Cytogenetic (CHIAS and RAPD), structural (SEM), phytochemical (GC-MS and EDS) and antioxidant assays on *in vivo* and *in vitro* plants of *Plectranthus zeylanicus* Benth.

Thesis
submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY in BOTANY

By

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DECLARATION

I, Betty K. P., hereby declare that the thesis entitled **"Cytogenetic (CHIAS and RAPD), structural (SEM), phytochemical (GC-MS and EDS) and antioxidant assays on *in vivo* and *in vitro* plants of *Plectranthus zeylanicus* Benth."** submitted to the University of Calicut, for the award of the degree of **DOCTOR OF PHILOSOPHY** is a record of original research work done by me under the supervision and guidance of **Dr. John E. Thoppil**, Reader in Botany, University of Calicut and that it has not formed the basis for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled **"Cytogenetic (CHIAS and RAPD), structural (SEM), phytochemical (GC-MS and EDS) and antioxidant assays on *in vivo* and *in vitro* plants of *Plectranthus zeylanicus* Benth."** submitted to the University of Calicut, for the award of the degree of **DOCTOR OF PHILOSOPHY** is an authentic record of original research work done by **BETTY K. P.**, during the period of her study (2004 - 2008) at the Genetics & Plant Breeding Division, Department of Botany, University of Calicut under my supervision and guidance and that it has not formed the basis for the award of any degree or diploma.

Dr. John E. Thoppil
Supervising Teacher

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CONTENTS

ABBREVIATIONS

2,4-D	2, 4-Dichlorophenoxyacetic acid
ACL	Average Chromosome Length
AFLP	Amplified Fragment Length Polymorphism
BA	Benzyl Adenine
BAP	Benzyl Amino Purine
bp	base pair
CHIAS	Chromosome Image Analysis System
CS	Coefficient of Similitude
CTAB	Cetyl Trimethyl Ammonium Bromide
DI	Disparity Index
dNTP	deoxy Nucleotide Tri Phosphate
EDS	Energy Dispersive Spectroscopy
EDTA	Ethylene Diamine Tetra Acetic acid
GC - MS	Gas Chromatography - Mass Spectrometry
GC	Gas Chromatography
IAA	Indole 3-Acetic acid
IBA	Indole 3-Butyric acid
kb	kilobase
KIN	Kinetin
mM	milli molar
MS	Murashige and Skoog
NAA	α-Naphthyl Acetic acid
NBT	Nitro Blue Tetrazolium
ng	nanogram
nm	nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RCL	Range of Chromosome Length
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species
rpm	rotations per minute
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
TAE	Tris Acetic acid EDTA
TBA	Thio Barbituric Acid
TBARS	Thio Barbituric Acid Reactive Substances
TCL	Total Chromosome Length
TE	Tris EDTA
TF%	Total Forma percentage
VC	Variation Coefficient
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar

INTRODUCTION

There is nothing lovelier on this planet than a flower, not more essential than a plant. The love of nature is inborn in mankind, for man himself together with the animals and plants form the body of this world. In ancient days we lived very closely with nature by listening and feeling it in one form or another. This long association and interdependence of plants and animals provide the energies to balance us as the denizens of the Earth. Although the plants that clothe the earth stand mute and unable to travel, they have used their heritage to dominate the landscape and to control and modify the environment.

Humans depend on plants in nearly every aspect of life. Worldwide tens of thousands of species of higher plants and several hundred lower plants are currently used by humans for a wide diversity of purposes as food, fuel, fibre, oil, herbs, spices, industrial crops, building materials, textiles, medicinals, ornamentals, forage and fodder, industrial raw materials *etc*. Photosynthesis provides the biological and chemical energy that fuels our world and is responsible for the oxygen and carbon dioxide cycling that makes our very existence on earth possible. The green invasion of the land thus revolutionized the atmosphere and they are universally recognized as an inevitable resource for the planet. In whatever direction we look, we find ourselves looking upon some object that reminds us of our debt to the world of plants. This tremendous indebtedness of mankind to plants is a sufficient reason in itself to justify the study of plants.

From time immemorial man has been in a long struggle to achieve mastery over the powerful forces of nature. Ancient people have always turned to plants in their daily life for food, shelter, clothing, weapons, healing and even for relief from the hardships of life. But modern man is destroying the harmony of this relationship by overexploitation of the natural resources and unconcerned interference in its spontaneity. This deleterious environment challenges the human body, resulting in

diverse diseases. The wish to put nature back in our lives arises in this perplexing and alarming situation. The complexity of the newly deriving diseases in the current depleting environment causes rapid expansion in the number of medical prescriptions and also accelerates the acceptance of various forms of alternative medicine, most of them employing herbs of some form. The past decade has witnessed a tremendous resurgence in the interest and use of medicinal plant products and also in neutraceuticals in which phytochemical constituents possess long term health promoting or medicinal qualities. The healing techniques and preventive methods of Ayurveda include herbal food supplements to help us in the access of our body's innate healing intelligence, strengthen the immune system and bring balance to mind and body. Today's pharmacopoeia contains many pure extracts and chemical analogues of products with foundation in herbal remedies. The demand for plant based natural products is increasing day by day with the increase in health awareness in the modern civilization. Products like natural colourants, fragrances, cosmetics and pharmaceuticals are in great demand. Recognition of traditional systems of medicines because of their efficacy without side effects and availability at low costs resulted in a great demand for medicinal plants and their products.

Higher plants are still 'the sleeping giants of drug development' (Farnsworth and Morris, 1976), 'a virtually untapped reservoir of potentially useful sources of drugs' (Farnsworth, 1984) that will continue to serve mankind in the 21st century as they have done since the dawn of history (Tyler, 1986). In recent years, numerous studies have been conducted to substantiate and confirm the worth of a number of herbal products in providing significant protection against various toxins and also in the treatment of various ailments. The plant-based formulations have proved biologically more compatible with human system. They are comparatively less toxic and have fewer side effects even on long usages than the synthetics. Some herbal remedies seem to have promise and are certainly worth further exploration.

Nature has bestowed on us a very rich botanical wealth, which includes a large number of medicinal and aromatic plants. Throughout the centuries, herbalists

have used specific herbs or combinations of herbs to treat various diseases. In the tropics alone it has been estimated that 25000-30000 species are in use (Heywood, 1992) and up to 25000 species have been used in traditional medicines. Sometimes the whole plants, including the root system, are used for medicinal purposes. Natural products, their derivatives and analogs represent over 50% of all drugs in clinical use, with higher plant derived natural products representing 25% of the total (Balandrin *et al*., 1993). The World Health Organization estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts (Farnsworth, 1988). In modern medicine also, plants occupy a very significant place as raw material for some important drugs, although synthetic drugs and antibiotics brought about a revolution in controlling different diseases. Judicious use of medicinal herbs can even cure deadly diseases that have long defied synthetic drugs (Bhattacharjee, 2000).

