.12 | 0-0.10 |
| Caryophyllene oxide | 0.15-0.17 | 0-0.10 |
| Patchoulene | 0.06-0.07 | 0-0.04 |
| Octanoic acid | T | 0-t |
| 3-Phenylpropyl acetate | 0.21-0.43 | 0.05-0.22 |
| Nonanoic acid | t-0.10 | 0-t |
| Guaicol | T | 0-0.08 |
| (E)-cinnamyl alcohol | 0.15 | 0.05-0.13 |
| (E)-ethyl cinnamate | 0.11-0.27 | t-0.14 |

| Compound | Leaf oil | Bark oil |
|---|---|---|
| Benzyl benzoate | 0.07-0.15 | t-0.38 |
| Methyl alaninate | t-0.05 | - |
| Guaicyl cinnamate | T | t |
| Decanoic acid | T | 0-t |
| Undecanoic acid | 0-0.04 | 0-0.11 |
| Dodecanoic acid | t-0.04 | 0-t |
| Benzoic acid | 0.07-0.11 | 0.07-0.10 |
| Saicylic acid | t-0.10 | 0.10-0.20 |

Source: Li *et al.*, 1998

Note: t- trace.

## 2.5 Harvest

There are two crops: the first crop is harvested form February to March (in the south) and from April to May (in the north); the second crop is harvested from July to September (in the south), from September to October (in the north). The bark extracted from the first crop has more scales than that of the second one, and the second crop bark is of better quality. In many growing areas in China, harvesting starts from four to five years after planting and is continued with every three to four year cycle.

Table 4. Area and production details of cinnamon in Kerala (Area in hectares; Production in tones; Productivity in kg/hectares )

| **Year** | **Area** | **Production** | **Productivity** |
|---|---|---|---|
| 1999-00 | 718 | 1661 | 2313 |
| 2000-01 | 701 | 1658 | 2365 |
| 2001-02 | 727 | 1658 | 2281 |
| 2002-03 | 739 | 1659 | 2245 |
| 2003-04 | 780 | 1662 | 2131 |
| 2004-05 | 820 | 1665 | 2030 |
| 2005-06 | 868 | 1669 | 1923 |
| 2006-07 | 869 | 1669 | 1921 |
| 2007-08 | 868 | 1668 | 1922 |
| 2008-09 | 878 | 1668 | 1900 |

*Source: Bureau of Economics and Statistics, New Delhi, 2009.*

Table 5. Commodity / item wise export of spices from India

| **Item** | **2004-05** | | **2005-06** | | **2006-07** | | **2007-08(E)** | | **2008-09(E)** | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Qty** | **Value** | **Qty** | **Value** | **Qty** | **Value** | **Qty** | **Value** | **QTY** | **Value** |
| Cinnamon | 56.07 | 33.20 | 125.22 | 123.39 | 30.93 | 40.56 | 64.10 | 71.69 | 17.75 | 29.67 |
| Cinnamon powder | 32.40 | 31.47 | 48.88 | 29.18 | 174.75 | 145.83 | 416.11 | 376.78 | 647.32 | 692.68 |
| Cassia | 64.33 | 27.75 | 236.69 | 89.22 | 441.55 | 245.70 | 155.41 | 83.25 | 137.47 | 92.87 |
| Cassia powder | 55.12 | 34.35 | 143.96 | 65.96 | 82.63 | 55.14 | 313.10 | 163.69 | 224.97 | 136.52 |
| Cinnamon oil | 1.08 | 7.37 | 1.35 | 10.68 | 0.87 | 15.74 | 2.85 | 26.74 | 2.40 | 24.45 |
| Cassia oil | 0.56 | 6.04 | 0.16 | 1.66 | 0.45 | 4.96 | 0.19 | 1.36 | 0.05 | 1.70 |
| Cinnamon oleoresin | 0.64 | 17.35 | 1.43 | 27.37 | 2.21 | 47.38 | 1.25 | 25.54 | 1.12 | 29.91 |
| Cassia oleoresin | 17.06 | 443.80 | 27.57 | 615.78 | 21.06 | 580.77 | 23.48 | 567.24 | 21.20 | 554.34 |

*Source: Spices Board, 2009*

Table 6. Commodity / item wise import of spices into India (Qty In Mt, Value Rs.Lakhs)

| Item | 2005-06 | | 2006-07 | | 2007-08(e) | | 2008-09(e) | |
|---|---|---|---|---|---|---|---|---|
| | **Qty** | **Value** | **Qty** | **Value** | **Qty** | **Value** | **Qty** | **Value** |
| Cinnamon | 215.93 | 322.21 | 304.39 | 446.45 | 207.67 | 341.08 | 223.52 | 478.55 |
| Cassia | 9730.14 | 2767.69 | 17001.84 | 5090.16 | 8993.53 | 3081.82 | 8600.00 | 3567.40 |
| Cinnamon oil | 0.96 | 6.84 | 18.15 | 120.48 | 11.88 | 73.66 | 11.46 | 66.94 |
| Cassia oil | 2.53 | 8.06 | 1.29 | 9.73 | 0.64 | 3.27 | 1.16 | 10.99 |

*Source: Spices Board, 2009.*

## 2.6 Cytology

The chromosome number of *Cinnamomum verum* is 2n=24. Later, *Chuang et al*. (1963) reported the same number in *Cinnamomum camphora*, *Cinnamomum japonicum*, *Cinnamomum linearifolium*, *Cinnamomum obstusifolium*, *Cinnamomum seiboldii* and *Cinnamomum verum*. Sharma and Bhattacharya (1959) reported the same in four species of *Cinnamomum*, *C. camphora*, *C. tamala, C. verum and C. iners*. Mehra and Bawa (1968, 1969) found the same number in *C. camphora, C. caudatum, C. cecidodaphne, C. impressinervium, C. obstusifolium and C. tamala.* Okada (1975) and Okada and Tanaka (1975) carried out cytological studies on Japanese Lauraceae, including five species of *Cinnamomum* and confirmed the same chromosome number in all of them.

## 2.7 Pests and Diseases

Cinnamon is reported to be infected by over 70 species of insect pests, especially in India and Sri Lanka. Some information on the insect pests of cinnamon

is offered by Singh *et al.* (1978), Rajapakse and Kulasekara (1982) Butani (1983), Kumaresan *et al.* (1988) and Premkumar *et al.* (1994). *Phytophthora* infections on cinnamon were reported by Rands (1922). However, information on diseases affecting this crop is few and scanty. The antimicrobial properties of essential oils present in cinnamon could be one of the reasons for fewer diseases recorded in this plant (Chandra *et al.*, 1982; Khanna *et al.*, 1988; Hili *et al.*, 1997; Baratta *et al.*, 1998). However, many of fungi have been recorded on dried and stored cinnamon (Balagopal *et al.*, 1973). Though many diseases have been recorded on cinnamon (*Cinnamomum verum*) only a few are serious, causing economic loses. These include stripe, canker, leaf spot, dieback, brown root rot and grey blight disease. Application of *Trichoderma* is an effective control measure for the leaf infection of the cassia caused by *Streptomyces*. Prevention of 'witches broom' disease include soaking the seeds in warm water ($70^0$C) containing an antibacterial medicine. To exterminate microorganisms, the chemicals suggested are Daconil-0.14%, Viben-0.07%, Anvil-0.15%, Tilt-0.006%, Sconee-0.02%, Formaldehyde-0.37%, Chloramphenicol-0.25% or pulverized petroleum and soap at a ratio of 1:1 at the concentration of 5% (Luc, 1999).

Major insect pests of cinnamon include foliage feeders such as the cinnamon butterfly (*Chilasa clytia* L.), shoot and leaf webber (*Scrolopha archimedias* Meyr.), leaf minor (*Conopomorpha civica* Meyr.) and chafen beetle (*Popilia complanata* Newman) in India and jumping plant louse (*Trioza cinnamomi* Boselli), cinnamon butterfly (*Chilasa clytia*), cinnamon blue bottle (*Grapheum sapidon* Felder) and leaf webber (*Orthaga vitalis* Walk) in Sri Lanka.

There are 14 insect species that are harmful to cassia gardens and forests, two of which are serious pests, *viz.*, *Liothrips sp*. (thrips) and *Anatkima*. The major destructive disease of cassia cinnamon nurseries is foot rot, caused by the soil borne fungus, *Fusarium oxysporum*. *Streptomyces* infection of leaves is another disease. Another major disease called 'witches broom' disease is caused by Mycoplasma Like Organism (MLO).

Very little published information is available on the management of the insect pests of cinnamon and cassia. The general recommendations suggested include spraying of quinalphos 0.05% for the management of leaf-feeding caterpillars and beetles. Monocrotophos 0.05% and quinalphos 0.05% are recommended for the management of leaf minor on cinnamon (Devasahayam, 2000).

**2. 8 Vegetative propagation**

Cinnamon can be propagated from seeds and cuttings of young three-leaved shoots. However, propagation by seeds is easier and the most common practice. Ripe seeds are collected from selected mother plants with desired characteristics such as erect stem with smooth bark, vigorous growth, easiness of peeling off stem bark, free from pest and diseases, good quality characteristics of the bark sweetness, pungency and flavour, etc. Sebastian *et al.* (1995) and Bhandari (1996) studied the effect of phytohormones on seed germination. Soaking of seeds in 150 ppm $GA_3$ or 1500 ppm thiourea resulted in significantly high germination (28%).

$GA_3$ at 50 ppm reduced the number of days taken for commencement of germination (13 days) when compared to the control (22 days). The subsequent seedling growth (root length, root number, dry weight of shoot, seedling vigour) was more in seedling raised from seeds treated with 1500 ppm thiourea. $GA_3$ at 300 ppm resulted in more leaves and greater shoot length. Bhandari (1996) found that gibberellic acid and kinetin showed stimulating activity in breaking seed dormancy while IAA and IBA had no such effect. ABA retarded germination.

Single node cuttings of Cinnamon with leaves can be rooted in 30 days under high humidity conditions (Anon., 1985). Application of IAA or IBA at 2000 ppm enhanced rooting. Variability in the rooting response of various cinnamon accessions (genotypes) are noted (Rema and Krishnamoorthy, 1993). Variation in rooting during different seasons has also been reported and this has been interpreted to be due to the differences to the endogenous levels of auxins, reducing and non reducing sugars, nitrogen and C:N ratio (Anon., 1996). Nageswary *et al.* (1999) studied the effect of biofertilizers on rooting of Cinnamon and found that phosphobacteria application (soil application and dipping the cutting in phosphobacteria containing slurry) gave a significantly high rooting percentage, longer roots and a greater number of roots per cutting. Nageswary *et al.* (2000) also reported that the use of IAA (100 and 500 ppm) gave better rooting (50%).

Another type of vegetative propagation is air layering. Banerjee *et al.* (1982) reported 80% rooting with the application of 100 ppm gallic acid during air layering. Hedge *et al.* (1989) obtained rooting with 2500 ppm NAA. IBA 3000 ppm when used in semi-hard wood cutting resulted in 70% rooting (Anon., 1990). Ranaware *et*

*al.* (1995) tried different rooting media and sphagnum moss was found to be the best. They also reported seasonal variations in rooting of air layers.

Cassia cinnamon also is usually grown from seeds, but can be grown from cuttings also. However, cassia plants grown from cuttings are not good, and the barks of such trees are not thick and essential oil contents in leaves and barks are less. Ripe fruits from strong mother trees producing thick bark of good aroma is selected for propagation.

The best time for planting cassia is October to November. Much genotypic variability was noted in the rooting response when semi-hard wood cuttings were rooted by treating with IBA, 500, 1000 and 2000 ppm (Anon., 1996; Rema *et al* 1997). Krishnamoorthy and Rema (1994) have reported 88 % and 50 % rooting during July and November respectively.

### 2.8.1 Crop improvement in cinnamon and cassia

The growers in Sri Lanka recognized eight types of cinnamon based on leaf morphology, bark pungency, grittiness of bark and leaves (Wijesekera *et al.*, 1975). In addition, 19 selections identified after screening 210 accessions by the Department of Export Agriculture of Sri Lanka are being popularized. In India, Indian Institute of Spices Research, Calicut, Kerala maintains 300 accessions of cinnamon. These are collected from Ancharakandi Estate, Kannur, the Mangalamcarp Estate of Wynad and also from different collection existing in Kerala. Of these, many lines are raised from seeds brought from Ceylon.

The Sreekundra Estate under Brook Bond Tea Ltd., at Valparai has 35 lines of Chinese cassia. They are developed from the open pollinated progenies of cassia trees introduced during the early 1950s from China. At the Aromatic and Medicinal Plants Research Station of Kerala, Agricultural University, Odakkali, Kerala, 236 cinnamon lines are being maintained. These lines are mainly derived from seed progenies of the material maintained at Ancharakandi. Flavonoid analysis indicated absolute similarity between *Cinnamomum verum* collections cultivated in India and those introduced from Sri Lanka, illustrating the common origin of both (Shylaja, 1984; Ravindran *et al.*, 1992). At the Indian Institute of Spices Research, Calicut, 291 lines of cinnamon were evaluated for quality characteristics and nine elite lines were identified. Clonal progenies of some of these lines with high quality parameters were evaluated in replicated trials. Four of the lines were poor in establishment and subsequently discarded. SL63 and IN189 were finally selected based on the regeneration capacity, fresh bark yield, dry bark yield, leaf oil, percentage of eugenol in leaf oil and cinnamaldehyde in bark oil, etc. (Krishnamoorthy *et al.*, 1996). These lines, named as Navasree (SL63) and Nithyasree (IN189), were released for cultivation and are being popularized in India.

Haldankar *et al.* (1994) screened 300 seedlings of cinnamon collected from Indian Institute of Spices Research, Calicut for isolating promising genotypes and four promising selections were further tested in field trials for yield and quality characteristics. One selection, B-iv exhibited the highest yield of fresh and dried bark (289.7 g and 84.5 g, respectively) bark oil (3.2%) with a good percentage of

cinnamaldehyde (70.2%). Leaf oil content was 2.28%, having 75.5% eugenol. This was released as 'Konkan Tej' for the Konkan region in Maharashtra area of India.

Pugalendhi *et al.* (1997) reported a cinnamon selection named YCD-1 from open pollinated seedlings. At the Regional Research Laboratory, Bhuvaneshwar, 2500 seed propagated plants were analyzed for quality characteristics and 20 selected elite plants were propagated vegetatively and field evaluated. Based on the evaluation results RRL (B) C-6 was selected as the most promising (having 94% eugenol in leaf oil and 83% cinnamaldehyde in the bark oil). This line was released for commercial cultivation (Paul *et al.*, 1996; Paul and Sahoo, 1993; Sahoo *et al.*, 2000).

Joy *et al.* (1998) evaluated 234 accessions of cinnamon maintained at the Aromatic and Medicinal Plants Research Station, Odakkali, based on growth, yield and quality parameters. They identified three superior accessions (ODC-130, ODC-10 and ODC-67) as the most promising. The best accession ODC-130 had given 18.34 kg fresh leaf per tree per year, 294.7 ml leaf oil per tree per year, and oil recovery of 1.6% and a Eugenol content of 93.7% in the oil. The eugenol yield per tree per year was 275.1 ml. This line was released under the name Sugandhani, exclusively for leaf oil production purpose.

### 2.8.2 Variability in cinnamon

Joy *et al.* (1998) investigated the genetic variability among 234 accessions of cinnamon maintained at the Aromatic and Medicinal Plants Research Station, Odakali. Among the cinnamon population investigated, 14% has deep flushes, 72%

medium coloured flushes and 14% light coloured or green flushes. In a study of 239 cinnamon plants, Krishnamoorthy *et al.*, (1988) and Gopalan (1997) reported that about 55% of trees had green flushes, while in the rest, the flushes had various degrees of purple colouration such as purple dominated with green, green dominated with purple, deep purple, etc. These workers also noted a correlation between flush colour and quality-the purple coloured plants having more bark oil (about 29% more).

Krishnamoorthy *et al.* (1988) observed significant variability for bark oil content in cinnamon germplasm. Krishnamoorthy *et al.* (1991) also reported significant variation in progeny performance of nine lines for plant height, number of branches per tree, fresh and dry weight of bark and percentage recovery of bark. Ponnuswamy *et al.* (1982) also conducted some studies on variability among seedling progenies of cinnamon.

In field plantations of cinnamon in the Orissa state, Paul and Sahoo (1993) recorded wide variations in many characteristics such as plant height (2.17-3.37m), stem girth ( 7-16.6cm), leaf oil (0.38-1.80%), eugenol in leaf oil (traces to 80-98%) and bark oil (0.05-2.18%). They also noticed plants having very low eugenol and high benzyl benzoate content ranging from 2.3% to 66.0%. The presence of benzyl benzoate and eugenol showed a negative relationship (Rao *et al.*, 1988; Paul and Sahoo, 1993).

Lin *et al.* (1997) reported allozyme variation in *Cinnamomum kanchirae*, a Taiwanese endangered species. Genetic diversity within and genetic differentiation among four geographic areas were investigated using 164 clones. Seven out of 11

loci examined were polymorphic. The mean, expected heterozygous loci per individual ranged from 13.9% of 21.6%, the number of alleles per locus ranged from 1.7 to 1.9 and the effective number of alleles per locus ranged from 1.34 to 1.54 at the area level. The large seed size and insect pollination impose a barrier to free gene flow, according to the above studies.

### 2.8.3 Improvement of Chinese cassia

Krishnamoorthy *et al.* (1999, 2001) evaluated cassia germplasm maintained at IISR, Calicut for morphological and quality parameters with an objective to select high yielding clones with high bark recovery and high regeneration capacity and growth.

Based on quality and other characteristics, four promising lines were identified. Two lines ($A_1$ and $C_1$) have high bark oleoresin (10.2% and 10.5% respectively) and two others D1 and D3 have high bark oil (4.7% and 4.9% respectively) and high cinnamaldehyde in bark oil (91.0% and 90.5% respectively). Taking the overall yield, chemical and flavor profiles, C1, D1, and D3 were selected for pre-release yield evaluation trials (Krishnamoorthy *et al.*, 1999, 2001).

## 2. 9 Plant Tissue Culture

Plant tissue culture is an experimental technique for culturing plant protoplasts, cells, tissues and organs in an artificial nutrient medium under aseptic and controlled conditions.

Haberlandt (1902) reported culture of isolated single palisade cells in Knop's salt solution enriched with sucrose. The cells remained alive one month, increased in

size, accumulated starch but failed to divide. Haberlandt is considered as the father of plant tissue culture. Hanning (1904), another German botanist cultured embryos of certain Crucifers and grew them to maturity.

Gautheret (1934) in France established *in vitro* cambium tissue. White (1939) developed the first long term callus cultures from excised stem and root tissues. In 1946, the first whole plants were developed through tissue culture from shoot tips of *Lupinus* and *Tropaeolum* by Ball. In 1955, Miller *et al.* discovered and isolated 6-furfuryl aminopurine and named it as kinetin. During 1958-59 Steward and co-workers in USA and Reinert in Germany rather simultaneously reported somatic embryo formation for the first time, in carrot cell suspension cultures. In 1960 Morel propagated orchids through meristem cultures. In 1962, the now popular MS medium was formulated by Murashige and Skoog. Subsequently totipotency was first demonstrated in single cells of *Nicotiana tabacum* plants (Vasil and Hildebrandt, 1965).

### 2.9.1 Micropropagation

In nature, the methods of plant propagation may be either asexual (apomictic- by multiplication of vegetative parts) or sexual (amphimictic- through generation of seeds). Sexually propagated plants demonstrate a high amount of heterogeneity since their seed progeny are not true-to-type unless they have been derived from inbred lines. Asexual reproduction, on the other hand, gives rise to plants which are genetically identical to the parent plant and thus permits perpetuation of the unique characters of the cultivars. Multiplication of genetically

identical copies of a cultivar by asexual reproduction is called clonal propagation. When clonal propagation is through tissue culture, it is popularly called micropropagation. Propagation through axillary bud multiplication is an easy and safe method for obtaining uniformity and it also assures the consistent production of true to type plants within a short span of time (George 1993; Salvi *et al.*, 2001).

### 2.9.2 Tissue culture propagation of woody species

Conventional breeding methods with woody perennial crops have been hampered because of the wide generation gap of 6-8 years before they can be assessed together with the difficulty associated with working on tissue culture of mature origin (Miah *et al.*, 2002). Micropropagation techniques for woody perennial have not been as successful as that of herbaceous plants due to the reasons such as low rate of shoot proliferation, persistent apical dominance, endogenous contaminants, phenolic exudations, difficulty in rooting, etc.

Perhaps the early researches on tissue culture of woody plants were on apples, Douglas fir, and Rhododendrons. Using the success of the apple, Rhododendron and maple programme as a basis, many recent advances have been made which facilitated the mass propagation of other woody species. It is suggested that taxonomic families behave somewhat alike in tissue culture. A large member of deciduous and evergreen *Rhododendron spp.* share common cultural requirements, the most notable of which is the requirement for the same cytokinin for adventitious shoot formation. A similar relationship exists between the trees and shrubs of the Rosaceae (including crab apples, pears, plums and hawthorns). Most rosaceous

species can be mass propagated on media very similar to that found to be optimal for apples.

Murashige (1990) of the University of California formulated three steps for micropropagation. They are explant establishment, multiplication of propagules and rooting and harvesting.

During the last few years, micropropagation techniques have been used for the rapid and large scale propagation of a number of fruit and forest trees (Hutchinson and Zimmerman, 1987). *In vitro* morphogenesis without a callus phase is regarded as the most faithful strategy to obtain plants with high speed as well as genetic fidelity. Martin *et al.* (2005) reported direct shoot induction in a woody medicinal plant, *Euphorbia nivulia* (Euphorbiaceae). Casado *et al.* (2002) also reported axillary bud multiplication of a woody medicinal plant, *Santolina canescens*. *In vitro* propagation using apical or axillary buds and nodal explants has been reported for some mulberry species (Ohyama & Oka 1987; Hossain *et al.*, 1992; Pattnaik *et al.*, 1996).

### 2.9.3 Direct organogenesis

Organogenesis refers to the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation. Direct organogenesis is regarded as the most reliable method for clonal propagation because it upholds genetic uniformity among the progenies (Beegam *et al.*, 2007). It has been suggested that the natural tendency which trees have towards diploidy

shown by tree species will ensure a normal karyotype in the regenerated plants (Mott, 1981).

In woody species, relatively little information is available on shoots formed directly on leaves without a callus stage (Oka and Oheyama, 1981; Simola, 1985; Preece *et al.*, 1993; Economou and Maloupa, 1995). *In vitro* techniques offer the possibility of rapid clonal propagation of important plants allowing production of genetically stable and true-to- type progeny (Hu and Wang, 1983). Propagation through axillary bud multiplication is an easy and safe method for obtaining uniformity and it also assures the consistent production of true-to- type plants within a short span of time (George, 1993; Salvi *et al.*, 2001).

Several woody species such as poplars, wild cherry, eucalyptus, red wood, radiate pine and teak are at present commercially micropropagated (Thorpe, 1990; Bajaj 1997). *In vitro* propagation (micropropagation) of forest tree species is an effective way to capture genetic gain and produce large amounts of plant material (Bonga and Park 2003).

*In vitro* propagation by meristem cultures or axillary buds is a standard practice in elite species requiring uniformity. The major attraction of this approach is that it ensures rapid and large scale multiplication free from pathogens either of bacterial or viral origin.

Enhanced axillary branching method involves the abolition of apical dominance resulting in de-repression and multiplication of axillary buds and has

become a very important micropropagation method due to simplicity of approach and a faster propagation rate ( Debergh and Read,1990; Wang and Charles,1991).

### 2.9.3.1 Direct regeneration in tree spices

Although micropropagation of woody spices has been previously reported (Huetteman and Preece, 1993; Kane, 2004), information on the tissue culture of *Cinnamomum* spp. is scanty. Earlier micropropagation works of *Cinnamomum* spp. were reported by Rai and Chandra (1987). They used the *in vitro* grown seedling explants for multiple shoot induction. The micropropagation of *Cinnamomum aromaticum* was reported by Inomoto and Kitani (1989). The explants used were nodal segments from seedlings. Sheeja *et al.* (2000) reported micropropagation of *Cinnamomum verum* from medium mature nodal segments.

Micropropagation protocols for *Cinnamomum camphora* were developed by Huang *et al.* (1998) and Babu *et al.* (1997). Soulange *et al.* (2007) reported multiple shoot induction in *Cinnamomum camphora* and bud break in *Cinnamomum verum* in the same medium. Shou *et al.* (2005) also reported micropropagation of *Cinnamomum camphora* var. *linaloolifera* by the formation of adventitious shoots from the delicate caudex.

Plant regeneration using protoplasts isolated from embryogenic suspension cultured cells of *Cinnamomum camphora* has recently been described (Du *et al.*, 2005). Shu-Hwa *et al.* (2002) reported the multiple shoot formation from seedling and mature explants of another species of cinnamon, *C. kanehirae* Hag. Mathew *et al.* (2001) reported multiple shoot induction in kokum (*Garcinia indica* Choisy) and

camboge (*Garcinia gummi-gutta* (L.) Rob.). Chabukswar and Deodhar (2006) also reported multiple shoot induction in *Garcinia indica* in WPM medium supplemented with 8.9 µM BA and 0.5 µM thidiazuron (TDZ). Thara *et al.* (2000) reported the formation of adventitious shoots form juvenile and mature explants of *Syzygium aromaticum* L. Mathew and Hariharan (1990) also reported the adventitious shoot formation from the nodal segments of *Syzygium aromaticum*. Rao *et al.* (1997) cultured nodal segments from seedlings and mature trees of *Syzygium aromaticum* for getting adventitious shoots. Eight to ten multiple shoots were produced from seedling explants and one to two shoots were produced from mature explants. He also reported the culture of nutmeg, tamarind and curry leaves. Micropropagation of curry leaf was also reported by Mathew *et al.* (1999).

#### 2.9.3.2 Explants

A major difficulty experienced in woody plants is the decontamination of the different organs to obtain sterile explants for culture. The part of the plant from which explants are taken depends mainly on the purpose of proposed culture. The regeneration potential of explants is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974).

The influence of plant material on the growth and development in tissue culture are related to many factors such as genotype, age of the plant, age of the tissue or organ, physiological state of the explant, the state of health of the plant, effect of season through out the year such as winter and summer, growth condition such as photoperiod, position of explant within the plant, size of the explant, wound surface area, method of inoculation, etc. The physiological state of the explant can

be artificially modified in many ways such as spraying the mother plants with regulators like cytokinins, putting the explant sources in sugar, BA, $GA_3$, etc. The regeneration potential of explants is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige, 1974).

According to Mohammed *et al.* (1992) and Thome *et al.* (1995), explant cells already in an active process of division contribute to increase in the adventitious bud induction on the explant. Juvenility is one of the most important factors influencing the in vitro response of many woody species (Bonga, 1987).

Mathew et al. (2001) reported the use of shoot tip and nodal explants from tender actively growing shoots for micropropagation in *Garcinia* sp. Seedling explants gave better establishment probably due to their juvenile nature and low endogenous microbial load (Thara et al., 2000). They also reported that explants collected from three year old clove showed poor establishment rate (20-45%) mainly due to polyphenol exudation and fungal contamination. The extent of loss was considerably low in the case of juvenile explants (15-30%). Only 64% of the survived cultures recorded bud break in the older explants while it was 95% in the case of juvenile explants. The explant source of callus and the growth regulator inducing the callus exhibited a significant influence on organogenesis (Martin, 2002).

Plants growing in the external environment are invariably contaminated with micro-organisms and pests. The contaminants are mainly confined to the outer surfaces of the plants, although, some microbes and viruses may be systemic within the tissues (George and Sherrington, 1984). Fungal contamination is a major

problem in the establishment of cultures in the tissue culture of tree species. Severe fungal contamination, predominantly endophytic was observed within 15-20 days of inoculation in 95-100% of cultures of *Garcinia gummi-gutta* (Mathew *et al.*, 2001). Among the biotic factors, microbial contamination was one of the serious constraints in tissue culture of cashew (*Anacardium occidentale*) (Thimmappaiah and Samuel, 2000). Litz and Conover (1978) reported as high as 95% contamination rate, when explants of cashew were collected from field grown trees. Rao *et al.* (1997) reported a high contamination rate in explants of mature trees of *Syzygium aromaticum*, a tree spice.

Poor establishment ratio in clove due to systemic fungal contamination has been also reviewed by Thara *et al.* (2000). Thara *et al.* (2000) reported fungal contamination even in the second and third subcultures which further reduced the performance of the mature explants and interference of phenolic exudates in clove cultures. To reduce fungal contamination, the explants were collected from regularly fungicide sprayed green house grown *Syzygium* plants.

To avoid phenolic exudation in clove, 0.05-0.1% PVP was incorporated in the culture medium (Thara *et al.*, 2000). Rao *et al.* (1997) included PVP, ascorbic acid, citric acid and activated charcoal in the medium against phenolic exudation. Shoot cultures of *Cinnamomum kanehirae* turned brown after continuous sub culturing for more than three months. This was overcome by adding 3.2 gm/l PVP to the medium (Shu-Hwa *et al.*, 2002).

For establishing an efficient *in vitro* shoot regeneration system the donor plant physiology and therefore the explant type is another important determinant for

responsiveness (Matt and Jehle, 2005). Tornero *et al.* (2000) found that the regeneration percentages increased at least two fold when young expanded apricot leaves were used instead of older ones. Adventitious shoot regeneration from vegetative shoot apices of different apple cultivars was significantly higher than by leaves (Caboni *et al.*, 2000).

#### 2.9.3.3 Culture Media and Conditions

Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media. Although the basic requirements of the cultured plant tissues are similar to those of whole plants, in practice, nutritional components promoting optimal growth of a tissue under laboratory conditions may vary with respect to the particular species. Media compositions are therefore formulated considering specific requirements of a particular culture system and a number of media have been devised for specific tissues and organs.

White's medium (1963) is one of the earliest plant tissue culture media originally formulated for root culture. The Murashige and Skoog (1962) medium is very popular, because most plants react to it favourably. However this nutrient solution is not necessarily always optimal for growth and development, since the salt content is so high. The B5 medium developed by Gamborg *et al.*, (1968) for the culture of soybean cell suspension has also been employed in many different investigations subsequently.

To counteract the high salt sensitivity of some woody species, Lloyd and McCown (1980) developed the so called WPM (Pierik, 1987). Woody Plant

Medium was developed for the culture of trees and shrubs such as *Betula*, *Kalmia*, *Rosa* and *Rhododendron* (Lloyd and McCown, 1981; McCown and Lloyd, 1981). WPM was used for the *in vitro* propagation of many trees such as *Cronus florida*, the state tree of Virginia (ornamental), *Liquidambar styraciflua*, (an ornamental, with softwood and gum resin) *Myrica esculenta* (Portugese- dried bark is medicinal) and *Syzygium aromaticum* (Sharma *et al.*, 2004; Thara *et al.*, 2000).

WPM was also reported to be successful in the micropropagation of *Vitis thunbergii*, *Garcinia indica*, *Camellia reticulata*, *Arbutus unedo*, *Populus* sp., *Garcinia mangostana*, juvenile and adult Squamosa and Cherry (Lu, 2005; Chabukswar and. Deodhar, 2006; Jose and Vieitez, 2003; Lemos and Blake, 1996; Tang *et al.*, 2002).

In order to study the influence of three media on *Myrica esculenta* which include PVP, 03% sucrose, the maximum number of shoots obtained in WPM. Swartz (1991) suggested that it is reasonable and preferable to attempt to grow plants on simple media with lower and balanced hormone combinations and from organized meristems. Since the nutritive media are favourable for the growth of micro organisms, plant tissue cultures must usually be established and maintained in aseptic conditions (George and Sherrington, 1984). Addition of activated charcoal in the media, promoted shoot growth and sustained leaf development in *Garcinia indica* (Mathew *et al.*, 2001). The explant of *Dalbergia latifolia* exudes polyphenols into the medium at all stages of culture. PVP (800 mg/l) was added to the cultures for prevention of browning (Rao, 1986).

The browning of callus in *Azadirachta indica* was satisfactorily checked by adding PVP to the medium during subcultures (Gautam *et al.*, 1993). The cultures were incubated at $25^0C \pm 2^0C$ under 14 hour photoperiod at 2500 lux.

Organogenesis has been reported in some leguminous tree species such as (*Dalbergia sisso* (Kumar *et al.*, 1991), *Acacia nilotica* subsp.*indica* (Dewan *et al.*, 1992), *Parkinsonia aculeate* (Mathur and Mukunthakumar, 1992) and *Bauhinia forficata* (Mello *et al.*, 2000) etc.

The direct regeneration is also reported in tree species such as is reported in *Camellia* sp. (Rajkumar and Marimuthu, 2000), *Anacardium occidentale* (Hedge *et al.*, 2000; Timmappaiah and Samuel, 2000; Keshavachandran *et al.*, 2000), *Ficus religiosa* (Deshpande *et al.*, 1998); *Eucalyptus torelliana* and *Eucalyptus camaldulensis* (Gupta *et al.*, 1983), *Morus alba* (Anis *et al.* 2003).

Direct adventitious shoot formation in *Cinnamomum verum* was achieved in MS medium supplemented with 0.1-1 mg/l kinetin and BAP (Rai and Chandra, 1987), in WPM medium supplemented with 0.5-4 mg/l BAP and kinetin (Mini *et al.*, 1997) and in WPM medium supplemented with 0.5-2 mg/lBAP and kinetin (Sheeja *et al.*, 2000). Bud break in *Cinnamomum verum* was achieved in MS medium supplemented with 1 mg/l BAP and 2.5 mg/l TDZ (Soulange *et al*, 2007).

Direct adventitious shoot formation of *Cinnamomum camphora* was achieved in WPM medium supplemented with BAP and zeatin alone, in BAP and kinetin combinations and in zeatin and kinetin combinations (Babu *et al.*, 2003) in MS medium supplemented with 3 mg/l BAP and 0.2 mg/l IBA (Hui, 2005) and in

MS medium supplemented with BAP (1 mg/l) and TDZ (0.05-2.5 mg/l) (Soulange *et al*, 2007).

Another species of cinnamon, *Cinnamomum kanehirae* is successfully micropropagated in WPM medium supplemented 2-6 mg/l BA (Shu-Hwa *et al.*, 2002).

Micropropagation studies were also conducted in other tree spices such as *Garcinia indica* and *Garcinia gummi-gutta* in MS medium supplemented with BAP, IAA, $GA_3$ and activated charcoal (Mathew *et al.*, 2001); clove in WPM medium supplemented with BAP and kinetin (Thara *et al.*, 2000) and *Murraya koenigii* in MS medium supplemented with BAP and NAA (Mathew *et al.*, 1999).

Mathew and Hariharan (1990) conducted *in vitro* studies in *Syzygium aromaticum*. She got more or less same results (6-8 shoots) both in MS medium and B5 medium supplemented with 3 mg/l BAP and 0.5 mg/l NAA.

For regeneration from sweet cherry leaves the N6 basal medium (Chu *et al.* 1975) and the woody plant medium (WPM, Lloyd and McCown, 1980) gave best results (Tang *et al.*, 2002; Bhagawat and Lane, 2004). Regeneration of a black cherry (*Prunus serotina* Ehrh.) was better on WPM than on medium of Driver and Kuniyuki (DKW, 1984; Hammatt and Grant, 1998).

Montecelli *et al.*, (2000) examined the influence of the different carbon sources glucose, sorbitol and sucrose on regenerating efficiency of three different apple explants. They found that sorbitol and sucrose induced the leaves to the same percentage of response in leaf explants.

### 2.9.3.4 Plant Growth Regulators

Plant growth regulators have a significant influence on shoot regeneration during the initial induction phase (Matt and Jehle, 2005). The growth, differentiation and organogenesis of tissues become feasible only on the addition of one or more plant regulators to a medium. The ratio of growth regulators required for root and shoot induction varies considerably with the tissue, which seems directly correlated to the quantum of hormones synthesized at endogenous levels within the cells of the explants (Razdan,2003). The age of the mother plant, the conditions under which it has been growing and the season at which explants are taken, are influenced by the level of naturally occurring auxins in them (Cassells, 1979).

Morphogenesis of *in vitro* cultured tissues as well as plant development were regulated by plant growth regulators especially auxins and cytokinins (Skoog and Miller, 1957). Plant growth regulators have a significant influence on shoot regeneration during the critical induction period.

Auxins promote cell division and root differentiation. IBA, NAA, IAA, 2, 4-D, etc. are very widely used as auxins in micropropagation and are incorporated into nutrient media to promote the growth of the callus, cell suspensions or organs and to regulate morphogenesis, especially in conjunction with cytokinin. Cytokinins like BAP, Kinetin, Zeatin, etc. are responsible for all cell division and shoot differentiation. BAP has been the most effective cytokinin for shoot tip meristem and bud cultures followed by Kinetin (Murashige, 1974). Cytokinin has been regularly incorporated into tissue culture for shoot regeneration (George and Sherrington, 1984). Nevertheless, the presence of cytokinin is generally required for

callus formation too (Hutchinson *et al.*, 1997). Callus, somatic embryo or shoot development depends on the concentration and type of growth regulator (Baker and Wetztein, 1998). BAP was proved to be a very effective plant growth regulator in Sandal (Abdul, 2005).

Park *et al.* (2008) reported adventitious shoot formation in *Salix pseudolasiogyne* in WPM medium supplemented with BAP, Zeatin and $GA_3$. Romano *et al.* (2002) has developed an *in vitro* propagation protocol based on axillary bud proliferation for mature female trees of *Ceratonia siliqua* in MS medium supplemented with BAP or Zeatin. Almost similar response was obtained with BAP and Zeatin. Several cytokinins such as Zeatin, 2 ip, Kinetin and BAP were used for the induction of multiple shoots in *Rhododendron ponticum* (Almeida *et al.*, 2005).

Nodal segments of *Rhododendron ponticum* produced multiple shoots in MS medium supplemented with cytokinins such as zeatin, BAP, kinetin and 2-iP along with IAA. Among the four cytokinins tested in combination with IAA, zeatin gave best results in terms of mean number of multiple shoots (Almeida *et al.*, 2005). Best results were obtained with 4 mg/l zeatin and 1mg/l IAA. Zeatin and 2-iP have been successfully used during shoot proliferation of several *Rhododendron* species and hybrids (Lapichino *et al.*, 1992; Briggs *et al.*, 1994; Hsia and Korban, 1998; Tomsone and Gertnere, 2003). Zeatin was also found to be effective for shoot initiation in *Vaccinium sp*. (Reed and Abdelnour-Esquivel, 1991) and for shoot proliferation in lingon-berry (Debnath and McRae, 2001) and high bush blue berry (Eccher and Noe, 1989).

In various legumes high concentration of cytokinin stimulated callus production and subsequent shoot bud formation and increased their survival (Malik and Saxena, 1992; Dornelas and Vieira, 1994). Other *in vitro* studies with legumes have determined the need of cytokinin for shoot differentiation and bud multiplication (Dewan *et al.*, 1992; Mohamed *et al.*, 1992).

Pattnaik and Chand (1997) in their study on rapid clonal propagation of three varieties of mulberries reported that the addition of a cytokinin was essential to enhance shoot development from apical shoot buds and to induce bud breaks in nodal explants. Of the two cytokinins tested, BAP was more efficient than kinetin in inducing shoots. The cytokinin and BAP showed the strongest effect with respect to the multiplication of axillary buds in *Holostemma ada-kodien* (Martin, 2002).

#### 2.9.3.5 Effect of additives

Growth additives such as activated charcoal, silver nitrate, silver thiosulphate, ascorbic acid, jasmonic acid and polyamines can not strictly be defined as plant hormones but they exert growth modulating effects and may play a novel mean of overcoming recalcitrance problems of woody plants (Gaspar *et al.*, 1996; Benson, 2000). The abscission of leaves of the *in vitro* regenerated shoots of *Holostemma ada-kodien* was prevented by the addition of silver nitrate (Martin, 2002).

### 2.9.4 Indirect regeneration

Adventitious shoot organogenesis is a type of *in vitro* propagation that can be used for the clonal reproduction of mass propagation of plants (Ringaile and Sigute,

2004). Factors influencing the efficiency of shoot regeneration of woody plants are the type of explants, the composition the basal medium and the mixture of phytohormones (plant growth regulators) and growth additives (Benson, 2000). Propagation by all methods of indirect organogenesis carries a risk that, the regenerated plants will differ genetically from each other and from the stock plant, because they are not formed on the tissues of the original mother plant, they are formed on the previously unorganized callus or cell cultures (George, 1993).

Callus production can be induced from a number of explants like leaf, roots, flower parts and parts of seed. Explants like tuber, shoot tips, hypocotyl, leaf and stem have been used to initiate callus with morphogenic potential (Chang and Chang, 1998; Manju and Subramanian, 1999; Kelkar and Krishnamoorthy, 1998).

Reports of organogenesis in a tree genus *Malus* from leaf tissue (James *et al.*, 1984, 1988; Welander, 1988; Fasolo and Predieri, 1990) and stem segments (Welander, 1988; Belaizi *et al.*, 1991) demonstrated adventitious shoot development directly from leaf tissue with a little callus formation. The auxins most often used for callus induction were 2,4-D, NAA, IAA and Picloram (Hee *et al.*, 1997; Myers and Simon, 1999, Rout *et al.*, 1999). Jaiswal and Narayan (1985) has reported the production of callus in MS medium supplemented with 1 mg/l 2,4-D from the stem cuttings of *Ficus religiosa* L. Internodal segments of *Dalbergia latifolia* produced callus on MS media containing IAA alone or IAA and IBA in combination (Rao, 1986). Regeneration of woody legumes from callus via. shoot organogenesis is achieved on *Sesbania grandflora* (Mohan Ram *et al.*, 1982), *Leucaena leucocephala* (Venketeswaran and Romano, 1982) and *Albizzia lebbeck* (Upadhyay and Chandra,

1983). Callus regeneration in trees coming under spices is not so far reported except in *Cinnamomum verum* (Mini *et al.*, 1997).

### 2.9.4.1 Callogenesis

Higher plant body is multicellular and is made of highly organized and differentiated structures like stem, leaf, root, etc. If the organized tissues are diverted into an unorganized proliferate mass of cells, they will form the callus tissue. The callus tissue which is important to plant tissue culture is produced experimentally from a small excised portion called the explant of any healthy plant on a nutrient medium containing specific phytohormones. In culture, the excised plant tissues loose their structural integrity and change completely to a rapidly proliferated unorganized mass of cells. Callus production was induced in stem and leaf segments of *Cinnamomum verum* in MS medium supplemented 2 mg/l 2, 4-D (Mini *et al.*, 1997). Embryogenic calli were produced from the hypocotyl region of *in vitro* developed seedlings.

2, 4-D at higher concentration induced callus production from clove and nutmeg leaves (Rao *et al.*, 1997). In Tamarind, callus initiation was observed in almost all explants cultured via cotyledon, internodes, hypocotyl and root segments excised from *in vitro* seedlings. The callus was white, cream to brown and soft but compact. 2, 4-D and kinetin were the growth regulators used in MS medium for callus induction. Vigorous callus formation noticed in MS medium supplemented with 2,4-D and BAP in *Cinnamomum verum* (Rai and Chandra, 1987).

Soulange *et al.* (2007) reported callus induction in *Cinnamomum camphora*. Leaf explants of *Cinnamomum camphora* responded in MS medium containing 1 mg/l BAP supplemented with TDZ (0.005-5 mg/l) by forming compact callus, while no response was obtained with leaf explants of *Cinnamomum verum* on the same media or on MS medium containing 3 mg/l BAP alone.

**2.9.4.2 Caulogenesis**

Caulogenesis refers to the regeneration of adventitious shoots. *In vitro* organogenesis in the callus tissue can be induced by transferring them to a suitable medium or a sequence of media that promote proliferation of shoot or root or both. Individual cells or groups of cells of smaller dimensions of callus for small nests of tissue scattered through out the callus tissue, the so-called meristamoids which become transformed into cyclic nodules from which shoot bud or root primordia may differentiate. In most calli, initiation of shoot buds may precede rhizogenesis or vise versa or the induced shoot bud may grow as rootless shoots. Callus tissue originating from juvenile or herbaceous material generally regenerate much better than material from adult or woody plants (Pierik, 1987). From the embryogenic calli of *Cinnamomum verum* produced from the hypocotyl of *in vitro* developed seedlings, adventitious shoots could be regenerated (Mini *et al*, 1997). These shoots could be rooted in the rooting medium and transfer to the field. Pierik (1987) reported indirect regeneration in a large number of woody plants such as *Ilex aquefolium, Coffea arabica, Corylus avellan*, *Populus* sp. etc.

Growth regulator concentrations in the culture medium are critical to the control of growth and morphogenesis (Skoog and Miller, 1957). Generally high

concentrations of cytokinin and a low concentration of auxin in the medium result in the induction of shoot morphogenesis and high auxin and low cytokinin concentration result in root regeneration. The first indication of callus differentiation and regenerative activity is the formation of green regions (Sears and Deckard, 1982). The occurrence of green spots in cereal callus cultures has been frequently noted and positive correlation between the presence of such spots and regeneration has been reported (Amer *et al.*, 1995). Jaiswal and Narayan (1985) reported indirect shoot regeneration of *Ficus religiosa* from stem segments derived callus in MS medium supplemented with 0.05 to 2 mg/l BAP.

### 2.9.5 *In vitro* rooting of microshoots

The microshoots produced *in vitro* should be rooted for getting a complete plantlet. Generally auxin favours root induction. Moderate to high concentration of all cytokinins inhibits rooting. Many plants require a specific rooting medium with required concentration of auxin or different auxins for root induction.

*In vitro* induction of roots from growing shoots has been achieved in standard media containing auxin and in media, in the absence of auxin depending on plant genotype (Rout *et al.*, 1988). Different plant species show marked variation in rooting potential and systematic trials are required for obtaining the suitable rooting medium for each species. Induction of adequate roots on *in vitro* grown microshoots are critical for the successful establishment of plantlets in the green house and field. For a long time rooting was considered as a single phase process, but numerous studies led to the division of the process of adventitious root formation into several

successive inter dependent phases (Jarvis, 1986; Gaspar *et al.,* 1992, 1994) and Moncousin (1987) identified three phases referred to as induction, initiation and expression.

IBA is the preferred auxin to induce rooting *in vitro* in many arborescent plants (Kumar and Seeni, 2000). *Elaeocarpus robustus* failed to produce roots without the exogenous supply of auxins (Roy *et al.*, 1998). The micro shoots showed cent percentage rooting in 23 days in half strength MS medium supplemented with 1 mg/l IBA and 0.5 mg/l IAA. *Monacelli et al.* (1999) reported that sucrose concentration higher than 1% improved rooting with combination of auxin (10 µM IBA) in a small tree *Vismia guianensis*. Sugar can enhance the rooting response in presence or absence of auxins as reported by Nanda *et al.*, 1971.

Root induction was observed in 86% of micro shoots developed from seedlings of *Cinnamomum verum* in White's Liquid Medium with IAA, IBA and IPA (0.1 mg/l each) within 20-25 days (Rai and Chandra, 1987). Sheeja *et al.* (2000) reported the rooting of adventitious shoots of *Cinnamomum verum* developed *in vitro* in WPM medium containing 0.5 mg/l each IBA and IAA. Root initials were induced within 45 days of incubation. Unlike other crops rooting of micro shoots in cinnamon was very poor (26.43%) (Sheeja *et al.*, 2000). Inomoto and Kitani (1989) reported rooting of micro shoots of *Cinnamomum cassia* in MS medium supplemented with NAA.

*In vitro* developed micro shoots of *Cinnamomum camphora* (camphor) were rooted in WPM supplemented with IBA and activated charcoal (Babu *et al.*, 1997). Huang *et al.* (1998) used NAA for root induction in *Cinnamomum camphora*. They

kept *in vitro* developed micro shoots in half MS medium supplemented with 0.2 mg/l IBA for 1-5 days and then transferred to half MS basal medium. Soulange *et al.* (2007) also reported 100% rooting of micro shoots of *Cinnamomum camphora* within two weeks in MS basal medium. The micro shoots of another species of *Cinnamomum, C. kanehirae* produced roots in WPM supplemented with 0.5-1 mg/l IBA (Shu-Hwa *et al.*, 2002). Rao *et al.* (1997) reported root induction in micro shoots of *Syzygium aromaticum* in half MS medium supplemented with NAA and IBA at 1.0 mg/l each.

Rhizogenesis in callus of *Syzygium aromaticum* was also observed occasionally without caulogenesis (Rao *et al.*, 1997) who also reported root induction in *Tamarindus indica* (MS media supplemented with IAA, NAA and IBA each at 0.2 mg/l) and *Murraya koenigii* (MS media supplemented with 1.0 mg/l IBA and 0.5 gm/l charcoal). Mathew *et al.* (1999) also reported rooting of *Murraya koenigii* microshoots in MS medium supplemented with IBA and NAA at 0.1 mg/l each.

Thara *et al.*, (2000) also reported *in vitro* rooting of *Syzygium aromaticum* microshoots in WPM supplemented with 2 mg/l IBA and 1 mg/l IAA after pulse treatment with IBA.

### 2.9.6 Somatic embryogenesis

Somatic embryogenesis is frequently regarded as the best system for the propagation of superior genotype mostly because both root and shoot meristems are present simultaneously in somatic embryos (Jin *et al.*, 2008). Of the various

methods, somatic embryogenesis and enhanced axillary branching are most extensively utilised in commercial micropropagation system and have been considered to be principal pathways of plant micropropagation (Vasil, 1994). However, *in vitro* regenerated shoots are unipolar in nature , which must then be rooted in a multistaged process (Rani and Raina, 2000). In contrast, somatic embryogenesis leads to the formation of a bipolar embryo, capable of producing of a complete plantlet (Thorpe, 1990).

Somatic embryogenesis has long been regarded as a stable system. However, results obtained in *Quercus* spp. revealed that somatic embryogenesis can have a multicellular origin that increases the risk for somaclonal variation ( Wilhelm, 2000)

Somatic embryogenesis and subsequent plant regeneration has been reported in most of the major crop species for reduction in the cost of production and rapid multiplication (Evans and Sharp, 1981). Somatic embryogenesis is an alternative to multiplication through axillary and apical buds (Razdan, 2003).

The process of somatic embryogenesis is a suitable method of micropropagation and has the potential of mass propagation commercially at low cost per unit (Miah et al., 2002). Somatic embryogenesis is a new advancement in vegetative propagation technology for plants, which has a major impact on tree breeding and high-value clonal forestry (Park, 2002; Sutton, 2002). The *in vitro* development of somatic embryo was first observed in carrot (*Daucus carota*) suspension cultures by Steward *et al.* (1958) although Reinert (1958, 1959) was also able to induce somatic embryogenesis in a callus cultured on a semi-solid medium.