Living plant cells are abundantly equipped with the raw materials for building thousands of compounds that have medicinal properties. Such active compounds include glycosides, alkaloids, essential oils, tannins, flavanoids, vitamins, mineral elements, antibiotics *etc*. Since natural flora represent an unlimited source of several novel compounds including drugs, the conservation and management of natural resources need our prime consideration. Conservative estimates suggest that there are about 30000 species of higher plants existing in this planet. The range of plants used in traditional medicinal preparation is so vast and diverse, that one may rightly wonder if there is such a thing as a 'non-medicinal' plant. But habitat transformation, overexploitation, pollution, climatic change *etc*. offer greatest challenges to the world's biological diversity. Continuous and often indiscriminate collection of medicinal plants in bulk from our forests have caused large scale loss of plant populations and reduced their distribution in the known habitats. The massive deforestation of forest systems on a global scale has recently caused a great alarm among the world community as a whole. The greenery, which was a boon to our existence, is now disappearing along with the rapidly expanding population. The population explosion coupled with the improved standard of living

lead to ruthless exploitation of these plants, resulting in habitat degradation, thereby extinction of them. The immediate and most effective way of protecting these rare, endangered and wild aromatic and medicinal plants is through their conservation in the natural habitat. Because the world's arable land is already utilized almost to its limit, it will be necessary to find new ways to improve plant species of economical or medicinal importance. Possibilities include enhancing resistance to disease, increasing tolerance to stresses, increasing the amount of an important nutrient, introducing other nutrients, *etc*. The net result will be plants that produce more, higher quality resources for the rising population.

The collection of medicinal and aromatic plants scattered in the natural flora is troublesome and also cannot cope up with the ever increasing and changing condition made by the indigenous systems of medicine and also by the modern pharmaceutical industries. Regular and sustained supply of these plants in accord with the mounting demand necessitates the domestication and propagation of them in a large scale. In this imperative context, the recent exciting developments in biotechnology have come as a blessing. Fast changing research scenario globally has established plant tissue culture as a fascinating tool of biotechnology. Tissue culture procedure has been worked out both for mass propagation as well as for inducing and exploiting some clonal variation and other genetic variability for desired chemical traits. Thus *in vitro* technique of micropropagation seems to be an apt answer for increasing efficiency and scale up of plant production. The economy of time, space and labour associated with tissue and organ culture has added to its importance. The technique of plant tissue culture, therefore, holds great promise to plant breeders, pharmaceutical industries and others, besides helping in conservation of our precious natural wealth.

The family Lamiaceae which consists of mostly aromatic herbs having medicinal importance is one of the most advanced angiosperm family that have large species diversity in India. Among the 250 genera and 6700 species reported so far (Mabberely, 1987), 70 genera and 425 species are available in India. The economic importance of the family stems chiefly from the copious presence of volatile oils

among many of its genera. Owing to such oils many species in a wide spectrum of genera have pharmaceutical and culinary uses. It is considered as the second largest source of culinary herbs (Richardson, 1992). In the lists of drugs supplied to herbalists, the Labiates are very strongly represented. Henrich (1992) pointed out that the family is of outstanding importance in its use in indigenous medical system, being ranked third in ethno-botanical importance in studies of medicinal plants used by North American and Indian cultures, and probably the same picture could be found in rural and primitive societies elsewhere in the world. Notable too, is the horticultural value of a few genera such as *Salvia* and *Coleus* whose cultivated species constitute decorative garden plants.

The characteristic features of the family include squarish stem with simple, opposite decussate leaves, zygomorphic bilabiate flowers arranged in verticillasters, superior ovary and gynobasic style. Most labiates accumulate terpenes, flavanoids and a range of other components mainly in epidermal glands of leaves, stems and reproductive structures.

The genus *Plectranthus* in the family is a large and widespread genus with about 80 species having several ethno-botanical uses. *Plectranthus zeylanicus* Benth. [synonym. *Coleus zeylanica* (Benth.) Cramer] is a native of Sri Lanka commonly cultivated as a medicinal plant (Nayar *et al*., 2006). The vernacular name of the plant is Iriweriya in Sinhala language and Iruveli in Malayalam. The plant is profusely branched, semi-succulent, strongly aromatic, softly tomentose herb with fibrous roots, leaves slightly fleshy, ovate or orbicular, and flowers small, blue or purple in terminal, panicled thyrsus (CSIR, 2004). The herb is identified as the source of an Ayurvedic drug Hribera (Iruveli, Valakah, Balam) in Kerala. According to Bhavaprakasanighantu, this drug is cooling, light, carminative and toxic and cures dyspepsia, indigestion, dysentery, vomiting, thirst, fever, dermatitis, ulcers and bleeding disorders (Chunekar, 1982). The entire plant is used in medicine. Gandhatailam and Eladitailam are some of the preparations using the drug (Sivarajan and Balachandran, 2002). The juice of the stem and leaves mixed with honey is taken as a remedy for diarrhoea (CSIR, 2004).

Many higher plants including *Plectranthus zeylanicus* accumulate extractable phytochemicals in sufficient quantities that are economically useful in diverse fields. Most of them can be obtained from plant materials by steam distillation or by extraction with organic or aqueous solvents. As the demand for natural products is increasing nowadays, the new technologies particularly methods for culturing plant cells and tissues will extend and enhance the usefulness of plants as renewable sources of valuable chemicals. It has become increasingly clear in recent years that through plant regeneration from *in vitro* cultures a vast reservoir of genetic variability is available. Regenerative callus culture is considered as a source of useful heritable variation and mass propagation of plants in a limited space. Callus tissues have a unique potential for evolving genetic variations and the plants regenerated from the callus may inherit these variations, which is expected to promote diversity and can be used to select medicinal plants that produce enhanced amount of important secondary metabolites. Recently micropropagation and enhancement of useful plant metabolites by elicitation and immobilization techniques has been reported in a number of medicinal plants. The genetic instability normally encountered during callus mediated regeneration will help in producing large number of selected superior chemotypes of the medicinal plants which has immense demand in the present Indian market.

The variability generated by tissue culture has been termed as somaclonal variation by Larkin and Scowcroft (1981). Plant regeneration from callus and protoplasts enhance the chance of recovering somaclonal variants because of the high incidence of cytological changes in callus tissue. The variability at cellular level can be raised to plant level resulting in the development of novel and desired variants. Somaclonal variants can be distinguished by their biochemical, physiological and genetic characteristics as well as by their morphological traits.

Chromosome variability is of well-known occurrence in cells of cultured tissues as well as in regenerants (Sacristan and Melchers, 1969; Bayliss, 1973). The study of chromosomal behaviour in cultures has proved to be an important parameter of investigation in recent years. The identification and characterisation of

individual chromosomes facilitate genetic improvement. Tissue cultured cells spontaneously accumulate many changes in both the number and structure of chromosomes. The cells in a callus vary in their potential to regenerate because of differences in the chromosome complement. In the calli cell population with heterogeneous chromosomal composition, plantlet regeneration occurs more often from diploid cells than from other cells.

Chromosome research requires a study of the morphology of individual chromosomes. Cytological techniques aid to determine the chromosome constitution and facilitate the recognition of individual chromosomes. Karyotype analysis with the help of computer based Chromosome Image Analysis System (CHIAS) proved to be a powerful tool to study chromosome morphology with great precision and speed. The imaging methods are indispensable in routine chromosome analysis such as karyotyping and detection of chromosomal aberrations and also in the basic chromosome research (Fukui and Nakayama, 1998).

In plant regeneration protocols, molecular tools are extremely powerful in the process of defining plant genetic variation. The minor genetic changes unable to disclose by cytological techniques could be detected by modern molecular techniques such as RFLP, RAPD *etc*. Molecular markers are polymorphic when there is DNA sequence variation between the individuals under study. These markers are therefore, simply an indicator of sequence polymorphism. These very sensitive tools reveal new types of variability that are not detected by phenotype or karyotype analysis. Repeated DNA sequences constitute a large part of higher plant genomes. A great deal of the variation observed between the genomes of related plant species and among plants of a single species occurs in this repetitive component. The PCR based Random Amplified Polymorphic DNA (RAPD) is the most common and effective molecular marker for detecting somaclonal variants. The method relies on the use of short oligonucleotide primers of arbitrary sequence, which are annealed to genomic DNA using low temperature conditions. Priming at a number of closely adjacent complementary sites allows the subsequent amplification of dispersed genomic sequences. This approach generates large number of fragments

providing opportunities to reveal polymorphism between genotypes for use in identification. DNA polymorphism generated by RAPD has been shown to be advantageous because of inessentiality of previous knowledge of the genome, speedy outcome, requirement of only minute amount of template DNA and technical simplicity of methodology. Since RAPD is a dominant marker, it is considered to be polymorphic if the presence and absence of the bands were observed in various individuals and monomorphic if the bands were present among all the individuals (Williams *et al*., 1990).

Many plants contain mixtures of volatile monoterpenes and sesquiterpenes, called essential oils in the glandular hairs that lend a characteristic odour to their foliage. They are highly concentrated, aromatic oily liquids obtained from a variety of aromatic plant materials including flowers, buds, seeds, leaves, bark, herbs, wood, fruits and roots. These aromatic compounds formed by plants as byproducts or as final metabolic products are stored in certain organs of the plant. They are widely distributed in about 60 families especially Apiaceae, Asteraceae, Myrtaceae, Rutaceae, Geraniaceae, Poaceae, Fabaceae and Lamiaceae (Schery, 1954; Sambamurthy and Subramanian, 1989). About 3000 essential oils are known, of which around 300 are commercially important and widely used in perfume, cosmetic, pharmaceutical, agricultural and food industries. It has long been recognised that some of them possess antimicrobial, antioxidant, antiviral, antifungal, antioxigenic, antiparasitic and insecticidal properties (Chemat *et al*., 2007). Essential oils are used in pharmacy or scientifically tested for medical purposes. There are hundreds of preparations - ointments, syrups, pills, *etc*. using essential oils or their constituents as remedies for pains, infections, eczema, bronchitis, skin diseases and many other problems. Some of them are used for perfuming soaps, deodorants, toilet preparations, for flavouring food and beverages. In India about 20% of the 4000 tones of chemicals estimated to be used annually in perfumes and flavours, are obtained from essential oils, which represent an important class of indigenously developed starting material for perfumery and flavour industry. The term aromatherapy, in brief, fragrance for health is the remedial treatment of mental and physical disorders by the application of essential

oils. Despite, the continuing inventions of synthetic aromatics, essential oils still remain as the most important part of fragrances (Ranade, 1993). The judicious use of essential oil and their components offer enormous scope in developing plant derived biosafe products of direct utility to humans (Khanuja, 2000).

The plant chemicals are often classified as either primary or secondary metabolites. The primary metabolites such as carbohydrates, lipids, proteins *etc*. are common to all plants and are involved in the primary metabolic processes of building and maintaining plant cells (Kaufman *et al*., 1999; Wink, 1999). The beneficial medicinal effects of plant materials typically result from the combinations of secondary products. Secondary metabolites biosynthetically derived from primary metabolites are more limited in distribution in the plant kingdom, being restricted to a particular taxonomic group. The individual chemicals isolated from essential oils are more often used than oils and the intimate knowledge of essential oil composition helps to evaluate the quality of oil that allows a better and specially directed application of it (Buchbauer, 2000). Analysis of the essential oil can be easily done using a recently developed technique of Gas Chromatography – Mass Spectrometry (GC-MS). GC is a tool for separating the volatile components while analysis depends upon retention characteristics under standard conditions. The mass spectrometer can be used as a detector for a gas chromatograph in which, the high degree of specificity of the mass spectrum is an aid to the identification of the sample. A GC-MS machine, which has computerized library search discs, can be regarded as the best single tool for essential oil analysis (Jose and Rajalakshmi, 2005).

Many species of the family Lamiaceae possess essential oils secreted by glandular hairs located on their aerial vegetative organs and some of their reproductive organs. Glandular trichomes are specialized epidermal cells known to be the primary sites of biosynthesis, secretion and accumulation of volatile oils and other secretions produced by the plants. The high resolution images of the glandular trichomes that help to reveal the structural details and complexities of glands can be obtained by Scanning Electron Microscopy (SEM). SEM, when used in conjunction

with an Energy Dispersive X-ray Spectrometer can perform an elemental analysis. Energy Dispersive X-ray Spectroscopy (EDS) is a chemical microanalysis technique, which utilizes X-rays that are emitted from the sample during bombardment by the electron beam to characterize the elemental composition of the analyzed volume without damaging the specimen.

Cell damage caused by free radicals appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline and brain dysfunction. The role of active oxygen and free radicals in tissue damage in various human diseases is becoming increasingly recognized (Halliwell and Aruoma, 1992). Active oxygen, in the different forms such as super oxide, hydrogen peroxide and hydroxyl radical, is a byproduct of normal metabolism and attacks biological molecules, leading to cell or tissue injury. Various beneficial compounds known as antioxidants controls free radical formation naturally. They act as free radical scavengers and slow down not only radical oxidation but also the accompanying damaging effects in the body.

Many plant derived substances, collectively termed as phytonutrients or phytochemicals are becoming increasingly known for their antioxidant properties. Phenolic compounds are commonly found in plants and they have been reported to have multiple biological effects, including antioxidant activity. Crude extracts of herbs and other plant materials rich in phenolics are increasingly of interest in the food industry. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection from coronary heart disease and cancer is also raising interest among scientists, food manufacturers and consumers, as the trend of the future is moving toward functional food with specific health effects (Loliger, 1991). The preservative effect of many spices and herbs suggests the presence of antioxidative and antimicrobial constituents. Several studies confirmed that many leafy spices, especially those belonging to the Lamiaceae family show strong antioxidant activity (Hirasa and Takemasa, 1998). A number of phenolic compounds with strong antioxidant activity have been identified in these plant extracts (Nakatani, 1997).

The economic importance of the phytochemicals necessitates further research in the field of medicinal and aromatic plants. The detailed study of the genetic constitution at both the chromosomal and molecular level together with the phytochemical analysis to detect the important phytochemical constituents having added biological activities is of great significance. Tissue culture provides a promising approach for the production of large number of plants with improved performance. In the present study an attempt is also made for the micropropagation of *Plectranthus zeylanicus*. The qualitative evaluation of the regenerants by means of cytogenetic, structural and phytochemical investigations helps to confirm the variability which is an asset in plant improvement. The screening of free radical scavenging capacity of the plant extract ensures the application of it in the defensive role against various diseases.

The study thus aims to fulfill the following objectives:-

- To establish a protocol for *in vitro* propagation of the medicinal and aromatic plant, *Plectranthus zeylanicus*.
- To analyze the possible variations of the *in vitro* derived plants from the parent plant by comparing them in various aspects such as:-
  - Karyomorphology
  - DNA profiling using RAPD
  - Essential oil and phenolics
  - Glandular trichomes
  - Elemental composition
  - Free radical scavenging ability.

REVIEW OF LITERATURE

Micropropagation

Plant improvement programmes primarily depend on the availability and efficient induction of genetic variability. The technique of plant cell and tissue culture offers an ample scope for *in vitro* rapid clonal multiplication (Murashige, 1977) and somaclonal variations for the recovery of novel genotypes (Scowcroft, 1984; Evans and Sharp, 1988). These variations may involve phenotypic alterations, chromosomal rearrangements, biochemical and molecular changes (Reddy, 1989). The heritable variations thus emerged, forms an essential ground for selection in quality improvement of plants.