Various initiation materials for somatic embryo induction has been tested in many studies, such as leaflets (Te- chato and Rungnoi, 2000; Das *et al.*, 2002) cotyledon (Lelu and Bornman, 1990), staminate and pistillate inflorescences and other floral and inflorescence tissues (Kiss *et al.*, 1992; Alemanno *et al.*, 1996; Merkle *et al.*, 1998; Merkle and Battle, 2000). However, immature zygotic embryos have been found to be a better initial material for somatic embryo induction in many species, such as Japanese Larch (*Larix leptolepis) by* Kim *et al.* (1999), sugi (*Cryptomeria japonica* D. Don by Igasaki *et al.* (2003), Canary Island date palm (Phoenix canariensis) by Huong *et al.* (1999) myrtle (*Myrtle communis* L.) by Canhoto *et al.*, 1999) and wild cherry (*Prunus avium) by* Garin *et al.* (1997).

Many reports show that 2, 4-D was a more effective auxin for somatic embryo induction in many species and various explants could be induced somatic embryos by using 2, 4-D (Carimi *et al.*, 1997; Garin *et al.*, 1997; Canhoto *et al.*, 1999) as in wild cherry (*Prunus avium*) by Garin *et al.* (1997). A combination of 2, 4-D or NAA with cytokinin was reported to be essential for the induction of somatic embryos (Gingas and Lineberger, 1986). The type and concentration of auxins employed are critical for the induction and formation of somatic embryos (Sharry *et al.*, 2006).

Regeneration of plants via somatic embryogenesis has been preferred as a method for multiplication of viable Germplasm in many woody plants (Bonga, 1987). The explant type, medium formulation and growth regulators are the most important factors contributing the induction of embryogenic callus and plant regeneration through somatic embryos (Buyukalaca and Mairtuna, 1996). There are

several reports on somatic embryogenesis in Tea (Abraham and Raman, 1986; Kato, 1986; Sood *et al.*, 1993). Owing to the wide variation expected without any loss in existing favourable traits by meiotic segregation, this technique is found useful in evolving autopolyploids (Kato, 1989).

Somatic embryogenesis and plant regeneration has been reported in cotton (Shoemaker *et al.*, 1986; Zhang *et al.*, 1991), *Santalum album* (Lakshmi *et al.*, 1980; Bapat *et al.*, 1985; Bapat *et al.*, 1990; Mujib *et al.*, 1997; Surajith *et al.*, 1998), and apple (James *et al.*, 1984; Kouider *et al.*, 1984 Paul *et al.*, 1994). Induction of somatic embryos from immature seeds in *C. camphora* was reported by Li *et al.* (2007).

#### 2.9.6.1 Induction of somatic embryos

According to Sharp *et al.* (1982), somatic embryogenesis is initiated either by pre embryogenic determined cells (PEDCs) or by induced embryogenic determined cells (IEDCs). In PEDCs, the embryogenic pathway is predetermined and the cells appear to only wait for the synthesis of an inducer (or removal of an inhibitor) to resume independent meiotic divisions in order to express their potential. IEDCs on the other hand, require redetermination to the embryogenic state by exposure to specific growth regulators such as 2, 4-D.

Auxins and cytokinins at various concentrations and combinations have been used for initiation of somatic embryogenesis (Lilien *et al.*, 1994) with some plants both embryo induction and subsequent maturation occur on the first medium and a second medium is required for plantlet development. According to Kohlenback

(1978) abnormalities known as embryonal budding and embryogenesis clump formation may occur and if relatively high level of auxin is present after the embryonic cells have been differentiated. The presence of auxin in the medium is generally essential for embryo initiation.

Different explants were used for the induction of somatic embryos. Somatic embryos were obtained from immature cotyledons, leaf, root and anther explants (Haridas, *et al.*, 2000). *In vitro* developed hypocotyl pieces and juvenile leaves of Camellia sinensis (Haridas, *et al.*, 2000), nucellus derived callus of *Citrus macroptera* (Miah *et al.*, 2002) and seeds of *Santalum album* (Abdul, 2005) have developed somatic embryos *in vitro*.

Physiological maturity of cotyledonary explants influenced the somatic embryogenic frequency (Haridas *et al.*, 2000). Zhang *et al.* (2001) has reported that the addition of activated charcoal promoted production, maturation and germination of somatic embryos in an elite Chinese cotton (*Gossypium hirsutum*). Different basal media and growth regulators were used for the *in vitro* development of somatic embryos. Somatic embryogenesis for different plants have been achieved using a variety of media ranging from relatively dilute White's medium (1963) to a more concentrated formulations of Gamborg *et al.* (1968) Schenk and Hilderbrandt (1972) and MS (Jasrai *et al.*, 2003).

Denchev *et al.* (1991) reported the role of 2,4-D, Kn and adenine for the direct induction of somatic embryos from the leaves of *Medicago sativa.* Induction of somatic embryos has been reported in MS medium with biotin for *Camellia sinensis* (Haridas, *et al.*, 2000), in Murashige and Tucker (MT, 1969) medium

supplemented with malt extract for Sweet orange (Pasqual *et al.*, 1984) and some other *Citrus* sp. (Pimental and Villegas, 1993), and in MS medium with low NAA and 2 iP for *Euphorbia pulcherrima* (Jasrai *et al.*, 2003).

Pasqual and Ando (1988) used 2, 4-D and Kn for getting embryogenic callus from nucellus of sweet orange. Addition of 2, 4-D stimulated the proliferation of embryogenic callus, but was disadvantageous to the differentiation and germination of somatic embryos. 2, 4-D and NAA has been reported to induce different soybean somatic embryogenesis (Shoemaker *et al.*, 1991; Trick *et al.*, 1997).

#### 2.9.6.2 Maturation and germination of somatic embryos

Germination of somatic embryo can occur only when it is mature enough to have functional shoot and root apices capable of meristamatic growth (Razdan, 2003). High auxin level can inhibit development and growth of the shoot meristem and often embryos mature when transferred to a medium lacking auxin. The addition of a low level of cytokinin in combination with ABA may prove beneficial for embryogenesis (Razdan, 2003).

Various physical factors may also affect embryo maturation (Razdan, 2003). Progressive increase in sucrose levels is also used to achieve maturation. Chen *et al.* (1990) have observed that $GA_3$ was effective for conversion of embryoids to plantlets in *Citrus*. Addition of $GA_3$ was effective in conversion of embryoids into plantlets (Iganacimuthu *et al.*,1999).

Omission or lowering of growth regulator concentrations in culture media has improved embryo development and germination in many medicinal plants

(Arumugam and Bhojwani, 1990; Kumar, 1992; Wakhlu *et al.*, 1990). Germination of somatic embryos was also obtained in basal MS medium (Purohit *et al.*, 1994; Hirai *et al.*, 1997; Choi et al, 1997).

### 2.9.7 Acclimatization and field establishment

The leaves of *in vitro* developed plantlets have poorly developed cuticle or wax layer, because the relative humidity (RH) is around 90-100% in *in vitro* conditions. This weaker cuticle layer results in extra water loss through evaporation, when the plant is transferred to less humid *ex vitro* conditions. Leaves of an *in vitro* developed plant, often then, soft and photosynthetically not very active, are not well adapted for the external climate. They have smaller and fewer palisade cells to use light effectively and have large mesophyll air spaces. Stomata do not operate properly in tissue culture derived plants; open stomata in tissue culture plants cause the most significant water loss during the first few hours of acclimatization. They may not have proper vascular connections between the shoots and roots which reduce water conduction.

#### 2.9.7.1 Hardening

*In vitro* developed cinnamon plantlets were transplanted to soilrite in cups, covered with polybags and kept in humid chamber for 30-40 days and subsequently transferred to polybags containing a mixture of garden soil, soilrite and sand in equal proportion and showed 80% establishment in the field (Mini *et al.*, 1997). Sheeja *et al.* (2000) have reported 43% establishment of *C. verum* plantlets in soilrite.

Babu *et al.* (2003) reported the hardening and field establishment of *Cinnamomum camphora*. Soulange *et al.* (2007) also reported the establishment of *in vitro* developed plantlets in pots containing top soil and compost (2:1). In *Murraya koenigii* 80% success was reported in the establishment of micropropagated plantlets in the green house (Mathew *et al.*, 1999).

Successful field establishment was reported in some tree species such as *Morus laevigata* (Hossain *et al.*, 1992; Anis *et al*, 2003; Pattnaik and Chand, 1997), *Azadirecta indica* (Eeswara *et al.*, 1998 and Morimoto *et al.*, 2006), *Camellia sinensis* (Rajkumar and Marimuthu, 2000), *Pterocarpus marsupium* (Anis *et al.*, 2005), *Sterculia urens* (Hussain *et al.*, 2007), *Calophyllum apetalum* (Nair and Seeni, 2003).

**2.10 Molecular Characterisation**

Cytological and cytophotometric analysis for *in vitro* assessment of clonal fidelity has been reported in several species (Nayak and Sen, 1995, 1997). The complexity of somaclonal variation requires the use of several approaches so that plants can be correctly evaluated. This attains a special interest in species with extended growing periods, such as forest trees, where identifying variants as early as possible is essential to avoid the propagation of mutant plants (Olmos *et al.*, 2002).Identification of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants (Soniya *et al.*, 2001). Molecular analysis of somaclonal variation provides an opportunity to

eliminate the influence of environmental factors and to provide a quantitative measure of somaclonal variability (Veilleux and Johnson, 1998).

After such a long period of developing plants in culture it would be disastrous if they turn out to be abnormal. In this context, Dunstan and Thorpe (1986) have suggested that the commercial application of tissue culture to perennial crops must await adequate quality checks and field testing with proper controls to prevent large scale loss as experienced with oil palm (Jaligot *et al.*, 2000) and poplars (Rani *et al.*, 1995). Therefore it is essential that the assessment of genetic integrity of micropropagated woody plant species is done before a particular protocol for a specific genotype is released for mass multiplication.

Different molecular techniques can be employed to detect somaclonal variability (Henry, 1998). During the last decade several novel DNA markers (RAPD, RFLP, SSR, ISSR, etc.) have been rapidly integrated into the tools available for genomic analysis (Priya and Maridas, 2008; Petit *et al.*, 1998; Avise, 2004.)

The use of molecular markers as a means of evaluating genetic stability of *in vitro* grown plants is very frequent now a days because these markers can characterise somaclonal variation with greater precision and less effort than cytological or morphological analysis (Polanco and Ruiz, 2002). Among these random amplified polymorphic DNA (RAPD) markers, despite their drawbacks (Hedrick, 1992) are an efficient technique to assess genetic stability of *in vitro* regenerated conifers including *C. libani* (Isabel *et al.*, 1996; De Verno *et al.*, 1999; Piola *et al.*, 1999; Tang *et al.*, 2001). Recently randomly amplified polymorphic DNA markers ( RAPDs) have been applied for characterization of micropropagated

forest trees ( Isabel *et al.*, 1993; Rani *et al.*, 1995; Barrett *et al.*, 1997; Rahman and Rajora, 2001) and used to obtain rapid information about genetic similarities and dissimilarities in micropropagation (Bindiya and Kanwar, 2003).

Many strategies can be used to evaluate plant genetic structure from *in vitro* derived plant clones, including cytogenetic analysis, isozyme markers and different DNA markers, etc., but most of them have limitations. Karyological analysis can not reveal alteration in specific genes or in small chromosome arrangements. Isozyme markers provide an appropriate method to detect genetic changes; however; these markers are susceptible to ontogenic variation and are limited in number and only DNA segments coding for soluble proteins can be sampled. RFLP (Restriction Fragment Length Polymorphism) markers are reliable for sampling various genome regions and are potentially unlimited in number. However, this technique is slow, expensive and requires large quantities of tissue. RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. The advantages of this technique are

a) large number of samples can be quickly and economically analysed using micro-quantities of material

b) the DNA amplicons are independent from the ontogenic expression and

c) many genomic regions can be sampled with a potentially unlimited number of markers

Molecular techniques help researchers not only to identify the genotypes, but also in assessing and exploiting the genetic variability through molecular markers

(Witkus *et al.*, 1994). Biochemical and molecular markers have been used to enhance understanding of plant population genetic structure.

Among several efficient methods for revealing genetic variability within and among plant populations, some of the most widely applied are isozyme electrophoresis (Hamrick and Allard, 1972; Hamrick and Godt, 1990), random amplified DNA polymorphism-RAPDs (Wolff and Rijn, 1993; Wachira *et al.*, 1995; Brummer *et al.*, 1995; Swoboda and Bhalla, 1997; Palacious and Candelas, 1997) and restriction fragment length polymorphisms-RFLPs (Klein *et al.*, 1988; Hong *et al.*, 1993; Yanesita *et al.*, 1997).

Of several PCR- based techniques, RAPD offers a simple and economical mean for rapid identification of a large number of accessions (Welsh and Mc Clelland, 1990; Williams *et al.*, 1990). They are also effective for cultivar identification (Ronning *et al.*, 1995a). RAPD is a DNA polymorphism assay on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams *et al.*, 1990). RAPD markers have been used widely in studying the genetic diversity of somaclonal variations in various plant species (Rani *et al.*, 1995; Soniya *et al.*, 2001). RAPDs are dominant molecular markers developed by Welsh and Mc Clelland (1990) and Williams *et al.* (1990). They are random pieces of DNA amplified from the genome by a PCR based technique. RAPD profiling uses single short oligonucleotide primers (10 bp.) and Taq DNA polymerase to amplify DNA fragments between priming sites. Amplified DNA fragments may be visualized on gel as bands and are scored as presence/absence character states (Prathepha, 2000). Although somewhat problematic, RAPD markers can be important tools when

appropriate care is taken to evaluate marker consistency and to analyse the data with recognition of their limitations (Lynch and Milligan, 1994; Perez *et al.*, 1998).

The advantage of the RAPD techniques are its simplicity, reduced running time and low cost. Moreover, it does not make use of radioactive probes, not requiring previous knowledge of DNA sequence to design the primers and requires only small amounts of DNA. RAPD markers can provide robust classification criteria that could be useful in species separation and systematics (Kassinee *et al.*, 2005).

Allelic variation among individuals is detected as the presence or absence of the multiplication product visualized as a band after PCR and electrophoresis (Rafalski *et al.*, 1993). Several studies have used RAPDs to assess levels and patterns of variation (Chalmers *et al.*, 1992; Huff *et al.*, 1993; Landry *et al.*, 1993; Nesbitt *et al.*, 1995; Prathepha and Baimai, 1999). RAPD markers have recently been applied in woody species (Goto *et al.*, 1998) to assess the reproduction of some segments of the genome, as rapid appraisal of tissue culture-derived plants (Rani and Raina, 1998).

Rashmi *et al.* (2004) has studied genetic relationships and genetic variability within the population of Acacia tree species, based on RAPD markers. In Citrus, PCR-based markers have been used for

1) Genetic mapping (Cai *et al.*, 1994)

2) To study genetic relationship among species and cultivars (Luro *et al.*, 1995; Omura *et al.*, 1993; Machado *et al.*, 1996; Federici *et al.*, 1998)

3) To discriminate citrus hybrids (Elisiario *et al.*, 1999) and

4) To identify citrus mutants and periclinal chimeras (Deng *et al.*, 1995; Sugawara *et al.*, 1995)

The development of PCR- based markers has revolutionized the repertoire of genotype identification. RAPDs or genetic markers resulting from PCR amplification of genomic DNA proved to be valuable in Citrus genotype identification (Deng *et al.*, 1995) and estimation of relationships (Machado *et al.*, 1996).

Identification of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants. Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions (Larkin and Scowcroft, 1981; Muller *et al.*, 1990). Any genetic change induced by *in vitro* conditions of tissue culture is expected to generate stable plants carrying interesting heritable traits. Randomly amplified polymorphic DNA (RAPD) based detection of genetic polymorphism (Welsh and McClelland, 1990; Williams *et al.*, 1990) has found successful application in describing somaclonal variability is regenerated individuals of several plant species (Isabel *et al.*, 1993; Hashmi *et al.*, 1997). RAPD analysis could also be useful for studying the genetic influence of different hormonal combinations during morphogenesis (Soniya *et al.*, 2001).

# 3. MATERIALS AND METHODS

## 3.1 *Cinnamomum verum* Bercht. and Presl

**(Syn. *C. zeylanicum* Blume)**

Major emphasis in the present study was given in multiplying the commonly used cinnamon, *Cinnamomum verum*, while *C. cassia* has been used only for preliminary experimentation in order to find its *in vitro* responses for comparison and the methods used for the two species are separately dealt with.

### 3.1.1 Source of plant material

Cinnamon (*Cinnamomum verum* Berchthold and Presl) trees and seedlings grown at Indian Institute of Spices Research (IISR) campus, Calicut, Kerala, were selected as the source of explants.

Detailed description of the plants *C. verum* and *C. cassia* is given in the Review of Literature pp 9 to 12.

### 3.1.2 Explants used

Shoot tips and nodal segments of three maturity levels were used for direct regeneration; explants from 2-3 year old seedlings, juvenile explants from mature trees (8-9 year old) and mature explants from mature trees. For collecting juvenile explants from the mature trees, the trunk of the trees were chopped and the newly emerging sprouts were collected (Figure 13).

Four to five month old immature seeds were used as explants for getting somatic embryos and organogenic calli. Leaves and internodes were also used as explants for getting callus.

### 3. 1. 3 Glass ware

The glass ware and the equipments used for the present investigation include culture tubes, conical flasks, beakers, reagent bottles, measuring cylinders, pipettes, standard flasks and petridishes of Borosil grade.

For establishment of cultures, test tubes were used (150 mm long and 25 mm diameter). Cultures were subcultured to Erlenmeyer's conical flask (250 ml) for multiple shoot induction.

All the glass wares (Borosil grade) were washed with a detergent solution (teepol), rinsed with distilled water and were placed in hot air oven for 2 hrs at 100°C.

### 3.1. 4 Basal Medium

Cinnamon being a perennial tree, the basal medium tried for establishment of cultures and multiple shoot induction was WPM (Woody Plant Medium, Lloyd and Mc Cown 1980). Varying hormonal combinations with WPM were tried to optimize the media for culture initiation for direct regeneration, multiple shoot induction, rooting, callus production, induction of somatic embryos and regeneration of somatic embryos into plantlets. The hormonal combinations used with the standardized multiple shoot induction medium were also tried with different basal

media such as MS (Murashige and Skoog, 1962), and White's medium (1963) to standardize the basal medium (Table 7).

### 3.1.4.1 Preparation of Culture Medium

Stock solutions of macronutrients (50x), micronutrients (100x), vitamins and growth hormones (50 mg/100 ml) were prepared and kept in refrigerator at 10-16°C. For preparing the medium, the required quantity of stock solutions of macronutrients, micronutrients, and growth regulators were taken and sucrose was added as carbon source. 20 gm/l sucrose was added to the medium for inoculation and 30 gm/l was added to the medium for subculture and made up to the final volume with double distilled water after adding growth regulators and additives. The pH of the medium was adjusted to 5.8, using 0.1N NaOH/HCl before autoclaving. For jelling the medium agar-agar (Qualigens) was used at a concentration of 7.5 gm/l.

The culture vessels were washed with a detergent solution, rinsed with distilled water and dried in hot air oven. The melted medium was poured into pre-sterilized culture tubes (10 ml) and conical flasks (200 ml). The culture vessels were closed with plugs made up of non-absorbent cotton and cotton gauze.

The medium sterilized in autoclave for 20 minutes at 121°C and 1.08 kg/cm$^2$ (16 psi) pressure were stored in the media storage room. Petridishes, forceps, spatula and other accessories used for inoculation were wrapped with brown paper and autoclaved. Autoclaved petridishes, scalpels, scissors, spatula, etc., were again dipped in alcohol and flamed on a spirit lamp at the time of inoculation.

Table 7. Chemical composition of WPM, MS and White's medium (mg/l)

| **Medium Constituents** | **WPM Medium** | **MS Medium** | **White's Medium** |
|---|---|---|---|
| $(NH_4)_2SO_4$ | ----- | ----- | ------- |
| $KNO_3$ | ----- | 1900 | 80.00 |
| $KH_2PO_4$ | 4,250 | 170 | ------- |
| $MgSO_4.7H_2O$ | 9,250 | 370 | 720 |
| $NH_4NO_3$ | 10,000 | 1650 | ------- |
| $NaH_2PO_4.H_2O$ | ----- | ----- | 16.50 |
| KCl | ----- | ----- | 65.0 |
| $Na_2SO_4$ | ----- | ----- | 200 |
| $K_2SO_4$ | 24,750 | ----- | ------ |
| $CaCl_2.2H_2O$ | 1,812 | 440 | ------ |
| $Ca\ (NO_3)_2.4H_2O$ | 13,900 | ----- | 300 |
| $Na_2EDTA$ | 932.5 | 37.3 | ------ |
| $FeSO_4.7H_2O$ | 695 | 27.8 | 2.50 |
| $MnSO_4.4H_2O$ | 557.5 | 22.3 | 7.00 |
| $ZnSO_4.7H_2O$ | 215 | 8.60 | 3.00 |
| $H_3BO_3$ | 155 | 6.20 | 1.50 |
| KI | ----- | 0.83 | 0.75 |
| $Na_2MoO_4H_2O$ | 6.25 | 0.25 | ------ |
| $CoCl_2$ .6H2O | ------ | 0.025 | ------ |
| $CuSO_4$ .$5H_2O$ | 6.25 | 0.025 | ------ |
| Glycine | 50 | 2.00 | 3.00 |
| Nicotinic acid | 12.5 | 0.50 | 0.50 |
| Pyridoxine-HCl | 12.5 | 0.50 | 0.10 |
| Thiamine HCl | 25 | 0.10 | 0.10 |
| Myo-inositol | 2,500 | 100 | ------ |
| Cystenic HCl | ------ | ------ | 1.00 |
| Calcium Pantothenate | ------ | ------ | 1.00 |

The cytokinins used were 6-Benzyl amino purine (BAP), Kinetin (Kn) and Zeatin (ZN). The auxins used were Indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and 2, 4-Dichlorophenoxy acetic acid (2,4-D).

Table 8. Stock solutions of WPM

| **Stock** | **Composition** | **Concentration of the stock solution** | **Quantity for 1 litre medium** |
|---|---|---|---|
| I | $NH_4NO_3$ | x 20 | 10 ml |
| | $Ca(NO_3)_2.4H_2O$ | | |
| II | $K_2SO_4$ | x 20 | 20 ml |
| III | $KH_2PO_4$ | x 100 | 10 ml |
| | $H_3BO_3$ | | |
| | $Na_2MoO_4.H_2O$ | | |
| IV | $MgSO_4.7H_2O$ | x 100 | 10 ml |
| | $MnSO_4.H_2O$ | | |
| | $ZnSO_4.7H_2O$ | | |
| | $CuSO_4.5H_2O$ | | |
| V | $FeSO_4.7H_2O$ | x 100 | 10 ml |
| | $Na_2$ EDTA | | |
| VI | Thiamine HCl | 0.025 | 10 ml |
| | Nicotinic acid | 0.0125 | |
| | Pyridoxine HCl | 0.0125 | |
| | Glycine | 0.050 | |
| VII | Myo-Inositol | 2.5 | |

Table 9. Growth regulators used to study the *in vitro* response of *C. verum*

| Sl. No. | Growth regulators | Range |
|---|---|---|
| | **BAP** | **50 mg/100 ml** |
| 1 | Kn | ,, |
| 2 | ZN | ,, |
| 3 | IBA | ,, |
| 4 | NAA | ,, |
| 5 | 2,4-D | ,, |

## 3.1.5 Direct regeneration

### 3.1.5.1 Explants

Explants from 2-3 year old seedlings and juvenile and mature explants from mature trees were taken for direct regeneration. The trunk of mature trees was incised at places and the new flushes that sprouted from the cut portions were taken for studying the response of juvenile tissues of mature plants.

### 3.1.5.2 Establishment of aseptic cultures

The major hurdle to the establishment of cultures of cinnamon and cassia was fungal contamination. The rate of contamination was less in explants collected from fungicide sprayed green house grown mother plants.

### 3.1.5.3 Preparation of explants

Since explants from all the tree spices are generally slow in responding to the tissue culture media, several pre-treatment methods were tried to overcome the inhibitory effects and induce faster growth. They are:

1. Removal of shoot tips one week before collecting the successive nodal segments to reduce apical dominance and to activate the growth of axillary buds before inoculation.

2. Incubation in BAP and IBA solutions (0.01-1%) overnight as a pulse treatment for shoot and root induction respectively.

3. Incubation in ascorbic acid solution (0.01-1%) to reduce the effect of phenolics.

4. Treatment with 5-10 % hydrogen peroxide to break bud dormancy.

5. Collection of explants into picric acid and 8-hydroxy quinolinine sulphate was done to reduce fungal contamination.

#### 3.1.5.4 Sterilization of explants

The branches of *C. verum* collected from seedlings and mature trees were defoliated without making any injury to apical buds and axillary buds and were washed in running tap water, followed by washing in distilled water with a detergent solution.

Shoot tips and nodal segments, 3-4 cm in length were selected as explants and were treated with 3% fungicide solution (Dithane M-45 or Bavistin) with a wetting agent (Tween 20 or Tween 80) for half an hour. The fungicide treated explants were washed with distilled water and further sterilization was done inside the Laminar air flow chamber, first by rinsing the explants with 70% alcohol for 30 seconds, then treated with 0.1% mercuric chloride solution with a wetting agent (Tween 20 or Tween 80) for 6-10 minutes depending on the maturity of the explants. The $HgCl_2$ treated explants were rinsed 4-5 times with sterile distilled water.

### 3.1.5.5 Selection of basal medium and initial establishment of cultures

WPM was selected as the basal medium because of the woody nature of the species. The growth regulators used were BAP and Kn. BAP was used at a range of 1-3 mg/l and Kn was used at a range of 0.5-1 mg/l for the initial establishment of cultures. BAP and Kn were used separately and in combination (Table 10).

Table 10. Media combinations used for the initial establishment of cultures of shoot tips and nodal segments in *C. verum*

| Sl. No. | Basal medium | Growth regulators | |
|---|---|---|---|
| | | BAP (mg/l) | Kn (mg/l) |
| 1 | WPM | 0.5 | 0 |
| 2 | | 1 | 0 |
| 3 | | 0 | 0.5 |
| 4 | | 0 | 1 |
| 5 | | 1 | 0.5 |
| 6 | | 2 | 0.5 |
| 7 | | 3 | 0.5 |
| 8 | | 1 | 1 |
| 9 | | 2 | 1 |
| 10 | | 3 | 1 |

The growth regulator concentration produced best results for shoot induction in WPM were tried with other basal media such as MS and White's media (Table 11).

Table 11. Standardisation of basal medium for the initial establishment of cultures in *C. verum*

| Sl. No. | Basal medium | Growth regulators (mg/l) | |
|---|---|---|---|
| | | BAP | Kn |
| 1 | WPM | 2 | 0.5 |
| 2 | MS | 2 | 0.5 |
| 3 | White's | 2 | 0.5 |

### 3.1.5.6 Inoculation and incubation

Inoculation was conducted inside the Laminar air flow chamber. The inner regions of the Laminar air flow cabinet was swabbed with alcohol and the UV light inside the unit was switched on for 20 minutes, after keeping all the equipments necessary for inoculation inside the chamber. Before starting inoculations the hands and arms were cleaned thoroughly using soap and water, then rinsed with 96% alcohol. Before inoculation, the cut ends of the explants, which were in contact with the sterilizing agent were removed and were inoculated aseptically.

Inoculated cultures were incubated at 25±2ºC under 14 hour photoperiod at a light intensity of 2500 lux (μmol/$m^{-2}s^{-1}$) with 55-60% relative humidity.

### 3.1.5.1.7 Subculture

Established cultures were subcultured to fresh medium in every two to three weeks. While subculturing, the dead tissues and the old medium were removed from the cultures and if necessary a fresh cut was made to get better accessibility of cultures to the medium.

### 3.1.6 Standardisation of multiple shoot induction medium

Aseptic cultures established were subcultured with an aim to induce multiple shoots. Twenty five media combinations were tried for the induction of multiple shoots from shoots tips and nodal segments of *Cinnamomum verum*. The explants collected for the experiment were of three maturity levels, seedling explants from 2-3 year old plants (Experiment-1), juvenile explants of mature trees (Experiments-2) and mature explants of mature trees (Experiment-3). All the three experiments were repeated twice and run in seven replicates.

The cytokinins used for multiple shoot induction were BAP, Kn and ZN. They were tried alone and in combination at a concentration of 1 mg/l, 2mg/l, 3mg/l, 4mg/l and 5ml/l (Table 12).

Number of shoots induced on the $40^{th}$ day was observed for all the three trials for three groups of explants and the mean value was calculated to study the effect of different growth regulators.

#### 3.1.6.1 Growth regulators and additives used

##### 3.1.6.1.1 BAP

To standardize the concentration of BAP for initial establishment of cultures, the range of BAP used was 0.5-3.0 mg/l. BAP was tried alone or in combination with Kn. To standardise the multiple shoot induction medium, the range of BAP used was 1-4 mg/l.

Table 12. Medium combinations tried for multiple shoot induction in seedling explants (Experiment 1), juvenile explants of mature trees (Experiment 2), and mature explants (Experiment 3) in *Cinnamomum verum*

| **WPM+ 1 mg/l cytokinin** | | |
|---|---|---|
| **Sl. No.** | **Basal Medium** | **Growth regulators (mg/ l)** |
| 1 | WPM | 1 BAP |
| 2 | | 1 Kn |
| 3 | | 1 ZN |
| 4 | | 0.5 BAP + 0.5 Kn |
| 5 | | 0.5 BAP + 0.5 ZN |
| **WPM+ 2 mg/l cytokinin** | | |
| 6 | WPM | 2 BAP |
| 7 | | 2 Kn |
| 8 | | 2 ZN |
| 9 | | 1 BAP + 1 Kn |
| 10 | | 1 Kn + 1 ZN |
| **WPM+ 3 mg/l cytokinin** | | |
| 11 | WPM | 3 BAP |
| 12 | | 1 BAP + 2 Kn |
| 13 | | 2 BAP + 1 Kn |
| 14 | | 2 Zn + 1 Kn |
| 15 | | 2 Kn + 1 ZN |
| **WPM+ 4 mg/l cytokinin** | | |
| 16 | WPM | 4 BAP |
| 17 | | 3 BAP + 1 Kn |
| 18 | | 3 BAP + 1 ZN |
| 19 | | 2 Kn + 2 ZN |
| 20 | | 2 BAP + 2 Kn |
| **WPM+5 mg/l cytokinin** | | |
| 21 | WPM | 4 BAP + 1 Kn |
| 22 | | 4 BAP + 1 ZN |
| 23 | | 3 Zn + 2 Kn |
| 24 | | 3 Kn + 2 BAP |
| 25 | | 3 Kn + 2 ZN |

#### 3.1.6.1.2 Kinetin

Kn was also used at a range of 0.5-1 mg/l for shoot induction and 1-4 mg/l for multiple shoot production. Kinetin was used alone or in combination with BAP.

**3.1.6.1.3 $GA_3$**

1- 3 mg/l $GA_3$ was incorporated into the multiple shoot induction medium to enhance the growth of the cultures, since it has an ability to break bud dormancy and is also used for elongation of internodes.

**3.1.6.1.4 Coconut milk**

The standardised medium for multiple shoot induction in cinnamon (WPM with 3 mg/l BAP and 1 mg/l Kinetin) was supplemented with 5-25% coconut milk to study its effect in multiple shoot induction. Coconut milk collected from tender coconut was boiled to deproteinize, filtered and refrigerated at 4º C.

**3.1.6.1.5 Adenine sulphate**

Adenine is added to the standardised multiple shoot induction medium to promote shoot formation since it has a cytokinin-like activity. One to three mg/l was incorporated into the medium.

**3.1.6.1.6 Ascorbic acid**

Apart from its role as a vitamin, ascorbic acid is also used as an antioxidant in plant tissue culture. AA at 1 -3 mg/l was added to the medium.

**3.1.6.1.7 Polyvinyl pyrrolidone (PVP)**

PVP is added at a range of 1-3 mg/l to the standardized multiple shoot induction medium to tap its antioxidant properties and to prevent the oxidative browning of explant tissues.

## 3.1.7 Indirect regeneration

### 3.1.7.1 Callogenesis

Induction of callus from an explant tissue is referred to as callogenesis.

### 3.1.7.2 Explants

The explants used were of internodal regions (1-1.5 cm), leaves (1$cm^2$ pieces with midrib) and seeds (4-5 month old) for callus induction.

### 3.1.7.3 Sterilization and inoculation

The sterilization procedure applied for all the three types of explants mentioned above was similar to that of shoot tips and nodal segments; but the duration of mercuric chloride treatment varied. Internodal segments were sterilized for five minutes; leaves were treated for three minutes whereas, the seeds were treated for eight minutes.

### 3.1.7.4 Inoculation and Incubation

Before inoculating the internodal segments for callus induction, the cut ends which were in contact with mercuric chloride solution were removed and the explants were split lengthwise into two. They were placed on the medium in such a way that the cut ends were in contact with the medium.

The leaves were cut into convenient size and treated with 0.1% mercuric chloride solution for 3 minutes. Then they were cut into 1$cm^2$ pieces and inoculated in a manner that the adaxial surfaces were in contact with the medium.

The seed coats of the sterilized seeds were removed aseptically and the embryo with the cotyledons were inoculated upright.

**3.1.7. 5 Culture media**

The basal media used for the standardization of callus induction from leaves and internodes were WPM and MS medium. Different concentrations of 2,4-D ranges from 1-3 mg/l were used for callus induction (Table 13).

Table 13. Media combinations tried for callus induction from leaves and internodes in *C. verum*

| **Sl. No.** | **Basal Medium** | **Concentration of 2, 4-D (mg/l)** |
|---|---|---|
| 1 | WPM | 1 |
| 2 | WPM | 2 |
| 3 | WPM | 3 |
| 4 | MS | 1 |
| 5 | MS | 2 |
| 6 | MS | 3 |

WPM and MS medium supplemented with different concentrations of BAP (0.5-3 mg/l) and Kinetin (0.5–3 mg/l) was used for the culture of seeds for getting organogenic calli (Table 14).

Table 14. Media combinations tried for the production of organogenic calli and somatic embryos from immature seeds of *C. verum*

| Sl. No. | Basal medium | Growth regulators (mg/l) | |
|---|---|---|---|
| | | BAP | Kn |
| 1 | WPM | 0.5 | 0 |
| 2 | | 1.0 | 0 |
| 3 | | 0 | 0.5 |
| 4 | | 0 | 1.0 |
| 5 | MS | 0.5 | 0 |
| 6 | | 1.0 | 0 |
| 7 | | 0 | 0.5 |
| 8 | | 0 | 1.0 |

### 3.1.7.6 Subculture

Table 15. Media combinations tried for the regeneration of shoots from organogenic calli produced from immature seeds in *C. verum*

| Sl. No. | Basal medium | Growth regulators | |
|---|---|---|---|
| | | BAP (mg/l) | Kn (mg/l) |
| 1 | WPM | 0 | 0 |
| 2 | | 0.5 | 0 |
| 3 | | 1 | 0 |
| 4 | | 0 | 0.5 |
| 5 | | 0 | 1 |
| 6 | | 0 | 2 |
| 7 | | 1 | 0.5 |
| 8 | | 2 | 0.5 |
| 9 | | 3 | 1 |
| 10 | | 4 | 1 |

The calli produced from the internodal segments and leaves were subcultured for regeneration. The organogenic calli obtained from the hypocotyl region

(cotyledonary axis) were also subcultured for regeneration. Ten media combinations were tried (Table 15).

### 3.1.8 Rhizogenesis

*In vitro* developed shoots from juvenile and mature explants were subcultured for root induction. WPM alone and WPM supplemented with different concentration and combinations of auxins and charcoal were tried to study the rooting pattern (Table 16).

Table 16. Media combinations tried for the induction of roots in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators and additives** | | |
|---|---|---|---|---|
| | | **IBA (mg/l)** | **NAA (mg/l)** | **Charcoal (gm/l)** |
| 1 | WPM | 0 | 0 | 0 |
| 2 | | 0 | 0 | 2 |
| 3 | | 1 | 0 | 0 |
| 4 | | 1 | 0 | 2 |
| 5 | | 0 | 1 | 0 |
| 6 | | 0 | 1 | 2 |
| 7 | | 2 | 0 | 0 |
| 8 | | 2 | 0 | 2 |
| 9 | | 0 | 2 | 0 |
| 10 | | 0 | 2 | 2 |
| 11 | | 0.5 | 0.5 | 0 |
| 12 | | 0.5 | 0.5 | 2 |
| 13 | | 1 | 1 | 0 |
| 14 | | 1 | 1 | 2 |

## 3.1.9 Somatic embryogenesis

### 3.1.9.1 Explants

The explants used for the induction of somatic embryos were healthy immature (4-5 month old) seeds (Figure 14 A).

### 3.1.9.2 Sterilization and inoculation

The seeds were collected, washed with a detergent solution and treated with a fungicide solution (3% Dithane M-45 or Bavistin). Then they were treated with 0.1% mercuric chloride solution for 8 minutes. The seed coats were removed aseptically and the embryos with the cotyledons were inoculated upright.

### 3.1.9.3 Culture medium

The basal media used for the inoculation of immature seeds were WPM and MS. Eight media combinations were tried for the induction of somatic embryos (Table 14). The somatic embryos produced were subcultured on WPM supplemented with different combinations and concentrations of BAP and Kinetin (Table 17).

### 3.1.9.4 Plant Growth Regulators and additives used

#### 3.1.9.4.1 BAP

BAP was used at a range of 1-4 mg/l for the standardisation of the medium for the maturation and germination of somatic embryos into plantlets. BAP was used alone and in combination with Kn.

Table 17. Media combinations tried for the maturation and germination of somatic embryos into plantlets *in C. verum*

| Sl. No. | Basal medium | Growth regulators | |
|---|---|---|---|
| | | BAP (mg/l) | Kn (mg/l) |
| 1 | WPM | 0 | 0 |
| 2 | | 0.5 | 0 |
| 3 | | 1 | 0 |
| 4 | | 0 | 0.5 |
| 5 | | 0 | 1 |
| 6 | | 0 | 2 |
| 7 | | 1 | 0.5 |
| 8 | | 2 | 0.5 |
| 9 | | 3 | 1 |
| 10 | | 4 | 1 |

**3.1.9.4.2 Kinetin**

Kinetin was used at a range of 0.5-2 mg/l for the maturation and germination of somatic embryos. Kn was used alone or in combination with BAP.

**3.1.9.4.3 Sucrose**

30-40 gm/l sucrose was added to the regeneration medium of somatic embryos whereas 20 gm/l sucrose was added to the medium which was used for induction of somatic embryos.

**3.1.9.4.4 Coconut milk**

5-25 % coconut milk was added to the optimised medium to study its effect on the maturation and germination of somatic embryos.

### 3.1.10 Hardening

*In vitro* developed plantlet 3-5 cm in height, with 3-4 healthy roots were taken out from the culture vessel and washed carefully to remove the traces of agar and sucrose present in the roots without damaging them. Plantlets were treated with 3% fungicide solution and were transplanted to soilrite in tea cups. These tea cups were covered with plastic bags and kept in mist chamber. After one week some pores were made in plastic bags to reduce the humidity. After two weeks of transplantation, the plantlets with soilrite were transplanted to poly bags containing a mixture of soilrite, sand and garden soil and were kept in green house. Hoagland's solution (Table 21) was used to irrigate the transplanted plantlets.

Table 18. Growth regulators and additives used for the induction of multiple shoots and roots in *C. verum*

| **Sl. No.** | **Growth regulators** | | **Range (mg/l)** |
|---|---|---|---|
| 1 | **Cytokinins** | Benzyl amino purine | 1-4 |
| | | Kinetin | 1-4 |
| | | Zeatin | 1-4 |
| | | Gibberellic acid | 1-3 |
| 2 | **Auxins** | Indole -3-butyric acid | 1-2 |
| | | Naphthalene acetic acid | 1-2 |
| 3 | **Charcoal** | | 2000 |

Table 19. Additives and antioxidants used for shoot induction in *C. verum*

| Sl. No. | Additives and antioxidants | Range |
|---|---|---|
| 1 | Coconut milk | 2-25 % |
| 2 | Charcoal | 2 gm |
| 3 | Adenine sulphate | 1-3 mg / l |
| 4 | Polyvinyl pyrrolidone | 1-3 mg / l |
| 5 | Ascorbic acid | 1-3 mg / l |

Table 20. Growth regulators used for the induction and maturation of somatic embryos in *C. verum*

| Sl. No. | Growth regulators | Range |
|---|---|---|
| 1 | Kinetin | 0.5-1 mg/l |
| 2 | Benzyl amino purine | 0.3 mg/l |
| 3 | Abscisic acid | 1-3 mg/l |
| 4 | Coconut milk | 5 – 25 (%) |
| 5 | Sucrose | 20 – 40 (gm/l) |

Table 21. Composition of Hoagland's solution (Epstein, 1972)

| Inorganic nutrients | Compounds | Elements | Concentration (mg/l) |
|---|---|---|---|
| *Macro salts* | $KNO_3$ | N, K | 22.23 |
| | $Ca(NO_3)_4H_2PO_4$ | Ca | 160.00 |
| | $NaH_2PO_4$ | P | 62.00 |
| | $MgSO_47H_2O$ | S, Mg | 32.64 |
| *Micro salts* | KCl | Cl | 1.72 |
| | $H_3BO_3$ | B | 0.27 |
| | $NaSO_4H_2O$ | Na | 0.11 |
| | $ZnSO_47H_2O$ | Zn | 0.131 |
| | $CuSO_45H_2O$ | Cu | 0.132 |
| | $Na_2MoO_4$ | Mo | 0.05 |
| | FeEDTA | Fe | 1.12 |

### 3.1.11 Molecular characterisation

Five plantlets produced *in vitro* through direct regeneration and five plantlets produced *in vitro* through somatic embryogenesis were compared with the mother plant by analyzing the RAPD data.

**3.1.11.1 Genomic DNA extraction**

Genomic DNA was extracted from leaves by a modified CTAB method (Rogers and Bendich, 1988).

- Leaves were washed in distilled water, dried by absorption of moisture with sterile filter papers (1-1.5 gms) were frozen in liquid nitrogen and ground to fine powder in mortar and pestle, mixed with 15 ml of CTAB extraction buffer with 2 β mercaptoethanol at pH 8.0 (Table 22).
- Mixtures were incubated at $65^0$C for 3 hours in a water bath with gentle shaking at regular intervals.
- To the mixture equal volume of chloroform was added, mixed well to make an emulsion, followed by centrifugation at 10,000 rpm for 5 mins at $4^0$C.
- To the upper aqueous phase collected in a corex tube, 1/10 volume of CTAB/NaCl solution was added. The mixture was gently shaken followed by the addition of equal volume of chloroform.
- The mixture was centrifuged at 10,000 rpm for 5 mins at $4^0$C.

- The upper aqueous phase was collected and to it added double volume of CTAB precipitation buffer (Table 22), mixed gently, sealed with parafilm and kept at $37^0$C for overnight incubation in water bath.

- Next day the mixture was centrifuged at 8,000 rpm for 10 mins at $4^0$C. Pellet containing nucleic acid were dissolved in 1 ml high salt TE buffer (Table 22).

- The solution was transferred to a fresh tube and 1 ml isopropanol was added gently and kept at $-20^0$C for 30 minutes to precipitate nucleic acids.

- Nucleic acids were recovered as pellets by centrifugation at 8000 rpm for 10 mins at $4^0$C. The pellets were washed in 80% ethanol, and resuspended in 0.5 ml TE pH at 8.0 (Table 22).

- The solution was transferred to a fresh eppendorf tube. RNA was removed from the isolated nucleic acids by incubating the solution with 3 µl RNase A (Sigma) and the solution incubated for 2 hours at $37^0$C.

- 0.5 ml chloroform was added to this solution and centrifuged at 10,000 rpm for 5 minutes at $4^0$C, and the supernatant was collected. The step was repeated twice.

- To the supernatant double volume of absolute alcohol and 50 µl 3 M solution of sodium acetate was added and kept for over night incubation at $-20^0$C.

- The pellet was collected by centrifuging the solution at 12,000 rpm for 15 minutes at $4^0$C, washed with 70% ethanol, air dried and resuspended in 100 µl 1X TE buffer at pH 8.0.

### 3.1.11.2 RAPD - PCR Analysis

RAPD assay was carried out in 25 µl reaction mixture containing 2.5 µl 10X amplification buffer (100 mM Tris HCl pH-8 at 25$^0$C, 15 mM $MgCl_2$, 500 mM KCl and 1.0% Triton X-100) 0.5 µl of dNTP mixture (10 mM each in 50 µl), 1.0 U of Taq DNA polymerase (Finnzyme, Finland), 15 pmoles (1.2 µl) of 10-mer primer (Biogene, USA) and 50ng of genomic DNA. The reaction mixture was overlaid with 20 µl of mineral oil in order to avoid evaporation.

Table 22. Stock solutions required for Genomic DNA extraction

| Sl. | Solutions | Composition | Amount |
|---|---|---|---|
| 1 | Tris Buffer<br>pH 8 | Tris, 1 M<br>$H_2O$ | 12.11 gm<br>100 ml |
| 2 | EDTA | $Na_2$ EDTA, 0.1M<br>$H_2O$ | 18.61 gm<br>100 ml |
| 3 | CTAB Extraction Buffer<br>pH 8.0<br>Stored at room temperature | CTAB 2% w/v<br>Tris Buffer 100 mM<br>$Na_2$ EDTA 20 mM<br>PVP 2%<br>NaCl 1.4 M<br>$H_2O$ | 2 gm<br>10 ml<br>4 ml<br>1 gm<br>8.2 gm<br>100 ml |
| 4 | CTAB – NaCl Solution | CTAB 10%<br>0.7 M NaCl<br>$H_2O$ | 2.5 gm<br>1.02 gm<br>25 ml |
| 5 | High Salt TE<br>pH 8.0<br>Stored at room temperature | Tris Buffer 10 mM<br>$Na_2$ EDTA 0.1 mM<br>NaCl 1 M<br>$H_2O$ | 1 ml<br>20 ul<br>5.85 gm<br>100 ml |
| 6 | CTAB Precipitation Solution<br>pH 8.0<br>Stored at room temperature | CTAB 1% w/v<br>Tris Buffer 50 mM<br>$Na_2$ EDTA 10 mM<br>$H_2O$ | 1 gm<br>5 ml<br>2 ml<br>100 ml |
| 7 | TE Buffer | Tris Buffer 10 mM<br>$Na_2$ EDTA 1 M<br>$H_2O$ | 1 ml<br>0.2 ml<br>100 ml |
| 8 | Sodium Acetate | Sodium Acetate 3 M<br>$H_2O$ | 40.8 gm<br>100 ml |

Amplification was performed in DNA Thermal Cycler Progene (Techne, USA). The sequential steps involved, 1 cycle of 2 min at $95^0$ C, 2 min at $35^0$ C and 2 min at $72^0$ C followed by 39 cycles of 1 min at $93^0$ C, 1 min at $36^0$ C and 2 min at $72^0$ C. The last cycle was followed by 7 min extension at $72^0$ C.

### 3.1.11.3 Agarose Gel Electrophoresis

The amplified products (25 µl) were electrophoresed in a horizontal gel apparatus (Bio-Rad, USA) using 1.2% agarose gel (containing 0.5 mg/ml ethidium bromide) in 1X TBE buffer pH 8.0 (Table 24) at 100 volts for approximately 2.5 hours. Agarose powder (2.4 gm) (Gibco-BRL) was mixed with 200 ml of 1X TBE and melted in a microwave oven and mixed with ethidium bromide (Table 24). Gels were casted in the form of 0.5 cm thick, horizontal slab. Amplification products were electrophoresed with 5 µl of 10X loading buffer (Table 24). Amplified products were co-electrophorosed with pGM as a DNA marker.

### 3.1.11.4 Analysis of amplification profile

Amplification profiles were compared with each other and bands of DNA fragment were scored as present (1) or absent (0). The data for all the 20 primers were used to estimate the similarity on the basis of the number of shared amplification products (Nei and Li, 1979). A dendrogram based on similarity coefficient was generated by using the unweighted pair group method arithmetic means (UPGMA).

Table 23: Random oligonucleotide decamer primers used in RAPD fragment polymorphism in *C. verum*

| Sl. No. | Primer | Primer Sequence (5'–3') |
|---|---|---|
| 1 | OPA-01 | CAG GCC CTT C |
| 2 | OPA-03 | AGT CAG CCA C |
| 3 | OPA-07 | GAA ACG GGT G |
| 4 | OPA-08 | GTG ACG TAG G |
| 5 | OPA-09 | GGG TAA CGC C |
| 6 | OPA-10 | GTG ATC GCA G |
| 7 | OPA-13 | CAG CAC CCA C |
| 8 | OPA-14 | TCT GTG CTG G |
| 9 | OPA-16 | AGC CAG CGA A |
| 10 | OPB-18 | CCA CAG CAG T |
| 11 | OPB-19 | ACC CCC GAA G |
| 12 | OPB-20 | GGA CCC TTA C |
| 13 | OPD-03 | GTC GCC GTC A |
| 14 | OPD-04 | TCT GGT GAG G |
| 15 | OPD-06 | ACC TGA ACG G |
| 16 | OPD-08 | GTG TGC CCC A |
| 17 | OPD-13 | GGG GTG ACG A |
| 18 | OPD-16 | AGG GCG TAA G |
| 19 | OPD-20 | ACC CGG TCA C |
| 20 | OPE-19 | ACG GCG TAT G |

Table 24. Stock solutions required for Agarose Gel Electrophoresis

| Sl. No. | Solutions | Compositions | Amount |
|---|---|---|---|
| 1 | TBE Buffer (10 X)<br>pH 8 | Tris Base<br>Boric Acid<br>$Na_2$ EDTA 0.5 mM<br>$H_2O$ | 21.6 gm<br>11 gm<br>8 ml<br>100 ml |
| 2 | Gel Loading Buffer | Bromo Phenol Blue 0.25 %<br>Xylene Cyanole 0.25 %<br>Sucrose 40% (w/v)<br>$H_2O$ | 250 mg<br>250 mg<br>40 gm<br>100 ml |
| 3 | Ethidium Bromide | Ethidium Bromide<br>$H_2O$ | 1.0 gm<br>100 ml |

#### 3.1.11.5 Data Analysis

Computer programme used for genetic data analysis was POPGENE (Ver 1.0; Yeh and Boyle, 1997) and PCO3D (Ver 1.2; Adams). Chemiimager 4000I Ver 4.04 (Alpha Innotech Corporation, USA) was used to document and analyse the RAPD gels. Statistical analysis was performed using Microsoft Excel 2003.