Cell culture technology has been applied to a number of medicinal plants to obtain pharmaceutically important drugs. Schieder (1985) has suggested that plant cell and tissue culture, may be an alternative to conventional methods for the improvement of medicinal plants.

Although more than 50 different devised media formulations have been used for the *in vitro* culture of tissues of various plant species, the formulation described by Murashige and Skoog (MS medium, 1962) is the most commonly used. The level and kind of plant growth regulators included in the culture medium largely determine the success of tissue culture. Root and shoot initiation and the process of differentiation from unorganised callus tissue are closely regulated by the relative concentrations of auxins and cytokinins in the medium (Skoog and Miller, 1957; Ammirato, 1983; Bajaj *et al*., 1988; Rout and Das, 1997a, 2004). Auxin : cytokinin ratios of ~10 yield rapid growth of undifferentiated callus, a ratio of ~100 favours root development and a ratio of ~4 favours the development of shoot morphogenesis (Murashige, 1980). Cytokinin levels were shown to be most critical for multiplication of many medicinal plants. The inclusion of low concentration of

auxins along with cytokinin triggered the rate of shoot proliferation in some genotypes (Roja *et al*., 1987; Ravishankar and Venkataraman, 1988; Barna and Wakhlu, 1988; Satheesh and Bhavanandan, 1988; Upadhyay *et al*., 1989; Jha and Jha, 1989; Tsay *et al*., 1989; Arora and Bhojwani, 1989; Sharma *et al*., 1993; Mao *et al*., 1995; Chen *et al*., 1995; Sharma and Singh, 1997; Rout and Das, 1997a, 1997b; Shasany *et al*., 1998; Saxena *et al*., 1998; Rout *et al*., 1999).

The tropical genus *Coleus*, differs from the related *Plectranthus* by having the stamens united, but this difference is not quite consistent, thus *Coleus* has been reduced to a synonym (Ryding, 1994). Much of the micropropagation research on the genus *Coleus* was concentrated on *Coleus forskohlii* and *Coleus blumei*. The establishment of *in vitro* cultures of *Coleus blumei* was published in 1977 by two different groups, Razzaque and Ellis (1977) and Zenk *et al*. (1977). Both groups used B5 medium (Gamborg and Eveleigh, 1968; Gamborg *et al*., 1968) for callus and suspension cultures. Razzaque and Ellis (1977) supplemented this basal medium with 1 mg/l 2,4-D and 0.1 mg/l kinetin. Zenk *et al*. (1977) tested 35 differently substituted phenoxyacetic acids (10^{-5} M) as sole addition and found that 2, 4-dimethyl phenoxyacetic acid had the highest effect. Successful plantlet regeneration in the callus and shoot tip cultures (Hervey and Robbins, 1978; Smith and Murashige, 1982) and large scale production of rosmarinic acid in cell cultures (Ulbrich *et al*., 1985) was reported in *C. blumei*.

Attempts to obtain high yielding plants of *C. forskohlii* by somaclonal variation or UV mutation were made by Mandler-Henger (1988). The cultures were established on B5 medium (Gamborg *et al*., 1968) with growth regulators 2, 4-D and kinetin for callusing and BAP and IAA for shooting and rooting respectively. A totally different method was used by Mersinger *et al*. (1988) who produced forskolin from suspension cultures of *C. forskohlii*.

Bayliss (1980) produced genetic variability through cell cultures. Since then the application of plant tissue culture for the induction of stable and heritable variations had been demonstrated in a range of economically important plant species (Evans and Sharp, 1985; Bajaj, 1986; Mathur *et al*., 1988).

Micropropagation from shoot tips of 20 to 30 days old aseptically germinated seedlings of *C. forskohlii* was reported by Sen and Sharma (1991). Multiple shoots were induced on MS medium supplemented with BAP. On testing auxins for their effect on shoot proliferation, only IAA together with BAP showed an effect. Another approach for micropropagation of *C. forskohlii* using nodal segments of mature plants was reported by Sharma *et al*. (1991). Callus induction and shoot proliferation was effected in phytohormone combinations of BAP and NAA as well as kinetin and IAA respectively. The *in vitro* selection method developed by Ibrahim *et al*. (1992) for *C. blumei* involved the initiation of the callus and regeneration of plantlets on MS medium with BA and NAA.

The interest in root cultures of *C. forskohlii* was based on the occurrence of forskolin in root tubers and the close correlation between root differentiation and production of forskolin. Krombholz *et al*. (1992) established root cultures of *C. forskohlii* from primary callus and suspension cultures and also by transformation of young leaves of *C. forskohlii* with *Agrobacterium rhizogenes*.

Shoot tip cultures, callus cultures and excised root tip cultures from rooted *in vitro* micropropagated shoots were established and investigated for their forskolin content by Sen *et al*. (1992, 1993). Multiple shooting from the shoot tip explants was promoted by BAP (0.5 - 2.5 mg/l). Callus cultures were established on MS media and further cultivated on White's medium (White, 1963) with 1 mg/l BAP and 1 mg/l NAA where they became friable, whitish and rhizogenic. Excised root tips from *in vitro* propagated rooted shoots grew to an entangled mass of roots with primary and secondary laterals on ¼ MS basal medium with 0.5 mg/l IBA and 1% sucrose.

An attempt was made with the help of high producing cell suspension cultures of *C. blumei* to elucidate the biosynthetic pathway of rosmarinic acid and to isolate the enzymes involved in the biosynthesis (Petersen and Alfermann, 1988; Petersen *et al*., 1993, 1994, 1995). The highest production of coleonol (forskolin) was described by Tripathi *et al*. (1995) in callus cultures of *C. forskohlii*.

Mukherjee *et al*. (1996, 2000a) demonstrated the production of forskolin in *in vitro* cultures of *C. forskohlii* transformed with *Agrobacterium tumefaciens*. Increased forskolin yield was obtained in transformed root, rhizogenic calli and cell suspension cultures of *C. forskohlii* when treated with various concentrations of auxins, auxin conjugates, cytokinins and giberellic acid (Mukherjee *et al*., 2003). The induction of hairy roots by infection with *Agrobacterium rhizogenes* and subsequent much higher forskolin production was described in other scientific experimentations done in *C. forskohlii* (Zhou *et al*., 1996; Sasaki *et al*., 1998). Zagrajski *et al*. (1997) pointed out the effectiveness of nodal explant as the best regeneration system in *C. blumei*.

A better alternative for micropropagation using flowers of *C. forskohlii* was suggested by Suryanarayanan and Pai (1998). Substantial callus formation was initiated from florets, stem and shoot tips on MS medium fortified with NAA and BA. Subculturing of the callus into another growth regulator supplemented medium was required for organogenesis in shoot tip and stem cultures but not required in floral culture.

An experiment was conducted to standardize the type of propagation material (terminal, middle or basal cuttings) and growth regulators for *in vitro* propagation of *C. forskohlii* (Sundharaiya *et al.,* 2000). The terminal cuttings supplemented with IBA (500ppm) recorded the highest survival percentage. A high frequency shoot organogenesis and plant establishment protocol has been developed for *C. forskohlii* from leaf derived callus cultures (Malathy and Pai, 1999; Reddy *et al.,* 2001). They were successfully raised somaclones exceeding the parent plants in forskolin content.

Multiple shoot emergences from shoot tip explants was observed in *C. forskohlii* by Bhattacharyya and Bhattacharya (2001) on MS medium augmented with kinetin and IAA. Asamenew and Narayanaswamy (2001) proposed another protocol for callus and plant regeneration in *C. forskohlii* with growth regulators IAA and BAP.