#### 3.1.11.6 Presentation of data

For general illustrations, tables, figures, photographs and histograms were used to get a brief idea of the direct regeneration, callogenesis, caulogenesis, rhizogenesis, and somatic embryogenesis. Data were presented to compare the response of different explants in different media compositions.

## 3.2 *Cinnamomum cassia* Blume

### 3. 2.1 Source of plant material

Five year old cassia trees grown at Indian Institute of Spice Research (IISR) campus, Calicut, Kerala were selected as the source of explants.

### 3. 2.2 Direct regeneration

Shoot tips and nodal segments were collected as explants for direct regeneration.

#### 3. 2. 2.1 Establishment of aseptic cultures

Explants were collected from fungicide sprayed field grown trees with a view to reduce contamination rate.

### 3. 2. 2.2 Preparation of explants

Explant sterilization procedures were same as that of *Cinnamomum verum.* The duration given for mercuric chloride (0.1%) treatment varied from 8-12 minutes depending on the hardness of the bark.

### 3. 2. 2.3 Inoculation and Incubation

Inoculation was conducted inside the Laminar air flow chamber. The Laminar air flow cabinet was cleaned with alcohol and the UV light inside the unit was switched on for 20 minutes, after keeping all the equipments necessary for inoculation. They include sterilized petridishes, forceps, blade holders, cotton, test tubes for taking alcohol, spirit lamp, surgical blades, alcohol bottle, etc. Before starting inoculation operations, the hands and arms were cleaned thoroughly using soap and water, then rinsed with 96% alcohol. The working table of the laminar air flow unit was swabbed with 70% alcohol. Before inoculation, the cut ends of the explants, which were in contact with the sterilizing agent were removed and were inoculated aseptically.

Inoculated cultures were incubated at 25±2ºC under 14 hour photoperiod and a light intensity of 2500 lux (μmol/$m^{-2}s^{-1}$) with 55-60% relative humidity.

### 3. 2. 2.4 Selection of culture medium

The basal medium (WPM) and growth regulator combinations (BAP and Kn) gave best results for initial establishment and multiple shoot induction in *Cinnamomum* verum, were selected as the culture medium for *Cinnamomum cassia* for the establishment of cultures and for multiple shoot induction.

### 3. 2. 2.5 Medium for initial establishment of shoot tips and nodal segments

WPM medium supplemented with 2 mg/l BAP and 0.5 mg/l Kn was used for initial establishment of culture.

### 3. 2. 2.6 Medium for multiple shoot induction

WPM supplemented with 3 mg/l BAP and 1 mg/l Kn was used for multiple shoot induction.

### 3. 2. 2.7 Medium for root induction

The media combinations tried for root induction are WPM supplemented with 2 gm/l charcoal and WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and 2 gm/l charcoal.

As the explants were taken from a single plant established at IISR, there was a real scarcity for explants of *Cinnamomum cassia* at the time of executing the work and hence all the experiments done in *C. verum* could not be done in *C. Cassia*.

# 4. RESULTS

The response of different explants of cinnamon such as shoot tips, nodal segments, leaves, internodes and immature seeds to various media combinations were studied. The various results obtained were direct regeneration, callus induction, callus regeneration, *in vitro* rooting and somatic embryogenesis. The plantlets were hardened and transferred to the field.

Five directly regenerated plantlets and five plantlets developed through somatic embryogenesis were profiled for RAPD polymorphism using twenty primers and were compared with the mother plant. The flow chart of the experiments done in the present work is represented as Fig. 9.

The results were illustrated with the help of tables, figures, photographs and histograms.

## 4.1 *Cinnamomum verum* Bercht. and Presl

### 4.1.1 Direct regeneration

#### 4.1.1.1 Responses of explants to pre-preparations

Pre-treatment methods employed to improve the growth response, such as removal of shoot tips to reduce apical dominance, incubation in BAP, IBA, ascorbic acid, hydrogen peroxide and collection of explants into picric acid and 8-hydroxy quinolinine sulphate in order to reduce contamination did not prove to be very useful.

#### 4.1.1.2 Surface sterilization of explants

Cinnamon explants when collected from the field grown trees showed over 90% contamination on inoculation. Explants from fungicide sprayed green house grown mother plants showed a further reduction in contamination rate to 80%. Mature explants showed a high rate of contamination (80-90%); juvenile explants of mature trees as well as seedling explants showed a comparatively lower contamination rate (60-70%). Contamination was observed even after 30-40 days of establishment of cultures and induction of shoots, since the vegetative buds in shoot tips and axils of leaves are covered with scales. Presence of scales prevents the proper contact of sterilizing agent with the vegetative buds present in the explants.

#### 4.1.1.3 Standardisation of Basal Medium and growth regulators for the initial establishment of cultures

##### 4.1.1.3.1 Inoculation and incubation

Shoot tips and nodal segments of *C. verum* were inoculated into WPM supplemented with 10 different combinations of cytokinins. BAP and Kn alone and in combination were tried at a range of 0.5-3 mg/ml to induce shoots. Woody Plant Medium (WPM) was selected as the basal medium since the medium showed better response.

Cultures of shoot tips and nodal segments of *Cinnamomum verum* were established *in vitro* from explants of different maturity levels as follows:

1) Explants from 2-3 year old seedlings,
2) Juvenile explants from mature trees and
3) Mature explants from mature trees.

Bud break and shoot induction were achieved in all the media combinations tried but the percentage of response was different in different combinations and concentrations of growth regulators. The highest percentage was achieved in WPM supplemented with 2 mg/l BAP and 0.5 mg/l Kn. BAP was found to be superior to Kn for bud break and shoot induction.

Bud break was observed within 6-12 days in seedling explants, 10-17 days in juvenile explants of mature plants and 12-22 days in mature explants of mature plants. To avoid the interference of phenolic compounds, the cultures were subcultured in every two weeks.

Table 25. Effect of BAP and Kn on percentage of shoot induction in different types of explants of *Cinnamomum verum* in WPM

| **Initial establishment of cultures** | | | | | | |
|---|---|---|---|---|---|---|
| **Sl. No.** | **Basal medium** | **Growth regulators** | | **Percentage of cultures showing response on 30th day** | | |
| | | **BAP (mg/l)** | **Kn (mg/l)** | **Seedling explants** | **Juvenile explants of mature trees** | **Mature explants** |
| 1 | **WPM** | 0.5 | 0 | 31. 58 | 26. 09 | 22. 73 |
| 2 | | 1 | 0 | 34. 78 | 28. 57 | 23. 81 |
| 3 | | 0 | 0.5 | 29. 41 | 25. 00 | 21. 74 |
| 4 | | 0 | 1 | 29. 41 | 23. 81 | 21. 43 |
| 5 | | 1 | 0.5 | 33. 33 | 27. 78 | 23. 53 |
| 6 | | 2 | 0.5 | 61. 54 | 56. 25 | 43. 48 |
| 7 | | 3 | 0.5 | 45. 83 | 43. 75 | 42. 86 |
| 8 | | 1 | 1 | 52. 94 | 47. 06 | 28. 57 |
| 9 | | 2 | 1 | 58. 33 | 52. 63 | 41. 18 |
| 10 | | 3 | 1 | 42. 86 | 31. 25 | 25. 00 |

Eventhough both shoot tips and nodal segments were inoculated initially, only nodal segments responded well while shoot tips responded poorly and hence for further experiments for getting multiple shoot induction, nodal segments were used.

Table 26. Response of shoot tips and nodal segments from mature trees of *C. verum* in different basal media and growth regulators

| Sl. No. | Basal medium | Growth regulators (mg/l) | | % of cultures showing response |
|---|---|---|---|---|
| | | BAP | Kn | |
| 1 | WPM | 2 | 0.5 | 44 |
| 2 | MS | 2 | 0.5 | 38 |
| 3 | White's | 2 | 0.5 | No response |

Establishment, bud break and the growth of the buds were the criteria for the selection of suitable basal media. For initial establishment and bud break of explants WPM and MS medium showed almost same results, but later on, WPM was found to be more suitable than MS medium for the growth and development of cultures and multiple shoot induction. White's medium (1963) did not show any favorable response. Hence, WPM which showed better response was selected as the basal medium for further studies.

#### 4.1.1.4 Standardisation of medium for multiple shoot induction

The established cultures were subcultured to 25 different media combinations to standardise the multiple shoot induction medium. Three trial experiments for three types of explants *i.e.*, seedlings (Experiment-1), juvenile explants of mature trees (Experiment-2) and mature explants (Experiment-3) were

conducted and the average was taken. The summary of three experiments conducted for multiple shoot induction in *Cinnamomum verum* is given in Table 81.

The cytokinins, BAP, Kn and ZN were used alone and in various combinations at a range of 1-4 mg/l. The number of multiple shoots produced *in vitro* varied, according to the concentrations and combinations of the cytokinins used.

The induction of multiple shoots started from 15-20 days of subculture. The number of shoots produced varied in all the three types of explants, seedlings, juvenile explants of mature trees and mature explants.

After two-three weeks of establishment, the cultures were subcultured after giving a fresh basal cut to remove the dead tissues and the portion of the stem filled with phenolic exudates.

Seven cultures (regenerated shoots) were used for each treatment and all the three experiments for multiple shoot induction were repeated twice.

#### 4.1.1.5 Multiple shoot induction in seedling explants (Experiment-1)

##### 4.1.1.5.1 Effect of WPM + 1 mg/l cytokinin

The combinations tried were WPM supplemented with 1 mg/l BAP, 1 mg/l Kn, 1 mg/l ZN, 0.5 mg/l BAP + 0.5 mg/l Kn and 0.5 mg/l BAP + 0.5 mg/l ZN (Table 27).

Of the five combinations tried, the highest mean of 8.67 multiple shoots were produced in WPM supplemented with 0.5 mg/l BAP and 0.5 mg/l Kn. The lowest mean was 7.71 in WPM supplemented with 1 mg/l Kn.

Table 27. Effect of **1mg/l** cytokinin on multiple shoot induction from **seedling** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 1 | WPM | 1 BAP | 9.33 | 8.48 |
| | | | 7.67 | |
| | | | 8.33 | |
| | | | 8.33 | |
| | | | 8.33 | |
| | | | 9.33 | |
| | | | 8.00 | |
| 2 | | 1 Kn | 7.33 | 7.71 |
| | | | 8.00 | |
| | | | 7.67 | |
| | | | 8.33 | |
| | | | 8.00 | |
| | | | 7.33 | |
| | | | 7.33 | |
| 3 | | 1 ZN | 8.33 | 7.95 |
| | | | 9.33 | |
| | | | 8.33 | |
| | | | 7.67 | |
| | | | 6.67 | |
| | | | 7.67 | |
| | | | 7.67 | |
| 4 | | 0.5 BAP + 0.5 Kn | 8.33 | 8.67 |
| | | | 9.67 | |
| | | | 8.33 | |
| | | | 7.67 | |
| | | | 9.33 | |
| | | | 7.67 | |
| | | | 9.67 | |
| 5 | | 0.5 BAP + 0.5 ZN | 8.33 | 8.52 |
| | | | 9.67 | |
| | | | 8.33 | |
| | | | 7.67 | |
| | | | 8.33 | |
| | | | 7.67 | |
| | | | 9.67 | |

#### 4.1.1.5. 2 Statistical Analysis

The data on multiple shoot induction from seedling explants (Experiment-1), juvenile explants of mature trees (Experiment-2) and mature explants (Experiment-3) were subjected to standard ANOVA (Analysis of Variance) and Post Hoc (Duncan) test using the SPSS (16.0) software to study the variations and significance of different experiments. The data were subjected to square root transformation before the statistical analysis.

The Standard Deviation (SD), Standard Error (SE) and Coefficient of Variation (CV) were also computed for the different sets of experiments.

All the three experiments are repeated twice with seven replicates.

The mean, SE and CV (%) for the data of multiple shoot induction from seedling explants in WPM supplemented with 1 mg/l cytokinin are given in the Table 28. The lowest CV value obtained was 16.67 %. The lower the CV value, the better is the estimate or consistency. The ANOVA and Duncan test for multiple shoot induction in seedling explants are given in Tables 29 and 30. The Repeat 1 in Experiment 1 as well as the overall $R^2$ was significant at 1% level (F=5.131). Here the $R^2$ was significant at 63%. The Duncan test of Repeat 1 in Experiment 1 gave 3 subsets significant at 1% level indicating that the grouping are very prominent and the results are good.

Table 28. Multiple shoot induction in **seedling** explants showing the mean and standard error with CV values in WPM medium supplemented with **1 mg/l** cytokinin

| **Experiment 1 (1 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 7 | 4.50±0.327 | 20.58 |
| 2 | 8 | 4.00±0.645 | 48.40 |
| 3 | 9 | 3.70±0.761 | 65.03 |
| 4 | 10 | 4.29±0.969 | 59.74 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 7 | 5.67±0.76 | 32.84 |
| 2 | 8 | 4.36±0.427 | 36.65 |
| 3 | 9 | 3.18±0.685 | 71.45 |
| 4 | 10 | 2.50±0.5 | 40.00 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 7 | 4.27±0.764 | 59.32 |
| 2 | 8 | 3.80±0.49 | 40.76 |
| 3 | 9 | 2.43±0.481 | 52.35 |
| 4 | 10 | 6.00±0.447 | 16.67 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 1 mg/l cytokinin | | | |

Table 29. ANOVA of multiple shoot induction in **seedling** explants in WPM supplemented with **1 mg/l** cytokinin

| Source | Df | Mean Square | F | Sig. |
|---|---|---|---|---|
| Corrected Model | 12 | 7.310 | 3.076 | 0.011 |
| Experiment-1 | 4 | 3.945 | 1.660 | 0.195 |
| Repeat-1 | 3 | 12.193 | 5.131** | 0.008 |
| Repeat-2 | 5 | 5.461 | 2.298 | 0.080 |
| Error | 22 | 2.376 | | |
| Total | 35 | | | |
| $R^2 = 0.627$ | | | | |
| ** 1% significance | | | | |

Table 30. Duncan test for multiple shoot induction in **seedling** explants in WPM supplemented with **1 mg/l** cytokinin

| Repeat-1 | N | Subset | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 10 | 4 | 2.50 | | |
| 9 | 11 | 3.18 | 3.18 | |
| 8 | 14 | | 4.36 | 4.36 |
| 7 | 6 | | | 5.67 |
| Sig. | | 0.420 | 0.170 | 0.129 |

#### 4.1.1.5. 3 Effect of WPM + 2 mg/l cytokinin

Table 31. Effect of **2 mg/l** cytokinin on multiple shoot induction from **seedling** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 6 | WPM | 2 BAP | 10.00 | 9.71 |
| | | | 8.33 | |
| | | | 10.67 | |
| | | | 9.33 | |
| | | | 10.33 | |
| | | | 9.67 | |
| | | | 9.67 | |
| 7 | | 2 Kn | 9.67 | 9.14 |
| | | | 9.33 | |
| | | | 8.00 | |
| | | | 9.33 | |
| | | | 10.33 | |
| | | | 8.33 | |
| | | | 9.00 | |
| 8 | | 2 ZN | 9.00 | 9.24 |
| | | | 8.00 | |
| | | | 9.00 | |
| | | | 8.67 | |
| | | | 10.33 | |
| | | | 10.33 | |
| | | | 9.33 | |
| 9 | | 1 BAP + 1 Kn | 10.00 | 10.29 |
| | | | 10.67 | |
| | | | 9.33 | |
| | | | 9.00 | |
| | | | 11.00 | |
| | | | 9.67 | |
| | | | 12.33 | |
| 10 | | 1 Kn + 1 ZN | 11.33 | 10.00 |
| | | | 10.00 | |
| | | | 10.67 | |
| | | | 8.67 | |
| | | | 11.00 | |
| | | | 9.67 | |
| | | | 8.67 | |

Table 32. Multiple shoot induction in **seedling** explants showing the mean and standard error with CV values in WPM medium supplemented with **2 mg/l** cytokinin

| **Experiment 1 (2 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 8 | 3.14±0.634 | 53.38 |
| 2 | 9 | 5.00±0.681 | 38.54 |
| 3 | 10 | 3.00±0.951 | 83.90 |
| 4 | 11 | 4.00±0.655 | 46.30 |
| 5 | 12 | 6.00±0.577 | 16.67 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 9 | 4.00±0.764 | 57.28 |
| 2 | 10 | 4.60±0.6 | 41.24 |
| 3 | 11 | 4.00±1.528 | 66.15 |
| 4 | 12 | 4.25±1.109 | 52.16 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 8 | 4.44±0.603 | 40.77 |
| 2 | 9 | 3.88±0.895 | 65.26 |
| 3 | 10 | 4.13±0.718 | 49.18 |
| 4 | 11 | 3.33±0.76 | 55.92 |
| 5 | 12 | 3.50±1.5 | 60.60 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 2 mg/l cytokinin | | | |

The combinations tried were WPM supplemented with 2 mg/l BAP, 2 mg/l Kn, 2 mg/l ZN , 1 mg/l BAP + 1 mg/l Kn and 1 mg/l Kn + 1 mg/l ZN (Table 31).

Of the five combinations tried, the highest mean of multiple shoots produced was 10.29 in WPM supplemented with 1 mg/l BAP and 1 mg/l Kn and lowest mean of shoots produced was 9.71 in WPM supplemented with 2 mg/l BAP.

The mean, SE and CV (%) are given in the Table 32. Here the lowest CV obtained was 16.67%, indicating that the estimate was good. The ANOVA for multiple shoot induction in WPM supplemented with 2 mg/l cytokinin was also done and given in Table 33.

Table 33. ANOVA of multiple shoot induction in **seedling** explants in WPM supplemented with **2 mg/l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** | **Sig.** |
|---|---|---|---|---|
| Corrected Model | 17 | 3.223 | 0.643 | 0.814 |
| Experiment-1 | 6 | 3.953 | 0.789 | 0.591 |
| Repeat-1 | 5 | 2.362 | 0.471 | 0.792 |
| Repeat-2 | 6 | 1.804 | 0.360 | 0.894 |
| Error | 17 | 5.012 | | |
| Total | 35 | | | |
| $R^2 = 0.391$ | | | | |

#### 4.1.1.5. 4 Effect of WPM + 3 mg/l cytokinin

Table 34. Effect of **3 mg/l** cytokinin on multiple shoot induction from **seedling** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 11 | WPM | 3 BAP | 12.33 | 11.90 |
| | | | 11.33 | |
| | | | 12.67 | |
| | | | 13.67 | |
| | | | 10.33 | |
| | | | 13.33 | |
| | | | 9.67 | |
| 12 | | 1 BAP + 2 Kn | 13.00 | 13.19 |
| | | | 11.67 | |
| | | | 13.00 | |
| | | | 13.00 | |
| | | | 13.67 | |
| | | | 13.33 | |
| | | | 14.67 | |
| 13 | | 2 BAP + 1 Kn | 14.33 | 14.05 |
| | | | 14.00 | |
| | | | 14.33 | |
| | | | 13.67 | |
| | | | 12.33 | |
| | | | 15.00 | |
| | | | 14.67 | |
| 14 | | 2 ZN + 1 Kn | 14.33 | 13.48 |
| | | | 13.33 | |
| | | | 12.67 | |
| | | | 13.33 | |
| | | | 13.67 | |
| | | | 13.00 | |
| | | | 14.00 | |
| 15 | | 2 Kn + 1 ZN | 12.67 | 12.90 |
| | | | 13.33 | |
| | | | 12.00 | |
| | | | 11.67 | |
| | | | 12.33 | |
| | | | 12.67 | |
| | | | 15.67 | |

The combinations tried were WPM supplemented with 3 mg/l BAP, 1 mg/l BAP + 2 mg/l Kn, 2 mg/l BAP + 1 mg/l Kn, 2 mg/l ZN + 1 mg/l Kn and 2 mg/l ZN + 1 mg/l ZN (Table 34).

Table 35. Multiple shoot induction in **seedling** explants showing the mean and standard error with CV values in WPM medium supplemented with **3 mg/l** cytokinin

| **Experiment 1 (3 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 5.00±0.707 | 28.28 |
| 2 | 11 | 2.67±0.333 | 21.61 |
| 3 | 12 | 3.71±0.68 | 48.49 |
| 4 | 13 | 3.43±0.782 | 60.35 |
| 5 | 14 | 5.00±0.894 | 40.00 |
| 6 | 15 | 3.60±1.249 | 77.58 |
| 7 | 16 | 4.75±1.315 | 55.37 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 11 | 4.67±0.615 | 32.25 |
| 2 | 12 | 2.00±0.577 | 50.00 |
| 3 | 13 | 3.14±0.459 | 38.69 |
| 4 | 14 | 5.11±0.633 | 37.18 |
| 5 | 15 | 4.75±1.436 | 60.46 |
| 6 | 16 | 2.67±1.202 | 77.98 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 4.50±1.258 | 55.93 |
| 2 | 12 | 3.43±0.841 | 64.87 |
| 3 | 13 | 3.86±0.508 | 34.84 |
| 4 | 14 | 3.67±0.76 | 50.74 |
| 5 | 15 | 4.43±0.922 | 55.08 |
| 6 | 16 | 5.67±0.882 | 26.95 |

CV – Coefficient of Variation
Medium combination – WPM + 3 mg/l cytokinin

The highest mean of multiple shoots produced was 14.05 in WPM supplemented with 2 mg/l BAP and 1 mg/l Kn. The lowest mean of multiple shoots produced was 11.90 in WPM supplemented with 2 mg/l Kn.

The mean, SE and CV (%) are given in the Table 35. The lowest CV of 21.61% was computed showing a good estimate. The ANOVA and Duncan test (Table 37) for multiple shoot induction in seedling explants are presented in Tables, 36 and 37. The overall $R^2$ value was significant at 0.597 and Duncan test showed one subset.

Table 36. ANOVA of multiple shoot induction in **seedling** explants in WPM supplemented with **3 mg/l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** | **Sig.** |
|---|---|---|---|---|
| Corrected Model | 19 | 4.398 | 1.169 | 0.384 |
| Experiment-1 | 6 | 0.737 | 0.196 | 0.973 |
| Repeat-1 | 7 | 5.337 | 1.419 | 0.269 |
| Repeat-2 | 6 | 4.113 | 1.093 | 0.410 |
| Error | 15 | 3.762 | | |
| Total | 35 | | | |
| $R^2 = 0.597$ | | | | |

Table 37. Duncan test for multiple shoot induction in **seedling** explants in WPM supplemented with **3 mg/l** cytokinin

| **Experiment-1** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 11 | 3 | 2.67 |
| 13 | 7 | 3.43 |
| 15 | 5 | 3.60 |
| 12 | 7 | 3.71 |
| 16 | 4 | 4.75 |
| 10 | 4 | 5.00 |
| 14 | 5 | 5.00 |
| Sig. | | 0.125 |

#### 4.1.1.5. 5 Effect of WPM + 4 mg/l cytokinin

The combinations tried were WPM supplemented with 4 mg/l BAP, 3 mg/l BAP + 1 mg/l Kn, 3 mg/l BAP + 1 mg/l ZN, 2 mg/l Kn + 2 mg/l ZN and 2 mg/l BAP + 2 mg/l Kn (Table 38).

The highest mean of multiple shoots produced was 17.05 in WPM supplemented with 3 mg/l BAP and 1 mg/l Kn. This medium combination was found as the best medium for multiple shoot induction (Fig. 11). The shoots produced were healthy and vigorous. WPM supplemented with 3 mg/l BAP and 1 mg/l ZN could induce an average of 15.48 shoots per culture. The lowest mean of shoots observed was 13.62 in WPM supplemented with 4 mg/l BAP.

Table 38. Effect of **4 mg/l** cytokinin on multiple shoot induction from **seedling** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators** | **Average number of multiple shoots produced on 40th day** | |
|---|---|---|---|---|
| | | | **Average of Experiment 1 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 16 | WPM | 4 BAP | 12.67 | 13.62 |
| | | | 13.67 | |
| | | | 13.00 | |
| | | | 14.33 | |
| | | | 12.33 | |
| | | | 15.33 | |
| | | | 14.00 | |
| 17 | | 3 BAP + 1 Kn | 18.00 | 17.05 |
| | | | 17.67 | |
| | | | 16.67 | |
| | | | 17.00 | |
| | | | 15.67 | |
| | | | 17.67 | |
| | | | 16.67 | |
| 18 | | 3 BAP + 1 ZN | 16.00 | 15.48 |
| | | | 14.67 | |
| | | | 14.00 | |
| | | | 16.00 | |
| | | | 15.33 | |
| | | | 16.67 | |
| | | | 15.67 | |
| 19 | | 2 Kn + 2 ZN | 14.00 | 14.86 |
| | | | 16.00 | |
| | | | 14.67 | |
| | | | 14.33 | |
| | | | 14.67 | |
| | | | 14.67 | |
| | | | 15.67 | |
| 20 | | 2 BAP + 2 Kn | 15.33 | 15.24 |
| | | | 15.00 | |
| | | | 15.67 | |
| | | | 15.33 | |
| | | | 14.00 | |
| | | | 15.67 | |
| | | | 15.67 | |

The mean, SE and CV(%) was given in Table 39. The lowest CV value obtained was 35.46%. The ANOVA done is given in Table 40. The $R^2$ value was 53 %, which was significant.

Table 39. Multiple shoot induction in **seedling** explants showing the mean and standard error with CV values in WPM medium supplemented with **4 mg/l** cytokinin

| **Experiment 1 (4 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 12 | 3.00±1.155 | 66.67 |
| 2 | 14 | 4.67±0.919 | 48.20 |
| 3 | 15 | 4.20±0.917 | 48.79 |
| 4 | 16 | 4.45±0.529 | 39.39 |
| 5 | 17 | 3.60±1.249 | 77.58 |
| 6 | 18 | 3.50±2.5 | 101.03 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 12 | 3.67±1.333 | 62.92 |
| 2 | 13 | 5.50±1.5 | 38.56 |
| 3 | 14 | 5.00±0.913 | 36.52 |
| 4 | 15 | 3.30±0.597 | 57.24 |
| 5 | 16 | 3.71±0.778 | 55.50 |
| 6 | 17 | 4.43±0.896 | 53.50 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 13 | 3.20±0.8 | 55.91 |
| 2 | 14 | 2.80±0.97 | 77.43 |
| 3 | 15 | 4.57±0.841 | 48.69 |
| 4 | 16 | 5.00±0.627 | 35.46 |
| 5 | 17 | 4.67±0.803 | 42.10 |
| 6 | 18 | 3.00±1 | 47.13 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 4 mg/l cytokinin | | | |

Table 40. ANOVA of multiple shoot induction in **seedling** explants in WPM supplemented with **4 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** | **Sig.** |
|---|---|---|---|---|
| Corrected Model | 20 | 3.713 | 0.791 | 0.692 |
| Experiment-1 | 6 | 3.105 | 0.661 | 0.682 |
| Repeat-1 | 6 | 2.691 | 0.573 | 0.746 |
| Repeat-2 | 6 | 3.785 | 0.806 | 0.582 |
| Error | 14 | 4.695 | | |
| Total | 35 | | | |
| $R^2 = 0.530$ | | | | |

**4.1.1.5. 6 Effect of WPM + 5 mg/l cytokinin**

The combinations tried were WPM supplemented with 4 mg/l BAP + 1 Kn, 4 mg/l BAP + 1 mg/l ZN, 3 mg/l ZN + 2 mg/l Kn, 3 mg/l Kn + 2 mg/l BAP and 3 mg/l Kn + 2 mg/l ZN (Table 41).

The highest mean of multiple shoots produced was 13.38 in WPM supplemented with 4 mg/l BAP and 1 mg/l Kn. The lowest mean was 11.95 in WPM supplemented with 3 mg/l Kn and 2 mg/l ZN. The best medium for multiple shoot induction in seedling explants of *Cinnamomum verum* was observed as WPM supplemented with 3 mg/l BAP and 1 mg/l Kn.

Table 41. Effect of **5 mg/l** cytokinin on multiple shoot induction from **seedling** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 21 | WPM | 4 BAP + 1 Kn | 11.67 | 13.38 |
| | | | 14.33 | |
| | | | 13.67 | |
| | | | 14.67 | |
| | | | 13.33 | |
| | | | 14.00 | |
| | | | 12.00 | |
| 22 | | 4 BAP + 1 ZN | 11.67 | 12.95 |
| | | | 13.33 | |
| | | | 14.00 | |
| | | | 12.67 | |
| | | | 13.00 | |
| | | | 12.33 | |
| | | | 13.67 | |
| 23 | | 3 ZN + 2 Kn | 14.67 | 12.10 |
| | | | 12.67 | |
| | | | 12.33 | |
| | | | 12.00 | |
| | | | 11.33 | |
| | | | 11.67 | |
| | | | 10.00 | |
| 24 | | 3 Kn + 2 BAP | 12.33 | 12.52 |
| | | | 10.67 | |
| | | | 13.67 | |
| | | | 11.67 | |
| | | | 14.33 | |
| | | | 13.00 | |
| | | | 12.00 | |
| 25 | | 3 Kn + 2 ZN | 11.67 | 11.95 |
| | | | 11.00 | |
| | | | 13.33 | |
| | | | 11.67 | |
| | | | 13.33 | |

| | | | | |
|---|---|---|---|---|
| | | | 12.67 | |
| | | | 10.00 | |

Table 42. Multiple shoot induction in **seedling** explants showing the mean and standard error with CV values in WPM medium supplemented with **5 mg/l** cytokinin

| **Experiment 1 (5 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 9 | 3.75±1.181 | 63.01 |
| 2 | 10 | 3.75±0.854 | 45.55 |
| 3 | 11 | 3.83±1.167 | 74.62 |
| 4 | 12 | 3.17±0.749 | 57.89 |
| 5 | 13 | 3.67±0.667 | 31.47 |
| 6 | 14 | 4.40±0.51 | 25.91 |
| 7 | 15 | 5.67±0.76 | 32.84 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 4.67±1.116 | 58.52 |
| 2 | 11 | 4.00±1.091 | 72.18 |
| 3 | 14 | 3.62±0.375 | 29.31 |
| 4 | 15 | 4.00±0.463 | 32.73 |
| 5 | 16 | 3.50±2.5 | 101.03 |
| 6 | Total | 4.00±0.343 | 50.73 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 9 | 5.67±0.882 | 26.95 |
| 2 | 10 | 4.67±1.453 | 53.90 |
| 3 | 11 | 4.50±1.323 | 58.80 |

| | | | |
|---|---|---|---|
| 4 | 12 | 5.00±0.516 | 25.30 |
| 5 | 13 | 1.60±0.4 | 55.88 |
| 6 | 14 | 3.25±0.491 | 42.74 |
| 7 | 15 | 4.50±0.922 | 50.18 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 5 mg/l cytokinin | | | |

The mean, SE and CV (%) were given in the Table 42. The lowest CV obtained was 25.3 %. The Repeat 1 of Experiment 1 was significant at 5 % level (F= 3.444) and also Repeat 2 showed 1% level (F = 4.695) of significance. The overall $R^2$ value (81%) was also highly significant. The Duncan test of Repeat 2 in Experiment 1 showed three subsets significant at 1% level, indicating that, the groupings are very prominent and the experiment has good variability.

Table 43. ANOVA of multiple shoot induction in **seedling** explants in WPM supplemented with **5 mg/l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 19 | 5.993 | 3.438 |
| Experiment-1 | 7 | 4.050 | 2.324 |
| Repeat-1 | 6 | 6.002 | 3.444* |
| Repeat-2 | 6 | 8.182 | 4.695** |
| Error | 15 | 1.743 | |
| Total | 35 | | |
| $R^2$ = 0.813 | | | |
| * 5% level of significant<br>** 1% level of significance | | | |

Table 44. Duncan test for multiple shoot induction in **seedling** explants in WPM supplemented with **1 mg/l** cytokinin.

| **Repeat -2** | **N** | **Subset** | | |
|---|---|---|---|---|
| | | **1** | **2** | **3** |
| 13 | 5 | 1.60 | | |
| 14 | 8 | 3.25 | 3.25 | |
| 11 | 4 | | 4.50 | 4.50 |
| 15 | 6 | | 4.50 | 4.50 |
| 10 | 3 | | 4.67 | 4.67 |
| 12 | 6 | | 5.00 | 5.00 |
| 9 | 3 | | | 5.67 |
| Sig. | | 0.082 | 0.093 | 0.251 |

### 4.1.1.6 Multiple shoot induction in juvenile explants of 7-8 year old mature trees (Experiment-2)

The above media combinations tried for multiple shoot induction in seedling explants were tried with juvenile explants of mature trees. Juvenile explants were collected from the new branches emerged from the chopped portions of mature trees (Fig. 13). The results obtained were tabulated in the Tables 45, 49, 53, 56 and 60. Of the 25 media combinations tried, the highest mean of multiple shoots produced was 14.48 in WPM supplemented with 3 mg/l BAP and 1 mg/l Kn (Fig. 15 A). The lowest mean produced was 4.81 in WPM supplemented with 1 mg/l Kn.

The number of multiple shoots regenerated from juvenile explants of mature trees was lesser than that of seedling explants. In cultures developed from seedling explants, the exudation of phenolic compounds was not noticeable. In juvenile explants of mature plants, the exudation of phenolic compounds was noticeable (Fig. 15 B).

#### 4.1.1.6.1 Effect of WPM + 1 mg/l cytokinin

Table 45. Effect of **1 mg/l** cytokinin on multiple shoot induction from **juvenile** explants of mature trees in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 1 | WPM | 1 BAP | 5.00 | 5.33 |
| | | | 5.67 | |
| | | | 5.67 | |
| | | | 5.33 | |
| | | | 5.67 | |
| | | | 5.00 | |
| | | | 5.00 | |
| 2 | | 1 Kn | 4.33 | 4.81 |
| | | | 5.33 | |
| | | | 4.67 | |
| | | | 5.00 | |
| | | | 4.00 | |
| | | | 5.00 | |
| | | | 5.33 | |
| 3 | | 1 ZN | 5.00 | 5.05 |
| | | | 4.33 | |
| | | | 5.00 | |
| | | | 5.67 | |
| | | | 5.33 | |
| | | | 5.00 | |
| | | | 5.00 | |
| 4 | | 0.5 BAP + 0.5 Kn | 5.33 | 6.10 |
| | | | 6.00 | |
| | | | 5.67 | |
| | | | 6.67 | |
| | | | 6.33 | |
| | | | 6.33 | |
| | | | 6.33 | |
| 5 | | 0.5 BAP + 0.5 ZN | 4.33 | 5.33 |
| | | | 6.00 | |
| | | | 5.00 | |
| | | | 5.67 | |
| | | | 5.67 | |
| | | | 5.67 | |
| | | | 5.00 | |

Table 46. Multiple shoot induction in **juvenile** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **1 mg/l** cytokinin

| **Experiment 2 (1 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 4 | 3.25±0.861 | 74.92 |
| 2 | 5 | 4.08±0.596 | 50.61 |
| 3 | 6 | 3.91±0.579 | 49.13 |
| 4 | 7 | 5.50±0.289 | 10.49 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 4 | 5.33±0.715 | 32.85 |
| 2 | 5 | 3.30±0.539 | 51.61 |
| 3 | 6 | 3.79±0.613 | 60.50 |
| 4 | 7 | 4.25±1.031 | 48.52 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 4 | 3.56±0.884 | 74.47 |
| 2 | 5 | 3.25±0.463 | 49.32 |
| 3 | 6 | 5.10±0.504 | 31.27 |
| 4 | 7 | 4.50±0.957 | 42.56 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 1mg/l cytokinin | | | |

Of the five combinations tried, the highest mean of 6.1 multiple shoots were produced in WPM supplemented with 0.5 mg/l BAP and 0.5 mg/l Kn. The lowest mean was 4.81 in WPM supplemented with 1 mg/l Kn (Table 45 ).

The mean, SE and CV (%) were given in the Table 46. The lowest CV obtained was 10.49 %. The ANOVA and Duncan test for the Experiment were

given in Tables, 47 and 48. The Duncan test of Experiment 2 and Repeat 2 in Experiment 2 gave one subset each .

Table 47. ANOVA of multiple shoot induction in **juvenile** explants of mature trees in WPM supplemented with **1 mg /l** cytokinin

| **Source** | **df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 10 | 3.879 | 0.920 |
| Experiment-2 | 3 | 1.443 | 0.342 |
| Repeat-1 | 4 | 3.124 | 0.741 |
| Repeat-2 | 3 | 2.947 | 0.699 |
| Error | 24 | 4.217 | |
| Total | 35 | | |
| $R^2 = 0.277$ | | | |

Table 48. Duncan tests for multiple shoot induction in **juvenile** explants of mature trees in WPM supplemented with **1 mg /l** cytokinin

| **Experiment-2** | N | Subset 1 |
|---|---|---|
| 4 | 8 | 3.25 |
| 6 | 11 | 3.91 |
| 5 | 12 | 4.08 |
| 7 | 4 | 5.50 |
| Sig. | | 0.066 |

| **Repeat-2** | N | Subset 1 |
|---|---|---|
| 5 | 12 | 3.25 |
| 4 | 9 | 3.56 |
| 7 | 4 | 4.50 |
| 6 | 10 | 5.10 |

| | | | Subset |
|---|---|---|---|
| **Experiment-2** | Sig. | N | 0.126 |

#### 4.1.1.6.2 Effect of WPM + 2 mg/l cytokinin

Table 49. Effect of **2 mg/l** cytokinin on multiple shoot induction from **juvenile** explants of mature trees in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 6 | WPM | 2 BAP | 7.00 | 6.67 |
| | | | 7.33 | |
| | | | 5.67 | |
| | | | 6.33 | |
| | | | 7.00 | |
| | | | 6.67 | |
| | | | 6.67 | |
| 7 | | 2 Kn | 4.67 | 6.00 |
| | | | 5.00 | |
| | | | 8.00 | |
| | | | 7.67 | |
| | | | 6.00 | |
| | | | 5.33 | |
| | | | 5.33 | |
| 8 | | 2 ZN | 5.67 | 6.29 |
| | | | 7.33 | |
| | | | 6.33 | |
| | | | 6.67 | |
| | | | 6.33 | |
| | | | 5.67 | |
| | | | 6.00 | |
| 9 | | 1 BAP + 1 Kn | 8.33 | 8.29 |
| | | | 8.33 | |
| | | | 8.00 | |
| | | | 8.67 | |
| | | | 8.33 | |
| | | | 8.00 | |
| | | | 8.33 | |
| 10 | | 1 ZN +1 Kn | 7.67 | 7.33 |
| | | | 6.67 | |

| | | | | |
|---|---|---|---|---|
| | | | 7.33 | |
| | | | 7.33 | |
| | | | 6.67 | |
| | | | 7.33 | |
| | | | 8.33 | |

Table 50. Multiple shoot induction in **juvenile** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **2 mg/l** cytokinin

| **Experiment 2 (2 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 5 | 4.10±0.752 | 58.00 |
| 2 | 6 | 3.80±0.8 | 47.08 |
| 3 | 7 | 4.22±0.641 | 45.55 |
| 4 | 8 | 3.60±0.812 | 50.47 |
| 5 | 9 | 4.00±1.033 | 63.25 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 2 | 5 | 4.83±0.792 | 40.19 |
| 3 | 6 | 5.60±0.6 | 23.96 |
| 4 | 7 | 2.71±0.522 | 50.92 |
| 5 | 8 | 3.56±0.729 | 61.40 |
| 6 | 9 | 4.43±0.751 | 44.88 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 5 | 2.67±0.989 | 90.71 |
| 2 | 6 | 4.60±0.678 | 32.98 |
| 3 | 7 | 4.62±0.513 | 40.04 |
| 4 | 8 | 3.78±0.722 | 57.33 |
| 5 | 9 | 3.50±1.5 | 60.60 |
| CV – Coefficient of Variation | | | |

| Medium combination – WPM + 2 mg/l cytokinin |
| --- |

Table 51. ANOVA of multiple shoot induction in **juvenile** explants in WPM supplemented with **2 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 13 | 4.192 | 1.030 |
| Experiment-2 | 4 | 0.590 | 0.145 |
| Repeat-1 | 5 | 6.664 | 1.637 |
| Repeat-2 | 4 | 2.949 | 0.724 |
| Error | 21 | 4.072 | |
| Total | 35 | | |
| $R^2 = 0.389$ | | | |

Table 52. Duncan tests for multiple shoot induction in **juvenile** explants of mature trees in WPM supplemented with **2 mg /l** cytokinin

| **Repeat-2** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 5 | 6 | 2.67 |
| 9 | 2 | 3.50 |
| 8 | 9 | 3.78 |
| 6 | 5 | 4.60 |
| 7 | 13 | 4.62 |
| Sig. | | .196 |
| | | |
| **Experiment-2** | N | Subset |
| | | 1 |
| 8 | 5 | 3.60 |
| 6 | 5 | 3.80 |
| 9 | 6 | 4.00 |
| 5 | 10 | 4.10 |
| 7 | 9 | 4.22 |
| Sig. | | .626 |

Of the five combinations tried, the highest mean of multiple shoots produced was 8.29 in WPM supplemented with 1 mg/l BAP and 1 mg/l Kn and lowest mean of shoots produced was 6 in WPM supplemented with 2 mg/l Kn (Table 49).

The mean, SE and CV (%) were given in the Table 50. The lowest CV (%) obtained was 23.96. The ANOVA is depicted in Table 51. Duncan test of Experiment 2 and Repeat 2 have one subset each (Table 52).

#### 4.1.1.6.3 Effect of WPM + 3 mg/l cytokinin

The highest mean of multiple shoots produced was 10.9 in WPM supplemented with 2 mg/l BAP and 1 mg/l Kn. The lowest mean of multiple shoots produced was 9.14 in WPM supplemented with 2 mg/l Kn + 1 mg/l ZN (Table 53).

The mean, SE and CV (%) are given in the Table 54. The lowest CV obtained was 12.85% and the results of ANOVA is a good estimate compared to the other values in the same experiment.

Table 53. Effect of **3 mg/l** cytokinin on multiple shoot induction from **juvenile** explants of mature trees in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 11 | WPM | 3 BAP | 9.67 | 9.67 |
| | | | 9.00 | |
| | | | 10.33 | |
| | | | 9.67 | |
| | | | 9.67 | |
| | | | 10.33 | |
| | | | 9.00 | |
| 12 | | 1 BAP + 2 Kn | 9.33 | 9.76 |
| | | | 9.67 | |
| | | | 11.00 | |
| | | | 11.33 | |
| | | | 10.00 | |
| | | | 8.33 | |
| | | | 8.67 | |
| 13 | | 2 BAP + 1 Kn | 10.67 | 10.90 |
| | | | 9.67 | |
| | | | 10.67 | |
| | | | 11.33 | |
| | | | 11.67 | |
| | | | 11.00 | |
| | | | 11.33 | |
| 14 | | 2 ZN + 1 Kn | 10.00 | 10.05 |
| | | | 9.00 | |
| | | | 10.67 | |
| | | | 10.33 | |
| | | | 9.67 | |
| | | | 11.00 | |
| | | | 9.67 | |
| 15 | | 2 Kn + 1 ZN | 9.00 | 9.14 |
| | | | 9.00 | |
| | | | 8.67 | |
| | | | 10.33 | |
| | | | 9.67 | |
| | | | 8.33 | |
| | | | 9.00 | |

Table 54. Multiple shoot induction in **juvenile** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **3 mg/l** cytokinin

| **Experiment 2 (3 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 8 | 4.50±1.147 | 62.47 |
| 2 | 9 | 3.00±0.724 | 63.83 |
| 3 | 10 | 3.40±0.67 | 62.32 |
| 4 | 11 | 5.20±0.374 | 16.10 |
| 5 | 12 | 4.20±0.735 | 39.12 |
| 6 | 13 | 5.50±0.5 | 12.85 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **Coefficient of variation (CV%)** |
| 1 | 8 | 3.43±0.719 | 55.45 |
| 2 | 9 | 4.70±0.761 | 51.19 |
| 3 | 10 | 4.38±0.68 | 43.90 |
| 4 | 11 | 2.57±0.429 | 44.12 |
| 5 | 12 | 5.33±0.882 | 28.67 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **Coefficient of variation (CV%)** |
| 1 | 8 | 5.17±0.749 | 35.49 |
| 2 | 9 | 2.87±0.693 | 68.26 |
| 3 | 10 | 4.78±0.683 | 42.85 |
| 4 | 11 | 3.40±0.748 | 49.21 |
| 5 | 12 | 3.71±0.778 | 55.50 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 3 mg/l cytokinin | | | |

Table 55. ANOVA of multiple shoot induction in **juvenile** explants in WPM supplemented with **3 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 13 | 4.882 | 1.339 |
| Experiment-2 | 5 | 4.061 | 1.114 |
| Repeat-1 | 4 | 3.326 | 0.912 |
| Repeat-2 | 4 | 2.817 | 0.773 |
| Error | 21 | 3.645 | |
| Total | 35 | | |
| $R^2 = 0.453$ | | | |

#### 4.1.1.6.4 Effect of WPM + 4 mg/l cytokinin

The highest mean of multiple shoots produced was 14.48 in WPM supplemented with 3 mg/l BAP and 1 mg/l Kn. This medium was selected as the best medium for multiple shoot induction (Fig. 15). The lowest mean of shoots observed was 11.86 in WPM supplemented with 4 mg/l BAP (Table 56).

The mean, SE and CV (%) are given in the Table 57. The lowest CV obtained was 25.30 %. The ANOVA showed that it was significant at 5% level (F=3.455). The Repeat 2 in Experiment 2 and the overall $R^2$ value (73%) was also highly significant (Table 58). The Duncan test of Experiment 2 showed two subsets significant at 5% level and that of Repeat 1 showed one subset (Table 59).

Table 56. Effect of **4 mg/l** cytokinin on multiple shoot induction from **juvenile** explants of mature trees in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 16 | WPM | 4 BAP | 12.67 | 11.86 |
| | | | 10.67 | |
| | | | 11.67 | |
| | | | 12.33 | |
| | | | 12.33 | |
| | | | 12.33 | |
| | | | 11.00 | |
| 17 | | 3 BAP + 1 Kn | 15.67 | 14.48 |
| | | | 14.67 | |
| | | | 14.33 | |
| | | | 13.33 | |
| | | | 14.67 | |
| | | | 14.33 | |
| | | | 14.33 | |
| 18 | | 3 BAP + 1 ZN | 14.00 | 13.24 |
| | | | 13.33 | |
| | | | 12.67 | |
| | | | 12.67 | |
| | | | 14.00 | |
| | | | 12.33 | |
| | | | 13.67 | |
| 19 | | 2 Kn + 2 ZN | 11.67 | 12.19 |
| | | | 12.67 | |
| | | | 13.00 | |
| | | | 13.33 | |
| | | | 12.67 | |
| | | | 11.00 | |
| | | | 11.00 | |
| 20 | | 2 BAP + 2 Kn | 13.33 | 12.57 |
| | | | 12.67 | |
| | | | 13.00 | |
| | | | 13.33 | |
| | | | 12.67 | |

| | | | | |
|---|---|---|---|---|
| | | | 12.67<br>10.33 | |

Table 57. Multiple shoot induction in **juvenile** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **4 mg/l** cytokinin

| **Experiment 2 (4 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 5.25±1.109 | 42.23 |
| 2 | 11 | 3.00±2.000 | 115.47 |
| 3 | 12 | 4.43±0.812 | 48.51 |
| 4 | 13 | 4.57±0.649 | 37.59 |
| 5 | 14 | 3.75±0.559 | 42.16 |
| 6 | 15 | 4.00±1.155 | 50.00 |
| 7 | 16 | 1.67±0.333 | 34.55 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 4.20±1.068 | 56.83 |
| 2 | 11 | 5.00±1.528 | 52.92 |
| 3 | 12 | 3.14±0.67 | 56.46 |
| 4 | 13 | 4.43±0.719 | 42.93 |
| 5 | 14 | 4.86±0.738 | 40.16 |
| 6 | 15 | 2.67±1.667 | 108.13 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 10 | 4.62±0.822 | 50.35 |
| 2 | 12 | 4.00±0.577 | 28.88 |
| 3 | 13 | 5.00±0.516 | 25.30 |
| 4 | 14 | 1.80±0.583 | 72.44 |
| 5 | 15 | 4.14±0.8 | 51.11 |
| 6 | 16 | 4.33±1.764 | 70.55 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 4 mg/l cytokinin | | | |

Table 58. ANOVA of multiple shoot induction in **juvenile** explants in WPM supplemented with **4 mg /l** cytokinin

| Source | Df | Mean Square | F |
|---|---|---|---|
| Corrected Model | 19 | 5.373 | 2.126 |
| Experiment-2 | 6 | 5.088 | 2.013 |
| Repeat-1 | 6 | 3.812 | 1.509 |
| Repeat-2 | 7 | 8.731 | 3.455* |
| Error | 15 | 2.527 | |
| Total | 35 | | |
| $R^2$ = 0.729 | | | |
| * - 5% level of significance | | | |

Table 59. Duncan tests for multiple shoot induction in **juvenile** explants of mature trees in WPM supplemented with **4 mg /l** cytokinin

| Repeat-1 | N | Subset |
|---|---|---|
| | | 1 |
| 15 | 3 | 2.67 |
| 16 | 3 | 3.00 |
| 12 | 7 | 3.14 |
| 10 | 5 | 4.20 |
| 13 | 7 | 4.43 |
| 14 | 7 | 4.86 |
| 11 | 3 | 5.00 |
| Sig. | | .075 |

| Experiment-2 | N | Subset | |
|---|---|---|---|
| | | 1 | 2 |
| 16 | 3 | 1.67 | |

| Repeat-1 | N | Subset | | |
|---|---|---|---|---|
| 11 | | 3 | 3.00 | 3.00 |
| 14 | | 8 | 3.75 | 3.75 |
| 15 | | 3 | 4.00 | 4.00 |
| 12 | | 7 | | 4.43 |
| 13 | | 7 | | 4.57 |
| 10 | | 4 | | 5.25 |
| Sig. | | | 0.067 | 0.085 |

**4.1.1.6.5 Effect of WPM + 5 mg/l cytokinin**

Table 60. Effect of **5 mg/l** cytokinin on multiple shoot induction from **juvenile** explants of mature trees in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 21 | WPM | 4 BAP + 1 Kn | 11.33 | 11.19 |
| | | | 10.67 | |
| | | | 10.33 | |
| | | | 12.33 | |
| | | | 10.67 | |
| | | | 11.33 | |
| | | | 11.67 | |
| 22 | | 4 BAP + 1 ZN | 10.00 | 10.95 |
| | | | 12.00 | |
| | | | 11.00 | |
| | | | 12.00 | |
| | | | 11.67 | |
| | | | 10.67 | |
| | | | 9.33 | |
| 23 | | 3 ZN + 2 Kn | 10.67 | 10.33 |
| | | | 9.67 | |
| | | | 9.33 | |
| | | | 10.67 | |
| | | | 10.33 | |
| | | | 11.00 | |
| | | | 10.67 | |
| 24 | | 3 Kn + 2 BAP | 11.00 | 10.29 |
| | | | 9.33 | |
| | | | 11.00 | |
| | | | 9.67 | |
| | | | 10.00 | |

| | | | | |
|---|---|---|---|---|
| | | | 10.00 | |
| | | | 11.00 | |
| 25 | | 3 Kn + 2 ZN | 10.67 | 9.95 |
| | | | 9.33 | |
| | | | 10.33 | |
| | | | 8.67 | |
| | | | 10.00 | |
| | | | 10.00 | |
| | | | 10.67 | |

Table 61. Multiple shoot induction in **juvenile** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **5 mg/l** cytokinin

| **Experiment 2 (5 mg)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 8 | 4.00±2.000 | 70.70 |
| 2 | 9 | 4.43±0.649 | 38.78 |
| 3 | 10 | 4.09±0.625 | 50.64 |
| 4 | 11 | 3.67±1.202 | 80.22 |
| 5 | 12 | 4.43±0.528 | 31.53 |
| 6 | 13 | 1.50±0.5 | 47.13 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 2 | 9 | 4.00±0.632 | 38.73 |
| 3 | 10 | 3.92±0.621 | 54.87 |
| 4 | 11 | 5.17±0.872 | 41.33 |
| 5 | 12 | 3.38±0.865 | 72.37 |
| 6 | 13 | 3.50±0.5 | 20.20 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 9 | 3.57±0.972 | 72.07 |
| 2 | 10 | 3.58±0.529 | 51.17 |

| | | | |
|---|---|---|---|
| 3 | 11 | 3.00±0.856 | 69.93 |
| 4 | 12 | 5.14±0.459 | 23.64 |
| 5 | 13 | 6.00±0.577 | 16.67 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 5 mg/l cytokinin | | | |

Table 62. ANOVA of multiple shoot induction in **Juvenile** explants in WPM supplemented with **5 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 14 | 3.332 | 0.714 |
| Experiment-2 | 5 | 1.156 | 0.248 |
| Repeat-1 | 5 | 1.074 | 0.230 |
| Repeat-2 | 4 | 5.279 | 1.131 |
| Error | 20 | 4.668 | |
| Total | 35 | | |
| $R^2 = 0.333$ | | | |

Table 63. Duncan tests for multiple shoot induction in **juvenile** explants of mature trees in WPM supplemented with **5 mg /l** cytokinin

| **Repeat-2** | **N** | **Subset** | |
|---|---|---|---|
| | | **1** | **2** |
| 11 | 6 | 3.00 | |
| 9 | 7 | 3.57 | 3.57 |
| 10 | 12 | 3.58 | 3.58 |
| 12 | 7 | 5.14 | 5.14 |
| 13 | 3 | | 6.00 |
| Sig. | | 0.138 | 0.094 |

| **Experiment-2** | **N** | **Subset** |
|---|---|---|
| | | **1** |

| Repeat-2 | N | Subset | |
|---|---|---|---|
| 13 | | 2 | 1.50 |
| 11 | | 6 | 3.67 |
| 8 | | 2 | 4.00 |
| 10 | | 11 | 4.09 |
| 9 | | 7 | 4.43 |
| 12 | | 7 | 4.43 |
| Sig. | | | 0.108 |

The highest mean of multiple shoots produced was 11.19 in WPM supplemented with 4 mg/l BAP and 1mg/l Kn. The lowest mean was 9.95 in WPM supplemented with 3 mg/l Kn and 2 mg/l ZN (Table 60).