Significant enhancement in forskolin content was observed in genetically transformed callus cultures of *C. forskohlii* in presence of casein hydrolysate (Mukherjee *et al.*, 2000b). The efficiency of genetic transformation in callus cultures of *C. blumei* using *Agrobacterium* was evaluated and appreciable improvement was noticed in rosmarinic acid production (Bauer *et al.*, 2002, 2004). Micropropagation of *Plectranthus vetiveroides* was obtained on MS medium supplemented with BA (Sivasubramanian *et al.*, 2002). Biotechnological production of rosmarinic acid with plant cell cultures from *C. blumei* has been proposed by Petersen and Simmonds (2003).

The growth and rosmarinic acid production by *C. forskohlii* hairy root cultures were examined by Li *et al.* (2005) in various liquid media such as MS and Gamborg B5 medium. Their inventions recommended methyl jasmonic acid as an effective elicitor for rosmarinic acid production. Anbazhagan *et al.* (2005) achieved callus induction and shoot emergence from the leaf explants of *C. forskohlii* on MS medium with 1mg BA/l and 2mg NAA/l. *In vitro* multiplication with same growth regulators was tried by Rajasekharan *et al.* (2005).

A rapid and higher effective method of micropropagation using terminal and axillary buds with MS medium having growth regulators BA and NAA had been reported in *C. blumei* (Rani *et al.*, 2006).

Variations shown by plants regenerated *in vitro* had been the subject of numerous scientific inquiries (D' Amato, 1977; Skirvin, 1978; Earle and Demarly, 1978; Larkin and Scowcroft, 1981; Benzion *et al*., 1986; Sun and Zheng, 1990; Ahmed and Sagi, 1993; Kaeppler and Phillips, 1993; Phillips *et al.*, 1994). There are reports of isolation of secondary metabolites in remarkable yield from tissue and cell suspension culture of higher plants (Ellis, 1988) either in levels equivalent to or higher than the parent plant (Basu and Chand, 1998).

Cytological Analysis

The passage of many plant tissues through an *in vitro* culture phase frequently causes chromosomal instability (Constantin, 1981). When plant cells are

allowed to proliferate in callus or suspension culture, there is a high probability of chromosomal variation between cells and of the emergence of cell lines with chromosome complements differing significantly from those of cells in the parent plant tissue. The occurrence of chromosomal changes in undifferentiated callus and suspension cultures has been reviewed by Bayliss (1980). Chromosomally variant cells in cultures of at least 55 plant species are mentioned in this review. Nearly all plants studied showed cells in culture with chromosome numbers and karyotypes different from those characteristic of cells of the intact plant. The changes which occur as a result of the instability are characterised as both structural rearrangements and numerical variations in the chromosomes. Mutations of chromosome complements in tissue culture are known to be common phenomena (D' Amato, 1975; Partanen, 1965; Sunderland, 1977). The chromosome complement changes in somatic cells of plants may be enhanced in tissue culture and plants of different ploidy may be produced (Mitra *et al*., 1960; Muir, 1965; Murashige and Nakano, 1965; Torrey, 1967; Wu and Jampates, 1986).

The studies on morphogenesis in long term plant tissue cultures demonstrated that during the prolonged period of subculture there was a progressive loss of organ-forming capacity in all tissue strains. This loss was paralleled by increasing abnormalities in the chromosomal constitution, including higher chromosome numbers and greater frequency of aneuploidy (Torrey, 1967). McClintock (1984) predicted tissue culture as one of the stress factor that could cause widespread genomic restructuring facilitated by transcriptional transposon activation, transposition of mobile elements and chromosome-breakage-fusion-bridges.

Calli obtained from root explants of *Zea mays* exhibited chromosomal abnormalities like inhibition of cell plate formation, chromosomal breakage, stickiness and clumping of chromosomes, asynchronous division, chromosomal grouping, laggards and micronuclei formation. Numerical variation in chromosomes including hypo- and hyper-diploid cells was observed (Mohanty *et al*., 1986).

Although the chromosomal constitution of certain plants seems to be highly stable *in vitro* (Bhaskaran, 1989) much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and chromosomal abnormalities. These chromosomal variations shown by the cultured cells may be due to the mixoploid nature of the source (explant) used or due to culture conditions (Phillips *et al*., 1994).

Several reports indicate that plants regenerated from callus or suspension cultures may show genetic changes ranging from increased phenotypic variability (Nishi *et al*., 1968; Williams and Collin, 1976; Lester and Berbee, 1977) through mitotic evidence of chromosomal rearrangements (Cummings *et al*., 1976) to complete polyploid or aneuploid plants (Murashige and Nakano, 1965; Sacristan and Melchers, 1969, 1977). In contrast, propagation techniques employing meristem culture successfully regenerate large numbers of genetically uniform plants (Murashige, 1974; Holdgate, 1977; Rao, 1977) suggesting that significant chromosomal changes occur principally in dedifferentiated callus or suspension cultures (Malnassy and Ellison, 1970).

It has been clear since early in the development of plant tissue culture methodology that a consequence of growth *in vitro* was the appearance of dividing cells with chromosome numbers and karyotypes not usually found within the intact plant (Partanen, 1963; Sheridan, 1975; D' Amato, 1975, 1977).

A correlation between nuclear state and potential for morphogenic expression has been observed. Calli derived from various explanted organs or cells in suspension show cytological alterations with prolonged subculture, the individual cells becoming progressively polyploid and aneuploid (D' Amato, 1977). Several literature reviews dealing with the genetically variable cells exhibiting alterations in chromosome number and structure in plant tissue cultures are available (Sacristan, 1971; Bayliss, 1973, 1975; D' Amato, 1978; Roy, 1980; Gupta and Ghosh, 1983; Bajwa and Wakhlu, 1986; Nair *et al*., 1993).

Plant regeneration appears to be linked with chromosomal behaviour of the source and callus culture (Navok, 1980; Jha and Roy, 1982; Henry *et al*., 1994;

Kumar and Mathur, 2004). Variation in chromosome structure and number disturbs the physiological and genetic balance of the callus leading to a loss in the capacity to regenerate plants (Torrey, 1967; Singh, 1986). Regenerative capacity of cell population decreases, as karyotypic abnormalities increase (Rice and Carlson, 1975).

Of the various changes attributable to somaclonal variation in tissue-cultured plants, cytogenetic changes are considered most prevalent. Chromosome based variation resulted from changes in chromosome number, chromosome rearrangements, breakage and lagging has been a common phenomenon in tissue-cultured plants of different species (Bayliss, 1980; D' Amato, 1985; Lee and Phillips, 1988; Phillips *et al*., 1994; Gupta, 1998). Chromosomal changes may also give rise to DNA variation that result in mutation at different levels (Vazquez, 2001).

Kaeppler *et al.* (2000), in their review article describes evidence indicating that epigenetic variation is an important mechanistic basis of somaclonal variation in plants. Epigenetics occurs in clonal expansion of a single cell leading to a diversity of cell types (Holliday, 1993). Recent epigenetic research suggests that DNA methylation is one of the major mechanisms. DNA methylation, being reversibly changeable over generations, inducing no base alteration and strongly affecting gene expression through regulation of chromatin structure appears to be one of the major mechanisms involved in epigenetic inheritance (Akimoto *et al*., 2007). In addition, the genes constantly silenced due to hypermethylation can be transcriptionally activated by demethylation, resulting in phenotypes and these changes are stably inherited. The biological function of DNA methylation has been proposed to be involved in gene silencing, often being associated with hypermethylation of promoter sequences (Paszkowski and Whitham, 2001; Bird, 2002).

Variations occurring in the plant genome in response to passage of plant cells through cycles of tissue culture and regeneration have been mentioned by Cullis and Creissen (1987). According to him, the genomic changes mainly occur in the highly repetitive fraction of the genome and are limited to a specific subset of these sequences which may be localized at particular chromosomal sites.

The occurrence of variability in chromosome number in *in vitro* cultures and the probable reasons for the variations were reviewed by Chatterjee and Prakash (1993). Chromosomal abnormalities especially chromosome doubling is a common feature associated with tissue culture (Morel, 1971). Chromosomal changes found in plants regenerated from tissue culture can be induced by media components, culture age, explant tissue and genotype. Chromosome breakage and its consequences (deficiencies, duplications, translocations and inversions) are events quite frequently observed in plant tissue culture. Such breakpoints are often associated with late replicating chromosome regions (Peschke and Phillips, 1992). Rearrangements involving chromosome breaks at heterochromatin was described by Sacristan (1971) in *Crepis capillaris* cultures. Since then, breakpoints involved in chromosome alterations associated with heterochromatic regions have been detected in regenerated plants from several species (McCoy *et al*., 1982; Lapitan *et al*., 1984; Johnson *et al*., 1987).

Cytogenetic instability is a common observation in plants regenerated from maize callus cultures (Lee and Phillips, 1987; Benzion and Phillips, 1988). Among the screened cell population, cells having different ploidy status and structurally altered chromosome complement have been noticed frequently in the callus and also in plants regenerated from this callus (Edallo *et al*., 1981; McCoy and Phillips, 1982; Fluminhan *et al*., 1996). Cytological analysis of the cultured cells carried out by Sengupta *et al*. (1986) revealed a heterogenous population of dividing cells with both euploid and aneuploid chromosome numbers. Among the species reported, polyploidy is the most frequently observed chromosomal abnormality in the regenerants. In the cytogenetic study performed in eight callus lines, a high level of polyploidization of all lines during callogenesis and in the subsequent culture, irrespective of their diploid or tetraploid origin was reported (Fras and Maluszynska, 2003). Although diploid, triploid and tetraploid plants were regenerated from the investigated callus lines, the majority of the regenerants were diploid. In the regenerative callus culture of *Dianthus*, 93% of regenerated shoots were diploids, despite their origin from a callus with higher ploidy level cells, indicating that diploid cells have a higher ability to regenerate shoots (Nontaswatsri and Fukai,

2005). Strong selection of cytogenetically normal plants was observed in some plants in the process of regeneration from calluses, which had karyological changes (Swedlund and Vasil, 1985; Hahne and Hoffmann, 1986). On the other hand, considerable variations in chromosome numbers among regenerants were also reported (Bennici and D' Amato, 1978; Asakura *et al*., 1995; Aida and Shibata, 2002). The investigation carried out on cytological stability of callus of pearl millet exposed different types of chromosomal abnormalities in the cell populations including hypo-haploidy, aneuploidy and polyploidy. The regenerants were all diploids (Mythili *et al*., 1995). There are many well documented examples of numerical abnormalities with altered ploidy levels and structural rearrangements in the chromosome complement of cell populations associated with *in vitro* culture and plant regeneration (Krikorian *et al*., 1983; Renfroe and Berlyn, 1985).

Cytological methods have been employed to assess the somaclonal variants within garlic callus culture (Dolezel and Novak, 1985; Novak *et al*., 1986) and also within subsequent regenerants (Novak, 1980; Al-Zahim *et al*., 1999). Sinha *et al*. (1987) reported numerical variation in chromosomes in the long term callus cultures raised from cotyledons of *Sesbania grandiflora*. Shanker and Mohanram (1993) disclosed changes in chromosome numbers both in the callus cells and in shoot buds regenerated from the callus. Cytological analysis of fresh callus and 12 months old callus developed in *Capsicum annuum* exhibited variations in chromosome structure and number with cells showing metaphase clumping, chromosome bridges, anueploidy and polyploidy (Nair and Kumar, 1998). Piola *et al*. (1999) described the occurrence of chromosomal instability and somaclonal variation with plant regeneration from unorganised callus producing adventitious buds rather with cultures derived from axillary meristems. Detailed morphometrical analysis carried out in the somaclonal variant of *Ocimum basilicum* revealed only slight structural variations of individual chromosomes (Tajo and Thoppil, 2003). Chromosomal instability, both structural and numerical was found in callus culture of *Pisum sativum* (Kumar and Mathur, 2004). The frequency of altered chromosome constitution increased with increasing concentrations of plant growth regulators.

Karyological examination of *in vivo* and *in vitro* plants that helps to identify the chromosome associated somaclonal variation is an area of immense interest among researchers. Some workers have reported the karyological data of the genus *Plectranthus* earlier. Thoppil (1993) studied the karyomorphology of *Plectranthus zeylanicus*, in which he identified the diploid chromosome number as 2n = 28.

Random Amplified Polymorphic DNA (RAPD) Analysis

The polymerase chain reaction (Saiki *et al*., 1988) has been the basis of a growing range of newer techniques in plant identification based on detection of DNA sequences. The amplification of DNA using arbitrary 10-base oligonucleotide primers has been described as a strategy to detect RAPD in many eukaryotic organisms (Williams *et al*., 1990). Polymorphisms generated by random amplified polymorphic DNA (RAPD) analysis have been used for fingerprinting (Connolly *et al*., 1994) and evaluating genetic relationships among cultivars (Stiles *et al*., 1993). RAPD - PCR has entered plant research in a revolutionary way due to its technical simplicity and genetic informativeness. This molecular marker using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Xena de Enrech, 2000).

Among the main concerns of the *in vitro* cloning are the occurrence of somaclonal variations and their methods of detection. RAPD or any other polymerase chain reaction (PCR) based analysis would be an attractive method for the detection of somaclonal variations. Polymorphism at the DNA level revealed by PCR based approaches such as RAPD have found extensive applications in plant genetics (Wilkie *et al*., 1993; Yang and Quiros, 1993; Yu and Pauls, 1993; Lashermes *et al*., 1993). DNA based marker will be more accurate in determination of relationships between accessions that are too close to be accurately differentiated by other variables. RAPDs have been used to analyze genetic variation in several species (Hu and Quiros, 1991; Chalmers *et al*., 1992; Demeke *et al.*, 1992; Halward *et al*., 1992; Lanham *et al*., 1992; Vierling and Nguyen, 1992). RAPD method for generating DNA fingerprints elucidates genetic differences at the DNA sequence

level which result from evolutionary mechanisms such as DNA deletions, additions, substitutions, repetitions and translocations.

Molecular markers such as RAPD, RFLP, AFLP, *etc*. have proven to be useful in identifying and estimating the genetic diversity among closely related cultivars and wild species. They contribute much for establishing taxonomic discrimination and phylogenetic relationships among taxa. The initial evaluation of genetic variation in the species among zones, among populations and among individuals can be effected by RAPD markers. For the identification of pigeonpea cultivars and their related wild species, RAPD markers were used and the level of polymorphism revealed the immense potential of RAPD in the genetic fingerprinting of pigeonpea (Ratnaparkhe *et al*., 1995). Phylogenetic relationships in *Ocimum* (Singh *et al*., 2004a), *Salvia* (Khalil *et al.*, 2005*) etc*. have been determined by analysing their genetic pattern using RAPD.

The usefulness of RAPD technique for detecting genetic variation among cultivars and identifying germplasms is well established (Welsh and McClelland, 1990; Hu and Quirios, 1991; Halward *et al*., 1992; Wilde *et al*., 1992). Genetic variation in rice cultivars was investigated at the DNA level using RAPD method. The extensive polymorphism assisted selection for genetic improvement in rice breeding (Yu and Nguyen, 1994).