The mean, SE and CV(%) are given in the Table 61. The lowest CV obtained was 16.67%. The ANOVA and Duncan test are given in Tables 62 and 63. Experiment 2 showed one subset where as Repeat 2 showed two subsets.

### 4.1.1.7 Multiple shoot induction in mature explants (Experiment-3)

The same media combinations tried for multiple shoot induction in seedling explants were also tried with mature explants . The results obtained were presented in Tables 64, 68, 72, 75 and 78. Of the 25 media combinations tried, WPM supplemented with 3 mg/l BAP and 1 mg/l Kn gave best results as in the case of seedling and juvenile explants. The highest mean of multiple shoots produced was 12.05. The lowest mean of multiple shoots produced was 3.33 in WPM supplemented with 1mg/l Kn. Exudation of phenolic compounds was higher in cultures developed from mature explants.

The best medium for multiple shoot induction in *C.verum* was found to be WPM supplemented with 3 mg/l BAP and 1 mg/l Kn in all the three types of explants. The highest mean of multiple shoots produced was 17.05 in seedlings, 14.48 in juvenile explants of mature trees and 12.05 in explants of mature trees. Multiple shoots were induced in all the media combinations tried except in WPM basal medium.

The time taken for multiple shoot induction also varied depending on the maturity of the explants. The seedling explants took 15-20 days for the emergence of multiple shoots, juvenile explants of mature trees, 20-26 days and mature explants of mature trees, 20-35 days.

#### 4.1.1.7.1 Effect of WPM + 1 mg/l cytokinin

Of the five combinations tried, the highest mean of 4.86 multiple shoots was produced in WPM supplemented with 0.5 mg/l BAP and 0.5 mg/l Kn. The lowest mean was 3.33 in WPM supplemented with 1 mg/l Kn (Table 64).

The mean, SE and CV (%) for the data on multiple shoot induction from mature explants in WPM supplemented with 1 mg/l cytokinin are given in the Table 65. The lowest CV value obtained was 10.88% giving a good estimate. The ANOVA and Duncan test are given in Tables 66 and 67.

Table 64. Effect of **1 mg/l** cytokinin on multiple shoot induction from **mature** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | **Average of Experiment 1 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 1 | WPM | 1 BAP | 4.33 | 4.05 |
| | | | 3.67 | |
| | | | 4.33 | |
| | | | 4.00 | |
| | | | 3.67 | |
| | | | 3.67 | |
| | | | 4.67 | |
| 2 | | 1 Kn | 4.00 | 3.33 |
| | | | 3.00 | |
| | | | 3.33 | |
| | | | 3.00 | |
| | | | 2.33 | |
| | | | 3.67 | |
| | | | 4.00 | |
| 3 | | 1 ZN | 4.33 | 3.90 |
| | | | 4.00 | |
| | | | 4.00 | |
| | | | 3.67 | |
| | | | 3.33 | |
| | | | 4.67 | |
| | | | 3.33 | |
| 4 | | 0.5 BAP + 0.5 Kn | 5.00 | 4.86 |
| | | | 4.67 | |
| | | | 4.67 | |
| | | | 5.00 | |
| | | | 4.67 | |
| | | | 5.67 | |
| | | | 4.33 | |
| 5 | | 0.5 BAP + 0.5 ZN | 4.67 | 4.19 |
| | | | 3.67 | |
| | | | 5.00 | |
| | | | 3.67 | |
| | | | 3.33 | |
| | | | 4.67 | |
| | | | 4.33 | |

Table 65. Multiple shoot induction in **mature** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **1 mg/l** cytokinin

| **Experiment 3 (1 mg/l cytokinin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 3 | 4.86±0.595 | 32.39 |
| 2 | 4 | 3.93±0.597 | 56.87 |
| 3 | 5 | 3.92±0.596 | 52.68 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 2 | 3.67±0.882 | 41.63 |
| 2 | 3 | 4.33±0.667 | 46.19 |
| 3 | 4 | 4.36±0.599 | 51.38 |
| 4 | 5 | 2.71±0.680 | 66.38 |
| 5 | 6 | 5.00±1.000 | 28.28 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 2 | 4.50±0.500 | 15.71 |
| 2 | 3 | 3.38±0.498 | 41.66 |
| 3 | 4 | 3.85±0.629 | 58.88 |
| 4 | 5 | 4.10±0.722 | 55.68 |
| 5 | 6 | 6.50±0.500 | 10.88 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 1mg/l cytokinin | | | |

Table 66. ANOVA of multiple shoot induction in **mature** explants of cinnamon in WPM supplemented with **1 mg /l** cytokinin

| Source | Df | Mean Square | F |
|---|---|---|---|
| Corrected Model | 11 | 4.008 | 0.961 |
| Experiment 3 | 3 | 4.284 | 1.027 |
| Repeat-1 | 4 | 2.679 | 0.642 |
| Repeat-2 | 4 | 3.197 | 0.767 |
| Error | 23 | 4.170 | |
| Total | 35 | | |
| $R^2 = 0.315$ | | | |

Table 67. Duncan tests for multiple shoot induction in **mature** explants in WPM supplemented with **1 mg /l** cytokinin

| **Repeat-2** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 3 | 8 | 3.38 |
| 4 | 13 | 3.85 |
| 5 | 10 | 4.10 |
| 2 | 2 | 4.50 |
| 6 | 2 | 6.50 |
| Sig. | | 0.068 |

| **Repeat-1** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 5 | 7 | 2.71 |
| 2 | 3 | 3.67 |
| 3 | 9 | 4.33 |
| 4 | 14 | 4.36 |
| 6 | 2 | 5.00 |
| Sig. | | 0.153 |

| **Experiment 3** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 6 | 2 | 2.00 |
| 5 | 12 | 3.92 |
| 4 | 14 | 3.93 |

| Repeat-2 | N | Subset |
|---|---|---|
| 3 | 7 | 4.86 |
| Sig. | | .052 |

#### 4.1.1.7.2 Effect of WPM + 2 mg/l cytokinin

Table 68. Effect of **2 mg/l** cytokinin on multiple shoot induction from **mature** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 6 | WPM | 2 BAP | 6.67 | 5.29 |
| | | | 5.00 | |
| | | | 5.33 | |
| | | | 4.67 | |
| | | | 5.33 | |
| | | | 5.67 | |
| | | | 4.33 | |
| 7 | | 2 Kn | 5.00 | 4.62 |
| | | | 3.67 | |
| | | | 5.33 | |
| | | | 4.67 | |
| | | | 4.33 | |
| | | | 4.67 | |
| | | | 4.67 | |
| 8 | | 2 ZN | 5.67 | 5.19 |
| | | | 5.67 | |
| | | | 4.33 | |
| | | | 5.67 | |
| | | | 4.33 | |
| | | | 5.67 | |
| | | | 5.00 | |
| 9 | | 1 BAP + 1 Kn | 4.67 | 5.67 |
| | | | 5.67 | |
| | | | 5.33 | |
| | | | 6.00 | |
| | | | 5.67 | |
| | | | 6.67 | |
| | | | 5.67 | |
| 10 | | 1 ZN + 1 Kn | 4.67 | 5.38 |
| | | | 5.67 | |
| | | | 5.00 | |

| | | | | |
|---|---|---|---|---|
| | | | 5.00 | |
| | | | 5.67 | |
| | | | 5.67 | |
| | | | 6.00 | |

Table 69. Multiple shoot induction in **mature** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **2 mg/l** cytokinin

| **Experiment 3 (2 mg/l cytokinin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 4 | 3.64±0.592 | 53.93 |
| 2 | 5 | 4.30±0.667 | 49.09 |
| 3 | 6 | 3.78±0.641 | 50.85 |
| 4 | 7 | 4.60±1.166 | 56.70 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 4 | 4.43±0.751 | 44.88 |
| 2 | 5 | 4.08±0.583 | 49.53 |
| 3 | 6 | 3.45±0.666 | 63.97 |
| 4 | 7 | 4.40±0.927 | 47.14 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 3 | 3.50±1.500 | 60.60 |
| 2 | 4 | 3.33±0.764 | 68.80 |
| 3 | 5 | 5.09±0.456 | 29.74 |
| 4 | 6 | 3.45±0.638 | 61.30 |
| 5 | 7 | 4.50±1.500 | 47.13 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 2 mg/l cytokinin | | | |

Of the five combinations tried, the highest mean of multiple shoots produced was 5.67 in WPM supplemented with 1 mg/l BAP and 1 mg/l Kn and lowest mean of shoots produced was 4.62 in WPM supplemented with 2 mg/l Kn (Table 68).

The mean, SE and CV (%) value for the data are given in the Table 69. The lowest CV value obtained is 29.74 %. The ANOVA done is shown in Table 70. Duncan test was also done (Table 71). Repeat 1 and Repeat 2 gave one subset each.

Table 70. ANOVA of multiple shoot induction in **mature** explants in WPM supplemented with **2 mg /l** cytokinin

| Source | Df | Mean Square | F |
|---|---|---|---|
| Corrected Model | 10 | 2.867 | 0.618 |
| Experiment 3 | 3 | 2.028 | 0.437 |
| Repeat-1 | 3 | 1.186 | 0.256 |
| Repeat-2 | 4 | 3.845 | 0.829 |
| Error | 24 | 4.639 | |
| Total | 35 | | |
| $R^2 = 0.205$ | | | |

Table 71. Duncan test for multiple shoot induction in **mature** explants in WPM supplemented with **2 mg /l** cytokinin

| **Repeat-1** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 6 | 11 | 3.45 |
| 5 | 12 | 4.08 |
| 7 | 5 | 4.40 |
| 4 | 7 | 4.43 |
| Sig. | | 0.425 |
| | | |
| **Repeat-2** | **N** | **Subset** |
| | | **1** |
| 4 | 9 | 3.33 |
| 6 | 11 | 3.45 |
| 3 | 2 | 3.50 |
| 7 | 2 | 4.50 |
| 5 | 11 | 5.09 |
| Sig. | | 0.320 |

#### 4.1.1.7.3 Effect of WPM + 3 mg/l cytokinin

Table 72. Effect of **3 mg/l** cytokinin on multiple shoot induction from **mature** explants in *C. verum*

| Sl. No. | Basal medium | Growth regulators | Average number of multiple shoots produced on 40th day | |
|---|---|---|---|---|
| | | | Average of Experiment 1 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 11 | WPM | 3 BAP | 5.67 | 6.33 |
| | | | 7.00 | |
| | | | 6.00 | |
| | | | 6.33 | |
| | | | 5.33 | |
| | | | 6.33 | |
| | | | 7.67 | |
| 12 | | 1 BAP + 2Kn | 7.33 | 7.62 |
| | | | 7.67 | |
| | | | 7.00 | |
| | | | 7.67 | |
| | | | 7.67 | |
| | | | 8.00 | |
| | | | 8.00 | |
| 13 | | 2 BAP + 1 Kn | 8.67 | 8.95 |
| | | | 8.67 | |
| | | | 9.33 | |
| | | | 7.67 | |
| | | | 9.67 | |
| | | | 9.33 | |
| | | | 9.33 | |
| 14 | | 2 ZN + 1 Kn | 7.67 | 7.67 |
| | | | 8.33 | |
| | | | 7.33 | |
| | | | 7.33 | |
| | | | 8.33 | |
| | | | 7.00 | |

| | | | | |
|---|---|---|---|---|
| | | | 7.67 | |
| 15 | | 2 Kn + 1ZN | 7.33 | 6.76 |
| | | | 6.00 | |
| | | | 6.67 | |
| | | | 7.33 | |
| | | | 7.00 | |
| | | | 6.67 | |
| | | | 6.33 | |

Table 73. Multiple shoot induction in **mature** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **3 mg/l** cytokinin

| **Experiment 3 (3 mg/l cytokinin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 5 | 4.00±1.049 | 58.63 |
| 2 | 6 | 3.17±0.91 | 70.32 |
| 3 | 7 | 3.50±0.655 | 52.91 |
| 4 | 8 | 5.43±0.719 | 35.03 |
| 5 | 9 | 4.00±0.447 | 25.00 |
| 6 | 10 | 4.33±1.764 | 70.55 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 5 | 4.80±1.020 | 47.50 |
| 2 | 6 | 3.00±0.577 | 33.33 |
| 3 | 7 | 3.78±0.619 | 49.10 |
| 4 | 8 | 3.20±0.696 | 68.78 |
| 5 | 9 | 5.20±0.860 | 37.00 |
| 6 | 10 | 5.00±2.000 | 56.56 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 6 | 4.50±0.885 | 48.18 |
| 2 | 7 | 3.80±0.512 | 42.61 |
| 3 | 8 | 3.83±0.638 | 57.68 |
| 4 | 9 | 4.00± 1.265 | 70.70 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 3 mg/l cytokinin | | | |

The highest mean of multiple shoots produced was 8.95 in WPM supplemented with 2 mg/l BAP and 1 mg/l Kn. The lowest mean of multiple shoots produced was 6.33 in WPM supplemented with 3 mg/l BAP (Table 72).

The mean, SE and CV (%) for the data are given in the Table 73. The lowest CV value obtained is 25 %. The ANOVA of the experiment is given in Table 74.

Table 74. ANOVA of multiple shoot induction in **mature** explants in WPM supplemented with **3 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 17 | 3.089 | 0.600 |
| Intercept | 1 | 79.773 | 15.500 |
| Experiment 3 | 6 | 4.442 | 0.863 |
| Repeat-1 | 6 | 3.846 | 0.747 |
| Repeat-2 | 5 | 1.445 | 0.281 |
| Error | 17 | 5.147 | |
| Total | 35 | | |
| $R^2 = 0.375$ | | | |

**4.1.1.7.4 Effect of WPM + 4 mg/l cytokinin**

The highest mean of multiple shoots produced was 12.05 in WPM supplemented with 3mg/l BAP and 1mg/l Kn. The lowest mean of shoots observed was 9 in WPM supplemented with 4 mg/l BAP (Table 75).

The mean, SE and CV (%) for the data are given in the Table 76. The lowest CV value obtained is 20.20%. The results of ANOVA done is given in Table 77. Experiment 3 showed 1% level of significance (F = 2.190) whereas Repeat 1 was

significant at 5 % level (F = 3.292). The overall $R^2$ value (81%) was also highly significant.

Table 75. Effect of **4 mg/l** cytokinin on multiple shoot induction from **mature** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators** | **Average number of multiple shoots produced on 40th day** | |
|---|---|---|---|---|
| | | | **Average of Experiment 1 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 16 | WPM | 4 BAP | 9.00 | 9.00 |
| | | | 9.33 | |
| | | | 7.00 | |
| | | | 8.67 | |
| | | | 10.00 | |
| | | | 10.00 | |
| | | | 9.00 | |
| 17 | | 3 BAP + 1 Kn | 12.67 | 12.05 |
| | | | 12.67 | |
| | | | 12.67 | |
| | | | 11.67 | |
| | | | 11.67 | |
| | | | 12.00 | |
| | | | 11.00 | |
| 18 | | 3 BAP + 1 ZN | 9.67 | 10.05 |
| | | | 9.67 | |
| | | | 9.33 | |
| | | | 9.33 | |
| | | | 10.00 | |
| | | | 11.33 | |
| | | | 11.00 | |
| 19 | | 2 Kn + 2 ZN | 8.00 | 9.24 |
| | | | 9.67 | |

| | | | | |
|---|---|---|---|---|
| | | | 10.00 | |
| | | | 9.67 | |
| | | | 9.00 | |
| | | | 7.33 | |
| | | | 11.00 | |
| 20 | | 2 BAP + 2 Kn | 10.33 | 9.38 |
| | | | 9.00 | |
| | | | 9.67 | |
| | | | 8.00 | |
| | | | 9.33 | |
| | | | 9.00 | |
| | | | 10.33 | |

Table 76. Multiple shoot induction in **mature** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **4 mg/l** cytokinin

| **Experiment 3 (4 mg/l cytokinin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 7 | 5.00±1.000 | 28.28 |
| 2 | 8 | 4.83±0.703 | 35.65 |
| 3 | 9 | 3.00±0.598 | 56.33 |
| 4 | 10 | 2.20±0.735 | 74.68 |
| 5 | 11 | 5.67±0.667 | 28.80 |
| 6 | 12 | 4.50±1.323 | 58.80 |
| 7 | 13 | 4.00±2.000 | 70.70 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 6 | 3.50±0.500 | 20.20 |
| 2 | 8 | 4.00±1.000 | 43.30 |
| 3 | 9 | 3.38±0.460 | 38.52 |
| 4 | 10 | 4.12±0.972 | 66.70 |
| 5 | 11 | 5.33±0.760 | 34.93 |
| 6 | 12 | 3.40±1.030 | 67.71 |
| **Repeat 2** | | | |

| Sl. No. | Medium Combination | Mean±SE | CV% |
|---|---|---|---|
| 1 | 8 | 4.60±1.122 | 54.57 |
| 2 | 9 | 2.71±0.474 | 46.27 |
| 3 | 10 | 4.57±0.948 | 54.86 |
| 4 | 11 | 4.38±0.420 | 27.12 |
| 5 | 12 | 5.00±1.049 | 46.90 |
| 6 | 13 | 2.50±0.500 | 28.28 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 4 mg/l cytokinin | | | |

Table 77. ANOVA of multiple shoot induction in **mature** explants in WPM supplemented with **4 mg /l** cytokinin

| Source | Df | Mean Square | F |
|---|---|---|---|
| Corrected Model | 21 | 5.394 | 2.624 |
| Experiment 3 | 7 | 4.502 | 2.190** |
| Repeat-1 | 7 | 6.767 | 3.292* |
| Repeat-2 | 6 | 4.802 | 2.336 |
| Error | 13 | 2.055 | |
| Total | 35 | | |
| Corrected Total | 34 | | |
| $R^2$ = 0.809 | | | |
| * 5% level of significance<br>** 1% level of significance | | | |

#### 4.1.1.7.4 Effect of WPM + 5 mg/l cytokinin

The highest mean of multiple shoots produced was 9.05 in WPM supplemented with 4 mg/l BAP and 1mg/l Kn. The lowest mean was 7.76 in WPM supplemented with 3 mg/l Kn and 2 mg/l ZN (Table 78).

The mean, SE and CV (%) for the data are given in the Table 79. The lowest CV value obtained is 23.57 %. The ANOVA is shown in Table 80.

Table 78. Effect of **5 mg/l** cytokinin on multiple shoot induction from **mature** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators** | **Average number of multiple shoots produced on 40th day** | |
|---|---|---|---|---|
| | | | **Average of Experiment 1 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 21 | WPM | 4 BAP + 1 Kn | 8.33 | 9.05 |
| | | | 9.00 | |
| | | | 10.00 | |
| | | | 9.00 | |
| | | | 8.33 | |
| | | | 9.67 | |
| | | | 9.00 | |
| 22 | | 4 BAP + 1 ZN | 7.67 | 8.71 |
| | | | 9.33 | |
| | | | 7.67 | |
| | | | 9.33 | |
| | | | 9.67 | |
| | | | 9.33 | |
| | | | 8.00 | |
| 23 | | 3 ZN + 2 Kn | 8.00 | 8.57 |
| | | | 8.33 | |
| | | | 9.67 | |

| | | | | |
|---|---|---|---|---|
| | | | 8.00 | |
| | | | 9.33 | |
| | | | 8.67 | |
| | | | 8.00 | |
| 24 | | 3 Kn + 2 BAP | 7.33 | 8.43 |
| | | | 8.33 | |
| | | | 9.33 | |
| | | | 9.33 | |
| | | | 9.00 | |
| | | | 7.67 | |
| | | | 8.00 | |
| 25 | | 3 Kn + 2 ZN | 6.67 | 7.76 |
| | | | 7.67 | |
| | | | 8.00 | |
| | | | 8.67 | |
| | | | 7.67 | |
| | | | 8.00 | |
| | | | 7.67 | |

Table 79. Multiple shoot induction in **mature** explants of mature trees showing the mean and standard error with CV values in WPM medium supplemented with **5 mg/l** cytokinin

| **Experiment 3 (5 mg/l cytokinin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 6 | 4.00±3.000 | 106.08 |
| 2 | 7 | 3.20±1.114 | 77.81 |
| 3 | 8 | 3.56±0.626 | 52.75 |
| 4 | 9 | 4.30±0.651 | 47.86 |
| 5 | 10 | 5.00±0.577 | 28.28 |
| 6 | 11 | 2.50±0.500 | 28.28 |
| **Repeat 1** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 6 | 2.50±0.500 | 28.28 |
| 2 | 7 | 6.00±1.000 | 23.57 |

| | | | |
|---|---|---|---|
| 3 | 8 | 4.36±0.692 | 59.40 |
| 4 | 9 | 3.44±0.530 | 46.22 |
| 5 | 10 | 4.00±0.535 | 35.35 |
| **Repeat 2** | | | |
| **Sl. No.** | **Medium Combination** | **Mean±SE** | **CV%** |
| 1 | 6 | 2.33±1.333 | 99.10 |
| 2 | 7 | 4.50±1.057 | 57.51 |
| 3 | 8 | 3.75±0.726 | 54.75 |
| 4 | 9 | 4.42±0.543 | 42.56 |
| 5 | 10 | 4.00±1.528 | 66.15 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 5 mg/l cytokinin | | | |

Table 80. ANOVA of multiple shoot induction in **mature** explants in WPM supplemented with **5 mg /l** cytokinin

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 17 | 3.990 | 0.940 |
| Experiment 3 | 6 | 4.332 | 1.020 |
| Repeat-1 | 5 | 4.005 | 0.943 |
| Repeat-2 | 6 | 5.326 | 1.255 |
| Error | 17 | 4.245 | |
| Total | 35 | | |
| $R^2 = 0.485$ | | | |

### 4.1.1.5 Effect of growth regulators

**4.1.1.5.1 Effect of BAP**

BAP was used at a range of 1-4 mg/l. The highest mean of multiple shoots produced was 13.83 in seedling explants, 10.56 in juvenile explants of mature trees and 7.11 in mature explants. When used alone, BAP was found to be the most suitable growth regulator for multiple shoot induction in *Cinnamomum verum* than Kn and ZN.

When used in combination with Kn and ZN, there was an increase in the production of multiple shoots. BAP-Kn combination produced more multiple shoots than BAP-ZN combination with WPM (Table 81).

Table 81. Summary of Experiments conducted for multiple shoot induction in *Cinnamomum verum*

| Sl. No. | Basal medium | Growth regulators | Number of shoots produced on 40th day* | | |
|---|---|---|---|---|---|
| | | | Seedling explants | Juvenile explants of mature trees | Mature explants |
| 1 | WPM | 1 BAP | 8.48 | 5.33 | 4.05 |
| 2 | | 2 BAP | 9.71 | 6.67 | 5.29 |
| 3 | | 3 BAP | 11.90 | 9.67 | 6.33 |
| 4 | | 0.5 BAP + 0.5 Kn | 8.67 | 6.10 | 4.86 |
| 5 | | 0.5 BAP + 0.5 ZN | 8.52 | 5.33 | 4.19 |
| 6 | | 3 BAP + 1 Kn | 17.05 | 14.48 | 12.05 |
| 7 | | 4 BAP + 1 Kn | 13.38 | 11.19 | 9.05 |
| 8 | | 1 Kn | 7.71 | 4.81 | 3.33 |
| 9 | | 2 Kn | 9.14 | 6.00 | 4.62 |
| 10 | | 3 Kn + 2 BAP | 12.52 | 10.33 | 8.43 |
| 11 | | 1 BAP + 1 Kn | 10.29 | 8.29 | 5.67 |

| | | | | | |
|---|---|---|---|---|---|
| 12 | | 1 ZN | 7.95 | 5.05 | 3.90 |
| 13 | | 2 ZN | 9.24 | 6.29 | 5.19 |
| 14 | | 3 Kn + 2 ZN | 11.95 | 9.95 | 7.76 |
| 15 | | 1 BAP + 2 Kn | 13.19 | 9.76 | 7.62 |
| 16 | | 2 BAP + 1 Kn | 14.05 | 10.90 | 8.95 |
| 17 | | 2 BAP + 2 Kn | 15.24 | 12.57 | 9.38 |
| 18 | | 3 BAP + 1 ZN | 15.48 | 13.24 | 10.05 |
| 19 | | 4 BAP | 13.62 | 11.86 | 9.00 |
| 20 | | 4 BAP + 1 ZN | 12.95 | 10.95 | 8.71 |
| 21 | | 1 Kn + 1 ZN | 10.00 | 7.33 | 5.38 |
| 22 | | 1 Kn + 2 ZN | 13.48 | 10.05 | 7.67 |
| 23 | | 2 Kn + 1 ZN | 12.90 | 9.14 | 6.76 |
| 24 | | 2 Kn + 2 ZN | 14.86 | 12.19 | 9.24 |
| 25 | | 3 ZN + 2 Kn | 12.10 | 10.33 | 8.57 |
| * Means of 3 replicates | | | | | |

#### 4.1.1.5.2 Effect of Kn

Kn was used at a range of 1-4 mg/l. The highest mean of multiple shoots produced was 12.28 in seedlings, 9.28 in juvenile explants of mature plants and 6.28 in mature explants of mature trees. Kn produced more multiple shoots when it is in combination with BAP than ZN. When used alone, the effect of Kn was less than that of BAP and ZN.

#### 4.1.1.5.3 Effect of ZN

ZN was used in a range of 1-4 mg/l. The highest mean of multiple shoots produced was 12.39 in seedlings, 10.11 in juvenile explants of mature trees and 6.61 in mature explants. When ZN was used in combination with BAP more number of multiple shoots were produced than when used alone.

### 4.1.1.6 Effect of additives used

#### 4.1.1.6.1 $GA_3$

When $GA_3$ was incorporated into the multiple shoot induction medium, enhanced shoot elongation was achieved. The range of $GA_3$ added was 1-3 mg/l.

#### 4.1.1.6.2 Coconut milk

The optimized medium combination was supplemented with 5-25% coconut water. However, it did not induce any noticeable effect in the bud break, number and development of multiple shoots.

#### 4.1.1.6.3 Adenine sulphate

There was no enhancement in the bud break and growth of cultures by the addition of adenine sulphate.

#### 4.1.1.6.4 Polyvinyl pyrrolidone (PVP)

PVP is added at a range of 1-3 mg/l to the standardized multiple shoot induction medium due to its antioxidant properties which is used to prevent the oxidative browning of explant tissues. However there was no noticeable increase in the response of cultures and decrease in the production of phenolic exudates.

## 4. 1. 2 Indirect regeneration

Callus produced from leaves, internodes and the hypocotyl region of immature seeds were tried for getting indirect regeneration. However, regeneration was achieved only from organogenic callus produced from the hypocotyl of *in vitro* germinated seeds.

### 4. 1. 2.1 Callogenesis

#### 4. 1. 2.1.1 Establishment of aseptic cultures

Leaves, internodes and immature seeds were inoculated for the induction and proliferation of the callus. The contamination rate was very less (5-8%).

#### 4. 1.2.1.2 Standardisation of medium for callogenesis from leaves and internodes

WPM and MS medium supplemented with 2,4-D at a range of 1-3 mg/l were tried for callus induction from leaves and internodes. Of the different combinations tried with MS medium and WPM, MS medium supplemented with 2 mg/l 2,4-D produced maximum amount of callus. Internodes produced more amount of callus than leaves (Table 82, 83 and Fig. 25 A ).

Table 82. Effect of 2,4-D on callus induction from leaf explants in *C. verum*

| Sl. No. | Basal Medium | 2, 4-D (mg/l) | No. of days taken for callus induction | Response after 30 days of inoculation* |
|---|---|---|---|---|
| 1 | WPM | 1 | 18 | x |
| 2 | WPM | 2 | 15 | xx |
| 3 | WPM | 3 | 13 | xx |
| 4 | MS | 1 | 15 | xx |
| 5 | MS | 2 | 12 | xxx |
| 6 | MS | 3 | 10 | xx |

* x- very poor callusing xxx- moderate callusing
xx- poor callusing xxxx- profuse callusing

Table 83. Effect of 2,4-D on callus induction from internode explants

| Sl. No. | Basal Medium | 2, 4-D (mg/l) | No. of days taken for callus induction | Response after 30 days of inoculation* |
|---|---|---|---|---|
| 1 | WPM | 1 | 15 | x |
| 2 | WPM | 2 | 12 | xx |
| 3 | WPM | 3 | 11 | xx |
| 4 | MS | 1 | 13 | x |
| 5 | MS | 2 | 10 | xxxx |
| 6 | MS | 3 | 9 | xx |

* x- very poor callusing xxx- moderate callusing
xx- poor callusing xxxx- profuse callusing

**4.1. 2.1. 3 Standardisation of medium for callogenesis from immature seeds**

Four to five month old immature seeds (Fig. 14 B) were inoculated into WPM and MS medium supplemented with low concentrations of BAP and Kn (0.5-1 mg/l) to study the *in vitro* responses. Organogenic calli were produced within 25-45 days in all the media combinations tried. The best medium was found to be MS supplemented with 0.5 mg/l Kn (Fig. 24).

The organogenic calli obtained were slightly brownish, granular and friable (Fig. 25 B), whereas, callus obtained from leaves and internodes were creamy white in colour and compact in texture (Fig. 25 A). Along with organogenic callus somatic embryos were also induced (Table 84).

Table 84. Effect of BAP and Kinetin on the production of organogenic callus and somatic embryos from immature seeds of *Cinnamomum verum*

| Sl. No. | Basal medium | Growth regulators | | No. of days taken for induction of organogenic callus and somatic embryos | Percentage of cultures showing response |
|---|---|---|---|---|---|
| | | BAP (mg/l) | Kn (mg/l) | | |
| 1 | WPM | 0.5 | 0 | 35-45 | 5-12 |
| 2 | | 1.0 | 0 | 30-40 | 10-15 |
| 3 | | 0 | 0.5 | 30-40 | 15-20 |
| 4 | | 0 | 1.0 | 32-42 | 8-15 |
| 5 | MS | 0.5 | 0 | 30-34 | 25-30 |
| 6 | | 1.0 | 0 | 32-38 | 15-20 |
| 7 | | 0 | 0.5 | 25-30 | 30-35 |
| 8 | | 0 | 1.0 | 28-32 | 20-25 |

### 4.1. 2.1.4 Effect of 2, 4-D on callogenesis from leaf and internodal explants

1-3 mg/l 2,4-D when experimented with WPM and MS medium showed varying responses with respect to the amount of callus produced. MS medium supplemented with 2 mg/l 2,4-D produced maximum amount of callus from leaf and internodal explants. Callus induction started from the cut ends of leaves and internodes within 10-15 days of inoculation. Within 30-45 days, dedifferentiation of the differentiated cells of the explant took place and an undifferentiated creamy white mass of compact callus was produced in WPM and MS medium supplemented with 2 mg/l 2,4-D. Poor callusing was observed in WPM and MS medium supplemented with 1 mg/l 2, 4-D.

Internodal explants responded more favourably by producing more amount of callus than leaf explants in the same concentration of 2,4-D.

### 4.1.2.1.5 Effect of BAP and Kn on induction of organogenic callus from immature seeds

WPM supplemented with BAP and Kn at a range of 0.5 – 1 mg/l were tried for the induction of organogenic callus. Within 30-34 days 2-30 percent of cultures showed the induction of organogenic callus in WPM supplemented with 0.5 mg/l BAP. Within 25-30 days 30-35 percent of the cultures showed the induction of organogenic callus in WPM supplemented with 0.5 mg/l Kn. Kn induced better responses than BAP in the induction of organogenic callus (Table 84).

### 4. 1. 2. 2 Caulogenesis

The hard callus produced from leaf and internode explants and organogenic callus produced from immature seeds were subcultured for regeneration. Of the two types of calli, only organogenic callus could produce adventitious buds and develop adventitious shoots (Fig. 26 and 27). Ten different combinations of BAP and Kn were tried along with basal WPM. Some media combinations responded by further multiplying the callus and some by regenerating shoots from the callus (Table 118).

#### 4. 1.2. 2. 1 Standardisation of regeneration medium

Organogenic callus proliferated from the hypocotyl of the immature seeds were subcultured into ten different combinations including basal WPM for the regeneration of shoots. The best medium for shoot regeneration was found to be WPM supplemented with 3 mg/l BAP and 1 mg/l Kn.

The compact calli produced from the leaves and internodes were also subcultured into regeneration medium, but there was no response of regeneration in any of the media tried.

Table 85. Effect of BAP and Kinetin on indirect regeneration of shoots from organogenic callus produced from immature seeds

| **Sl. No.** | **Basal medium** | **Growth regulators** | | **Response** | **% of cultures showing response** |
|---|---|---|---|---|---|
| | | **BAP (mg/l)** | **Kn (mg/l)** | | |
| 1 | WPM | 0 | 0 | Multiplication of callus | **100** |
| 2 | | 0.5 | 0 | " | 100 |
| 3 | | 1 | 0 | ‘’ | 100 |
| 4 | | | 0.5 | ,, | 100 |
| 5 | | | 1 | ,, | 100 |
| 6 | | | 2 | ‘’ | 100 |
| 7 | | 1 | 0.5 | " | 100 |
| 8 | | 2 | 0.5 | " | 100 |
| 9 | | 3 | 1 | Regeneration of shoot | 46 |
| 10 | | 4 | 1 | Regeneration of shoot | 16 |

#### 4. 1. 2. 2. 2 Effect of BAP on indirect regeneration

BAP was used at a range of 1- 4 mg/l with WPM. Multiplication of the callus took place at lower concentrations of BAP (0.5-1mg/l).

#### 4.1. 2. 2. 3 Effect of Kn on indirect regeneration

The range of Kn used was 1-2 mg/l and only multiplication of callus took place.

#### 4.1. 2. 2. 4 Effect of BAP and Kinetin on indirect regeneration

Regeneration of shoots from organogenic callus was achieved in WPM supplemented with 3mg/l BAP and 1mg/l Kn and 4 mg/l BAP and 1 mg/l Kn. In all the other media with BAP and Kn combinations, only multiplication of callus took place (Table 85).

### 4.1.3 Rhizogenesis

The microshoots of *Cinnamomum verum* produced *in vitro* through direct and indirect regeneration were transferred to rooting medium for the induction of roots. The shoots developed from juvenile and mature explants were transferred to rooting medium to study the difference in response of rooting. Shoots of 3-4 cm length were taken for root induction. The number, length and other features of the roots varied depending on the combinations and concentrations of the rooting media and maturity of the explants taken.

### 4.1.3.1 Standardisation of rooting medium

Basal WPM and WPM supplemented with different concentrations of IBA, NAA and activated charcoal was used for rhizogenesis. Roots were induced in all the media tried. Of the 14 different media combinations tried, the best medium for rooting was found to be WPM supplemented with 2 gm/l charcoal for shoots regenerated from juvenile as well as mature explants. WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and 2 gm/l charcoal also gave good results in rooting. After the induction of three to four roots, 4-5 cm long, the plantlets were transferred to basal WPM for further growth ( Fig. 28 and 29).

### 4.1.3.2 Statistical Analysis

The data on root induction in shoots regenerated from juvenile explants (Experiment-4) and root induction in shoots regenerated from mature explants (Experiment-5) were subjected to standard ANOVA (Analysis of Variance) and Post Hoc (Duncan) test using the SPSS (16.0) software to study the variations and the significance of different experiments. The data were subjected to square root transformation before the statistical analysis.

The Standard Deviation (SD), Standard Error (SE) and Coefficient of Variation (CV) were also computed for the different sets of experiments.

The experiments 4 and 5 were repeated twice.

### 4.1.3.3 Root induction in shoots regenerated from juvenile explants of *C. verum* (Experiment-4)

#### 4.1.3.3.1 Effect of WPM + 1 mg/l auxin

Table 86. Effect of **1 mg/l** auxin in root induction on shoots regenerated from **juvenile** explants in *C. verum*

| Sl.No. | Basal medium | Growth regulators (mg/ l) | No. of roots produced on the 30th day | |
|---|---|---|---|---|
| | | | Average of Experiment 4 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 1 | WPM | 1 IBA | 0.33 | 0.90 |
| | | | 1.00 | |
| | | | 0.67 | |
| | | | 1.33 | |
| | | | 1.33 | |
| | | | 0.67 | |
| | | | 1.00 | |
| 2 | | 1 NAA | 2.33 | 2.29 |
| | | | 2.33 | |
| | | | 3.00 | |
| | | | 2.00 | |
| | | | 2.33 | |
| | | | 1.33 | |
| | | | 2.67 | |
| 3 | | 0.5 IBA + 0.5 NAA | 3.67 | 3.38 |
| | | | 3.33 | |
| | | | 4.00 | |
| | | | 3.67 | |
| | | | 3.67 | |
| | | | 2.33 | |
| | | | 3.00 | |

The combinations tried were WPM supplemented with 1 mg/l IBA, 1 mg/l NAA, 0.5 mg/l IBA + 0.5 mg/l NAA (Table 86).

Of the three combinations tried, the highest mean of 3.38 roots were produced in WPM supplemented with 0.5 mg/l IBA and 0.5 mg/l NAA. The lowest mean was 0.9 in WPM supplemented with 1 mg/l IBA.

The mean, SE and CV (%) for the data of root induction in shoots regenerated from juvenile explants in WPM supplemented with 1 mg/l auxin are given in the Table 87. The lowest CV value obtained was 35.78 %. The ANOVA is given in Table 88.

Table 87. *In vitro* rooting of **juvenile** explants of *C. verum* showing the mean and standard error with CV values in WPM medium supplemented with **1 mg/l** auxin

| **Experiment 4 (1 mg/l auxin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean± SE** | **CV%** |
| 1 | 1 | 4.83±0.792 | 40.19 |
| 2 | 2 | 3.50±0.719 | 50.31 |
| 3 | 3 | 4.50±0.885 | 48.18 |
| **Repeat 1** | | | |
| 1 | 1 | 4.17±0.946 | 55.56 |
| 2 | 2 | 5.00±0.73 | 35.78 |
| 3 | 3 | 2.50±0.957 | 76.60 |
| 4 | 4 | 4.00±1.528 | 66.15 |
| **Repeat 2** | | | |
| 1 | 0 | 3.50±2.5 | 101.03 |
| 2 | 1 | 4.40±0.927 | 47.14 |
| 3 | 2 | 4.40±0.98 | 49.80 |
| 4 | 3 | 4.17±0.872 | 51.25 |
| 5 | 4 | 2.50±1.5 | 84.84 |
| CV – Coefficient of Variation | | | |

Medium combination – WPM + 1 mg/l auxin

Table 88. ANOVA of root induction in shoots regenerated from **juvenile** explants of *C. verum* in WPM supplemented with **1 mg /l** auxin.

| **Source** | **Df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 14 | 3.540 | 0.617 |
| Experiment-4 | 4 | 6.838 | 1.191 |
| Repeat-1 | 4 | 4.540 | 0.791 |
| Repeat-2 | 4 | 0.988 | 0.172 |
| Error | 6 | 5.740 | |
| Total | 21 | | |
| $R^2 = 0.590$ | | | |

#### 4.1.3.3.2 Effect of WPM + 2 mg/l auxin

The combinations tried were WPM supplemented with 2 mg/l IBA, 2 mg/l NAA and 1 mg/l IBA + 1mg/l NAA (Table 89).

Of the three combinations tried, the highest mean of roots produced was 8.48 in WPM supplemented with 2 mg/l NAA and the lowest mean of roots produced was 2.67 in WPM supplemented with 1 mg/l IBA + 1 mg/l NAA.

The mean, SE and CV (%) are given in the Table 90. The lowest CV value obtained was 15.71 %, indicating that the estimate was good. The ANOVA is given in Table 91 and the overall $R^2$ was significant at 91%.

Table 89. Effect of **2 mg/1** auxin in root induction on shoots regenerated from **juvenile** explants in *C. verum*

| Sl.No. | Basal medium | Growth regulators (mg/ l) | No. of roots produced on the 30th day | |
|---|---|---|---|---|
| | | | Average of Experiment 4 and 2 Repeats (3 cultures) | Total Average (21 cultures) |
| 1 | WPM | 2 IBA | 5.00 | 5.38 |
| | | | 5.67 | |
| | | | 6.00 | |
| | | | 4.67 | |
| | | | 6.00 | |
| | | | 5.00 | |
| | | | 5.33 | |
| 2 | | 2 NAA | 10.00 | 8.48 |
| | | | 7.67 | |
| | | | 8.00 | |
| | | | 8.33 | |
| | | | 9.00 | |
| | | | 9.00 | |
| | | | 7.33 | |
| 3 | | 1 IBA+1 NAA | 2.33 | 2.67 |
| | | | 3.33 | |
| | | | 2.33 | |
| | | | 3.00 | |
| | | | 2.33 | |
| | | | 2.33 | |
| | | | 3.00 | |

Table 90. *In vitro* rooting of **juvenile** explants of *C. verum* showing the mean and standard error with CV values in WPM medium supplemented with **2 mg/l** auxin

| **Experiment 4 (2 mg/l auxin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean± SE** | **CV%** |
| 1 | 2 | 4.33±0.882 | 35.29 |
| 2 | 3 | 3.00±2 | 94.27 |
| 3 | 4 | 5.00±1.528 | 52.92 |
| 4 | 5 | 3.75±1.250 | 66.67 |
| 5 | 7 | 5.00±1.155 | 40.00 |
| 6 | 8 | 4.00±2.000 | 70.70 |
| 7 | 9 | 4.50±0.500 | 15.71 |
| **Repeat 1** | | | |
| 1 | 2 | 4.00±1.528 | 66.15 |
| 2 | 3 | 4.00±1.528 | 66.15 |
| 3 | 4 | 5.00±1.000 | 28.28 |
| 4 | 5 | 2.50±1.500 | 84.84 |
| 5 | 6 | 6.00±1.000 | 23.57 |
| 6 | 7 | 2.33±0.333 | 24.76 |
| 7 | 8 | 5.25±0.854 | 32.53 |
| **Repeat 2** | | | |
| 1 | 2 | 4.00±1.291 | 64.55 |
| 2 | 3 | 4.00±1.155 | 50.00 |
| 3 | 4 | 3.00±1.000 | 47.13 |
| 4 | 5 | 4.33±1.764 | 70.55 |
| 6 | 7 | 5.67±0.882 | 26.95 |
| 7 | 9 | 2.00±1.000 | 70.70 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 2 mg/l auxin | | | |

Table 91. ANOVA of root induction in shoots regenerated from **juvenile** explants of *C. verum* in WPM supplemented with **2 mg /l** auxin

| Source | df | Mean Square | F |
|---|---|---|---|
| Corrected Model | 19 | 4.000 | .500 |
| Experiment- 4 | 3 | 7.333 | 0.917 |
| Repeat-1 | 3 | 11.333 | 1.417 |
| Repeat-2 | 5 | 5.014 | 0.627 |
| Error | 1 | 8.000 | |
| Total | 21 | | |
| $R^2$ =0 .905 | | | |

### 4.1.3.4 Root induction in shoots regenerated from mature explants of *C. verum* (Experiment-5)

The above mentioned combinations tried for root induction in shoots regenerated from juvenile explants were also tried for root induction in shoots regenerated from mature explants.

#### 4.1.3.4.1 Effect of WPM + 1 mg/l auxin

The combinations tried were WPM supplemented with 1 mg/l IBA, 1 mg/l NAA and 0.5 mg/l IBA + 0.5 mg/l NAA (Table 92).

Of the three combinations tried, the highest mean of 2.14 roots were produced in WPM supplemented with 0.5 mg/l IBA and 0.5 mg/l NAA. The lowest mean was 0.52 in WPM supplemented with 1 mg/l IBA.

The mean, SE and CV (%) are given in the Table 93. The lowest CV value obtained was 25 %. The ANOVA is given in Table 94 and the overall $R^2$ value was 51 %. The Duncan test in Repeat 2 of Experiment 5 showed one subset (Table 95).

Table 92. Effect of **1 mg/1** auxin in root induction on shoots regenerated from **mature** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators (mg/ l)** | **No. of roots produced on the 30th day** | |
|---|---|---|---|---|
| | | | **Average of Experiment 4 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 1 | WPM | 1 IBA | 0.67 | 0.52 |
| | | | 0.33 | |
| | | | 0.67 | |
| | | | 0.00 | |
| | | | 0.33 | |
| | | | 1.00 | |
| | | | 0.67 | |
| 2 | | 1 NAA | 1.67 | 1.52 |
| | | | 1.33 | |
| | | | 1.67 | |
| | | | 2.00 | |
| | | | 1.33 | |
| | | | 1.33 | |
| | | | 1.33 | |
| 3 | | 0.5 IBA + 0.5 NAA | 2.00 | 2.14 |
| | | | 2.67 | |
| | | | 2.00 | |
| | | | 2.00 | |
| | | | 2.00 | |
| | | | 2.33 | |
| | | | 2.00 | |

Table 93. In vitro rooting of **mature** explants of *C. verum* showing the mean and standard error with CV values in WPM medium supplemented with **1 mg/l** auxin

| **Experiment 5 (1 mg/l auxin)** | | | |
|---|---|---|---|
| **Sl No** | **Medium Combination** | **Mean± SE** | **CV%** |
| 1 | 0 | 4.00±0.577 | 25.00 |
| 2 | 1 | 4.25±0.901 | 60.00 |
| 3 | 2 | 4.00±0.667 | 50.00 |
| **Repeat 1** | | | |
| 1 | 0 | 4.75±1.109 | 46.67 |
| 2 | 1 | 3.33±0.843 | 62.04 |
| 3 | 2 | 3.90±0.657 | 53.31 |
| **Repeat 2** | | | |
| 1 | 0 | 3.00±0.913 | 60.87 |
| 2 | 1 | 5.14±0.738 | 37.98 |
| 3 | 2 | 3.29±0.714 | 57.45 |
| 4 | 3 | 4.33±1.453 | 58.13 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 1 mg/l auxin | | | |

Table 94. ANOVA of root induction in shoots regenerated from **mature** explants of *C. verum* in WPM supplemented with **1 mg /l** auxin

| **Source** | **df** | **Mean Square** | **F** |
|---|---|---|---|
| Corrected Model | 9 | 4.753 | 1.269 |
| Experiment-5 | 3 | 4.372 | 1.167 |
| Repeat-1 | 3 | 5.675 | 1.514 |
| Repeat-2 | 3 | 9.799 | 2.615 |
| Error | 11 | 3.747 | |
| Total | 21 | | |
| $R^2 = 0.509$ | | | |

Table 95. Duncan test for root induction in **mature** explants in WPM supplemented with **1 mg /l** auxin.

| **Repeat 2** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 0 | 4 | 3.00 |
| 2 | 7 | 3.29 |
| 3 | 3 | 4.33 |
| 1 | 7 | 5.14 |
| Sig. | | 0.147 |

#### 4.1.3.4.2 Effect of WPM + 2 mg/l auxin

The combinations tried were WPM supplemented with 2 mg/l IBA, 2 mg/l NAA and 1 mg/l IBA + 1mg/l NAA (Table 96).