During the last few years, various molecular DNA markers, which screen nuclear and organellar genomes have been utilized for the fast and unambiguous assessment of the genetic fidelity of micropropagated plants (Rani and Raina, 1998, 2000, 2002). Bouman *et al*. (1992) found intraclonal RAPD polymorphism among micropropagated *Begonia*, but at a lower frequency than phenotypic variations. Rani *et al*. (1995) observed RAPD variations among 23 morphologically similar micropropagated *Populus deltoides* plants originating from the same clone. RAPD technology has been used in the analysis of culture derived somaclones of two grasses, *Lolium* (Wang *et al*., 1993) and *Triticum* (Brown *et al*., 1993) and changes in banding pattern have been reported. Utility of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been amply demonstrated in a large array

of plants by many workers (Isabel *et al*., 1993; Gupta and Varshney, 1999; Latto *et al*., 2005). Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of the plants. Several groups have applied the RAPD technique to detect somaclonal variations (Munthali *et al*., 1996; Bohm and Zyprian, 1998; Al-Zahim *et al*., 1999; De Verno *et al*., 1999).

Godwin *et al*. (1997) analyzed eight rice somaclones generated from mature seed-derived callus cultures by RAPD method and identified that all somaclonal families differed significantly from the original material, indicating genomic alterations in all families. The genetic fidelity of the micropropagated plants were recently analyzed using RAPD as molecular marker for confirmation of genetic homogeneity of the *in vitro* raised plantlets (Piccioni *et al*., 1997). Rout *et al*. (1998) used 15 different decamers to assess the genetic stability of the micropropagated plants of *Zingiber officinale*. The amplified products exhibited monomorphisms among all the *in vitro* raised plants and were similar to those from the mother plants.

RAPDs have been used in the diversity studies in some medicinal plants. Padmesh *et al*. (1999) studied the diversity of *Andrographis paniculata*. They collected germplasm from different parts of India and South East Asia and a dendrogram was produced which formed 5 clusters with most of the genotypes from same geographical locations falling together in the same clusters. Shasany *et al*. (1999) worked out the relatedness and diversity among 23 accessions of garlic from different parts of India and Argentina and obtained high degree of polymorphism in the RAPD banding pattern.

Molecular marker, RAPD was used to identify the level of polymorphism of somaclonal variants in *Triticum* and *Saccharum* (Taylor *et al*., 1995a, 1995b). The variation expressed in phenotypes of *in vitro* derived *Musa acuminata* was found to be accompanied with the genetic variation as revealed by the DNA polymorphism in the RAPD profile (Vidhya and Nair, 2002).

Molecular genome analysis using RAPD proved to be suitable for screening genetic variation in *Stachys sieboldii* regenerants obtained at various phytohormone concentrations. High DNA polymorphism was demonstrated for two types of

S. sieboldii callus cultures and for plants regenerated from a callus culture (Kochieva *et al*., 2002). RAPD is referred as an appropriate tool for examining the clonal identity and for certifying genetic fidelity of *in vitro* propagated plants (Gupta and Rao, 2002; Carvalho *et al*., 2004). The results of the experiment carried out by Feuser *et al*. (2003) in pineapple provided a contribution towards the abilities of isozyme and RAPD markers to detect somaclonal variants in pineapple micropropagated plantlets when genotypic fidelity monitoring is necessary. The sensitivity of RAPD for revealing the genetic basis for somaclonal variation was demonstrated by Kim *et al*. (2003) and detected significantly higher level of genotypic polymorphisms between regenerants derived from a single genotype.

Genome characterization through RAPD showed distinct variation in profiles to confirm menthol tolerance and high menthol content character of the genotype that favoured the *in vitro* selection of *Mentha arvensis* clones (Dhawan *et al*., 2003). The changes in the banding pattern obtained in basil plants regenerated *in vitro* suggested the existence of genetic variation that might affect the biochemical synthesis of phytoproducts (Rady and Nazif, 2005).

Essential Oil Analysis

The main phytochemical constituents of the genus *Plectranthus* are diterpenoids, essential oils and phenolics. The major essential oil ingredients of *Plectranthus* include mono- and sesquiterpenes (Abdel-Mogib *et al*., 2002). Variations in the chemotypes of essential oil have been observed for several forms of plants gathered from ecologically dissimilar regions (Grayer *et al*., 1996; Silva *et al*., 2003). The effect of a range of nutritional and hormonal conditions on the synthesis and accumulation of secondary compounds *in vitro* has been the subject of intense investigation (Mantell and Smith, 1983). Many works on medicinal plants regarding the production of active principles by *in vitro* cultures developed from any plant part and optimization of the product yield by manipulating the medium composition and the physical factors are described (Rideau, 1987). Extensive investigations on secondary metabolism of plant species in culture revealed diverse findings. In *Mentha* species, absence of accumulation of terpenoids (Wang and

Staba, 1963; Becker, 1970; Suga *et al*., 1980), accumulation at reduced levels (Bricout *et al*., 1978) and cultures that match whole plants in their biosynthetic capabilities (Kireeva *et al*., 1978; Charlwood and Charlwood, 1983) were reported.

Callus cultures of *Pimpinella anisum*, *Foeniculum vulgare* and *Mentha piperita* did not produce appreciable amount of monoterpenes comparable with that of intact plants (Becker, 1970). A very low and insignificant level of monoterpene accumulation and composition was found in callus cultures of *Geranium* (Brown and Charlwood, 1986). Kulkarni *et al*. (1996) established intraclonal variations in terms of oil content and composition in plants derived from leaf cuttings of rose scented geranium. Both rooty and leafy callus cultures established in *Origanum vulgare* by Isabel *et al*. (1998) failed to synthesize mono- and sesquiterpenoids which are the major compounds in the parent plant. But there was increase in the quantity of other components.

Accumulation of essential oils in plant tissue culture is the result of *de novo* synthesis and in many cases the type and proportion of their components differ greatly from those in the parent tissues. Increased or decreased essential oil concentration was detected in different growth hormones. All the micropropagated plantlets of *Lavandula dentata* showed positive correlation between oil accumulation and the percentage of glandular hairs in the secretory stage. Quantitative changes in the major mono- and sesquiterpene components of plantlet oil were also noticed in response to the effect of varying growth regulator concentration in the culture medium (Sudria *et al*., 1999; Bonfill *et al*., 2002).

Higher production of secondary metabolites in the callus cultures of various plant species has been reported (Huang and Staden, 2002; Karam *et al*., 2003). Callus cultures of *Melissa officinalis* ssp. *altissima* were established and cultured on MS media supplemented with various phytohormones. Several new compounds were detected in callus cultures. Changes in oil production and composition as well as enhanced accumulation of sesquiterpenes/monoterpenes were observed depending upon the changes in phytohormone concentration (Binder and Mandour, 2000). Samresh *et al*. (2000) reported unchanging oil profile in *in vitro* raised plants

of *Ocimum basilicum* even after 8 subculturing passages. The effect of benzyl adenine (BA) and IBA on ultrastructures, gland formation and essential oil accumulation resulted in the finding that BA stimulates essential oil production and IBA decreases oil concentration of *in vitro* derived plantlets in *Lavendula dentata* with respect to control (Sudria *et al*., 2001). The variation in morphology and essential oil components in 63 regenerated plantlets of *Lavandula vera* was investigated by Tsuro *et al*. (2001). None of the regenerated plantlets produced as much essential oil as the original plant. The regenerated plantlets had a different fragrance characterised by higher levels of distinct components. Tissue culture raised plants of *Mentha arvensis* showed similar essential oil profile as that of the parent plant, but at early stages of growth there was distinct change in oil composition (Phatak and Heble, 2002).