Of the three combinations tried, the highest mean of roots produced was 6.05 in WPM supplemented with 2 mg/l NAA and the lowest mean of roots produced was 1.62 in WPM supplemented with 1 mg/l IBA + 1 mg/l NAA.

Table 96. Effect of **2 mg/1** auxin in root induction on shoots regenerated fro **mature** explants in *C. verum*

| **Sl.No.** | **Basal medium** | **Growth regulators (mg/ l)** | **No. of roots produced on the 30th day** | |
|---|---|---|---|---|
| | | | **Average of Experiment 4 and 2 Repeats (3 cultures)** | **Total Average (21 cultures)** |
| 1 | WPM | 2 IBA | 4.00 | 3.90 |
| | | | 3.33 | |
| | | | 3.67 | |
| | | | 4.33 | |
| | | | 3.67 | |
| | | | 4.33 | |
| | | | 4.00 | |
| 2 | | 2 NAA | 5.67 | 6.05 |
| | | | 6.00 | |
| | | | 6.00 | |
| | | | 6.00 | |
| | | | 5.33 | |
| | | | 7.33 | |
| | | | 6.00 | |
| 3 | | 1 IBA+1 NAA | 1.67 | 1.62 |
| | | | 1.67 | |
| | | | 2.00 | |
| | | | 1.33 | |
| | | | 2.00 | |
| | | | 1.33 | |
| | | | 1.33 | |

The mean, SE and CV (%) are given in the Table 97. The lowest CV value obtained was 28.28 %. The ANOVA is given in Table 98 and the overall $R^2$ value was significant at 66 %. The Duncan test in Repeat 1 of Experiment 5 showed one subset (Table 99).

Table 97. *In vitro* rooting of **mature** explants of *C. verum* showing the mean and standard error with CV values in WPM medium supplemented with **2 mg/l** auxin

| **Experiment 5 (2 mg/l auxin)** | | | |
|---|---|---|---|
| **Sl. No.** | **Medium Combination** | **Mean± SE** | **CV%** |
| 1 | 1 | 4.67±1.202 | 44.58 |
| 2 | 2 | 3.50±1.19 | 68.00 |
| 3 | 3 | 3.50±1.5 | 60.60 |
| 4 | 4 | 4.67±1.202 | 44.58 |
| 5 | 5 | 3.83±1.046 | 66.92 |
| 6 | 6 | 3.00±1.000 | 47.13 |
| **Repeat 1** | | | |
| 1 | 1 | 4.00±1.155 | 50.00 |
| 2 | 2 | 4.33±1.764 | 70.55 |
| 3 | 3 | 2.50±0.5 | 28.28 |
| 4 | 4 | 3.00±1.155 | 66.67 |
| 5 | 5 | 4.40±1.03 | 52.32 |
| 6 | 6 | 4.33±1.202 | 48.08 |
| 7 | 7 | 5.00±2.000 | 56.56 |
| **Repeat 2** | | | |
| 1 | 1 | 4.67±1.856 | 68.84 |
| 2 | 2 | 3.50±0.645 | 36.89 |
| 3 | 3 | 4.25±1.377 | 64.80 |
| 4 | 4 | 3.67±0.882 | 41.63 |
| 5 | 6 | 4.00±1.528 | 66.15 |
| 6 | 7 | 2.50±1.5 | 84.84 |
| CV – Coefficient of Variation<br>Medium combination – WPM + 1 mg/l auxin | | | |

Table 98. ANOVA of root induction in shoots regenerated from **mature** explants of *C. verum* in WPM supplemented with **2 mg /l** auxin

| **Source** | **Type III Sum of Squares** | **Df** | **Mean Square** | **F** |
|---|---|---|---|---|
| Corrected Model | 55.750[a] | 16 | 3.484 | 0.493 |
| Experiment-5 | 22.167 | 4 | 5.542 | 0.785 |
| Repeat-1 | 37.436 | 5 | 7.487 | 1.060 |
| Repeat-2 | 21.669 | 4 | 5.417 | 0.767 |
| Error | 28.250 | 4 | 7.062 | |
| Total | 420.000 | 21 | | |
| $R^2$ =0.664 | | | | |

Table 99. Duncan test for root induction in **mature** explants in WPM supplemented with **2 mg /l** auxin

| **Repeat 1** | **N** | **Subset** |
|---|---|---|
| | | **1** |
| 3 | 2 | 2.50 |
| 4 | 3 | 3.00 |
| 1 | 3 | 4.00 |
| 2 | 3 | 4.33 |
| 6 | 3 | 4.33 |
| 5 | 5 | 4.40 |
| 7 | 2 | 5.00 |
| Sig. | | 0.317 |

#### 4.1.3.4.3 Effect of Plant Growth Regulators

##### 4.1.3.4.3.1 Effect of IBA

IBA was used at a range of 0.5-2.0 mg/l. Normal roots were not induced when IBA was used alone. There was callus formation at the basal cut end of the shoot. The roots produced were thicker.

When IBA was used along with charcoal normal roots were produced. WPM supplemented with 1 mg/l IBA and 2gm/l charcoal produced a highest mean of 2.71 roots on shoots developed from juvenile explant and 1.62 roots on shoots developed from mature explants. When 2 mg/l IBA was used along with 2 gm/l charcoal, the highest mean of roots produced was 2.05 on shoots developed from juvenile explants and 1.33 on shoots developed from mature explants. The number and length of the roots varied depending on the maturity of the explant.

##### 4.1.3.4.3.2 Effect of NAA

NAA was used at a range of 0.5 – 2.0 mg/l. When NAA was used alone, the roots produced were thick and stout. NAA could induce more number of roots than IBA. Use of NAA induced more callus production than IBA.

Addition of charcoal to NAA supplemented medium could avoid callus formation and produced normal roots. The highest mean of 2.71 roots were produced on shoots regenerated from juvenile explants in WPM supplemented with 1 mg/l NAA and 2 gm/l charcoal. WPM supplemented with 2mg/l NAA and 2gm/l charcoal produced a highest mean of 3.14 roots.

In the case of shoots regenerated from mature explants, the highest mean of roots produced was 1.81 in the medium containing 1 mg/l NAA and 2 gm/l charcoal and 2.05 in the medium containing 2 mg/l NAA and 2 gm/l charcoal. Same medium could produce more number of roots in shoots regenerated from juvenile explants than in shoots regenerated from mature explants. The number and length of the roots varied depending on the maturity of the explant.

#### 4.1.3.4.3.3 Effect of IBA and NAA

When WPM was supplemented with 0.5 mg/l IBA and 0.5 mg/l NAA, a highest mean of 3.38 roots were produced on cultures of juvenile explants and 2.14 roots were produced on cultures of mature explants.

WPM along with 1 mg/l IBA and 1 mg/l NAA could induce a highest mean of 2.67 roots on cultures of juvenile explants and 1.62 roots on cultures of mature explants. All the cultures showed callus production at the basal cut end of the shoots.

When charcoal was added to above media combinations, normal healthy roots were produced without any callus formation. WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and 2 gm/l charcoal was also found to be a good rooting medium for cinnamon.

#### 4.1.3.4.3.4 Effect of Charcoal

Of the 14 media combinations tried for rooting, basal WPM supplemented with 2 gm/l charcoal gave the best result in the rooting of microshoots of cinnamon, both on shoots regenerated from juvenile explants (7 roots) as well as on shoots regenerated from mature explants (5 roots). Basal WPM could induce only 1- 2 roots

per culture on cultures developed from juvenile explants as well as on cultures developed from mature explants.

Table 100. Effect of IBA and NAA on *in vitro* rooting of shoots regenerated from **juvenile** and **mature** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators (mg/ l)** | **Average No. of roots produced on 30th day** | |
|---|---|---|---|---|
| | | | **Shoots developed From juvenile explants** | **Shoots developed from mature explants** |
| 1 | WPM | 0 | 0.43 | 0.19 |
| 2 | | 1 IBA | 0.90 | 0.52 |
| 3 | | 1 NAA | 2.29 | 1.52 |
| 4 | | 0.5 IBA + 0.5 NAA | 3.38 | 2.14 |
| 5 | | 2 IBA | 5.38 | 3.90 |
| 6 | | 2 NAA | 8.48 | 6.05 |
| 7 | | 1 IBA+1 NAA | 2.67 | 1.62 |

Table 101. Effect of IBA, NAA and charcoal on *in vitro* rooting of shoots regenerated from **juvenile** and **mature** explants in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators (mg/ l)** | **Average No. of roots produced on 30th day** | |
|---|---|---|---|---|
| | | | **Shoots developed from juvenile explants** | **Shoots developed from mature explants** |
| 1 | WPM | 2 CH | 5.33 | 3.90 |
| 2 | | 1 IBA + 2 CH | 2.71 | 1.62 |
| 3 | | 1NAA + 2 CH | 2.71 | 1.81 |
| 4 | | 0.5 IBA + 0.5 NAA + 2 CH | 4.76 | 2.52 |
| 5 | | 2 IBA + 2 CH | 2.05 | 1.33 |
| 6 | | 2 NAA + 2 CH | 3.14 | 2.05 |
| 7 | | 1 IBA + 1 NAA + 2 CH | 2.29 | 0.81 |

Table 102. Effect of charcoal on root induction and basal callus formation in shoots regenerated from mature explants of *Cinnamomum verum*

| Sl.No. | Basal medium | Growth regulators (mg/ l) + charcoal (gm/l) | Response of cultures on 30-40 days | |
|---|---|---|---|---|
| | | | Percentage of Rooting | Remarks |
| 1 | WPM | 0 | 17 | No basal callus formation |
| 2 | | 2 CH | 100 | 8-10 cm long roots were produced without callus. Best medium for rooting |
| 3 | | 1 IBA | 50 | No callus formation |
| 4 | | 1 IBA + 2 CH | 100 | ,, |
| 5 | | 1 NAA | 100 | Callus formation at the basal cut ends of the shoot |
| 6 | | 1NAA + 2 CH | 100 | No callus formation |
| 7 | | 2 IBA | 100 | Thicker roots, 1-2 cm long with basal callus formation |
| 8 | | 2 IBA + 2 CH | 83 | No callus formation |
| 9 | | 2 NAA | 100 | Thicker roots, 0.5- 1 cm long, with vigorous callus formation at the cut end and basal portion of shoots |
| 10 | | 2 NAA + 2 CH | 100 | No callus formation |
| 11 | | 0.5 IBA + 0.5 NAA | 100 | Root induction along with basal callus formation |
| 12 | | 0.5 IBA + 0.5 NAA + 2 CH | 100 | No callus formation. Good medium for rooting |
| 13 | | 1 IBA+1 NAA | 100 | 0.5-0.75 cm long roots were produced with callus |
| 14 | | 1 IBA+1 NAA + 2 CH | 56 | No callus formation |

The induction of callus in the rooting medium is more favoured by the addition of NAA than IBA in *Cinnamomum verum*. Addition of 1 mg/l NAA

induced callus production in cultures, whereas addition of 1 mg/l IBA did not induce callus at the basal region. At higher auxin concentration (2 mg/l), callus was induced in cultures and addition of 2 mg/l NAA induced vigorous callus formation at the basal cut ends of the shoots as well as along the basal portion of the stem. When charcoal was supplemented with 2 mg/l NAA, callus was not produced. Calli were not induced in basal WPM and all other media with charcoal.

## 4.1. 4 Somatic Embryogenesis

Somatic embryos were produced *in vitro* from the hypocotyl region of 4-5 months old immature seeds.

### 4. 4. 1 Induction of somatic embryos

When immature embryos (Fig. 34) of cinnamon were inoculated on WPM medium supplemented with 0.5 mg/l Kn, about 20-30 percent of the cultures showed direct production of somatic embryos from the hypocotyl region (Fig. 35) within 30-40 days of inoculation (Table 84). The somatic embryos produced were easily separable. They were creamy white in colour and globular in shape (Fig. 36 A and B). The number of somatic embryos ranges from 75 to 100 in each culture tube (36 A).

Induction of somatic embryos could also be achieved in MS medium supplemented with low concentrations of cytokinins (Table 84).

### 4.4.2 Standardisation of the medium for the maturation and germination of somatic embryos

The somatic embryos produced were subcultured on 10 different combinations of WPM containing BAP (0.5-4.0 mg/l) and Kn (0.5-2.0 mg/l). Of the 10 combinations tried, eight combinations including WPM basal medium did not develop plantlets from somatic embryos. However, multiplication of somatic embryos occurred. Subculture of somatic embryos resulted the formation of secondary somatic embryos along with maturation of primary embryos (Table 103).

The somatic embryos showed indications of embryo development (Fig. 38 A and B ) after 2-3 subcultures on WPM supplemented with 3 mg/l BAP and 1 mg/l Kn and WPM supplemented with 4 mg/l BAP and 1 mg/l Kn. Of the two media, the best medium for maturation and germination of somatic embryos was found to be WPM supplemented with 3 mg/l BAP and 1 mg/l Kn. 10-15 percent of the embryos produced have matured and developed into plantlets (Table 103).

From the white, globular embryos, cluster of cup shaped embryos (Fig. 36 B) were developed. The cup-shaped structures developed into torpedo structures, then cotyledonary stages (Fig. 38 A) and developed into plantlets (Fig. 39) within 30-40 days. About 20% of somatic embryos germinated into complete plantlets.

### 4. 4. 3 Effect of plant growth regulators

#### 4. 4. 3.1 Effect of BAP

BAP was used at a range of 0.5-4 mg/l. Multiplication of the somatic embryos could be achieved in WPM supplemented with 0.5mg/l and 1 mg/l BAP. Multiplication of somatic embryos could also be achieved in WPM supplemented with 1mg/ l BAP and 0.5 mg/l Kn and 2 mg/l BAP and 0.5 mg/l Kn. Plantlet

formation from somatic embryos could be achieved in WPM supplemented with 3mg/l BAP and 1mg/l Kn and 4 mg/l BAP and 1 mg/l Kn.

Table 103. Effect of BAP and Kinetin on germination of somatic embryos into plantlets in *C. verum*

| **Sl. No.** | **Basal medium** | **Growth regulators** | | **Response** | **% of cultures showing response** |
|---|---|---|---|---|---|
| | | **BAP (mg/l)** | **Kn (mg/l)** | | |
| 1 | WPM | 0 | 0 | Multiplication of embryos | **100** |
| 2 | | 0.5 | 0 | " | 100 |
| 3 | | 1 | 0 | ‘’ | 100 |
| 4 | | | 0.5 | ,, | 100 |
| 5 | | | 1 | ,, | 100 |
| 6 | | | 2 | ‘’ | 100 |
| 7 | | 1 | 0.5 | " | 100 |
| 8 | | 2 | 0.5 | " | 100 |
| 9 | | 3 | 1 | Germination of embryos into plantlets | 46 |
| 10 | | 4 | 1 | Germination of embryos into plantlets | 16 |

#### 4. 4. 3.2 Effect of Kinetin

Kn was used at a range of 1-2 mg/l. When WPM was supplemented with 0.5, 1, and 2 mg/l Kn multiplication of somatic embryos took place. When used along with BAP, Kn could induce better responses.

#### 4. 4. 3.3 Effect of BAP and Kn

The synergistic effect of BAP and Kn gave better results in the multiplication of somatic embryos and development of somatic embryos into plantlets.

#### 4. 4. 2.4 Effect of $GA_3$

When $GA_3$ was added at a range of 1-3 mg/l, to the standardized media (WPM supplemented with 3 mg/l BAP and 1 mg/l Kn), it did not show any enhancement on maturation and germination of somatic embryos.

#### 4. 4. 2.5 Effect of sucrose

A high rate of multiplication of somatic embryos was noticed in medium containing 30 gm/l sucrose than in medium containing 20 gm/l sucrose.

### 4.1.5 Hardening

*In vitro* developed plantlets through direct regeneration, indirect regeneration and somatic embryogenesis were transplanted to the field with 80-90% success after a hardening period of 4-5 weeks.

The plantlets with 4-5 leaves were taken out carefully without damaging the roots. The old medium on the plantlet was washed off and the plantlets were

immersed in fungicide solution (3%) for 5-10 minutes. The fungicide treated plantlets were transplanted to soilrite in tea cups ( Fig. 40 A) and kept in green house. The humidity was maintained by covering the cups with poly bags for two weeks. After one week, holes were made in the poly bags to reduce the humidity. The plantlets along with soilrite were transferred after two weeks of transplantation to potting mixture containing garden soil, soilrite and sand in equal proportion in poly bags (Fig. 40 B) and kept in the green house. After the emergence of 3-4 new leaves the plantlets were transplanted in the field (Fig. 41). Proper shade was provided up to 3 months after transplantation. At *in vitro* conditions and early stages of hardening the leaves were somewhat linear in shape. However, later on the leaves were turned into normal size and shape under greenhouse conditions.

### 4.1.6 RAPD results

Analysis of RAPD data of mother plant, five directly regenerated plants and five plants regenerated through somatic embryogenesis showed low levels of genetic variation. Among the 45 primers tried, 20 primers which are repeatedly produced scorable amplifications were taken into consideration for further analysis. 20 primers studied amplified a total of 191 DNA fragments (Table 104). Out of them 153 were found to be polymorphic (80.10%) in one or other of 11 cinnamon plants studied. The levels of polymorphism were different with different primers among these species. Primers A16, A10, D20, D13 and B20 produced maximum numbers of amplified products (12) and E19 produced the least (6). Primer A1, A7, A8, A9, D3, D4, D8, D16 and B20 displayed 100% polymorphism for the products generated. On an average, the primers produced 9.2 products and 7.1 polymorphism

each. The number of amplified products from each plant varies significantly for all the 20 primers. The size of the amplified products ranged from 0.35kb to 2.6kb.

The similarity matrix obtained based on Nei and Leis method shows the coefficient of similarity value ranging from 0.41 to 0.81 with a mean value of 0.625. The observed value signifies the extent of genetic variation in these plants. Cluster analysis based on UPGMA reveals two major groups. Cluster A comprises of six plants (mother plant and five directly regenerated plants) and has coefficient of similarity value ranging from 0.50 – 0.81 with a mean value of 0.65.

Table 104. List of primers used for RAPD analysis

| **Sl. No.** | **Primer** | **Sequence 5'→3'** | **No. of bands** | **No. of polymorphic bands** |
|---|---|---|---|---|
| 1 | OPA-03 | AGT CAG CCA C | 10 | 1 |
| 2 | OPA-13 | CAG CAC CCA C | 9 | 8 |
| 3 | OPA-16 | AGC CAG CGA A | 12 | 8 |
| 4 | OPA-01 | CAG GCC CTT C | 11 | 11 |
| 5 | OPA-07 | GAA ACG GGT G | 8 | 8 |
| 6 | OPA-08 | GTG ACG TAG G | 8 | 8 |
| 7 | OPA-09 | GGG TAA CGC C | 8 | 8 |
| 8 | OPA-10 | GTG ATC GCA G | 12 | 8 |
| 9 | OPA-14 | TCT GTG CTG G | 8 | 5 |
| 10 | OPD-03 | GTC GCC GTC A | 10 | 10 |
| 11 | OPD-20 | ACC CGG TCA C | 12 | 8 |
| 12 | OPD-06 | ACC TGA ACG G | 8 | 6 |
| 13 | OPD-13 | GGG GTG ACG A | 12 | 8 |
| 14 | OPD-04 | TCT GGT GAG G | 10 | 10 |
| 15 | OPD-08 | GTG TGC CCC A | 10 | 10 |
| 16 | OPD-16 | AGG GCG TAA G | 8 | 8 |
| 17 | OPB-18 | CCA CAG CAG T | 9 | 8 |
| 18 | OPB-19 | ACC CCC GAA G | 8 | 6 |
| 19 | OPB-20 | GGA CCC TTA C | 12 | 12 |
| 20 | OPE-19 | ACG GCG TAT G | 6 | 4 |
| | Total No. of bands | | 191 | 153 |
| | Mean per primer | | 9.2 | 7.1 |

Cluster B with the remaining 5 (somatic embryonically generated plants) plants of the genus is comparatively more diverse than cluster A. The average coefficient similarity value of 0.53 with a distribution range from 0.43 to 0.66 reveals the extent of diversity in this cluster.

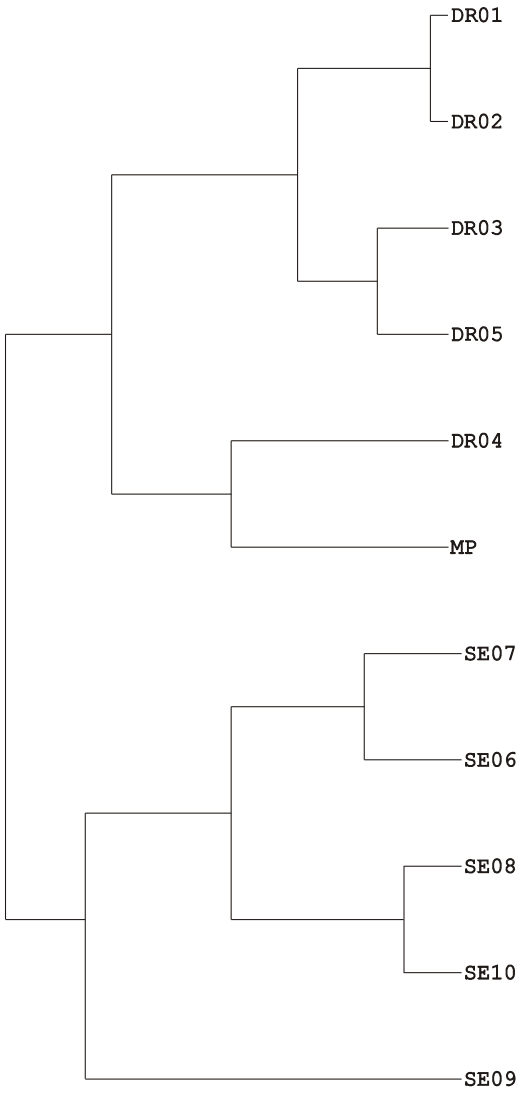


Fig. 47. Dendrogram showing genetic distances of RAPD data following the UPGMA method. **DR** indicates directly regenerated plants, **MP** indicates mother plant and **SE** indicates plants regenerated through somatic embryogenesis

Table 105. Genetic relationship among 11 plants of *Cinnamomum verum* (*Nei's genetic identity (above diagonal) and genetic distance (below diagonal*))

| | **DR01** | **DR 2** | **DR03** | **DR04** | **DR05** | **MP 6** | **SE06 7** | **SE07 8** | **SE07 9** | **SE09 10** | **SE10 11** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **DR 01** | 1.00 | 0.99 | 0.96 | 0.96 | 0.97 | 0.99 | 0.99 | 0.98 | 0.97 | 0.97 | 0.95 |
| **DR 02** | 0.02 | 1.00 | 0.96 | 0.96 | 0.97 | 0.99 | 0.99 | 0.98 | 0.98 | 0.97 | 0.95 |
| **DR 03** | 0.03 | 0.03 | 1.00 | 0.99 | 0.98 | 0.98 | 0.98 | 0.96 | 0.97 | 0.95 | 0.96 |
| **DR 04** | 0.03 | 0.03 | 0.07 | 1.00 | 0.99 | 0.98 | 0.98 | 0.97 | 0.97 | 0.96 | 0.97 |
| **DR 05** | 0.02 | 0.02 | 0.01 | 0.09 | 1.00 | 0.98 | 0.98 | 0.98 | 0.97 | 0.96 | 0.97 |
| **MP** | 0.02 | 0.02 | 0.01 | 0.01 | 0.04 | 1.00 | 0.98 | 0.98 | 0.97 | 0.97 | 0.97 |
| **SE 06** | 0.09 | 0.08 | 0.01 | 0.01 | 0.01 | 0.01 | 1.00 | 0.99 | 0.96 | 0.97 | 0.97 |
| **SE 07** | 0.01 | 0.01 | 0.03 | 0.02 | 0.01 | 0.01 | 0.05 | 1.00 | 0.98 | 0.97 | 0.97 |
| **SE 08** | 0.02 | 0.01 | 0.02 | 0.02 | 0.02 | 0.02 | 0.01 | 0.01 | 1.00 | 0.98 | 0.97 |
| **SE 09** | 0.02 | 0.02 | 0.04 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.01 | 1.00 | 0.96 |
| **SE 10** | 0.04 | 0.04 | 0.03 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.04 | 1.00 |

DR 01 – DR 05 indicate directly regenerated plants
MP indicates mother plant
SE 06 – SE 10 indicate plants regenerated through somatic embryogenesis

## 4.2 *Cinnamomum cassia*

### 4.2.1 Direct regeneration

#### 4.2.1.1 Establishment of aseptic cultures

Shoot tips and nodal segments of *Cinnamomum cassia* were used for the initial establishment of cultures. The medium used for initial establishment was WPM supplemented with 2 mg/l BAP and 0.5 mg/l Kn (Fig. 50 A). There was a heavy loss of cultures due to high contamination. The regenerated shoots could attain a length of 1-1.5 cm within 21 days of inoculation (Fig. 50 B).

When fungicide sprayed explants were collected, the contamination rate reduced up to 5%.

#### 4.2.1.2 Multiple shoot induction

The medium combination selected as the best medium for multiple shoot induction in *Cinnamomum verum*, *i.e.*, WPM supplemented with 3 mg/l BAP and 1mg/l Kn, was tried in *C. cassia* also for the induction of multiple of shoots. In cassia, the same medium induced up to 10 multiple shoots (Fig. 51) within 20-30 days of subculture.

#### 4.2.1.3 Rhizogenesis

Roots up to 2 could be induced in WPM supplemented with 2 gm/l charcoal. Rooting was also achieved in WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and 2 gm/l charcoal (Fig. 52).

All the experiments done in *C. verum* could not be done in *C. cassia* due to the scarcity of explants. However, the experiments conducted in *C. cassia* showed promising results and hence the plant can be multiplied in future through all routes of multiplication.

# 5. DISCUSSION

Cinnamon (*Cinnamomum verum* Bercht. and Presl), Cassia (*C. cassia* Blume) and their products have long been recognized for their sweet and delicate flavours and are widely used in food, pharmaceutical, soap and cosmetic industries. Food technologists and food manufacturers find it very difficult to imitate them completely with synthetic substitutes. Although cinnamaldehyde gives a very crude imitation of either cinnamon or cassia, it is nowhere near the natural flavour (Senanayake and Wijesekera, 2004). It has been noted that, cinnamon from the same root stock but grown in different climatic conditions, even within the same land have different chemical and organoleptic properties. The same situation applies to cassia and also, since these plants are cross pollinated, variation arises among the progenies.

Cinnamon is usually propagated through seeds. Eventhough vegetative propagation using cuttings rooted in high humid conditions is possible, the percent survival of such propagules in the field is very low *i.e*., less than 4% in some genotypes (Ravindran and Babu, 2004). Cinnamon shows great variation in morphology and the content of the fragrant material. Hence, an elite plant with desirable characteristics has to be multiplied *in vitro* for large scale production of the propagules (clonal propagation) for developing plantations of Cinnamon for industry. The present work is an attempt to develop protocols for effective and quick production of adequate clones of *C. verum* and *C. cassia in vitro.*

There has always been a great demand for natural cinnamon and cassia. Recently, Sri Lanka and other cinnamon and cassia growing countries, have embarked on programmes to propagate the best varieties using tissue culture techniques. There is bright prospect for large scale plantations evolving from selected plants through tissue culture, thereby producing a product of consistent quality (Senanayake and Wijesekera, 2004).

Various explants such as shoot tip, nodal segment, young leaf, internodes and immature seeds were successfully cultured in different media containing different combinations of auxins and cytokinins. Shoot tips and nodal segments at three maturity levels were used for direct regeneration in cinnamon *viz.*, explants from 2-3 year old seedlings, explants from juvenile shoots of mature trees (8-9 year old) and mature explants from mature trees. In the present study, the suitable basal medium for initial shoot induction in *C. verum* was found to be WPM supplemented with 2 mg/l BAP and 0.5 mg/l Kn. Shoot induction in *C. cassia* was also achieved in the same medium in the present study.

The best medium for multiple shoot induction was found to be WPM supplemented with 3 mg/l BAP and 1 mg/l Kn in all the three types of explants. A comparison of number of shoots produced was done in all the three types of explants studied, and the best result was obtained with seedling explants producing up to 19 microshoots. Compact calli were induced from young leaves and internodes in MS medium supplemented with 2 mg/l 2, 4-D. Organogenic calli and somatic embryos were initiated from the hypocotyl region of immature seeds in WPM supplemented with 0.5 mg/l Kn. Regeneration of shoots was achieved on the organogenic callus

and plantlets could be developed through the germination of somatic embryos in WPM supplemented with 3 mg/l BAP and 1 mg/l Kn. The regenerated shoots from organogenic callus were rooted in WPM supplemented with 2 gm/l charcoal. A comparison of root induction in micro shoots regenerated from juvenile as well as mature explants was done. The plantlets produced *in vitro* were hardened and could be established in the field. Five directly regenerated plants and five plants developed through somatic embryogenesis were profiled for RAPD polymorphism using twenty primers and compared with the mother plant. RAPD analysis showed low levels of genetic variation among mother plant and micropropagated plants developed through direct regeneration and somatic embryogenesis. All these aspects are discussed in detail with the available literature in the proceeding pages under separate headings.

The media combinations in which the cinnamon explants responded well, is tried with *Cinnamomum cassia* shoot tips and nodal segments, as it is well known that tissue culture is genotype dependent (Bhojwani and Razdan, 1996).

## 5.1 Direct Regeneration

### 5.1.1 Basal medium

A culture medium is composed of inorganic salts, an iron source, vitamins, amino acids, growth regulators and a carbohydrate supply (De 1997). Different basal media like WPM, MS and White's media were tried for the initial establishment of cultures. The significant feature of the MS medium is its high concentration of nitrate, potassium and ammonia. The White's medium (1963) is also being used by many researchers and has been proved to be one of the basic media for a variety of

tissue culture. WPM is widely used for the propagation of ornamental shrubs and trees in commercial laboratories (George and Sherrington, 1984). It has the same phosphate level as MS medium but less $Cl^-$.

In the present study, the suitable basal medium for initial shoot induction in *Cinnamomum verum* and *C. cassia* was found to be WPM. WPM is used for the *in vitro* culture of the same plant by Sheeja *et al.* (2000). The results obtained by Babu *et al*. (2003) in *C. camphora* and Shu-Hwa *et al*. (2002) in *C. kanehirae* are in agreement with the superiority of WPM over other basal media for the *in vitro* propagation of *Cinnamomum* spp. These observations are also supported by the results obtained in clove (Thara *et al*., 2000) and *Garcinia* sp. (Meera *et al*., 2006) where WPM was found to produce better response. However, in nutmeg, another tree spice, WPM could induce only poor responses (Rao *et al*., 1997). Earlier reports on nutmeg micropropagation by Mariska *et al*. (1989) also revealed similar culture response.

In contradictory to the above mentioned reports on *Cinnamomum*, Rai and Chandra (1987) developed *in vitro* cultures of *C. verum* using seedling explants in MS basal medium. MS medium was also found useful by Soulange *et al*. (2007) for the *in vitro* propagation of *C. verum*. Inomoto and Kitani (1989) also developed in vitro cultures of *C. cassia* in MS medium. Huang *et al*. (1998) used MS basal medium for the *in vitro* culture of *C. camphora*. Shou *et al*. (2005) could multiply seedling explants of *C. camphora* var. *linaloolifera* in MS medium. WPM is also found to be a good basal medium for shoot multiplication in other trees like

*Sterculia urens* (Mohammad *et al*., 2007), *Garcinia mangostana* (Goh *et al*., 2000), *Syzygium aromaticum* (Thara *et al*., 2000), etc.

In contrast to this, MS medium was found to be useful for the micropropagation of *Garcinia indica* and *G. cambogia* (Mathew *et al.*, 2001) and in the micropropagation of *Murraya koenigii* (Mathew *et al*., 1999). Mathew and Hariharan (1990) achieved multiple shoot induction in nodal segments of seedling explants of *Syzygium aromaticum* on half MS medium and Gamborg's medium. In the case of above mentioned tree spices MS medium was found to be useful for the production of multiple shoots from *in vitro* grown seedling explants. Concerted effects on tissue culture propagation of spices, especially tree spices have been few when compared to those on ornamentals, fruits, vegetables and other agricultural crops, though conventional vegetative propagation of tree spices has not been able to meet the demand of planting material (Rao *et al*., 1997).

In *C. verum* and *C. cassia*, nodal segments gave better responses than shoot tips with respect to the number of multiple shoots produced. Nodal segments of three maturity levels, such as seedling explants, juvenile explants of mature trees and mature explants were used for direct regeneration in *C. verum* in the present study. Sheeja *et al.* (2000) used semi mature nodal segments for the *in vitro* culture of the same species, whereas Soulange *et al*., 2007 used shoot tips from young seedling trees. Rai and Chandra (1987) developed *in vitro* cultures of cinnamon using nodal segments and shoot tips from *in vitro* grown seedlings. Inomoto and Kitani (1989) have reported micropropagation of *Cinnamomum cassia* by using nodal explants from seedlings. Shoot tips and nodal segments of mature trees of *C.*

*camphora* were used as explants for direct regeneration by Babu *et al.* (2003) whereas Soulange *et al*. (2007) and Huang *et al*. (1998) used shoot tips and nodal segments collected from young seedling trees. Shou *et al*. (2005) could also multiply seedling explants of *C. camphora* var. *linaloolifera.*

Other trees coming under spices, nodal segments of seedling plants of *Syzygium aromaticum* were used as explants for direct regeneration (Mathew and Hariharan, 1990) whereas actively growing tender shoots in *Syzygium aromaticum* were used as explants by Rao *et al*. (1997). Thara *et al*. (2000) used shoot tips and nodal segments from three-year old clove seedlings. Rao *et al*. (1997) reported the positive responses of orthotropic shoot segments in nutmeg, shoot tips and nodal segments of *in vitro* grown seedlings in tamarind, shoot tips and nodal segments of *Garcinia indica* and *G. gummi-gutta.* Tender shoots in mature trees of *Murraya koenigii* were used as explants for direct regeneration by Mathew *et al*. (2001).

Fungal contamination and exudation of phenolic compounds were the main hindrance for the initial establishment of cultures in *C. verum and C. cassia* in the present study. Browning and growth of contaminants were the major obstacles for culture establishment in mature female trees of *Ceratonia siliqua* (Romano *et al.*, 2002).

For standardization of multiple shoot induction medium, for *C. verum* 25 media combinations were tried with cytokinins, BAP, Kn and ZN. BAP, Kn and ZN were tried alone in a range of 1-4 mg/l and BAP-Kn, BAP-ZN and Kn-ZN combinations were also tried. The best hormonal combination in multiple shoot induction was found to be 3 mg/l BAP and 1 mg/l Kn in *C. verum* in the present

study. When this medium was tried for axillary bud multiplication in *C. cassia*, multiple shoots up to 10 were produced. This result confirmed the result obtained by Sheeja *et al*. (2000) in the same plant who got maximum number of shoots in a combination of 3 mg/l BAP and 1 mg/l Kn. The same hormonal combination for the induction of multiple shoots is used in *C. camphora* by Babu *et al*. (2003). In an earlier report on Cinnamon micropropagation, Rai and Chandra (1987) got better results with BAP and kinetin combination who have reported that when tried alone BAP was superior to Kn. In the reports of *in vitro* multiplication of *C. verum* (Soulange *et al*., 2007) and *C. camphora* (Soulange *et al*., 2007; Babu *et al*., 2003; Huang *et al*., 1998) the authors have reported the induction of multiple shoots from shoot tips and nodal segments when supplied with only cytokinins in the medium. Induction of multiple shoots was affected by the concentration of cytokinin (Rauf *et al.*, 2005).

When the above medium combination (WPM supplemented with 3 mg/l BAP and 1 mg/l Kn) was tried with *Cinnamomum cassia* in the present study, multiple shoots were produced. However, for the *in vitro* culture of *Cinnamomum cassia*, Inomoto and Kitani (1989) added low concentrations of an auxin, NAA along with the cytokinin BAP. Rai and Chandra (1987) reported vigorous shoot growth in medium containing Kn alone. For shoot elongation of *C. cassia*, Inomoto and Kitani (1989) transferred the cultures into the medium containing BAP alone. BAP (8.9 µM) and TDZ (0.5 µM) combination was used for multiple shoot induction in *Garcinia* sp. (Meera *et al*., 2006).

BAP-Kn combination was also found useful for the multiple shoot induction in *Syzygium aromaticum* (Thara *et al*., 2000). Mathew and Hariharan (1990) achieved multiple shoot induction in *Syzygium aromaticum* with BAP alone. Growth regulator combinations of BAP with Kn or NAA at lower levels proved to be successful in inducing multiple shoots in Teak (Gupta *et al*., 1980). Rao *et al*. (1997) has reported the production of multiple shoots in clove in MS medium supplemented with BAP (2-3 mg/l) combined with IAA and NAA (0.2 mg/l each). Increasing ZN concentration promoted shoot multiplication in *Rhododendron ponticum,* although this effect tends to decrease with higher ZN concentration (Almeida *et al*., 2005).

In contrast to the above mentioned works on *Cinnamomum*, Shou *et al*. (2005) used NAA along with BAP for multiple shoot induction in *C. camphora* var. *linaloolifera* in the presence or absence of $GA_3$**.** This may be because of the use of seedling explants or due to the synergistic effect of BAP, NAA and $GA_3$ or the difference in response of the variety, *C. camphora* var. *linaloolifera*. In another species of cinnamon *viz.*, *C. kanehirae*, the optimum concentration of BAP was found to be 2-6 mg/l for multiple shoot induction (Shu-Hwa *et al*., 2002).

In the present study, multiple shoots up to 19 were produced in seedling explants, up to 15 were produced in juvenile explants of mature trees and up to 14 were produced from the mature explants in the presence of cytokinins without the addition of any auxins in *C. verum*. Since organogenesis is an outcome of auxin-cytokinin balance and their synergistic effect, it is inferred that there was enough auxin in the explant itself to balance with the exogenously supplied cytokinins. Babu *et al*. (1997) reported up to 20 multiple shoots from mature trees in *C. camphora*. In

*C. camphora*, Soulange *et al*. (2007) reported the production of only six shoots. This may be according to them, due to high salt concentration of MS medium which might be less favourable to the *Cinnamomum*. In *C. verum*, the same medium could induce only bud break (Soulange *et al*., 2007).

Multiple shoots up to 10 were produced from nodal segments of seedlings and multiple shoots only up to two were induced from nodal segments of mature trees of clove (Rao *et al*., 1997). Tender shoot explants of *Murraya koenigii* produced multiple shoots up to 4-6 with BAP (1-2 mg/l) and NAA or IAA (0.1-0.5 mg/l) over a period of three weeks.

Soulange *et al*. (2007) conducted tissue culture studies in *Cinnamomum verum* and *Cinnamomum camphora* and compared the response obtained in *C. verum* with that of *C. camphora*. They have found the best medium combination for multiple shoot induction for *C. camphora* was 1 mg/l BAP and 2.5 mg/l TDZ. No multiple shoot formation was observed in *C. camphora* shoot tips cultured on medium containing BAP alone. The response of *C. verum* shoot tips to the same treatment was different from that of *C. camphora* and only bud break was observed in *C. verum* after eight weeks growth in the same medium. According to them the difference in response is due to the difference in explant source and the balance of growth regulators within the explants and in the medium. The growth regulators work in conjunction with each other and have synergistic effects. Gurib-Fakim (2002) reported the difference in response of *C. verum* to the same medium and attributed this to the morphological differences between these two species. Huang *et*

*al*. (1998) also used BAP-TDZ combination at different concentrations for micropropagation of *C. camphora.*

In most cases organ formation is far more easily induced in juvenile explants than in adult ones (Hackett, 1970). In the present study also seedling explants and juvenile explants of mature trees produced more number of shoots than the mature explants. This may be due to the high level of endogenous hormones present in juvenile tissues. Thus the original physiological status of the explant and their endogenous hormonal content are very important factors directing the *in vitro* response (Ammirato, 1986). Early response and multiple shoot formation of jackfruit from bud explants originated from trunk sprouts could be attributed to different degrees of juvenility and maturity present in an adult tree (Bonga 1982; Hartman and Kester, 1986)

In the present study, it is also found that nodal segment explants produced more number of shoots than shoot tips. This may be due to high level of auxin content in the shoot tips since shoot tips are considered to be the site of storage auxins. According to George and Sherrington (1984), shoot tip/nodal segment culture depends on stimulating axillary shoot growth by overcoming the dominance of shoot apical meristem by the incorporation of growth regulators into the medium. When nodal segments are excised from the plant body the apical dominance is removed and more number of shoots are produced from the axillary bud in the presence of exogenously supplied cytokinins. These findings are in accordance with the finding of Jorge *et al.* (1998) who found that cytokinin is directly responsible for reprogramming the shoot apex organization in cotton. In supporting this view, Nair

and Seeni (2003) demonstrated similar responses in *Calophyllum apetalum.* The number of shoots formed was always higher in nodes than in shoot tips in *Calophyllum apetalum* as also reported in *Aegle marmelos* in which the shoot tips were poor substitute for nodes possibly due to the increased apical dominance and lesser formation of axillary shoots (Kumar and Seeni, 1998).

When the concentration of the BAP was increased, 3-4 shoots sprouted in tamarind, though callus formation was noticed at the base of the shoots (Rao *et al*., 1997). In the present study also there was basal callus formation on *in vitro* cultured shoots at higher concentrations of BAP. Basal callus formation on *in vitro* cultured shoots was also reported in *Tylophora indica* (Sharma and Chandel, 1992). According to Marks and Simpson (1994) it may be due to the action of the accumulated auxin at basal cut ends, which can stimulate cell proliferation, especially in the presence of cytokinins.

In the present investigation, combination of cytokinins (BAP and Kn) showed better results in shoot multiplication than when used alone. George and Sherrington (1984) were of the opinion that adding more than one cytokinin to the medium results in improved production of shoots or shoots with better quality. The synergistic effect of BAP and Kn in producing more number of shoots in cultures was also reported in *Curcuma longa* (Salvi *et al.*, 2001).

From the present study it can be inferred that in general, combination of cytokinins in the medium produce more number of shoots than when they are used alone. The seedling explants and juvenile explants of mature trees responded better

than mature explants with respect to the number of shoots produced. Axillary buds of nodal explants produced more multiple shoots than the apical buds.

Among the various methods to micropropagate plants, enhanced axillary shoot multiplication in culture has become the most important propagation method (Pierik, 1987). This method is advantageous because it is simple and the propagation rate is relatively high (Pierik, 1990). Moreover, it is generally considered to be an *in vitro* culture system with low risk of genetic instability (Pierik 1990; Schoofs, 1992), because the organized meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Shenoy and Vasil, 1992).

## 5. 2 Indirect regeneration

When the organogenesis involves an intermediate callus phase, it is known as indirect regeneration. Callus production is an essential step in the regeneration of adventitious organs (Huetteman and Preece, 1993).

### 5. 2.1 Callogenesis

Callus refers to the actively dividing non-organized tissues developed either at the site of the injury or due to some diseases in an intact plant. In *in vitro* culture under the influence of exogenously supplied hormones, the explant is triggered off a growth sequence in which cell enlargement and cell division predominate to form an unorganized mass of cells.

A multicellular explant is made up of different types of cells. Some are dividing cells and some are non-dividing. But all the cells are derived from a single celled zygote through the process of cell division and cellular differentiation. So, the cells of the explant are present in differentiated state. When such an explant is brought into callus induction medium, most of the cells including the non-dividing mature cells within the explant start to divide and form a mass of undifferentiated callus tissue. The quiescent, vacuolated and highly differentiated cells such as pith, cortex, etc. are also stimulated to divide and proliferate into irregular mass of cells (De, 1997). Any tissue can be changed into callus if cultured on a suitably defined medium under controlled conditions (Nayar *et al*., 1992). This phenomenon is termed as 'dedifferentiation' and the cells of the callus tissue are termed as dedifferentiated cells. When such dedifferentiated cell mass is placed in a medium which is suitable for plant regeneration, the cells again form the whole plant or plant organ. This phenomenon is known as 'redifferentiation' and the coherent capacity of the plant cell to regenerate the whole plant is known as cellular totipotency. Localized groups of meristematic cells that arise in the callus tissue as a result of differentiation are known as meristemoids which may give rise to shoot and root primordia which in turn produce shoots, roots or both. Hormonal control of organ formation is demonstrated by Skoog and Miller (1957), with high auxin-low cytokinin ratio roots develop and with low auxin-high cytokinin ratio shoot buds develop; at intermediate levels undifferentiated callus tissue develops. These cellular masses are formed around the cut edges or on the ruptured surface and gradually, the whole tissue is involved in callus production. Callus increases in mass as the new cells are added by mitosis.

In the present study, two types of calli were formed from different explants of *C. verum*; compact callus from leaves and internodes and organogenic callus from hypocotyls of immature seeds. Production of compact callus was achieved from leaves and internodes in MS medium supplemented with 2 mg/l 2, 4-D. This is in agreement with the result obtained by Rao *et al*. (1997) in clove who got callus production with the addition of 2, 4-D in MS medium.

Similar results were obtained in some monocot plant species where profuse callusing was obtained with 2, 4-D (Henni *et al*., 1997; Myers and Simon, 1998; Zheng and Konzak, 1999; Lauzer *et al.*, 2000). Sometimes 2, 4-D alone is sufficient for callus formation (Martin, 2003). Superiority of 2, 4-D over NAA in inducing callus was also reported in *Averrhoea carambola* (Litz and Conover, 1980). This is in contradictory to the report of Soulange *et al.* (2007) in *C. camphora* where compact calli were produced with BAP in combination with TDZ. At the same time no response was obtained with leaf explants of *C. verum* in the same medium (Soulange *et al*., 2007).

The second type of calli produced in *C. verum* is organogenic produced from the hypocotyl region of immature seeds in MS medium supplemented with 0.5 mg/l Kn. Among the explants used for callusing *viz.*, leaves, internodes and hypocotyls, the most proliferative callus was obtained from hypocotyls of immature seeds in *C. verum*. Narayanaswamy (1990) reported that each tissue type requires a different formulation of growth regulators for callogenesis.

There are many reports on the production of organogenic calli from the seed explants such as *Bauhinia forticata* (Mello, 2000), *Citrus aurantium* (Beloualy,

1991), *Santalum album* (Abdul, 2005), *Ficus religiosa* (Jaiswal and Narayan, 1985), *Pyrus malus* (Rugini and Muganu, 1998), *Dalbergia latifolia* (Rao, 1986), etc.

In contradiction to the individual effect of auxin and cytokinin in *in vitro* callus induction, some reports state that an auxin-cytokinin combination was useful for the induction of callus. For callus induction in clove, tender leaf segments were cultured on MS medium employing several auxins (2, 4-D, IAA, IBA, PCPA) and cytokinins (Kn and BAP) at varying ratios (Rao *et al.*, 1997). Both auxins and cytokinins are required for indefinite growth and cell division in callus cultures (Martin, 2003). This view is also supported by the results obtained in tamarind, another tree spice in which callus induction was obtained from internodes and root segments excised from *in vitro* grown seedlings in MS medium supplemented with 2,4-D and Kn (Rao *et al*., 1997). Rao *et al*. (1997) reported that tender leaf segments of nutmeg, another tree spice, established capability of callus production on MS medium supplemented with 2,4-D and BAP as growth regulators.

In tamarind, callus was produced from the cotyledonary axis of the immature fruits as in the present study. The medium used was MS supplemented with 0.5 mg/l BAP, 0.5 mg/l Kn and 0.1 mg/l IAA and the callus induction was observed in cotyledons and hypocotyl. Rai and Chandra (1987) also reported vigorous callus formation from the cotyledons of *C. verum* in MS medium supplemented with 2, 4-D and BAP than in MS medium supplemented with 2,4-D alone or 2,4-D and Kn. In cinnamon organogenic callus was produced in WPM and in MS medium supplemented with 0.5 mg/l Kn (Mini et al., 1997) whereas, in tamarind alongwith cytokinins, BAP and Kn, there was incorporation of an auxin, IAA (Rao *et al*.,

1997). An increased concentration of auxins facilitated more callus formation (Martin, 2003).

It is generally agreed that an exogenous supply of growth regulators play an important role for the induction of callus. However, the exogenous growth regulator requirement depends strongly on the genotype and endogenous hormone content (Pierik, 1987). Any tissue can be changed into callus if cultured on a suitably defined medium under controlled conditions (Nayar *et al*., 1992). Auxins and cytokinins are effective for callus and organ formation in tissue culture of many plants (Yakauwa *et al.*, 1982).

High frequency callus mediated shoot regeneration can be utilized for the induction of somaclonal variations for the improvement of medicinal plants (Beegum *et al*., 2007). Shoot regeneration through an intermediate callus phase remains the simple way to induce somaclonal variations and, thus, the improvement of the species (Thorpe *et al*., 1991; Salvi *et al*., 2001).

Based on the hormonal type and concentrations used, calli raised in cultures showed variation in morphology, colour and quality. Development of callus with varying morphology with respect to hormones was reported in *Azadirachta indica* (Ramesh and Padhya, 1990) and *Withania somnifera* (Kulkarni and D'Souza, 2000).

### 5.2.2 Caulogenesis

Caulogenesis refers to the formation of adventitious shoots from the callus tissue. The present study was the first report on regeneration of adventitious shoots from callus of *Cinnamomum verum* (Mini *et al*., 1997). Adventitious shoots were

regenerated from nodular and fragile organogenic callus produced from the hypocotyl of immature seeds in WPM supplemented with 3 mg/l BAP and 1 mg/l Kn. WPM was optimal for regeneration of shoots in sweet cherry (Tang *et al.,* 2002; Bhagawat and Lane, 2004), black cherry (Hammatt and Grant, 1998) and *Salix pseudolasiogyne* (Park *et al.,* 2008). Callus regeneration is also reported in trees such as *Ficus religiosa* (Jaiswal and Narayan, 1985), *Dalbergia latifolia* (Rao, 1986), *Malus domestica* (Caboni *et al*., 2000), etc. Organogenesis was also reported from many other tree species such as *Dalbergia sissoo* (Kumar *et al*., 1991), *Acacia nilotica* subsp. *indica* (Dewan *et al*., 1992), *Ophiorrhiza prostrata* (Beegum *et al*., 2007), *Eucalyptus camaldulensis* (Mullins *et al*., 1997), *Sesbania grandifolia* (Detrez *et al*., 1994), *Parkinsonia aculeate* (Mathur and Mukunthakumar, 1992), etc.

Many earlier reports suggest that the compact or fragile nature of the callus is one of the indicators of the fate of the callus and many reports very well explain the regenerative capacity of the fragile callus (Zypman *et al.*, 1997; Pascal *et al*., 1993). So far, regeneration of adventitious shoots from callus in trees coming under spices has not been reported. This is the first report of callus regeneration in *C. verum*. Regeneration of shoots in *C. verum* was initiated with the appearance of small, round greenish structures in the callus. The green pigmentation of the callus indicates the fact that they are capable of photosynthesis, one of the vital metabolic activities of the plant cell. Past reports suggest positive correlation between the percentage of green spots and regeneration capacity of the callus (Amer and Borner, 1997). Malamy *et al*. (1992) speculated that the chlorophyll content of the callus played a role in enhancing the shoot formation process. However, there is a

contradictory report that regenerative potential of the calli is delayed due to the development of green pigments (Attree and Fowke, 1993). Two-three mm long shoot like outgrowth, was observed to develop from one of the calli in Nutmeg, with BAP concentration higher than 1 mg/l, but further development of the outgrowths were not obtained (Rao *et al*., 1997).

The initial phases of shoot regeneration-like formation of light green nodular structures in *Ficus religiosa* is same as that of the results obtained in C. verum in the present study, which subsequently differentiated into shoot buds after 4-5 weeks of subculture and developed further into shoots (Jaiswal and Narayan, 1985). 2,4-D at lower levels gave rise to creamy, friable but nodular callus in clove which upon transfer to medium having still lower levels of 2,4-D developed embryogenic nodules (Rao *et al*., 1997).

The results obtained in the present study for indirect regeneration is in agreement with the results obtained by Bapat and Rao (1984) in *Santalum album* and Mello *et al*. (2000) in *Bauhinia forticata. R*egeneration is achieved in these two plants from callus induced from hypocotyl segments with varying concentrations of cytokinins. It is typical of cytokinin to induce shoot formation in callus as well as in tissue and organ culture (Ashok *et al*., 2002). Similar results in *Rauwolfia* sp. were obtained by Chaturvedi and Mitra, 1974. BAP without auxin supplement induced shoot bud regeneration in *Aegle marmelos* (Varghese *et al*., 1993). The positive effect of cytokinins in shoot regeneration is reported in *Salix pseudolasiogyne* in which Park *et al.* (2008) found that BAP was superior to ZN. The effect of exogenous growth regulation, its concentration and combination strongly influence

*in vitro* morphogenic responses (Centeno *et al*., 1996). BAP promoted green compact callus in clove, but failed to regenerate (Rao *et al*., 1997).

The incorporation of $GA_3$ into the medium was reported to have promotive effect on shoot elongation. Coconut milk was reported to have great advantages in organ, tissue and cell cultures (Steward *et al*., 1969). In *Citrus* cell cultures, coconut milk stimulated embryogenesis in cell cultures (Button and Botha, 1975). This was successfully employed in different plant species (Subhadevi and Nataraja, 1984).

Contradictory to this, extensive efforts with 175 different auxin-cytokinin combinations on callus cultures of *Eucalyptus bancrofti* failed to induce morphogenetic response and generally woody plants show slow and poor responses in culture media (Bonga, 1997). The presence of auxin in the medium inhibited the initiation of shoot from callus (Lin *et al*., 2000) and 2, 4-D strongly antagonizes any organized development in *in vitro* cultures (Hu and Wang, 1983).

Many chemical factors other than the auxin and cytokinin were found to affect morphogenesis (Murashige, 1974). The promotive effect of coconut milk for the induction of regeneration from callus has been widely reported (Steward *et al*., 1969; Button and Botha, 1975; Subhadevi and Nataraja, 1984). The bud formation in the callus of *Ulmus campestris* was reported to be dependent on the proper balance of auxin and meso-inositol (Jacquiot, 1966).

Bonga (1997) stated that the morphogenesis leading to plantlet formation is difficult and who has also stated that the concentration commonly used for callus induction and maintenance are generally inhibitory to morphogenesis and in most

cases, a precise auxin-cytokinin balance is necessary to obtain proper root and shoot primordia. Chaturvedi *et al*. (1974, 1974a) reported that in *Citrus* cultures, organ formation generally followed cessation of unlimited proliferation.

The main factors influencing organogenesis are size of the explant, source of the explant, age of the explant, seasonal variation, oxygen gradient, quality and intensity of light, temperature, plant hormones, agar concentration, pH of the medium, ploidy level and age of the culture (De, 1997). The right choice of the explant is one of the most important factors in obtaining morphogenesis and the morphogenic potential of the calli often varies with the source of the explant (Baker, 1969; Mehra and Mehra, 1974).

Experiments for indirect regeneration were not conducted in *C. cassia.*

## 5.3 Rooting

*In vitro* developed microshoots of *Cinnamomum verum* was rooted in WPM supplemented with different combination of IBA, NAA and charcoal. The best medium for rooting of microshoots of *C. verum* was WPM supplemented with 2 gm/l charcoal. 3-4 roots, 4-5 cm long were produced without any callus formation on the 30$^{th}$ day of subculture. Almost same results were also obtained in WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and 2 gm/l charcoal. Rooting could be achieved in *Cinnamomum cassia* also in the same medium in the present study.

Many researchers used WPM as the basal medium for the induction of roots in plants such as *Garcinia mangostana* (Goh *et al*., 1988), *Cinnamomum camphora* (Babu *et al*., 2003), *Cinnamomum verum* (Sheeja *et al*., 2000), *Syzygium*

*aromaticum* (Thara *et al*., 2000), etc. As in the present study Sheeja *et al.* (2000) also used WPM as the basal medium for the induction in *C. verum*.

MS medium is also found suitable for rooting in many plants such as *Murraya koenigii* (Mathew *et al*, 1999), *Cinnamomum camphora* (Huang *et al*., 1998), *Holostemma ada-kodien* (Martin, 2002) and *Rotula aquatica* (Martin, 2003). Deshpande *et al*. (1998) reported root induction in micro shoots of *Anacardium occidentale* in MS medium. Huang *et al*. (1998) also used MS medium for rooting in *C. camphora*. Many other workers also used MS basal medium for the *in vitro* root induction (Jaiswal and Narayan, 1985; Rao *et al*., 1997; Deshpande *et al*., 1998).

Best medium for rooting in *C. verum* was found to be WPM supplemented with 2 gm/l charcoal. Almost same results were obtained in WPM supplemented with 0.5 mg/l IBA, 0.5 mg/l NAA and charcoal. The best medium for rooting in *C. verum* could also induce roots on shoots of *C. cassia.*

Root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration. The most commonly used auxins are IBA, NAA and IAA. In most of the plant systems, IBA was used for efficient rooting (Loreti *et al*., 1998; Roy *et al*., 1991). As in the present study Sheeja *et al.* (2000) also used a combination of auxins, for the induction of roots in *C. verum*, in which they used IBA alongwith IAA, whereas in the present study a combination of IBA and NAA is used for the induction of roots. However, Sheeja *et al*. (2000) could induce only root initials within 45 days of incubation. Unlike other crops, rooting of microshoots in cinnamon is very poor (26.43%).

In the present study addition of NAA produced more number of roots than IBA. This is in contradictory to the results obtained in *Actinidia deliciosa* by Kumar *et al*. (1997). In most of the plant systems, IBA was used for efficient rooting (Loreti *et al*., 1998; Roy *et al*., 1991). Purohit *et al*. (2002) reported that although auxins are essential for root induction, they may not be required for root growth and rooting is sometimes inhibited in the continuous presence of auxins. The initiation of root primordia often requires a high auxin concentration than the concentration of cytokinin which is required for the outgrowth of shoot primordia. Root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration.

Gupta *et al.* (1983) reported the positive effect of activated charcoal in root induction in *Eucalyptus torelliana.* Activated charcoal plays an important role in root initiation in *in vitro* grown shoots from mature trees of *Eucalyptus torelliana* and *E. camaldulensis*. A positive effect of charcoal in rooting is reported in *Cassia angustifolia* (Siddique and Anis, 2007).

Necessity of auxin in the medium for root induction is well accepted (Rout *et al*., 1999). According to Pasqua *et al*. (1993) rooting induced by IBA treatment occurs by direct origin of active division of cambial cells resulting in the development of a functional root system.

Callus formation was observed at the basal cut ends of *C. verum* in all the media containing IBA and NAA without charcoal. The basal callus formation could be avoided when charcoal was added to the medium. This may be due to the adsorption of excess auxin in the medium by the charcoal. This is in agreement with

the observations of Sheeja *et al.* (2000) in the same plant which produced basal callus in her study also. Increasing IBA concentration favoured callus induction especially from the cut ends of explants (Beegum *et al*., 2007).

*In vitro* developed roots turned to be reddish brown from white through golden yellow. The colour of the roots might be due to the accumulation of secondary metabolites. The positive effect of low salt media in rhizogenesis has been reported earlier in *Cercis canadensis* (Mackay *et al*., 1995) and *Sterculia urens* (Purohit and Dave, 1996; Jain and Babbar, 2000). Low salt concentration may be good for rooting due to the need of small amount of total nitrogen (George and Sherrington, 1984).

In the present study auxin supplementation in the rooting medium without charcoal induced basal callus formation. The cultures with basal callus formation showed less vigorous growth. The massive growth of callus at the base of the shoots may block the translocation of nutrients through the root and as a result leaves become yellow.

Ajith *et al*. (1999) reported that in *Syzygium bacatum* vascular connections with the stem was not established in roots which were differentiated from the basal calli, which in turn led to poor establishment under *ex vitro* conditions. Most of the roots differentiated from the calli did not have vascular connection with the stem and therefore establishment of plants during hardening stage was poor (Kumar and Seeni, 1998). The low survival rate could be due to an anomalous adventitious root system formed by indirect origin via callus phase (Churikui, 1985).

The stimulation of root induction by a combination of auxins has been observed in *Eucalyptus citriodora* (Sita, 1979), *Morus laevigata* (Hossain *et al.*, 1992) and *Artocarpus heterophyllus* (Amin and Jaiswal, 1993). In the present study, a combination of IBA and NAA gave better results than when they are used alone. Huang *et al*. (1998) used MS medium supplemented with NAA for rooting in *C. camphora*.

Application of an auxin was essential for adventitious root formation and no significant difference was observed between effectiveness of the auxins, IBA and NAA (Roy *et al.,* 1990). This is in contradiction to the results obtained on rooting in *C. verum* in the present study where addition of NAA produced more number of roots. Contrary to that, the shoots originated from the jackfruit seedling explants could be rooted without auxins (Roy *et al.,* 1990).

To conclude, combination of auxins, IBA and NAA produced more number of roots in *C. verum* than when they were used separately. Number of roots produced on shoots regenerated from juvenile explants was higher than the number of roots produced on shoots regenerated from mature explants in C. verum.

## 5.4 Somatic embryogenesis

Plant regeneration can be achieved in two ways, through organogenesis or somatic embryogenesis. Somatic embryos develop when somatic cells undergo developmental process similar to the development of zygotic embryos, passing through all stages *viz*., globular, walking stick, torpedo and heart stages of development (Williams and Maheshwaran, 1986). It is possible that plants derived

from somatic embryogenesis are of single cell origin (Haccius, 1978) avoiding the possibility of development of genetic chimeras which are common with those derived from organogenesis. Since non-zygotic embryos have no vascular connection with the maternal tissue, in principle, they can be more easily manipulated than plantlets derived through organogenesis. Stable transformation required that a single cell gives rise to a plant and the ideal transformation scheme is that via somatic embryogenesis (Rao, 1986). Somatic embryogenesis could be used for *Agrobacterium* mediated transformation and regeneration of transgenic plants. The advantage of this system is the availability of compact organized embryos which can be screened, multiplied and germinated easily (McGranahan *et al*., 1990). Somatic embryogenesis in cultured plant cells is the best demonstration of totipotency in plant cells. *In vitro* propagation via somatic embryogenesis is reported in plants such as *Quercus ilex (*Mauri and Manzanera, 2003), *Panax ginseng (*Arya *et al*., 1993), *Theobroma cacoa (*Li *et al*., 2007), *Prunus avium* (Garin *et al*., 1997), *Citrus sinensis* (Carimi *et al*., 1998), etc.

In the present study somatic embryos are induced from the hypocotyl of immature embryos of *Cinnamomum verum* in WPM supplemented with 0.5 mg/l Kn within 30-40 days of inoculation. Almost similar results were obtained in MS medium supplemented with 0.5 mg/l Kn. The present study is the first report on the production of somatic embryos in *Cinnamomum verum.*

In many plants such as *Garcinia indica* (Thencane *et al*., 2006), *Quercus rubra* (Gingas and Lineberger, 1986), *Q. suber* (Bueno *et al*., 1992), *Q. variabilis* (Kim *et al*., 1995) and in *Eucalyptus citriodora* (Muralidharan and Mascarenhas,

1987) immature zygotic embryos were used as the explants for the induction of somatic embryos. Only immature zygotic embryos were susceptible to somatic embryo induction in *Quercus ilex* (Mauri and Manzanera, 2003) as in *C. verum* in the present study.

Basal media like WPM and MS were tried for the induction of somatic embryos from immature seeds in *C. verum*. Somatic embryos could be induced in both of the basal media tried and comparatively, MS medium showed better responses. Li *et al.* (2007) also induced somatic embryogenesis in *Cinnamomum camphora* in MS medium

Murthy and Saxena (1998) could achieve induction of somatic embryos using MS medium in *Azadirachta indica*. MS basal medium is used for the induction of somatic embryos in *Acacia mangium* (Xie and Hong, 2001), *Euphorbia pulcherrima* (Jasrai *et al*., 2003), *Citrus sinensis* (Carimi *et al*., 1998), etc.

WPM is also used as the basal medium for the induction of direct embryogenesis from the zygotic embryos in Eastern black walnut (an ornamental yellow foliage tree, Neuman *et al*., 1993) and *Picea glauca* (Ellis *et al*., 1991). Neuman *et al*. (1993) reported induction of somatic embryos in WPM in *Juglans nigra.*

Trigiano *et al*. (1988) have reported the induction of somatic embryos in *Cercis canadensis* in SH (Schenk and Hildebrandt, 1972) medium containing 100 mg/1 myo-inositol, 1 mg/l thiamine and 1-5 mg/l 2, 4-D. Nair and Gupta (2006) also

reported the production of somatic embryos in SH (Schenk and Hildebrandt, 1972) medium in Piper nigrum without any growth regulators.

Ammirato (1987) and Narayanaswamy (1990) reported that the nutrient media used for initial proliferation of the tissue played a vital role in inducing embryogenesis. Low concentration of Kn was sufficient for the induction of somatic embryos in *C. verum*. This might be due to the presence of adequate endogenous auxin content in the explants to balance with the externally supplied cytokinin (0.5 mg/l). Cytokinins have an important role in promoting embryogenesis by stimulating divisions in the proembryogenic cells (Fujimura and Komamine, 1980). The formation of distinct root and shoot poles may be attributed to the presence of cytokinins, since it has been reported that cytokinins such as BAP stimulate shoot and tap root formation (Chang and Bamboos, 1991). Cytokinins in general are known to foster somatic embryo development. Promotory effect of BAP in somatic embryo maturation is well documented (Sahrawat and Chand, 2001). In contrast to this, cytokinins (BAP and kinetin) inhibited somatic embryogenesis in *Panax ginseng* (Arya *et al*., 1993).

The positive effect of TDZ in the induction of somatic embryogenesis is documented in *Juglans nigra* (Neuman *et al*., 1993) and in *Azadirachta indica*. (Ammirato, 1987; Murthy and Saxena, 1998) TDZ treatments at 2.5, 5 and 10 µM, increased the germination to more than 75% compared to 62% under control conditions.

Most protocols for somatic embryogenesis use a strong auxin such as 2, 4-D or a combination of auxin-cytokinin concentration in the primary culture medium to

support both cell proliferation and induction of embryogenesis (Chalupa, 1987; Kurten *et al*., 1990). Attree and Fowke (1991) reported induction of somatic embryos on a medium containing a combination of auxin and cytokinin. The importance of auxin in the induction of somatic embryos was reported by many workers (Ammirato, 1983, 1987; Das *et al*., 1997; Evans *et al*., 1983; Finner, 1994). According to Sofiari *et al*. (1997), NAA is more efficient than 2, 4-D for inducing somatic embryogenesis. Michalzuok *et al*. (1992) suggested that exogenously applied synthetic auxin stimulates the accumulation of endogenous IAA. This maintains the proliferation state of the callus and prevents subsequent embryo formation. Transferring the tissue to an auxin free medium result in the decline of total IAA levels, too low to set up an internal gradient for initiation and maintenance of polarized growth and subsequent embryo development. Ammirato (1987) is of the opinion that frequency of the somatic embryo induction is dependent on the type of auxins, explants used and amount of ammonium or sucrose in the medium.

However, auxins, 2,4-D, NAA and IAA at 1mg/l were effective in inducing secondary and tertiary somatic embryos in many species, which proliferated directly from the apical or cotyledonary portions of the primary somatic embryos (Arya *et al* ., 1993).

The formation of secondary somatic embryos from primary somatic embryos explains the totipotency of each cell in primary somatic embryos. As an experimental system it has certain advantages compared to primary somatic embryogenesis such as very high multiplication rate, independence of an explant source and repeatability. Additionally embryogenicity can be maintained for

prolonged periods of time by repeated cycles of secondary somatic embryogenesis (Nair and Gupta, 2006). Secondary somatic embryogenesis have also been reported in *Quercus ilex* (Mauri and Manzanera, 2003), *Prunus avium* (Garin *et al*., 1997), *Panax ginseng* (Arya *et al*., 1993), etc.

Skoog and Miller (1957) showed that the growth regulator concentrations in the culture medium are critical to the control of growth and morphogenesis and influence the developmental stages of somatic embryogenesis. The requirement, according to them may vary with the plant species and varieties. On the other hand, supply of exogenous growth regulators was not necessary for the induction of somatic embryos in *Quercus rubra* (Gingas and Lineberger, 1986). This is in contradiction to the results obtained in the present study wherein presence cytokinins were essential for the maturation and germination of somatic embryos.

Initially the embryos produced in *C. verum* were white, disc shaped or globular. When these embryos were subcultured to the same medium, secondary somatic embryos were produced in clusters. Secondary somatic embryogenesis is a process whereby new somatic embryos are initiated from originally formed somatic embryos or primary somatic embryos. When these somatic embryos were transferred to the maturation and regeneration medium *i.e.*, WPM supplemented with 3 mg/l BAP and 1 mg/l Kn, multiplication of the somatic embryos took place producing secondary somatic embryos. Hormonal balance could be among the determined factors in directing the course of somatic embryogenesis (Mujib *et al*., 1996) and the three stages of somatic embryogenesis (initiation, maturation and germination) often require sequential changes in the medium composition. After 2-3

subcultures in the same regeneration medium within 40-45 days of the production of somatic embryos, germination of embryos into plantlets started. The synergistic effect caused by both BAP and Kn might have caused for the germination and maturation of the somatic embryos. When these plantlets were transferred to the basal WPM, shoot and root elongation took place. However, the multiplication rate was low in the basal medium without the addition of growth regulators. Germination of embryos was also not observed without the addition of cytokinins in the medium in *C. verum.*

In the present study, in *C. verum*, high sucrose concentration favoured the maturation and germination of somatic embryos. This is in agreement with the results obtained by Wald et al. (1989) in *Mangifera indica* where high sucrose concentration in the medium favoured somatic embryo maturation and *Picea* sp. (Tremblay and Tremblay, 1991). Sucrose functions as both a carbon source and an osmoticum. Sucrose in high concentrations resulted in high osmolarity in maturation medium (Tremblay and Tremblay, 1995), which suppressed precocious germination and enhanced embryo maturation (Alfaro et al., 1996). However, negative effect of high sucrose concentration in plant tissues also was reported (Tremblay and Tremblay, 1995).

Evans *et al*. (1983) reported the benefit of reduced nitrogen in addition to nitrate nitrogen for induction of somatic embryogenesis. Coconut milk added to basal medium, enhanced embryo production in many varieties of mango (Jana *et al*., 1994). $GA_3$ was also found essential along with 2, 4-D in inducing somatic embryogenesis as gibberellins promoted cell division and elongation in tissue

cultures (Danielle and Williams, 1989). The enhancing effects of $GA_3$ in cell division, cell elongation and initiation of somatic embryogenesis were also reported by Danielle and Williams (1989), Nitsch and Nitsch (1969), Firoozabady and Boer (1993) and Litz *et al*. (1982) on *in vitro* cultured plant tissues.

In conclusion it is generally seen that immature tissues respond well than mature tissues and immature seeds are good explants for the induction of somatic embryos. Hay and Charest (1999) stated that the successful induction of embryogenic tissue occurs only from juvenile tissue.

## 5.5 Hardening

During micropropagation, plantlets are grown as heterotrophs in special conditions like high humidity, low temperature, etc. which result in the poor development of cuticle and improper vascular connections between root and stem. Hence after *ex vitro* transfer, the plants are easily prone to be impaired by sudden changes in environmental conditions and need a period of acclimatization to correct the abnormalities (Pospisilova *et al*., 1999).

The decrease in wax deposition due to high humidity during the *in vitro* culture is an important factor contributing to excessive water loss when they are transferred to the green house. *In vitro* induced roots are rarely functional, usually without root hairs. Hence micropropagated plants need 2-4 weeks of acclimatization (Preece and Sutter, 1991). Uncontrolled water loss through the leaf cuticle causes poor survival of the regenerated plants when transplanted to the field (Grout and

Aston, 1978). The substrata used for initial transplanting of the micropropagated plants are vermiculite, soilrite, saw dust, coarse sand, etc.

The *in vitro* developed *Cinnamomum verum* plantlets were initially transplanted to soilrite in tea cups with 80% success and later transferred to polybags containing soil, soilrite and sand in equal proportion with 90% success in the green house. After 4-5 weeks of acclimatization, the plantlets could be transplanted to the field with 100 % success. Hoagland's solution (Epstein, 1972) was used to nourish the plantlets during the hardening stage. Trolinder and Goodin (1987) watered *in vitro* raised cotton plantlets with ¼ strength Hoagland's solution (Hoagland, 1920). However, Sheeja *et al*. (2000) have reported only 43% establishment of *C. verum* in soilrite. Hardening of regenerated plantlets for successful field transfer is considered to be a major obstacle in clonal micro-propagation of Cinnamon (Chaturani and Subasinghe, 2006).

*In vitro* developed *C. camphora* plantlets could be established with 70% success on initial hardening in the incubation room. The plantlets could be established with over 90% success under the nursery conditions. Hardened plants have been planted in the field and established with 100% success (Babu *et al.*, 2003) in *C. camphora.*

Mathew *et al.* (1999) successfully transferred *in vitro* grown *Murraya koenigii* plantlets to the field with 80% survival. The *in vitro* regenerants of *Morus laevigata* were transferred to the field after acclimatization and 80% of the transplanted plants were successfully resumed growth (Hossain *et al.*, 1992). Eeswara *et al.* (1998) has reported transplantation of *in vitro* grown plantlets of

*Azadirachta indica* with 100% establishment in the field. Martin (2002) transferred *in vitro* grown plantlets of *Holostemma ada-kodien* with 90% survival on field transfer wherein the initial transplantation was in sterile soilrite and sand. The plantlets obtained through somatic embryogenesis were transferred into the soil mixed with biogas slurry in the ratio 1:1 and the rate of survival was 100% in *Citrus macroptera* (Miah *et al.*, 2002). Chandrasekhar *et al.* (2006) has reported field transfer of *Tylophora indica* plantlets developed through somatic embryogenesis with 70% success.

## 5.6 Molecular Characterisation

In 1981, Larkin and Scowcroft coined a general term, somaclonal variation for plant variants derived from any form of cell or tissue cultures. Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions (Larkin and Scowcroft, 1981; Muller *et al*., 1990; Masoud *et al*., 2008). Somaclonal variation may be an additional tool for crop improvement rather than an interesting scientific phenomenon (Evans and Sharp, 1986; Evans, 1989; Bajaj, 1990). The term ‘somaclonal variation’ is used to refer to the phenotypic and genotypic variations of both qualitative and quantitative traits that occur in plants regenerated from cell and tissue cultures (Jain 2001). To commercially propagate a crop by tissue culture and genetic transformation, it is important to know the genetic stability in the propagules produced (Jin *et al.*, 2008)

Theoretically micropropagated plants are supposed to maintain clonal fidelity (Ahuja, 1987; Wang and Charles, 1991; Rahman and Rajora, 2001).

However, there are numerous reports on the incidence of somaclonal variation among micropropagated plants (Maynard *et al*., 1991; Moore *et al*., 1992; Wakasa, 1979; Schoofs, 1992; Swartz, 1990; Martinelli, 1992; D'Amato, 1978; Skirvin 1978). For example, reports have indicated the occurrence of somaclonal variation in micropropagated bananas and plantains (Schoofs, 1992) raised through meristem culture. Various kinds of leaf chlorosis coupled with multiple apexing and dwarfing in strawberry (Martinelli, 1992) and spininess and albino strips in pineapple ( Moore *et al*., 1992 ) were some of the common off-type features observed to occur in micropropagated plants. Due to somaclonal variation generated during *in vitro* culture, it cannot be certified that the genetic organization of tissue culture derived material is identical to the explant from which it originates (Corniquel and Mercier, 1994).

The best test for assessing somaclonal variation is to fruit out the plants and conduct an extensive horticultural evaluation, which is unfortunately a long term endeavor with a woody fruit crop (Grosser *et al*., 1996). Molecular analysis of somaclonal variation provides an opportunity to eliminate influence of environmental factors and to provide a quantitative measure of somaclonal variability (Veilleux and Johnson, 1998).

Several approaches have been applied to identify variants among micropropagated plants. They are phenotypic variation (Vuylsteke *et al*., 1998), karyotypic analysis of metaphase chromosomes (Jha *et al*., 1992) and biochemical analysis (Damasco *et al*., 1996). The most important part of any *in vitro* propagation system is mass multiplication of plantlets that are genetically similar and

phenotypically uniform, otherwise, the advantage of desirable characters in elite clones may not be achieved.

The use of molecular markers as means of evaluating genetic stability of *in vitro* somaclonal variation with greater precision and less effort than cytological or morphological analysis (Polanco and Ruiz, 2002). Different molecular techniques can be employed to detect somaclonal variability (Henry, 1998). Among the different DNA markers available, restriction fragment length polymorphism (RFLP) is considered to be a reliable technique for detecting variability among *in vitro* raised plants (Valles *et al.*, 1993). However, randomly amplified polymorphic DNA (RAPD) analysis is often preferred because of reduced complexity. RAPD has been used for a number of crop species to detect genetic diversity among micropropagated plants (Isabel *et al.*, 1993; Valles *et al.*, 1993; Rani *et al*., 1995; Damasco *et al.*, 1996). Analysis of somaclonal variation at the nuclear genome level using RAPDs has advantages over RFLPs, as a single primer produces several loci, covering a large portion of the genome (Tulseiram *et al.*, 1992).

Allelic variation among individuals is detected as the presence or absence of the amplification product, visualized as a band after PCR and electrophoresis (Welsh and McClelland, 1990). Among these Random Amplified Polymorphic DNA (RAPD) markers, despite their drawbacks (Hedrick, 1992) are an efficient technique to assess genetic stability of *in vitro* regenerated conifers including *C. libani* (Isabel *et al*., 1996; De verno *et al*., 1999; Piola *et al*., 1999; Tang *et al*, 2001). Recently the RAPD technique has been reported to be a powerful tool to analyse variation among *in vitro* regenerated plants. (Isabel *et al*., 1993; Rani *et al*., 1995; Shoyama *et al*., 1997; Goto *et al*., 1998).

*Discussion*

In the present study, analysis of RAPD data of mother plant, five directly regenerated plants and five plants regenerated through somatic embryogenesis was done. The similarity matrix obtained based on Nei and Li (1979) method shows the coefficient of similarity value ranging from 0.41 to 0.81 with a mean value of 0.625. The observed value signifies the extent of genetic variation in these plants. Among the 45 primers tried, 20 primers produced 153 scorable bands. The maximum number of bands produced was 12 in primers OPA-16, OPA-10, OPD-20, OPD-13 and OPB-20. The minimum number of bands produced was 6 in OPE-19. The size of amplified products ranged from 0.35 kb to 2.6 kb. Cluster analysis based on UPGMA reveals two major groups. Cluster A comprises of 6 plants (mother plant and 5 directly regenerated plants) and has coefficient of similarity value ranging from 0.50 – 0.81 with a mean value of 0.65.

Cluster B with the remaining 5 plants (developed through somatic embryogenesis) of *C. verum* is comparatively more diverse than cluster A. The average coefficient similarity value of 0.53 with a distribution range from 0.43 to 0.66 reveals the extend of diversity in this cluster.

The RAPD analysis was used to find out genetic relationship among the progenies and mother plant in many species. Many plants such as *Robinia pseudoacacia* (Bindiya and Kanwar, 2003), *Melia azedarach* (Olmos *et al.*, 2002), *Populus deltoids* (Rani *et al.*, 1995) *Musa* spp. (Hwang and Ko, 1987; Stover, 1987) *Sorghum bicolor* Cai *et al.* (1990) showed polymorphism among micropropagated plants in RAPD analysis. RAPD markers have also been able to detect somaclonal variation among micropropagated plants of *Picea glauca* (Isabel *et al.*, 1996), peach

(Hashmi *et al.*, 1997), sugarcane (Taylor *et al*., 1995) and *Populus deltoides* clone L.34 (Rani *et al*., 1995).

In contrast to this, Gangopadhyay *et al.* (2004) has ascertained the genetic fidelity of the *in vitro* developed plantlets by using RAPD profiling. There was no difference in RAPD profiles between mother and tissue cultured plants. In *Curcuma longa* also, micropropagated plants and mother plants were compared by RAPD analysis which revealed monomorphic bands in all *in vitro* grown plants which confirmed genetic uniformity among somaclones of *C. longa.* Nanda *et al. (2004)* compared *in vitro* grown plantlets from mature nodal segments of *Acacia mangium* with that of mother plant using RAPD markers and no variation was detected among the micropropagated plants.

The RAPD results of *Robinia pseudoacacia* (Bindiya and Kanwar, 2003) showed 30 % variation among mother plant and micropropagated plants. Here 286 bands were produced from 19 random decamer primers. The number of bands produced per primer ranged from 10 to 32. The size of amplified products varied from 150 bp to 3484 bp. Similarity indices in RAPD analysis among mother plant and micropropagated plants of *Robinia pseudoacacia* ranged from 0.86 to 0.96 (Bindiya and Kanwar, 2003) whereas in *C. verum* in the present study it ranged from 0.50 – 0.81 with a mean value of 0.65.

Williams *et al.* (1990) suggested that the polymorphism in amplified bands might result from changes in either the sequence of the primer binding site (e.g. point mutations) or change which alter the size or prevent successful amplification of target DNA (e.g. insertions, deletions, inversions). On similar findings, Laia *et al*. (2000) reported a total of 62 amplification products from 15 random, 10-mer

primers out of which 39 products were polymorphic in *Eucalyptus sp.* Similarly Goto *et al* (1998) obtained 134 scorable bands using 30 decamer primers in micropropagated shoots of *Pinus thunburghii* and the number of bands for each primer varied from 2 to 7. Olmos *et al*. (2002) conducted RAPD analysis in micropropagated plants of *Melia azedarach* who used 10 primers in their study which detected a total of 46 polymorphic bands. The total diversity obtained by using these primers ranged from 0.62 to 0.89 with a mean value of 0.79. In Banana also (*Musa spp*), micropropagation using isolated buds and meristems has resulted in the production of higher than expected numbers of somaclonal variants with frequencies ranging from 3 to 25% (Hwang and Ko, 1987; Stover, 1987). Some other trees, shrubs and herbs showing variations among mother plant and micropropagated plantlets produced through direct regeneration is listed in Tables 103 and 104.

Table 103. Some trees and shrubs showing variations among mother plant and micropropagated plants produced through direct regeneration

| **Sl. No.** | **Plant species** | **Reference** | **Percentage of variation** |
|---|---|---|---|
| 1 | *Populus deltoids* | Rani *et al.* (1995) | 26% |
| 2 | *Robinia pseudoacacia* | Bindiya and Kanwar (2003) | 30% |
| 3 | *Robinia pseudoacacia* | Major *et al. (1998)* | • |
| 4 | *Pinus thunberghii* | Goto *et al.(1998)* | • |
| 5 | Peach | Hashmi *et al.* (1997) | • |
| 6 | Eucalyptus | Laia *et al.* (2000) | 39% |
| 7 | *Populus tremuloides* | Rahman and Rajora (2001) | • |
| 8 | *Melia azadarach* | Olmos *et al.* (2002) | • |
| 9 | *Piper longum* | Parani *et al.* (1997) | 10% |
| 10 | *Blackberries* | Swartz *et al.* (1983) | • |

- Percentage is not specified

Table 104. Some herbs showing variations among mother plant and micropropagated plantlets produced through direct regeneration

| Sl. No. | Plant species | Reference | Percentage of variation |
|---|---|---|---|
| 1 | Strawberry (Meristem culture) | Swartz *et al.* (1981); Anderson *et al.* (1982); Martinelli (1992) | • |
| 2 | Bananas and Plantains | Hwang (1986); Hwang and Ko (1987); Vuylsteke *et al.* (1998); Krikorian (1989); Schoofs (1992) | 20% some times upto 90 % |
| 3 | *Beta vulgaris* | Munthali *et al.* (1996) | • |
| 4 | Pineapple | Wakasa (1979) | • |
| 5 | Sugar beet | Moore *et al.* (1992) | • |
| 6 | *Lotus corniculatus* (Nodal cultures) | Orshinsky and Tomes (1984) | • |
| 7 | Celery | Orton (1983,1985); Browers and Orton (1982) | • |
| 8 | *Dendrobium* (meristem culture) | Vajraabhaya (1977) | • |

- Percentage is not specified

In the present study plantlets regenerated through somatic embryogenesis showed more variations than directly regenerated plants when compared with mother plant because somatic embryos were formed in *C. verum* from hypocotyl of immature seeds which were produced after cross pollination.

Ryan *et al.* (1987) found profound variation in many agronomic and quality traits among the somatic embryogenic regenerants of three hexaploid wheat cultivars Mohamand and Nabors (1990). In one of the most often cited examples of variations

among somatic embryo derived micropropagated plants is that of oil palm (Corley *et al*., 1986; Paranjothy *et al.*, 1990). These variations were noticed only after many years of transplantation in field conditions, in frequencies ranging 0-90% depending on the genotype. Somaclonal variations have reported in somatic embryo derived regenerants of sugarcane (Taylor *et al.*, 1995; Ahloowalia and Maretzki, 1983). Extensive heritable phenotypic variations have been reported in somatic embryo-derived regenerants of maize (Earle and Kuehnle, 1990; Philips *et al.*, 1988). A high frequency of morphological variants has been reported in sandal wood regenerants derived through somatic embryogenesis (Rao *et al.*, 1984).

In contradiction to the above results, Jayanthi and Mandal (2001) reported homogeneity among mother plant and 14 somatic embryo derived plants of *Tylophora indica.* They used 18 primers and all of them produced monomorphic bands. Several reports confirm the genetic integrity of somatic embryo regenerated plants such as *Pennisetum purpureum* (Haydu and Vasil, 1981), *Panicum maximum* (Hanna *et al*., 1989) and *Lolium multiflora* (Dale *et al*., 1981).

Induction of somaclonal variation has been successful in identifying potential new varieties in different crops such as wheat (Gao *et al*., 1991) and citrus (Grosser *et al.*, 1996). Variations in the shape and colour of flowers have, in some cases, resulted in the selection of novel flower types, as in *Dendrobium* (Vajraabhaya, 1977). In addition, somaclonal variation is likely to be a reflection of response to cellular stress in other situations as well. Therefore, understanding the mechanism of tissue culture variation will be useful in defining cellular mechanisms

acting in the process of evolution and in elucidating the mechanism by which plants respond to stress.

The cause of tissue culture induced variation is not fully understood and therefore, it cannot be controlled or predicted (Orbovic *et al*., 2008). However Hammerschlag (1992) suggested that somaclonal variation among tissue cultured plants can be either eliminated or minimized, if special efforts are made to distinguish between axillary and adventitious shoots that are produced during *in vitro* propagation and eliminating the inferior adventitious shoots.

Some of the factors that influence origin and frequency of somaclonal variation are plant genotype, ploidy level, explant tissue source, media components, number of subcultures, age of the donor plants and tissue culture procedure (Veilleux and Johnson, 1998). At the molecular level, variations in tissue culture – derived plants arise from changes in chromosome number or structure, or from more subtle changes in the DNA (Orbovic *et al.*, 2008). Various reasons have been assigned to the occurrence of variability among *in vitro* propagated plants. Smith (1998) reviewed the factors contributing to this variation and divided these into two, an intrinsic factor which largely depends on the genetic stability of the explant and an extrinsic factor depending on culture media and particularly growth regulators. Even using the same explant, the extent of this variation can be influenced by composition of culture media, culture periods, and the level of tissue organization during culture (Smith 1998). The incidence of somaclonal variation depends on the explant origin and regeneration method as well as the regenerant source (Ahuja, 1998).

It is well known that genetic variations occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of regenerated plants. The cause of variation is mostly attributed to changes in the chromosome number and structure. Variants selected in tissue cultures have been referred to as calliclones (from callus culture, Skirvin, 1978) or protoclones (from protoplast cultures, Shepard *et al*., 1980). Evidence from herbaceous species suggest, that somaclones are more likely in callus cultures. Also culture duration or repeated subculturing contribute to genetic instability (Fry *et al*., 1997).

The variations observed in the RAPD pattern may be due to different causes including loss/gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke *et al.*, 1991). The DNA of higher plants contains 5 – methylcytosine ($m^5C$) as up to 30% of the total of cytosine residues (Finnegan *et al.*, 1998). Both naturally occurring and induced differentiation / dedifferentiation processes and several environmental stresses, initiate perturbations in the level and distribution of DNA methylation (Jaligot *et al.*, 2002). These same conditions, which favour epigenetic instability, also occur during micropropagation processes and often result in disruptions of clonal fidelity in the micropropagated progenies (Lambe *et al*., 1997; Jaligot *et al*., 2000)

The positive correlation between *in vitro* culture, age and incidence of somaclonal variation is well documented in many plant taxa, as in wheat (*Hartmann,* 1989), *Brassica campestris* (Shirzadegan *et al*., 1991) and oat (McCoy *et al*., 1982). One of the reasons given for such phenomenon has been attributed to

the delayed activation of mobile genetic elements such as retroposons (Peschke *et al.*, 1987; Hirochika *et al.*, 1996).

RAPD markers for genetic stability analysis were used for *Quercus suber* (Gallego *et al*., 1997) and *Q. serrata* (Thakur *et al*., 1999; Ishii *et al*., 1999) but no aberrations were detected in the banding pattern. However, several studies in conifers indicated that these types of markers my not be ideal for assessing the mutability of the cell lines and derived plants, as compared to markers such as simple sequence repeats (SSRs) or microsatellites (Isabel *et al*., 1996; Fourre *et al*., 1997).

The results obtained in the study of Bindiya and Kanwar (2003) suggested that RAPD technique can be successfully used to assess genetic variations in micropropagated plants. It also demonstrates that genetic integrity of micropropagated plants should invariably be confirmed before transfer of hardened plants to field. Bindiya and Kanwar (2003), has demonstrated that somaclonal variations arose even from axillary bud explants of *Robinia pseudoacacia*, which is a tree legume.

Any genetic change induced by *in vitro* conditions of tissue culture is expected to generate stable plants carrying interesting heritable traits (Veilleux and Johnson, 1998).

# 6. SUMMARY AND CONCLUSION

Cinnamon (*Cinnamomum verum* Bercht. and Presl), Cassia (*C. cassia* Blume) and their products have long been recognized for their sweet and delicate flavours and are widely used in food, pharmaceutical, soap and cosmetic industries. Cinnamon bark oil contains no less than 90 identified compounds and over 50 very minute unidentified compounds. The mild and mellow aroma of both cinnamon bark and cassia oils is due to the synergistic effect of all these compounds. Food technologists and food manufacturers find it very difficult to imitate them completely with synthetic substitutes. Although cinnamaldehyde gives a very crude imitation of either cinnamon or cassia, it is nowhere near the natural flavour. Now a days the synthetic essential oils are rejected by leading food manufacturers. There has always been a demand for natural cinnamon and cassia. Since these plants are naturally cross pollinated, variation arises among the progenies.

According to the statistics of the Spices Board, Govt. of India, the area under cinnamon and cassia cultivation in Kerala is 326 hectares during 2005-2006. The import of cinnamon and cassia to India was 11313.5 t during 2005-2006. The export of cinnamon and cassia from India during 2006-2007 was 304.9 t, and cinnamon and cassia in powder form was 406.5 t. The export of cinnamon and cassia oil was 9.4 t and cinnamon and cassia oleoresin was 17.9 t during 2006-2007. The import of cinnamon and cassia oil to India was 19.7 t during 2006-2007.

The shortage of adequate planting material of elite trees is a major problem for cinnamon propagation. A few dozen elite cultivars obtained through selection possess considerable market relevance. However, conventional breeding methods are extremely time consuming because they depend on cross pollination, seed germination and selection as well as vegetative regeneration. The major drawback of seed propagation is that, cinnamon being a cross-pollinated plant, exhibits wide variability in yield, quality of produce and oil content and other morphological characteristics.

Modern plant biotechnology has adequate potential to reduce the time needed for traditional breeding programmes and to multiply adequate propagules that will have the same genetic make up as the mother plant within a short time. Thus it helps in maintaining uniformity within the population.

Major emphasis in the present study was given in multiplying the commonly used cinnamon, *Cinnamomum verum*, while *C. cassia* has been used only for preliminary experimentation in order to find its *in vitro* responses for comparison.

The present investigation is an attempt in this direction with the following objectives:

1. Standardization of micropropagation protocols for *Cinnamomum verum* and *Cinnamomum cassia.*
2. To develop callus regeneration protocol for *Cinnamomum verum*.
3. To develop a protocol for direct somatic embryogenesis in *Cinnamomum verum*

4. To standardize the hardening of *in vitro* developed plantlets and field establishment.

5. To compare the genotype of the regenerated plants with that of the mother plant using RAPD markers.

Vegetative buds in shoot tips and nodal segments were used to initiate cultures in *Cinnamomum verum*. Bud break and shoot induction was achieved in all the 10 media combinations tried but the percentage of response was different in various combinations and concentrations of growth regulators. The highest percentage was achieved in WPM supplemented with 2 mg/l BAP and 0.5 mg/l Kn. BAP was found to be superior to Kn for bud break and shoot induction.

Explants of three maturity levels were cultured for direct regeneration as follows:

1) Explants from 2-3 year old seedlings

2) Juvenile explants from 7-8 year old mature trees and

3) Mature explants from mature trees.

Twenty five media combinations were tried for multiple shoot induction. The best medium for multiple shoot induction was found to be WPM supplemented with 3 mg/l BAP and 1 mg/l Kn in all the three types of explants. The highest mean of multiple shoots produced was 17.05 in seedlings, 14.48 in juvenile explants of mature trees and 12.05 in mature explants of mature trees.

In the case of explant responses, the highest percentage was in seedling explants (68%). The juvenile explant of mature plants showed a higher percentage

of bud break (60%) than mature explants of mature trees (48%). Bud break was observed within 6-12 days in seedling explants, 10-17 days in juvenile explants of mature plants and 12-22 days in mature explants of mature plants.

The data on multiple shoot induction from seedling explants (Experiment-1), juvenile explants of mature trees (Experiment-2) and mature explants (Experiment-3) were subjected to standard ANOVA (Analysis of Variance) and Post Hoc (Duncan) test using the SPSS (16.0) software to study the variations and significance of different experiments. The data were subjected to square root transformation before the statistical analysis.

The Standard Deviation (SD), Standard Error (SE) and Coefficient of Variation (CV) were also computed for the different sets of experiments. All the three experiments were repeated twice with seven replicates.

When the best medium combinations for initial shoot induction and multiple shoot induction in *C. verum* were tried with *Cinnamomum cassia*, multiple shoots were produced.

WPM and MS medium supplemented with different concentrations of 2,4-D at a range of 1-3 mg/l were tried for callus induction from leaves and internodes. Of the 6 combinations tried, MS medium supplemented with 2 mg/l 2,4-D produced maximum amount of callus. Organogenic calli and somatic embryos were produced from the hypocotyls of 4-5 month old immature seeds in WPM and MS medium supplemented with BAP and Kn at a range of 0.5-1 mg/l. The best medium was

found to be MS supplemented with 0.5 mg/l Kn in which about 30-35% of cultures responded.

The compact calli produced from internodes and organogenic calli produced from immature seeds were subcultured for regeneration. Of the two types of calli, only organogenic calli could induce adventitious buds which develop into adventitious shoots in WPM supplemented with 3 mg/l BAP and 1 mg/l Kinetin.

The micro shoots of *Cinnamomum verum* produced *in vitro* through direct and indirect regeneration were transferred to rooting medium for the induction of roots. Shoots of 3-4 cm long were taken for root induction. The shoots developed from juvenile and mature explants were transferred to rooting medium to study the difference in response of rooting. The number and length of the roots varied depending on the combinations of medium and concentrations of auxins and maturity of the explants taken.

Basal WPM and WPM supplemented with different concentrations of IBA, NAA and activated charcoal were used for rhizogenesis. Roots were induced in all the media tried. Of the 14 different media combinations tried, the best medium for rooting was found to be WPM supplemented with 2 gm/l charcoal for shoots regenerated from juvenile as well as mature explants. WPM supplemented with 0.5 mg/l IBA , 0.5 mg/l NAA and 2 gm /l charcoal also gave good results in rooting. Root induction is also achieved in *C. cassia.*

The somatic embryos induced *in vitro* from the hypocotyl of immature (4-5 month old) seeds of *Cinnamomum verum* were cultured on different combinations

of BAP and Kn at a range of 0.5-4 mg/l along with basal WPM. Initially the embryos produced were creamy white in colour with globular shape. The embryos were produced in clusters and easily separable. The number of somatic embryos ranges from 75-100 in each culture tube. On subculture, alongwith maturation of somatic embryos, secondary somatic embryos were also produced in *C. verum.*

*In vitro* developed plantlets of *C. verum* were hardened and established in the field after a hardening period of 4-5 weeks. The plantlets with 4-5 leaves were taken out carefully without damaging the roots. The old medium on the plantlet was washed off and the plantlets were immersed in fungicide solution (3%) for 5-10 minutes. The fungicide treated plantlets were transplanted to soilrite in tea cups and kept in green house. The humidity was maintained by covering the cups with poly bags for two weeks. After one week, holes were made in the poly bags to reduce the humidity. The plantlets along with soilrite were transferred after two weeks of transplantation to potting mixture containing garden soil, soilrite and sand in equal proportion in poly bags and kept in the green house. After the emergence of 3-4 new leaves the plantlets were transplanted in the field.

Analysis of RAPD data of mother plant, five directly regenerated plants and five plants regenerated through somatic embryogenesis was done, which showed low levels of genetic variation. Among the 45 primers tried, 20 primers which repeatedly produced scorable amplifications were taken into consideration for further analysis. 20 primers studied amplified a total of 191 DNA fragments. The levels of polymorphism were different with different primers among these species. Primers A16, A10, D20, D13, and B20 produced maximum numbers of amplified

products (12) and E19 produced the least (6). Primer A1, A7, A8, A9, D3, D4, D8, D16 and B20 displayed 100% polymorphism for the products generated. On an average, the primers produced 9.2 products and 7.1 polymorphism each. The number of amplified products from each plant varies significantly for all the 20 primers. The size of the amplified products ranged from 0.35 kb to 2.6 kb.

The similarity matrix obtained based on Nei and Li method shows the coefficient of similarity value ranging from 0.41 to 0.81 with a mean value of 0.625. The observed value signifies the extent of genetic variation in these plants. Cluster analysis based on UPGMA reveals 2 major groups. Cluster A comprises of 6 plants (mother plant and 5 directly regenerated plants) and has coefficient of similarity value ranging from 0.50 – 0.81 with a mean value of 0.65.

Cluster B with the remaining 5 (somatic embryonically generated plants) plants of the genus is comparatively more diverse than cluster A. The average coefficient similarity value of 0.53 with a distribution range from 0.43 to 0.66 reveals the extent of diversity in this cluster.

Contamination and exudation of phenolic substances were major problems for the establishment of cultures in *C. verum and C. cassia*. In general, combination of cytokinins in the medium produced more number of shoots than when they are used alone. The seedling explants and juvenile explants of mature trees responded better than mature explants with respect to the number of shoots produced. Axillary buds of nodal explants produced more multiple shoots than the apical buds.

In the present study auxin supplementation in the rooting medium without charcoal induced basal callus formation. The cultures with basal callus formation showed less vigorous growth. Combination of auxins, IBA and NAA produced more number of roots in *C. verum* than when they were used separately. Number of roots produced on shoots regenerated from juvenile explants was higher than the number of roots produced on shoots regenerated from mature explants in *C. verum*. It is generally seen that immature tissues respond well than mature tissues and immature seeds are good explants for the induction of somatic embryos. Before using the *in vitro* developed plantlets for cultivation, genetic fidelity tests must be conducted for each batch and each time

All the experiments done in *C. verum* could not be done in *C. cassia* due to the scarcity of explants. However, the experiments conducted in *C. cassia* showed promising results and hence the plant can be multiplied in future through all routes of multiplication.

The present study was the first report on:

1. Regeneration of adventitious shoots from mature explants
2. Indirect regeneration of plantlets from callus and
3. Somatic embryogenesis in *Cinnamomum verum*

*In vitro* developed plantlets through direct regeneration and somatic embryogenesis were transplanted to the field with 80-90% success.

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* Original not seen

Fig. 1  Cinnamon quills purchased from Malaysian market

Fig. 2  Cinnamon quills purchased from Australian market

Fig. 3  Cinnamon quills purchased from Kuwait market

Fig. 4 *Cinnamomum verum* trees at IISR

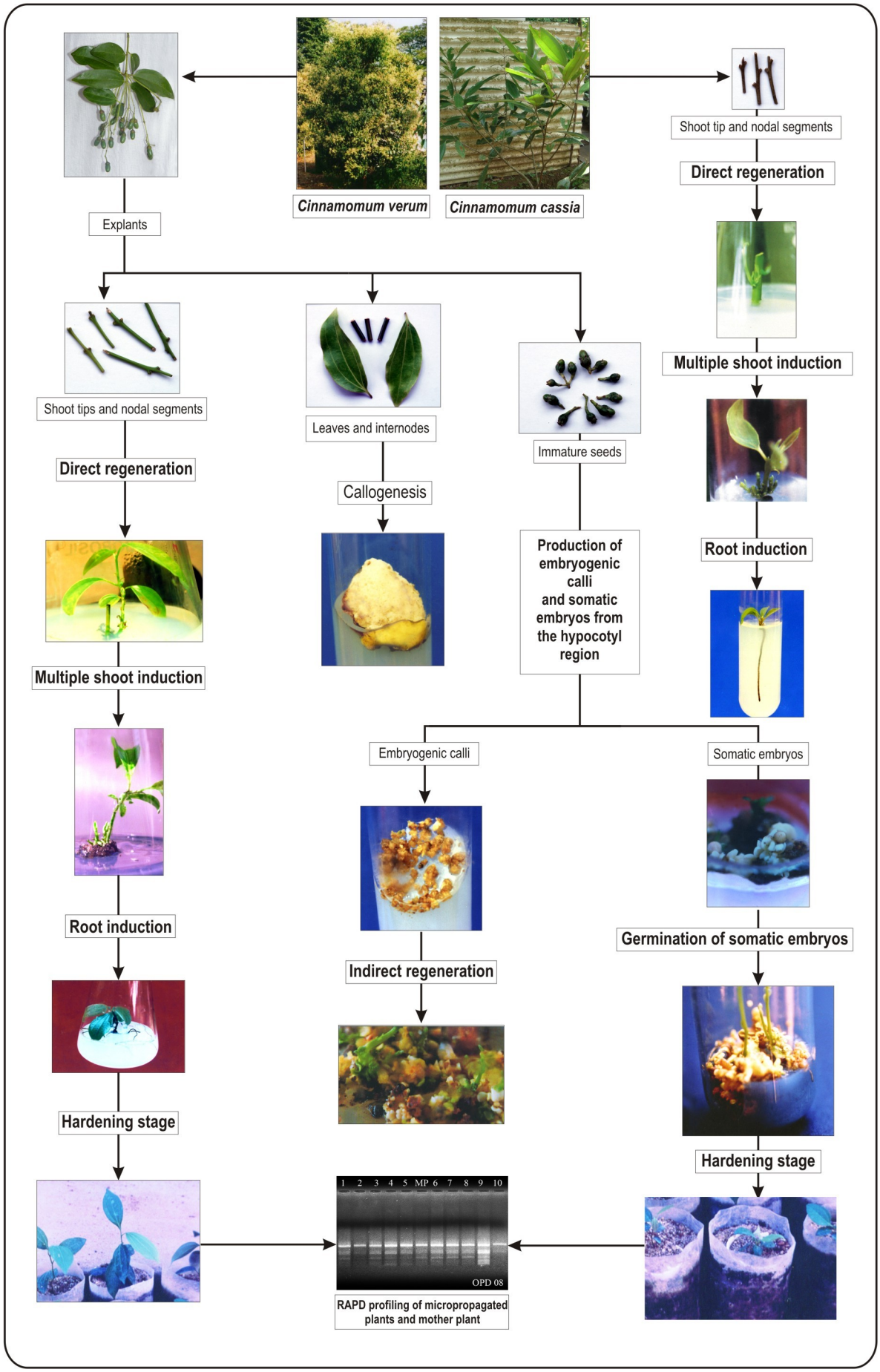
Fig. 5  Cinnamon tree with inflorescences

Fig. 6 Inflorescences of *C. verum* (panicle)

Fig. 7 *C. verum* - Twig with immature seeds

Fig. 8 *Cinnamomum cassia*

Fig. 9 Schematic representation of the important activities in the present study



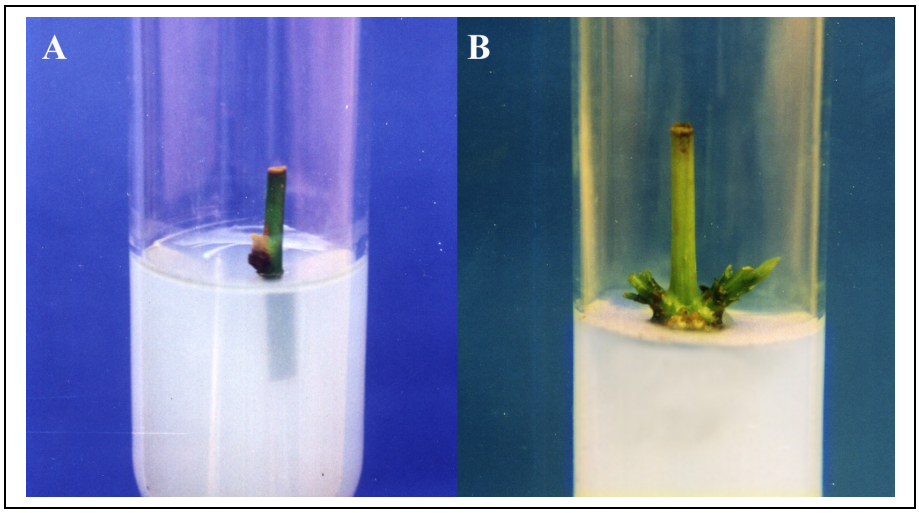



Fig.10 *C. verum*. A- *In vitro* bud break
B- Induction of shoots

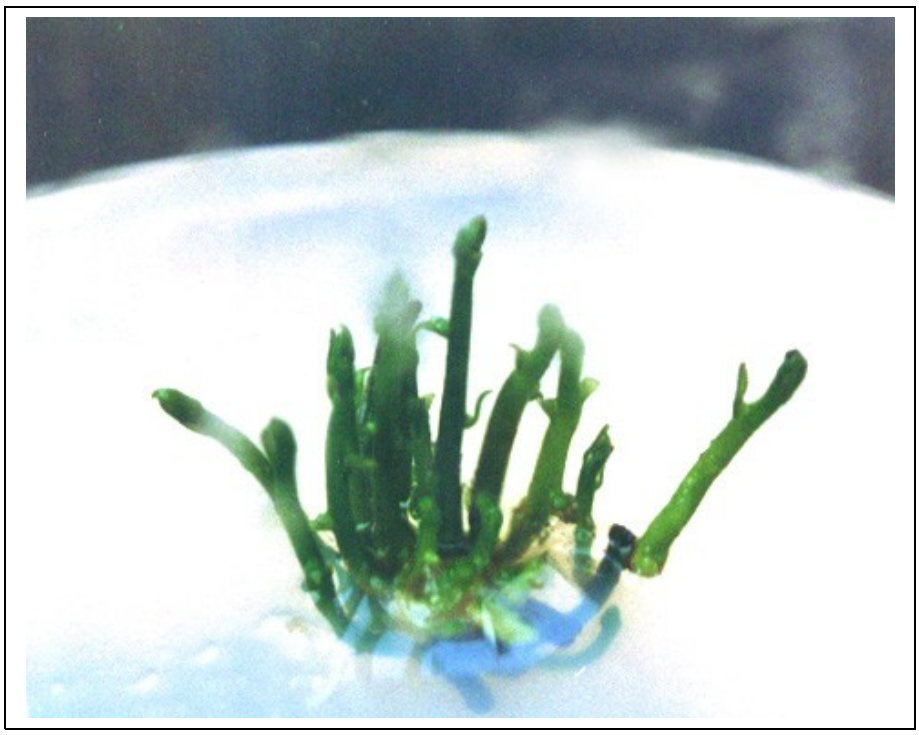


Fig. 11 *C. verum*. Multiple shoot induction in seedling explant

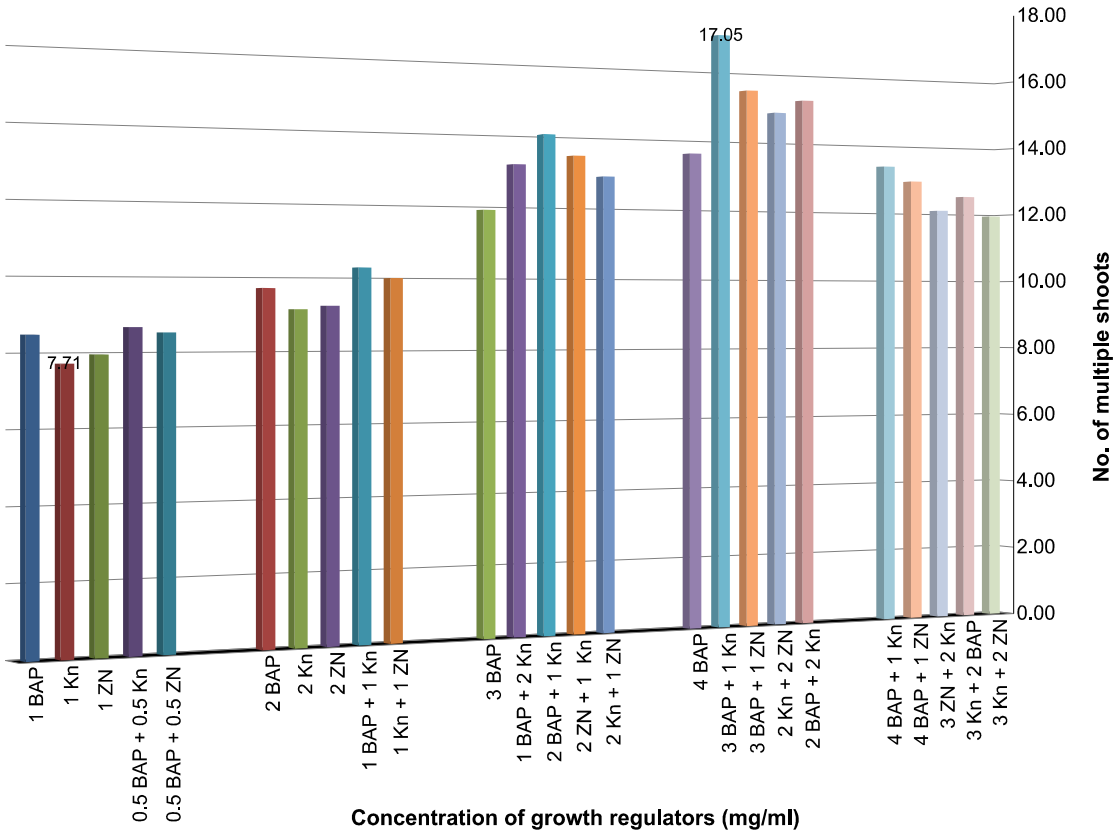



Fig. 12 Effect of BAP, Kn and ZN on multiple shoot induction of seedling explants of *C. verum*

Fig. 13 Emergence of juvenile branches from the chopped portion of the mature trees of *C. verum*

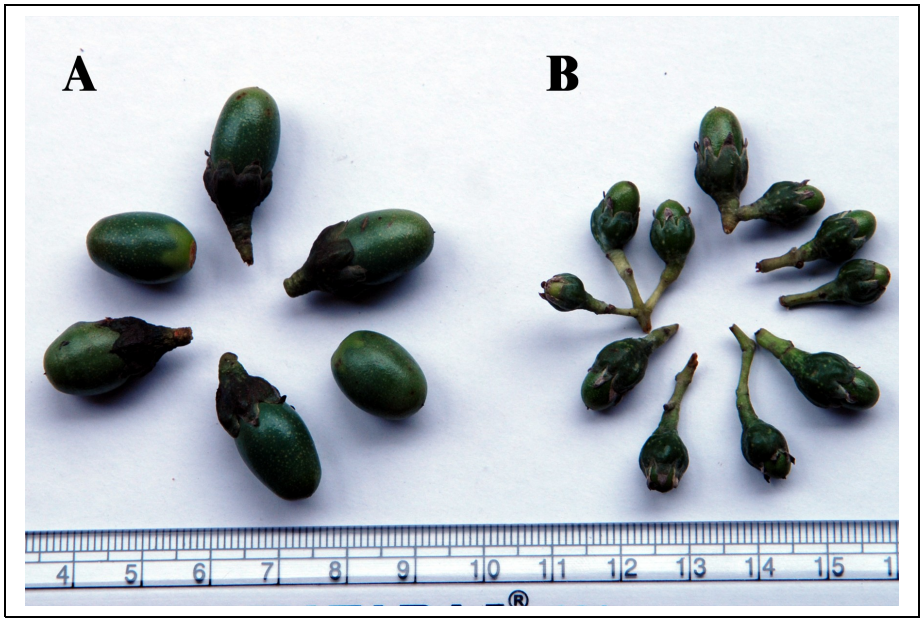



Fig. 14 *C. verum* - Mature (A) and immature (B) fruits

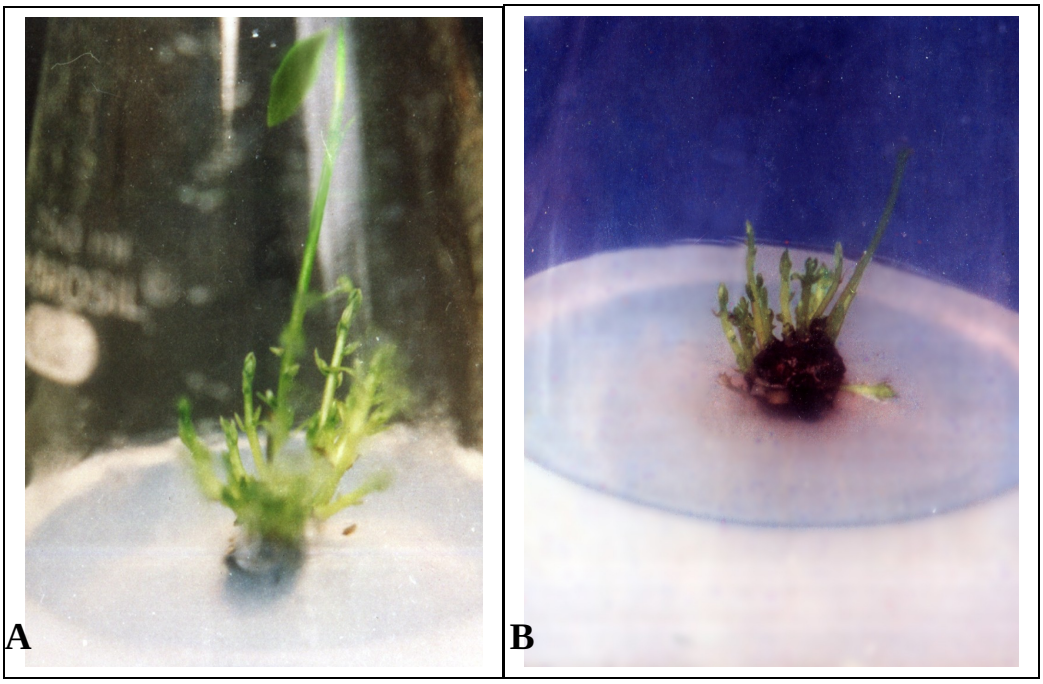



Fig. 15 *C. verum* A- Multiple shoot induction in juvenile explants of mature trees
B-Exudation of phenolic compounds from the culture

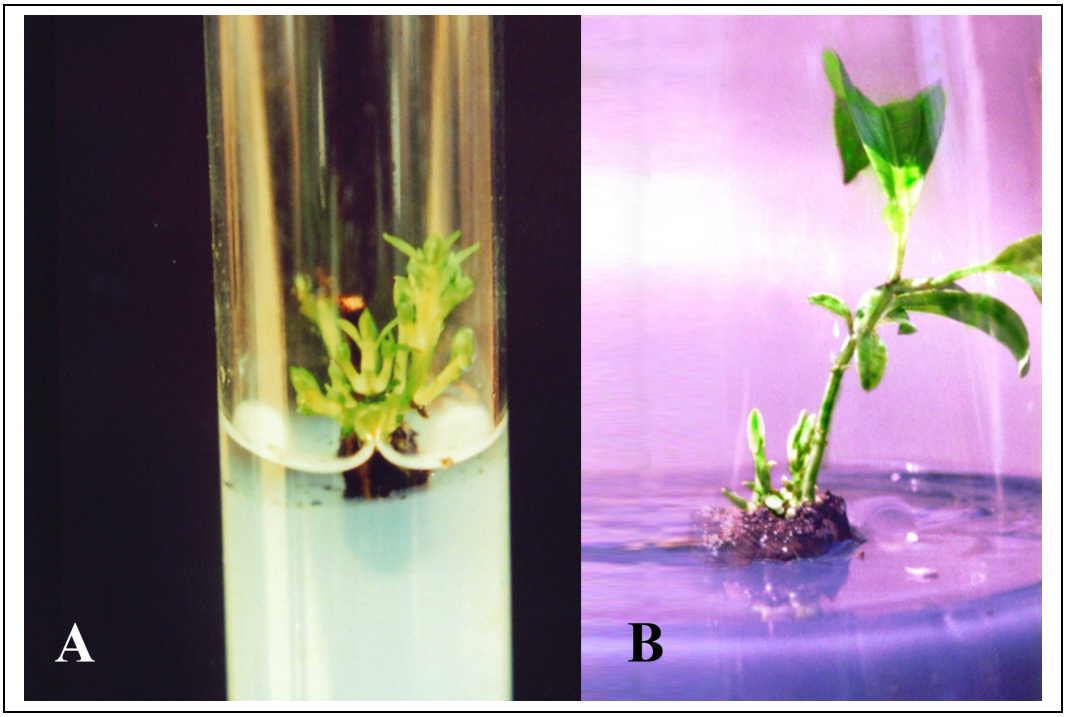



Fig. 16 *C. verum* A - Multiple shoot induction in mature explant
B - Exudation of phenolic compounds from the culture

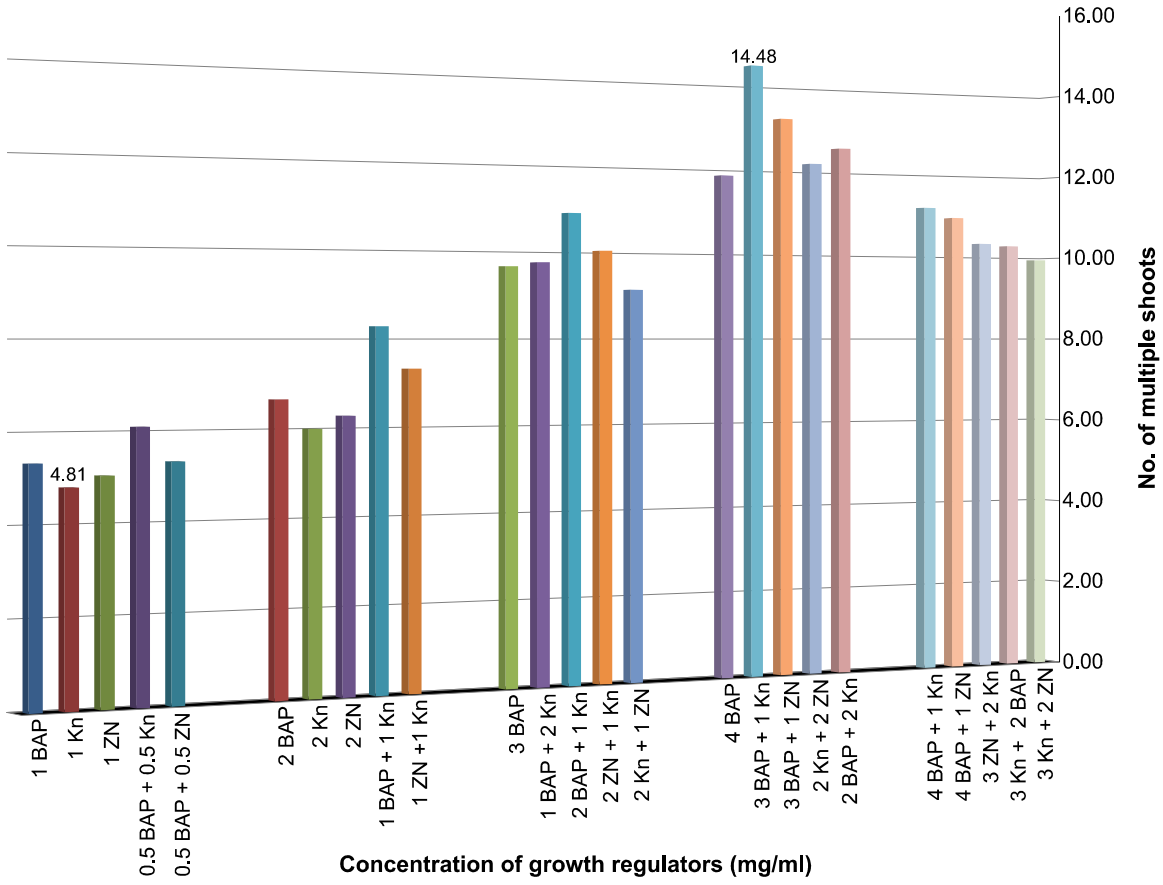



Fig. 17 Effect of BAP, Kn and ZN on multiple shoot induction of juvenile explants of *C. verum*

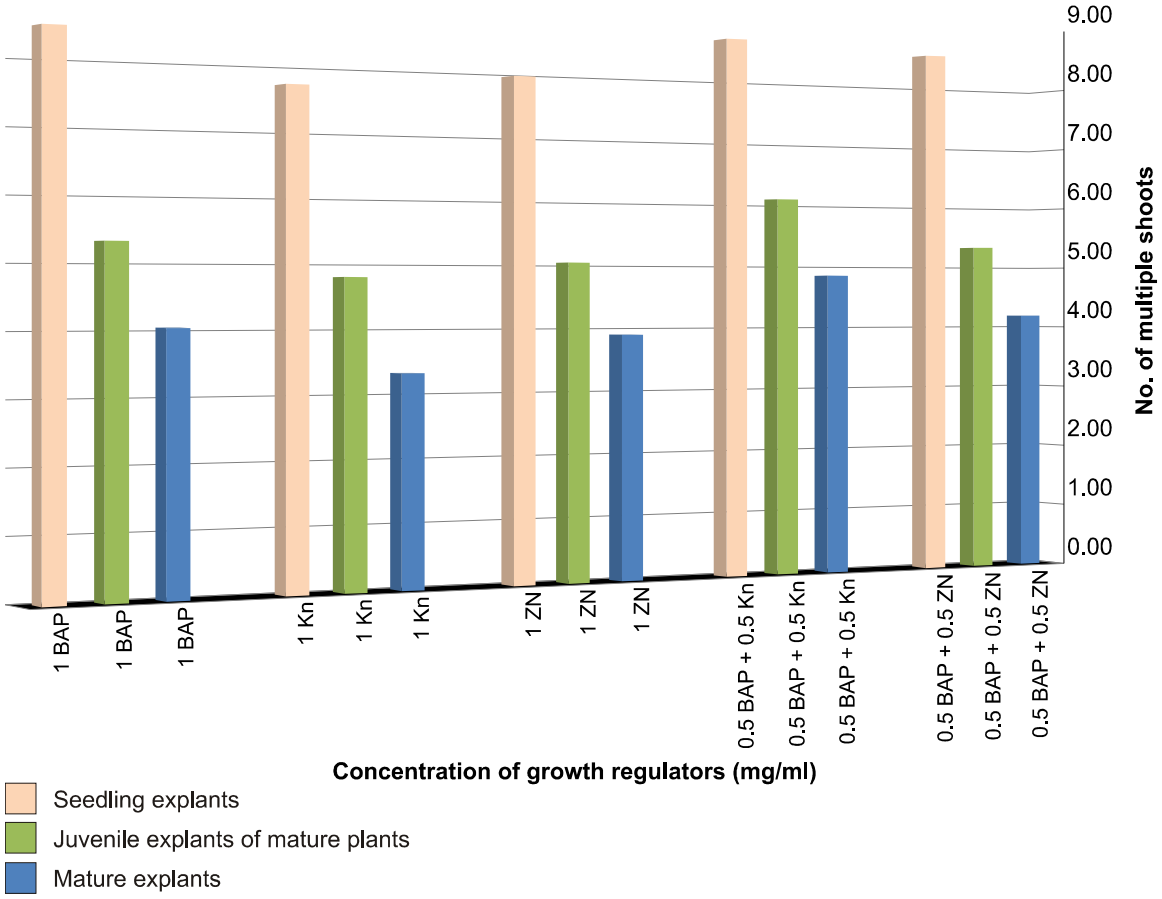



Fig. 18 Comparison of number of multiple shoots produced in seedling explants, juvenile explants of mature trees and mature explants of *C. verum* in WPM supplemented with **1 mg/l** of different cytokinins

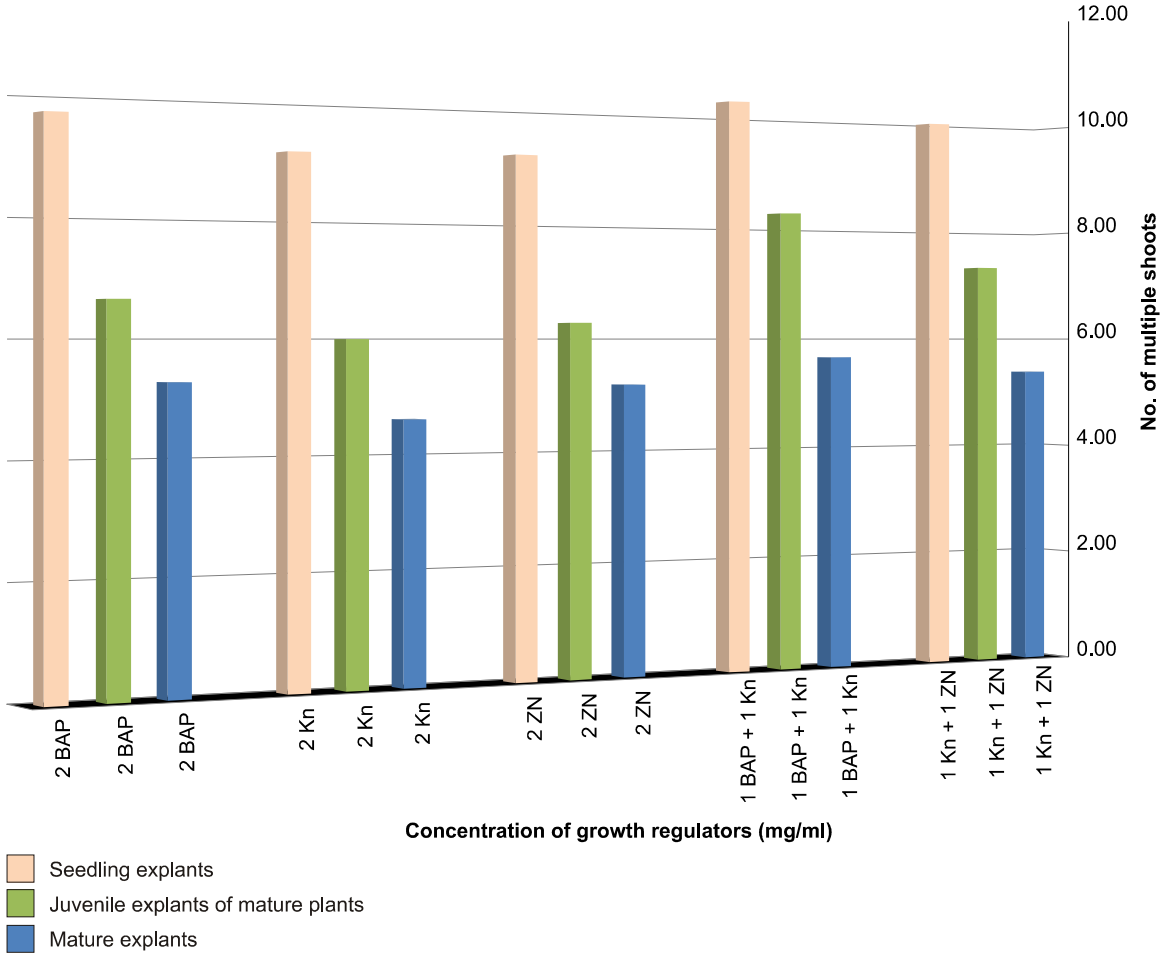



Fig. 19 Comparison of number of multiple shoots produced in seedling explants, juvenile explants of mature trees and mature explants of *C. verum* in WPM supplemented with **2 mg/l** of different cytokinins

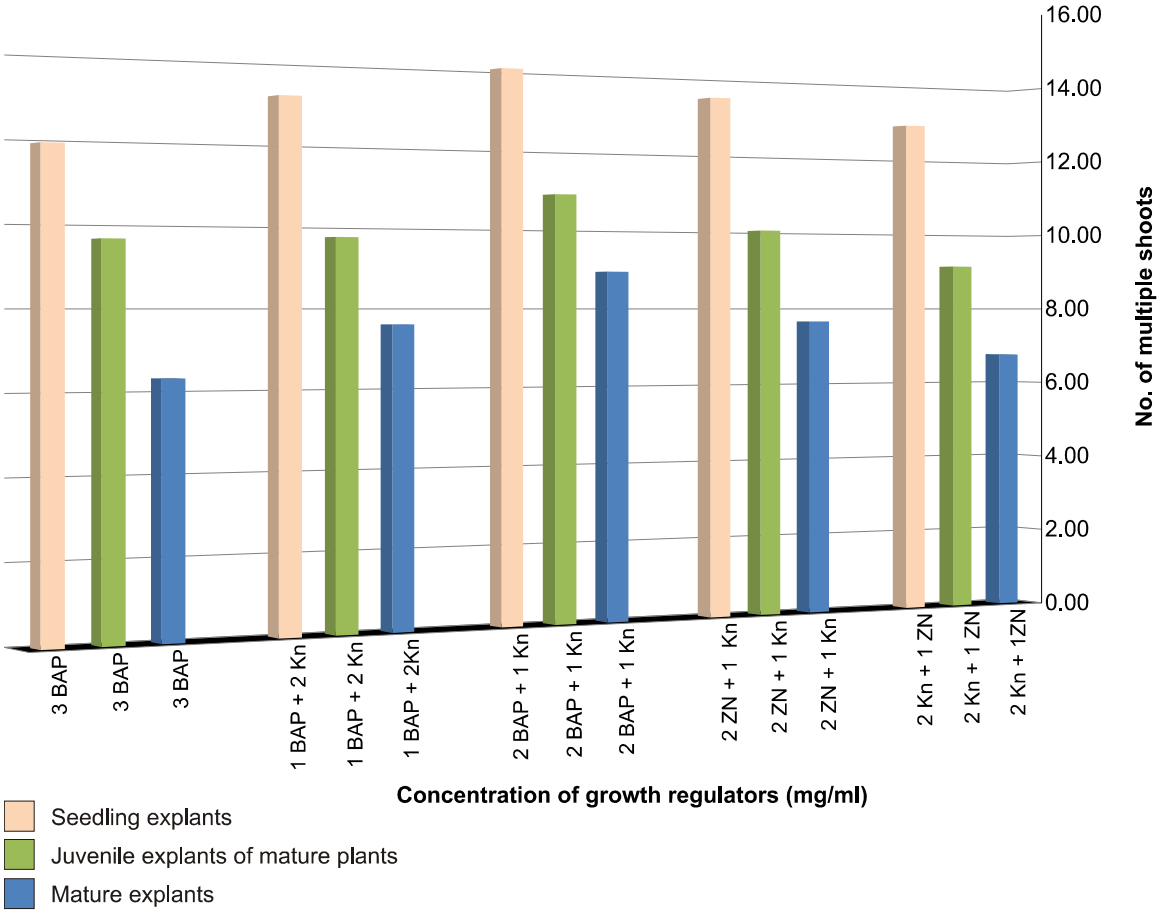



Fig. 20 Comparison of number of multiple shoots produced in seedling explants, juvenile explants of mature trees and mature explants of *C. verum* in WPM supplemented with **3 mg/l** of different cytokinins.

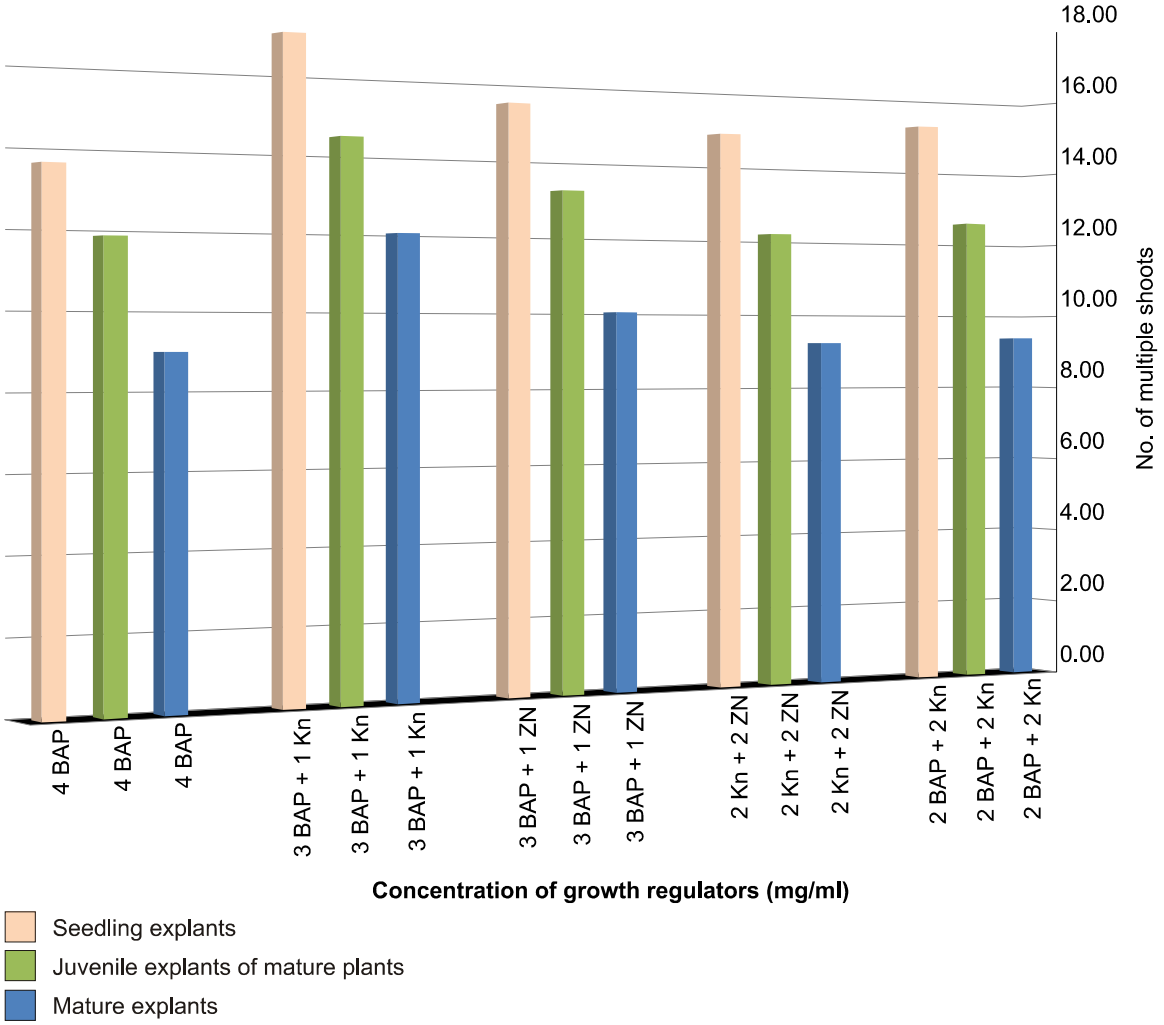



Fig. 21 Comparison of number of multiple shoots produced in seedling explants, juvenile explants of mature trees and mature explants of *C. verum* in WPM supplemented with **4 mg/l** of different cytokinins.

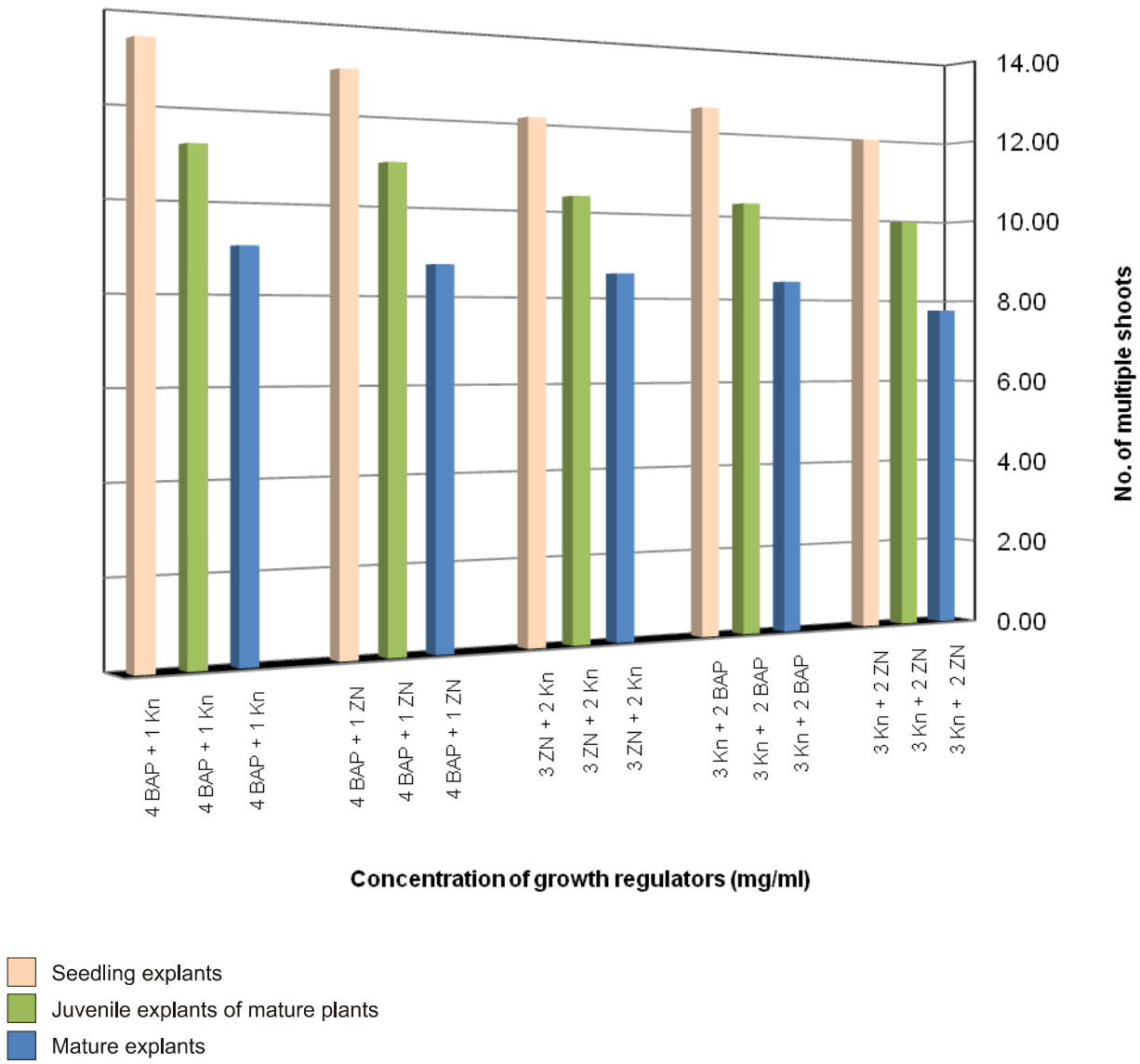



Fig. 22 Comparison of number of multiple shoots produced in seedling explants, juvenile explants of mature trees and mature explants of *C. verum* in WPM supplemented with **5 mg/l** of different cytokinins.

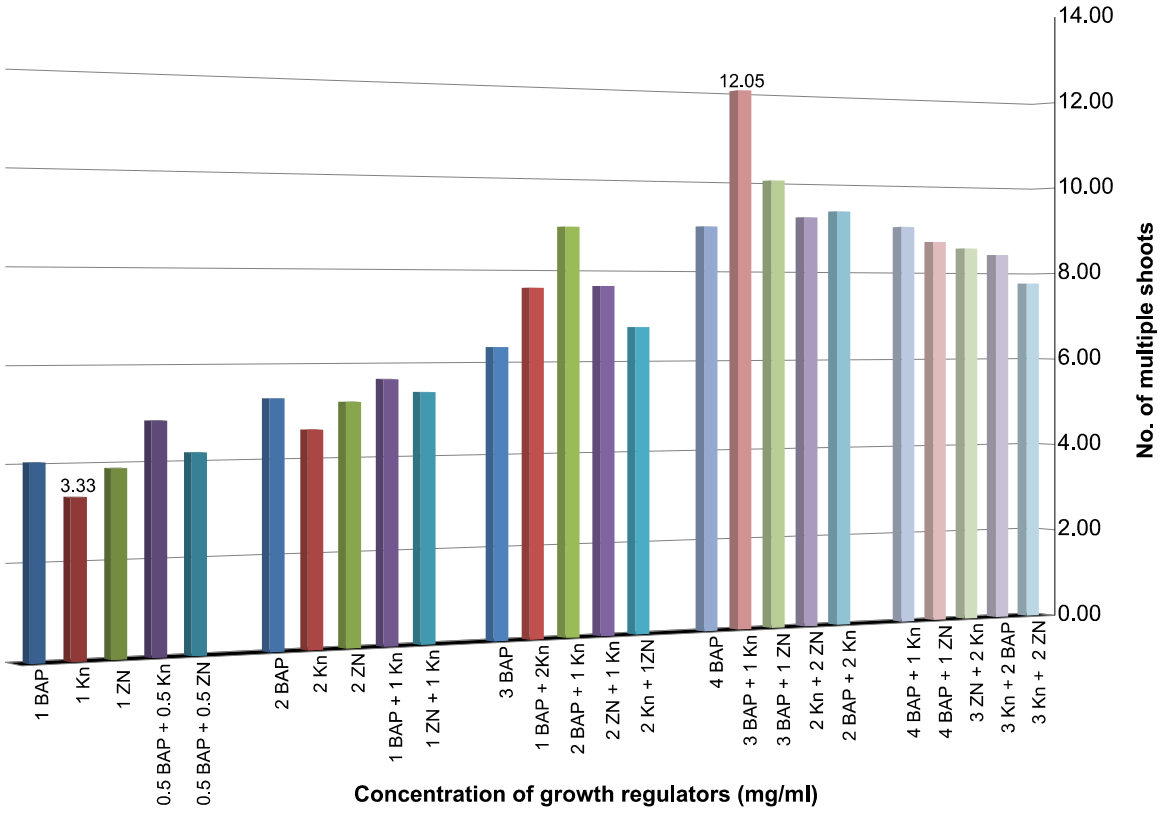



Fig. 23 Effect of BAP, Kn and ZN on multiple shoot induction of mature explants of *C. verum*

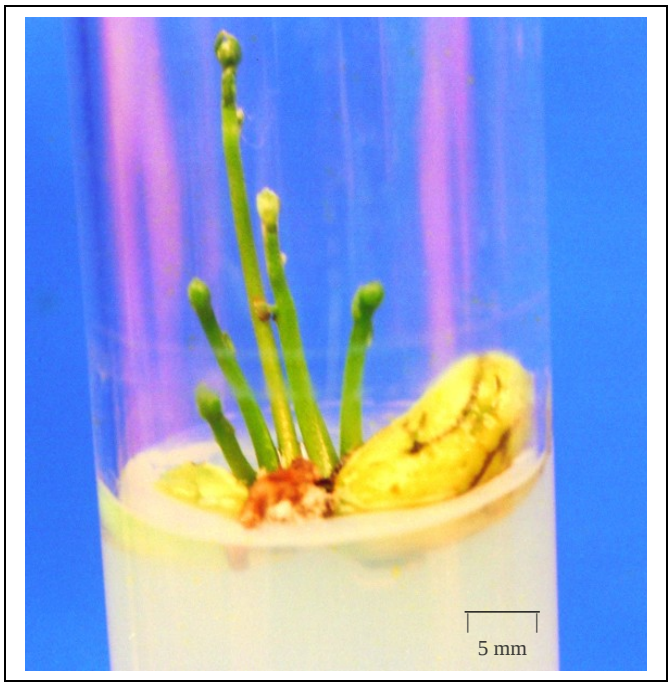



Fig. 24 *C. verum.* Proliferation of organogenic callus from the cotyledonary axis of immature seed

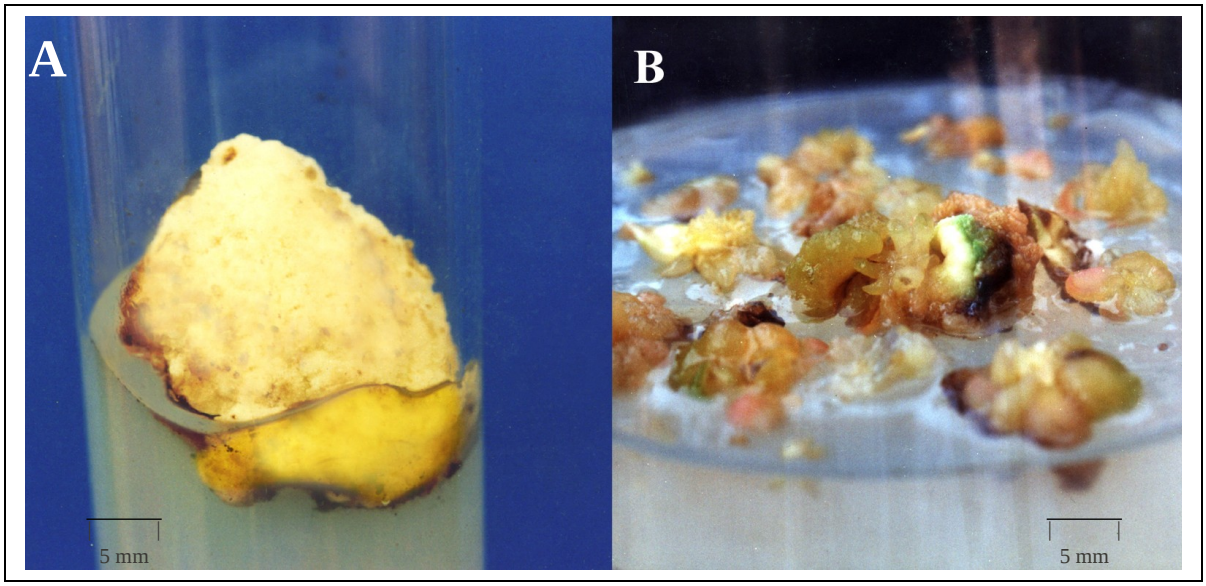



Fig. 25 *C. verum*. A - Compact callus developed from the internode explant
B - Organogenic callus produced from immature seeds (magnified)

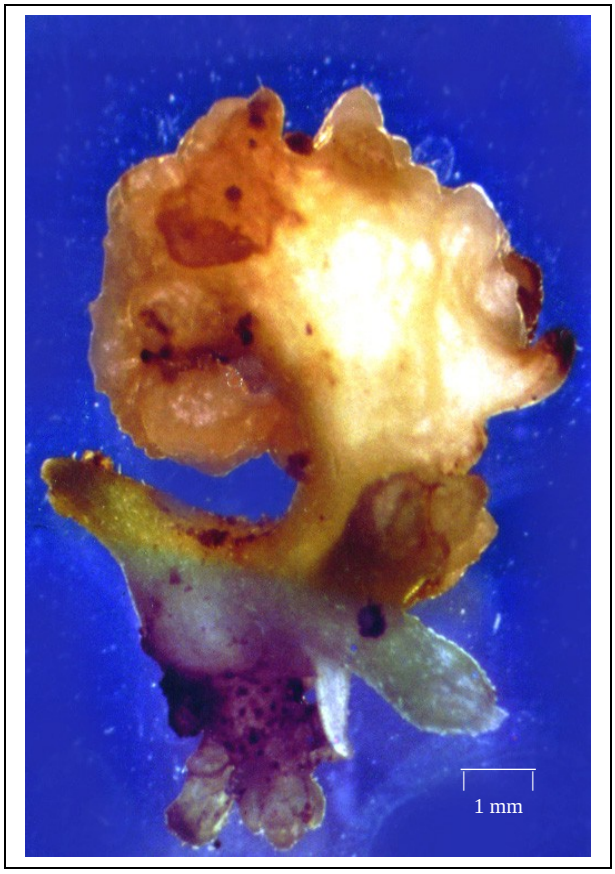



Fig. 26 *C. verum*. Initiation of shoot development from the organogenic callus (magnified)

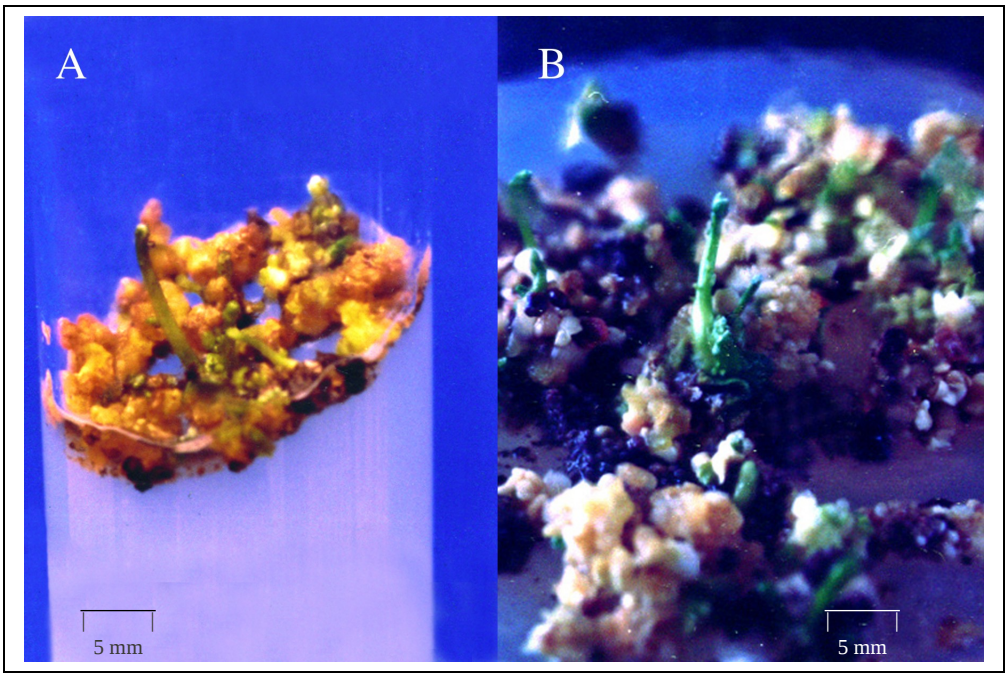



Fig. 27 (A) and (B) *C. verum.* Regeneration of shoots from the organogenic callus. Somatic embryos can also be seen in figure B

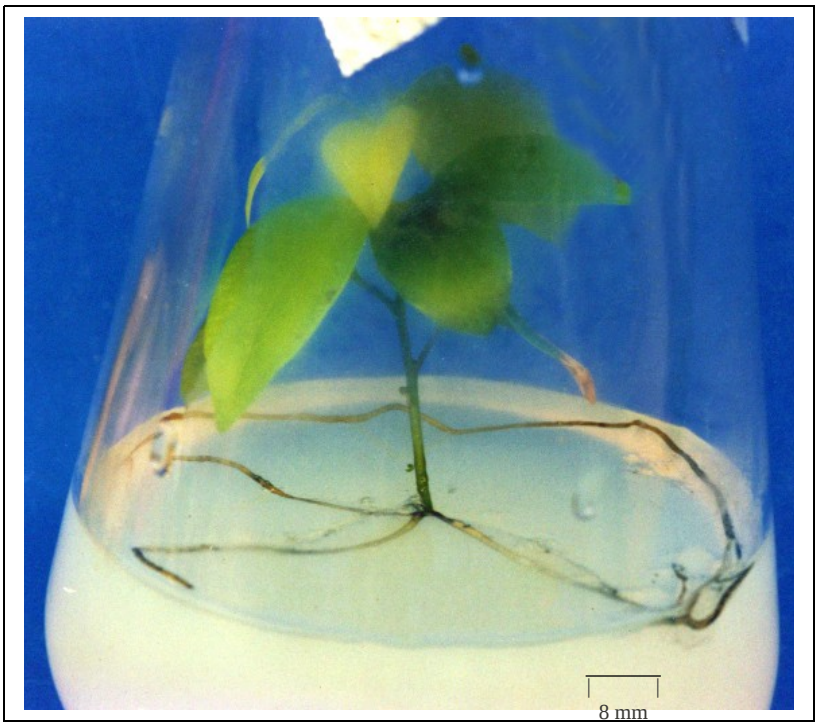



Fig. 28 *C. verum. In vitro* rooting in cultures of juvenile explants

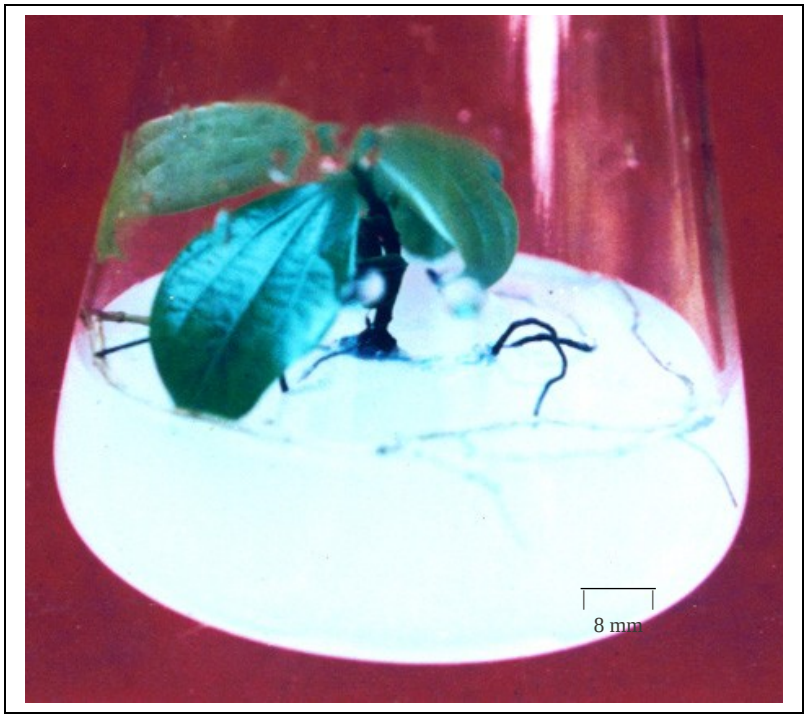



Fig. 29 *C. verum. In vitro* rooting in cultures of mature explants

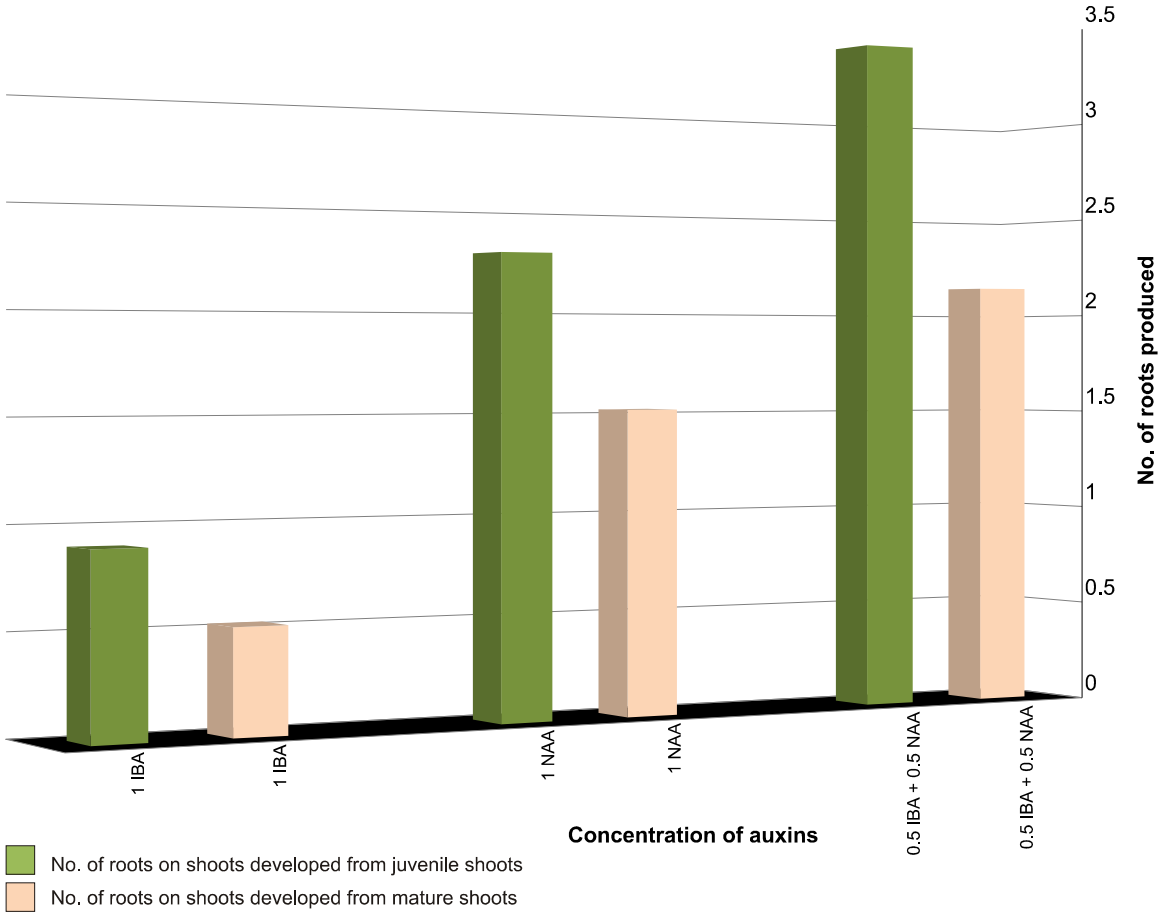



Fig. 30 Effect of IBA (**1 mg** /l) in root induction on shoots regenerated from juvenile and mature explants in *C. verum*

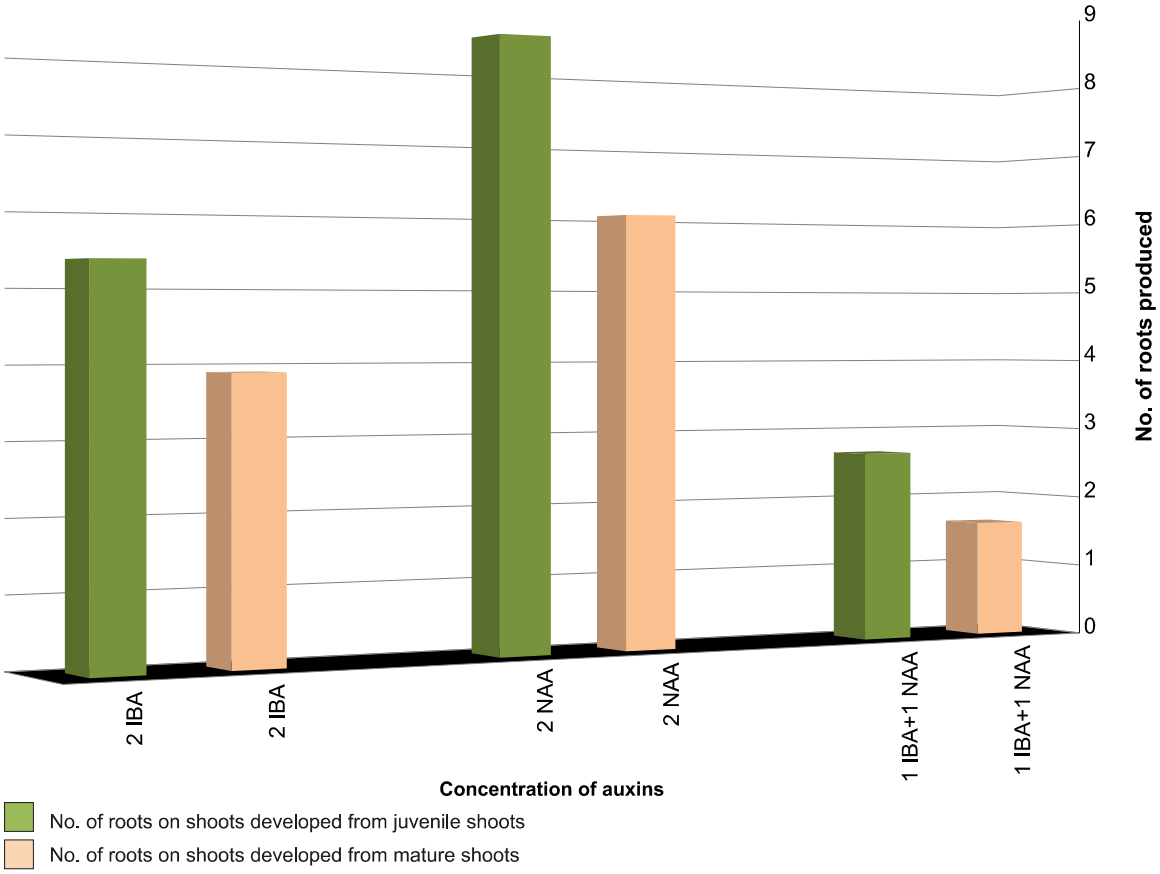



Fig. 31 Effect of IBA (2 mg/l) in root induction on shoots regenerated from juvenlie and mature explants in *C. verum*.

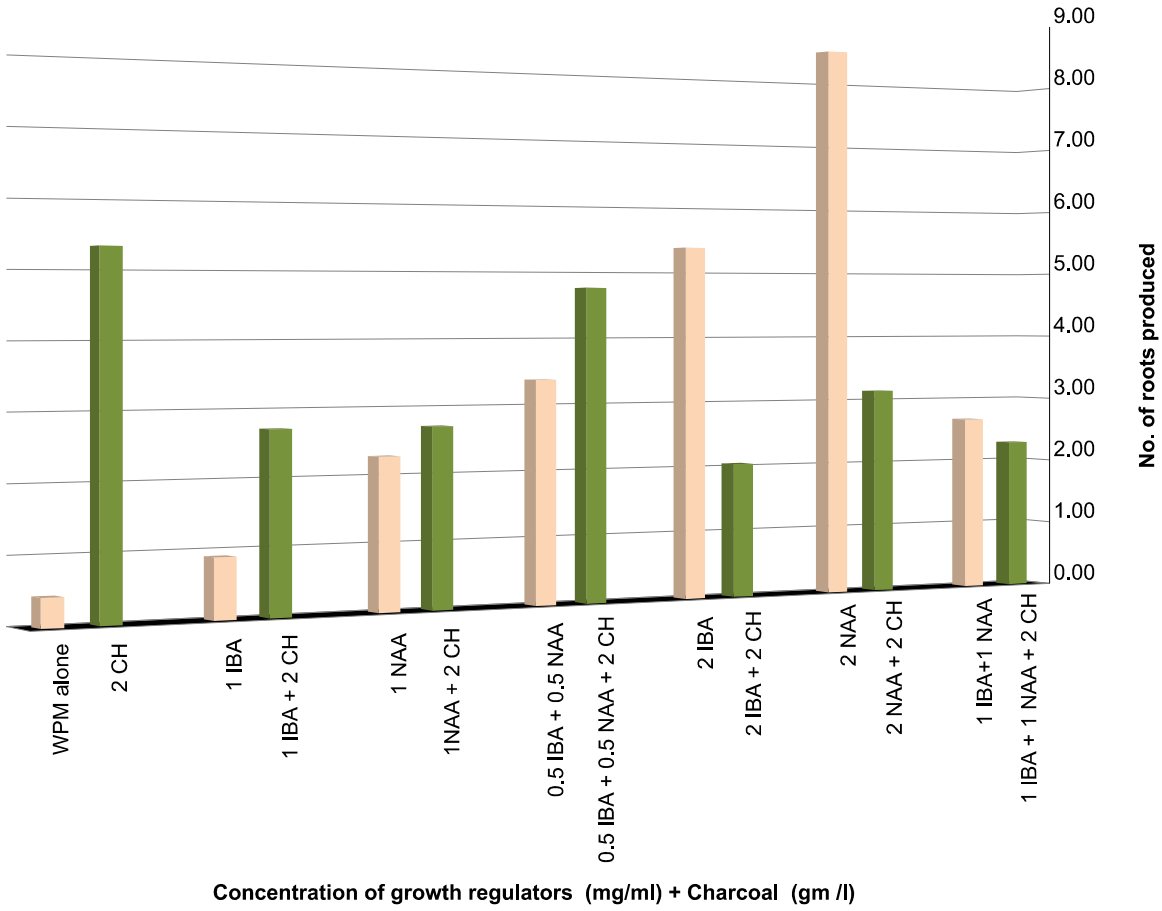



Fig. 32 Effect of charcoal on rooting of shoots regenerated from juvenile explants of *C. verum*

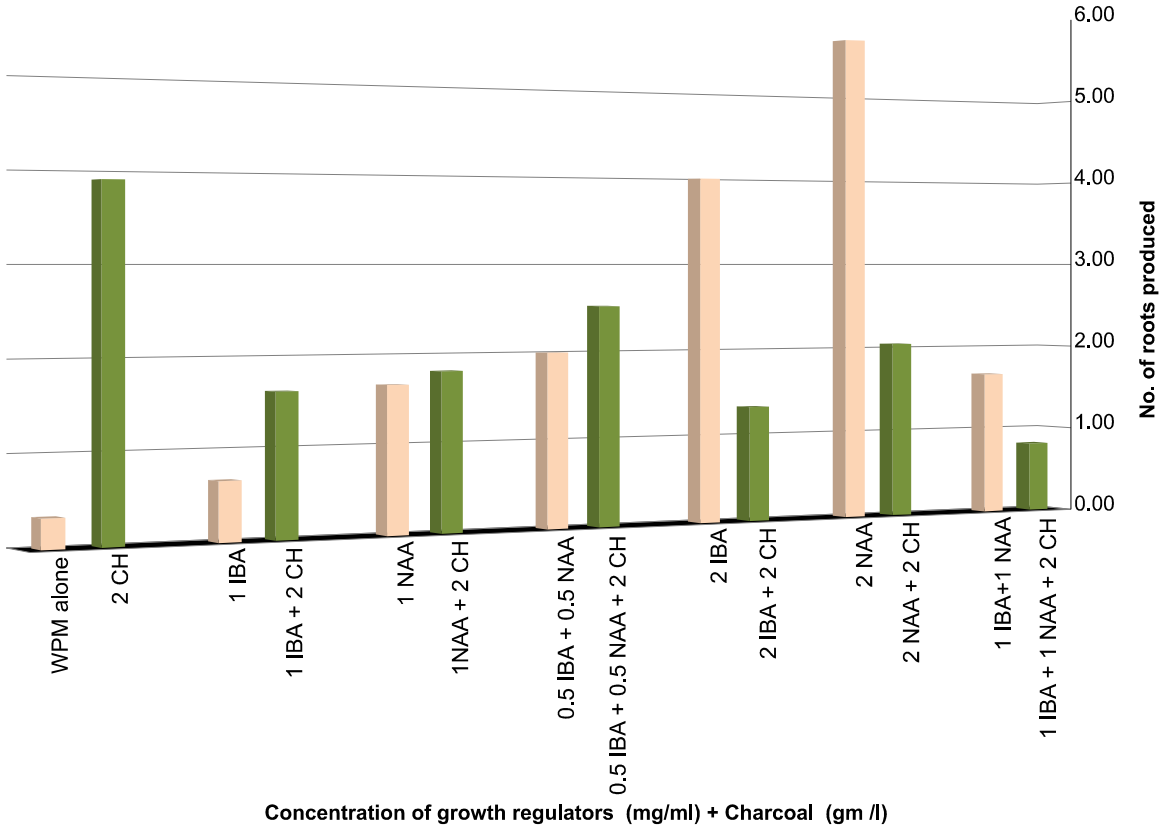



Fig. 33 Effect of charcoal on media combinations tried for rooting on shoots regenerated from mature explants of *C. verum*

Fig. 34 *C. verum.* Embryo with cotyledons of immature seeds

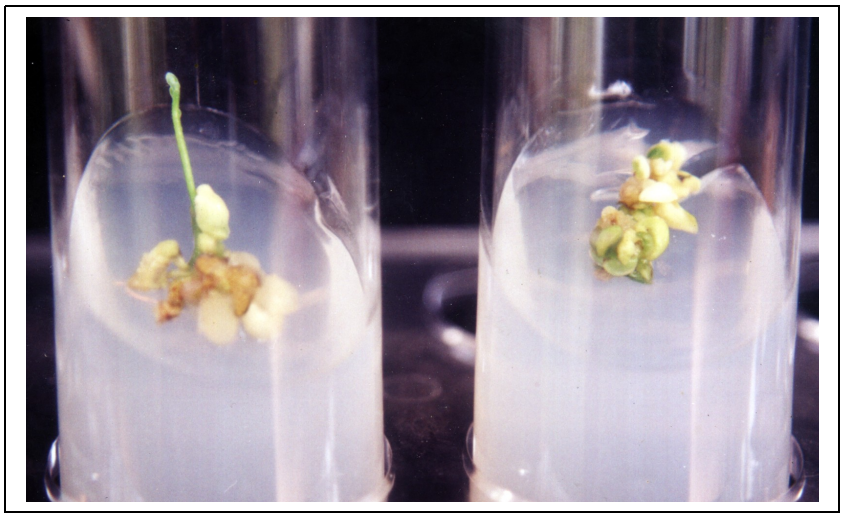


Fig. 35 *C. verum.* Initiation of organogenic callus and somatic embryos from the cotyledonary axis of immature seeds

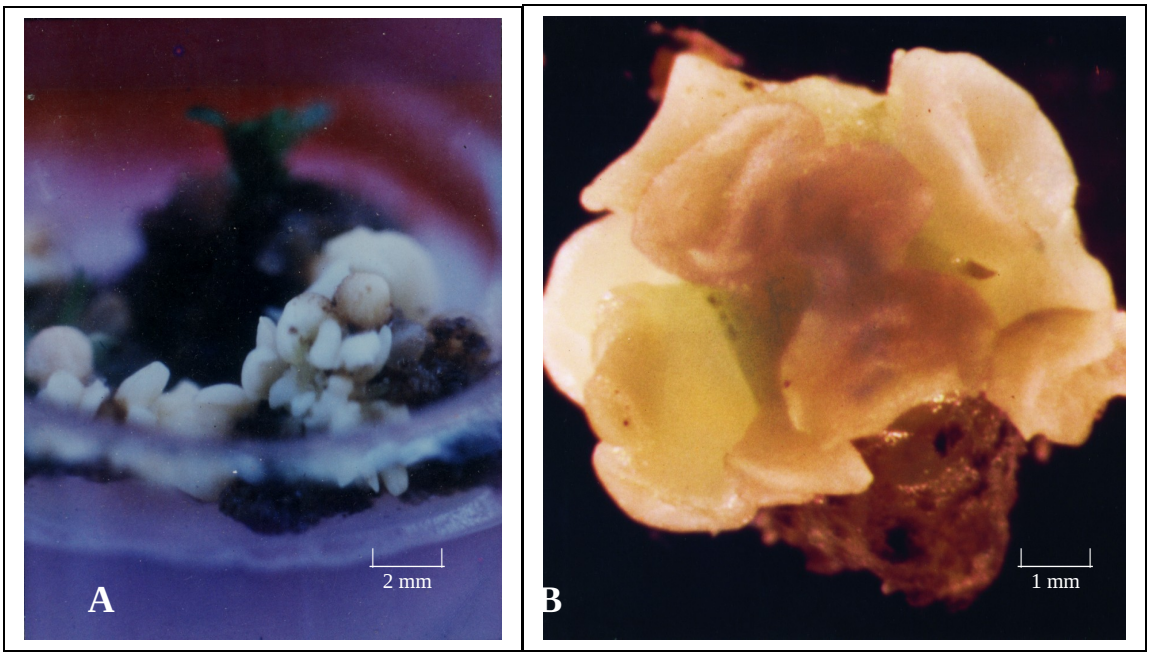



Fig. 36 *C. verum*. A - Proliferation of somatic embryos (magnified)
B - Cluster of cup shaped somatic embryos (magnified)

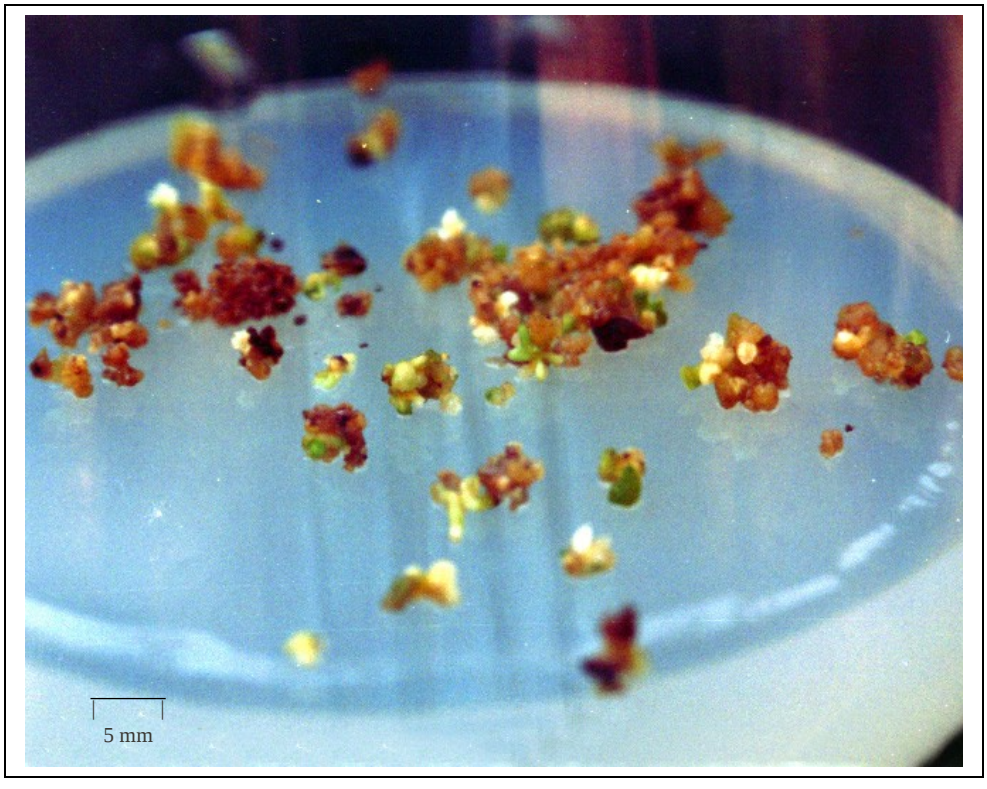



Fig. 37 *C. verum*. Regeneration of shoots from organogenic callus. Somatic embryos can also be seen

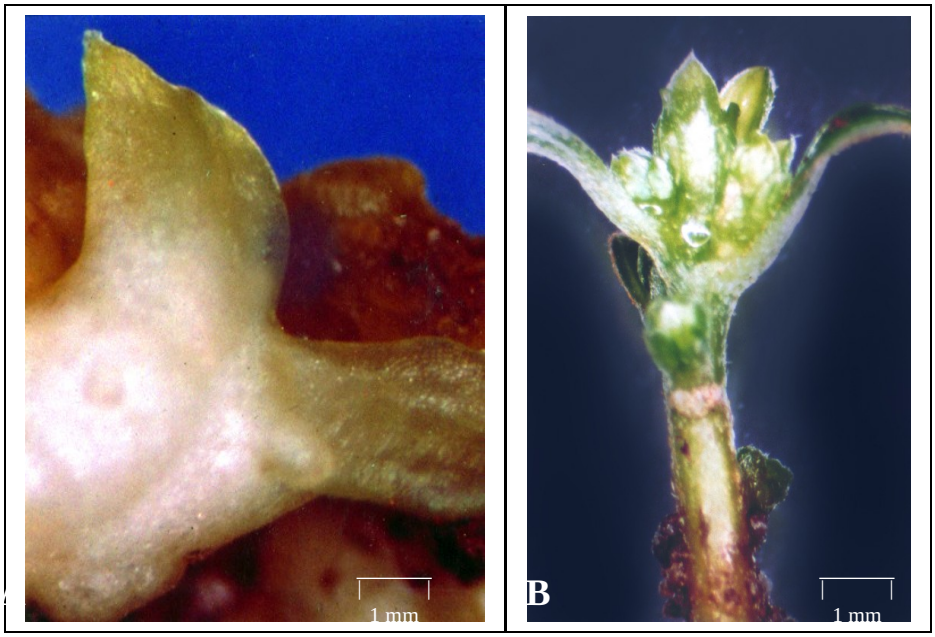



Fig. 38 *C. verum*. A - Cotyledonary stage of somatic embryo development (magnified)
B - Development of shoot from the somatic embryo (magnified)

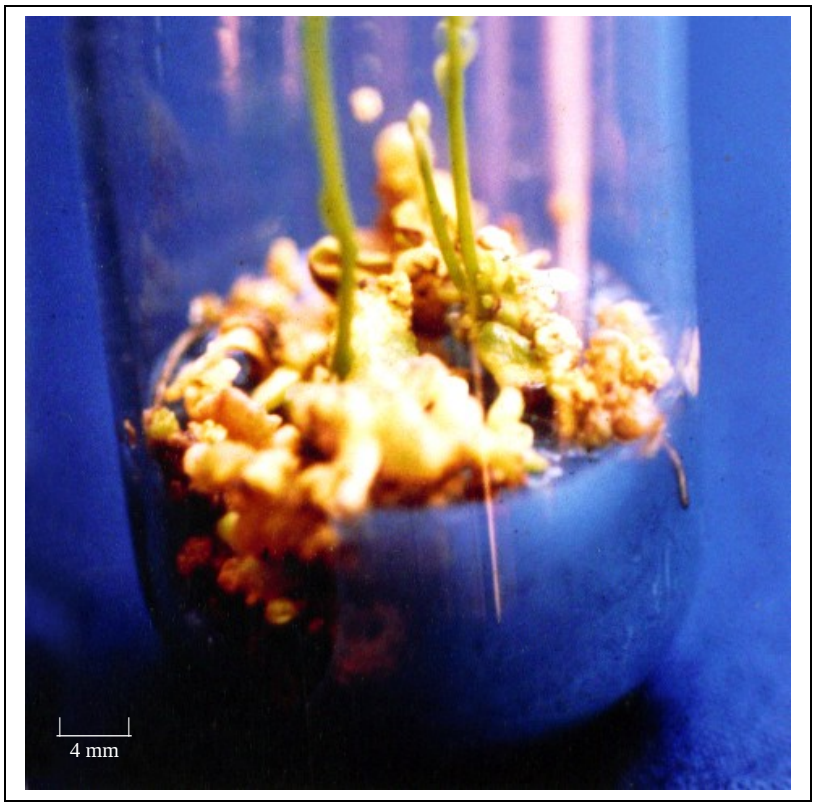



Fig. 39 *C. verum*. *In vitro* germination of somatic embryos into plantlets


Fig. 40 *C. verum.* Hardening stages
A - *In vitro* developed plantlet in soilrite
B - *In vitro* developed plantlets in polythene bags

Fig. 41 *C. verum*. Tissue cultured plant in the field

Fig. 42 *C. verum* – RAPD profiles of micropropagated plants and mother plant.
1 to 5 – Plants developed through direct regeneration
MP – Mother plant
6 to 10 – Plants developed through somatic embryogenesis.

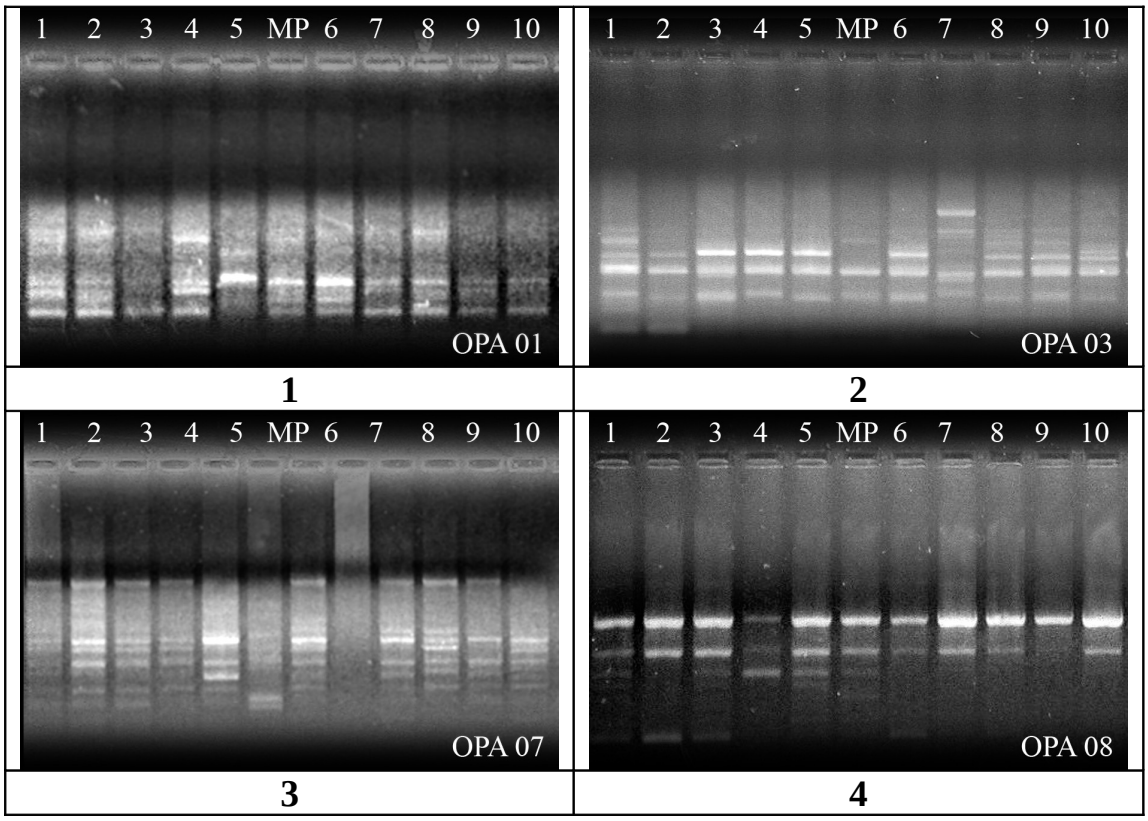


Fig. 43 *C. verum*– RAPD profiles of micropropagated plants and mother plant.
1 to 5 – Plants developed through direct regeneration
MP – Mother plant
6 to 10 – Plants developed through somatic embryogenesis.

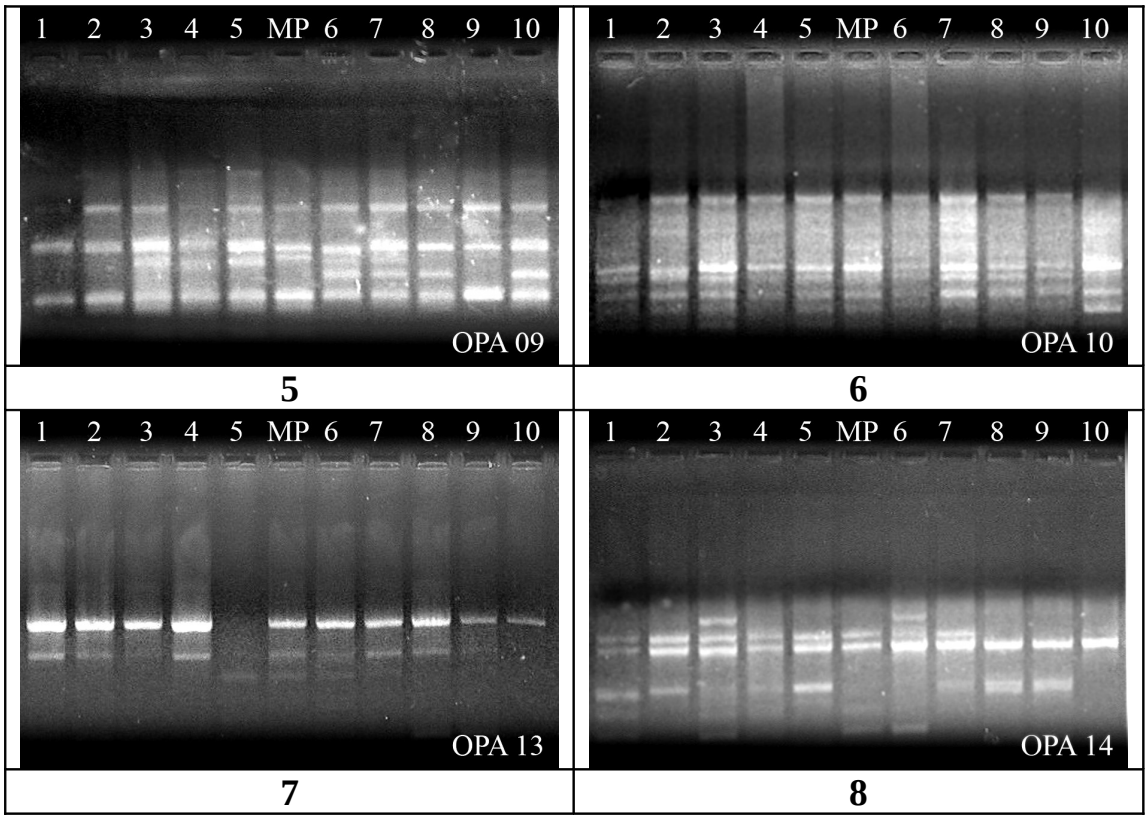


Fig. 44 *C. verum*– RAPD profiles of micropropagated plants and mother plant.
1 to 5 – Plants developed through direct regeneration
MP – Mother plant
6 to 10 – Plants developed through somatic embryogenesis.

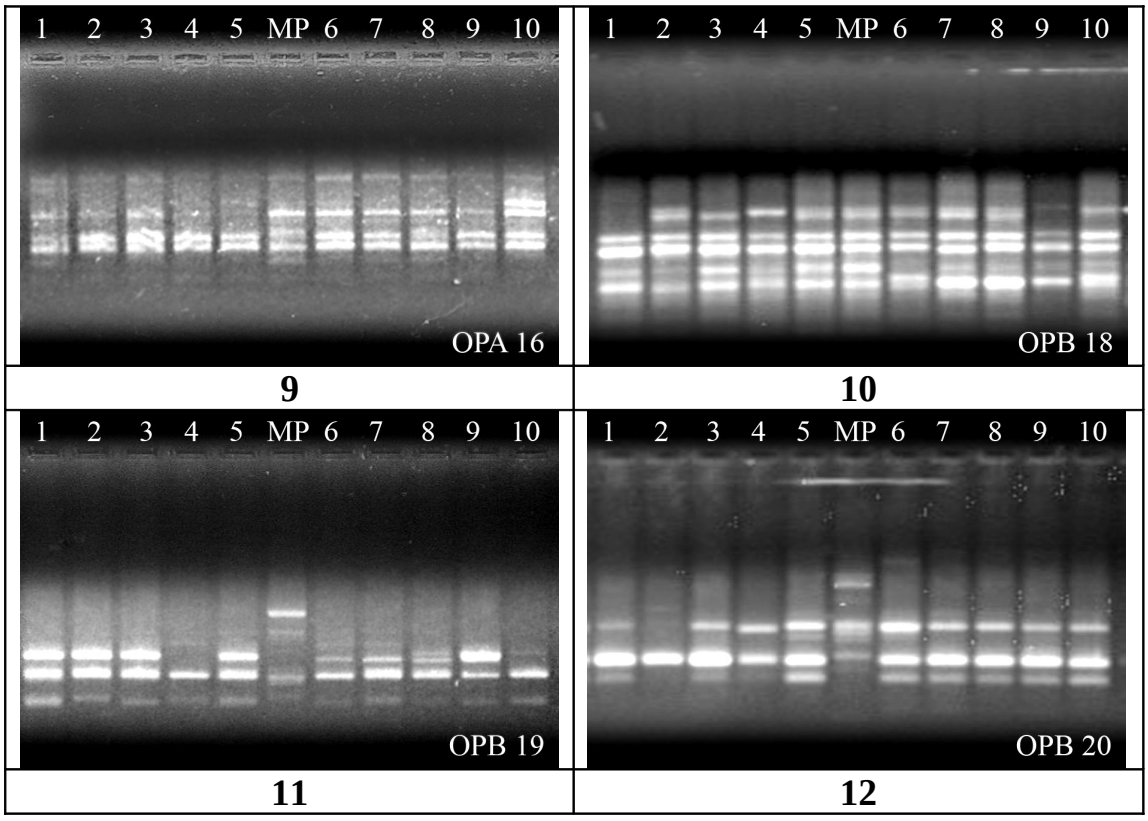


Fig. 45 *C. verum*– RAPD profiles of micropropagated plants and mother plant.
1 to 5 – Plants developed through direct regeneration
MP – Mother plant
6 to 10 – Plants developed through somatic embryogenesis.

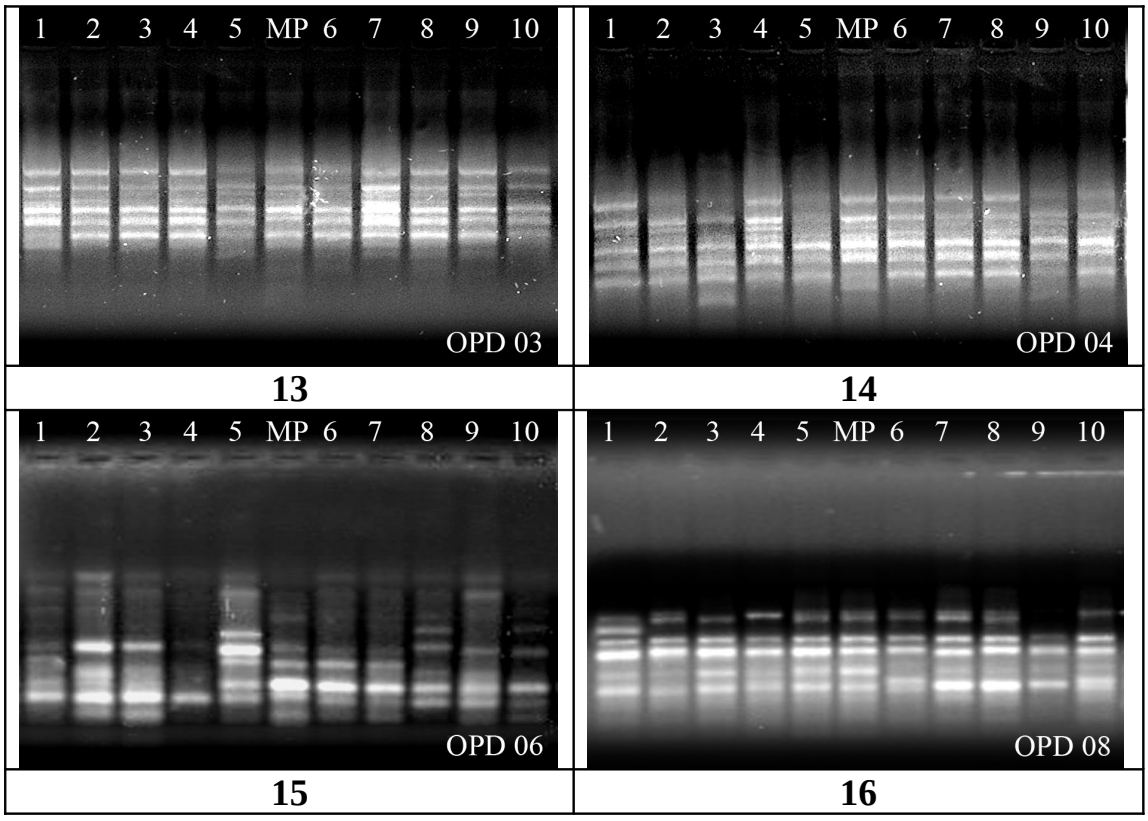


Fig. 46 *C. verum*– RAPD profiles of micropropagated plants and mother plant.
1 to 5 – Plants developed through direct regeneration
MP – Mother plant
6 to 10 – Plants developed through somatic embryogenesis.

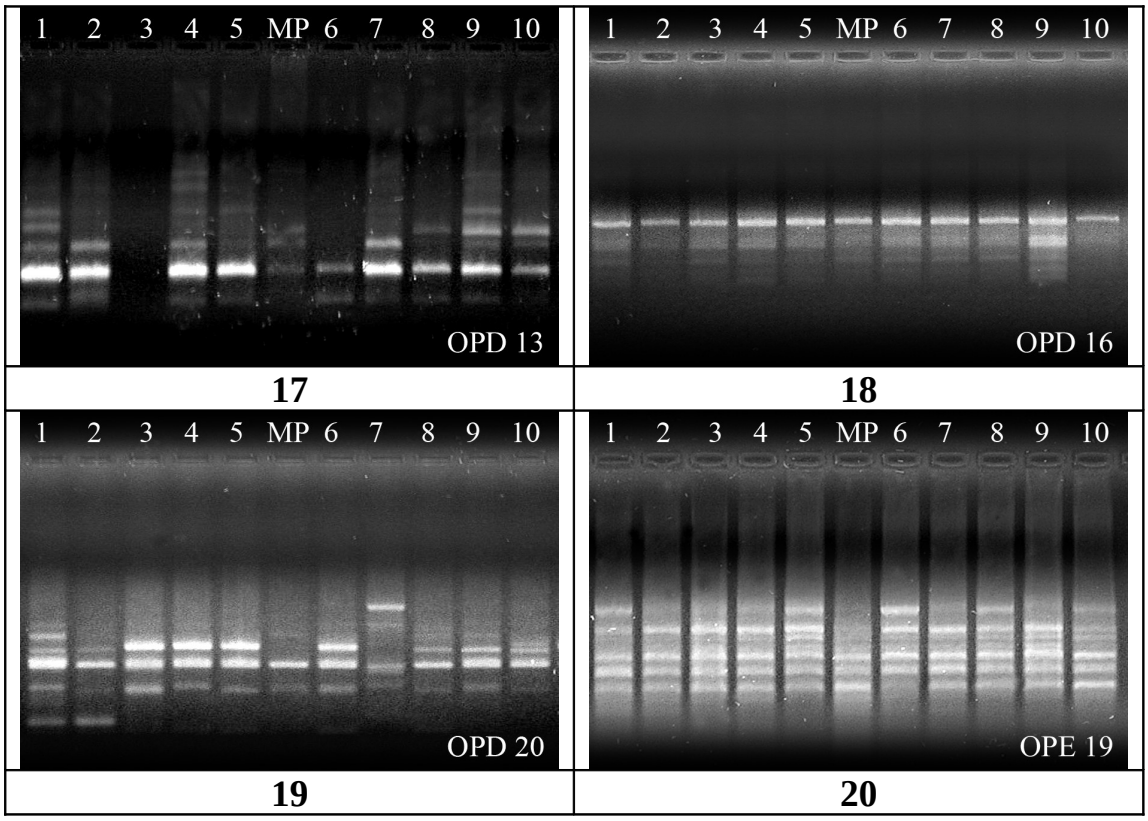


Fig.47
Dendogram

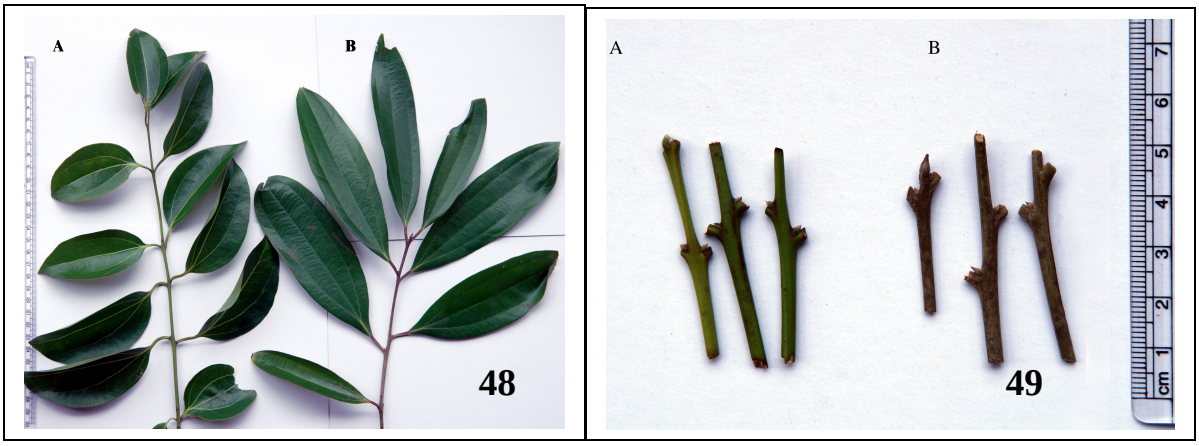



Fig. 48 Twig of *C. verum* (A) and *C. cassia* (B) with apical and axillary buds

Fig. 49 A - Shoot tip and nodal segment explants of *C. verum*
B - Shoot tip and nodal segment explants of *C. cassia*

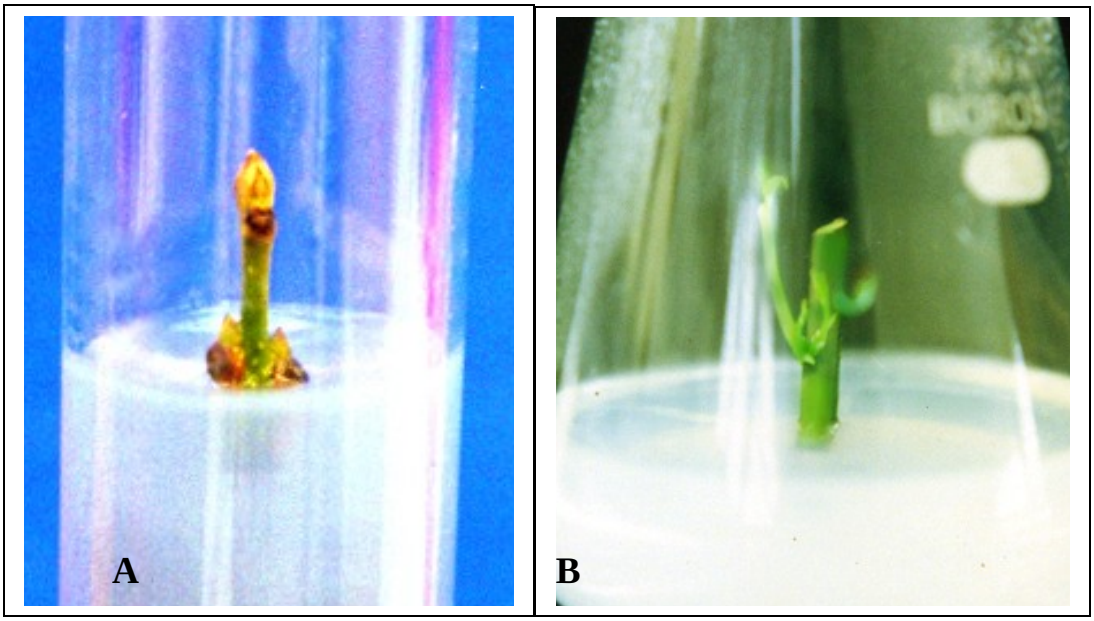



Fig. 50 *C. cassia.* A - *In vitro* bud break
B -*In vitro* induction of shoots

Fig. 51 *C. cassia* - Multiple shoot induction

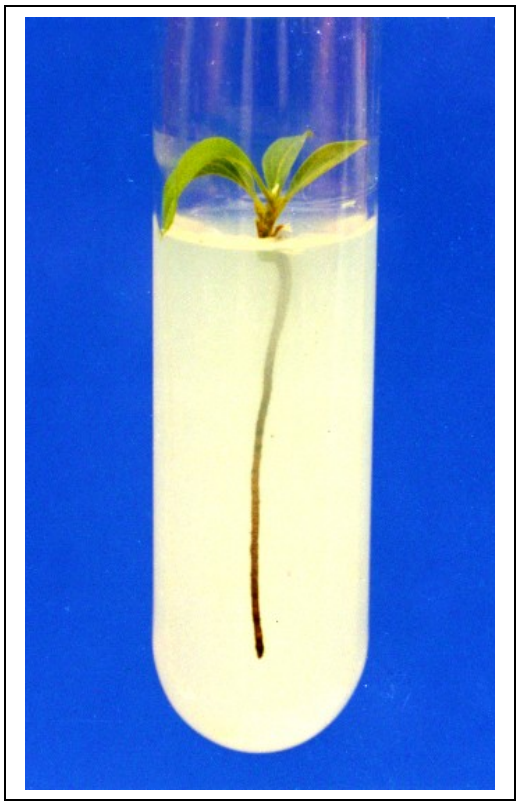


Fig. 52 *C. cassia* - *In vitro* rooting