In vitro culture of lavender plants were carried out to determine the effects of plant growth regulators on the multiplication, oil production and chemical composition of lavender essential oil (Badawy *et al*., 2003). Genetically variable clones with high oil content of patchouli were identified in *in vitro* culture (Mariska and Lestari, 2003). *In vitro* shoots of sage (*Salvia officinalis*) were established under 8 different hormonal supplementations. The respective essential oils were composed of more than 75 compounds and the percentage compositions of the oils were found to exhibit a narrow range of variation. However, the type and concentration of growth regulators apparently influenced the accumulation of essential oils (Gomes and Ferreira, 2003).

The essential oil composition and genetic variability of six commercial cultivars of thyme (*Thymus vulgaris*) were analysed by GC-MS and RAPD. All evaluated cultivars belong to the thymol chemotype with differences in the concentrations of thymol, γ-terpinene, p-cymene and other minor components (Echeverrigaray *et al.*, 2001). Morphological, chemical and genetic differences of *Ocimum gratissimum* accessions were studied to determine whether volatile oils and flavanoids can be used as taxonomic markers and to examine the relationship between RAPDs and these chemical markers (Vieira *et al.*, 2001). The genetical

distinctness shown by the accessions was found to be highly correlated to volatile oil constituents.

GC-MS analysis revealed carvacrol, α-terpinolene and p-cymene as the abundant components of *Plectranthus cylindraceus* oil. The presence of these components was further confirmed by ^{13}CNMR analysis (Marwah *et al*., 2007). The oil of *P. cylindraceus* was found to be chemotypically similar to the oils of *P. coleoides* (Buchbauer *et al*., 1993), *P. tenuiflorus* (Mwangi *et al*., 1993), *P. amboinicus* (Vera *et al*., 1993) and *P. melissoides* (Mallavarapu *et al*., 2005) and the major components identified were carvacrol and cymene. The phytochemical analyses of the extracts of *Plectranthus* spp. have revealed the presence of abietane diterpenoids and eudesmane sesquiterpenes (Orabi *et al*., 2000).

Thoppil (1993) reported the essential oil composition of *Coleus zeylanicus* with α-terpineol and δ-cadinene as the major components. Misra *et al*. (1994) examined the oil from the roots of 10 genotypes of *C. forskohlii* and reported the presence of 3-decanone, bornyl acetate, β-sesquiphellandrene and γ-eudesmol as major constituents. Chowdhury and Sharma (1998) identified 18 important compounds in the oil from *C. forskohlii* of which 22% were hydrocarbons and 69% oxygenated compounds with α-fenchyl acetate and α-pinene as the major components. The essential oil characterization studies conducted on the genus *Coleus* lead to the discovery of β-ionone and α-humulene as the major components in *C. laciniatus* and β-thujone and α-farnesene as the active principles in *C. parviflorus* (Thoppil and Jose, 1995). The composition of essential oil of the leaves of *C. zeylanicus* was analyzed by GC-FID, GC-MS and olfactoric evaluation. 80 compounds were detected with monoterpenes especially geraniol and nerol derivatives, hexane- and octane derivatives as the main constituents (Jirovetz *et al*., 1998).

Constituents of the essential oil of *P. amboinicus* contained carvacrol, p-cymene and γ-terpinene as the major ones (Mallavarapu *et al*., 1999). The root essential oil of *C. forskohlii* separated in the GC column included four classes of

compounds as monoterpenes, sesquiterpene hydrocarbons, sesquiterpene alcohols and diterpenes in varying proportions (Patil and Hulamani, 1999). The essential oil of fresh and dried leaves of *P. glandulosus* were analysed by GC and GC/MS. A high percentage of oxygenated monoterpenes was obtained (Ngassoum *et al*., 2001). Chemical investigations of the leaf essential oil of *C. amboinicus* by GC and GC-MS techniques indicated the presence of 6 components accounting for 97% of the total oil. The major components were thymol followed by carvacrol, 1,8-cineole, p-cymene, spathulenol, terpinene-4-ol and an unidentified component (Singh *et al*., 2002). The analysis of essential oils from leaves, stems and roots of *P. barbatus* gave α-pinene in the leaves, β-phellandrene in the stems and β-ocimene in the roots as the major constituents (Kerntopf *et al*., 2002).

Trichome Observations by SEM

Labiates carry a great diversity of epidermal hairs, many of which are non-glandular (El-Gazzar and Watson, 1970; Werker *et al*., 1985b). Glandular trichomes which store volatile oils vary in morphology between species, and more than one type can occur on a single leaf (Bruni and Modenesi, 1983; Venkatachalam *et al*., 1984; Werker *et al*., 1985b). The number of glandular trichomes per unit area of epidermis varies considerably amongst labiatae species. The presence of peltate and capitate glandular trichomes is a characteristic feature of Lamiaceae species. Different versions and combinations of these two morphological types also exist in different species. The two types can be distinguished by head size and stalk length. As a rule, in a capitate trichome, the length of the stalk should be more than half the height of the head (Abu-Asab and Cantino, 1987), whereas peltate trichomes are short with a uni- or bicellular stalk and a large secretory head with 4 to 18 cells arranged in one or two concentric circles (Werker, 1993). Capitate trichomes are extremely variable in stalk length, head shape and secretion process, and can be subdivided into various types (Werker *et al*., 1985a). In all the Lamiaceae species, the secretory material of peltate glands accumulate in the subcuticular space and the rupture of the cuticle lead to the release of the exudate. Whereas in capitate hairs,

the secretory product accumulates in the apical cells and the release of secretion probably occurs through cuticular micropores.

A survey of trichome types on vegetative and reproductive organs of several Lamiaceae species (Werker *et al*., 1985b) listed three kinds of capitate trichomes according to their morphology and secretion processes. The leaves, stems and reproductive structures of plants of *Origanum vulgare* carry both capitate and peltate hairs at densities of 10-20 peltate hairs per mm^2, in addition to non-glandular hairs (Werker *et al*., 1985a). Trichome density also varies with ontogeny and between different tissues. There are reports of higher trichome densities on the abaxial than the adaxial surfaces of labiate leaves, and in the vicinity of vascular bundles (Werker *et al*., 1985b). In *Satureja thymbra*, peltate trichomes with 12 head cells occupied 6% of the leaf surface (Bosabalidis, 1990).

The types of glandular hairs and their pattern of distribution on leaves of *Ocimum basilicum* at different stages were investigated by Werker *et al*. (1993). The density of the glandular hairs appeared to be very high on young meristematic leaves and on meristematic regions of older leaves. The glandular hairs consist of small capitate hairs and larger peltate hairs. Quantitative and qualitative variability in oil constituents of the glandular hairs with age of the hairs was observed in *Mentha piperita* (Maffei *et al.*, 1989).

The types of glandular trichomes, their ontogeny and pattern of distribution on the vegetative and reproductive organs of *Leonotis leonurus* at different stages of development were studied by light and scanning electron microscopy (Ascensao *et al.*, 1995). Two morphologically distinct types of glandular trichomes - capitate and peltate - with difference in secretion process were described. Uniseriate, multicellular and point-shaped non-glandular trichomes were also observed. Structural investigations of the secretory hairs of *Salvia aurea* leaves explained two types of glandular trichomes - peltate glands characterized by a short stalk and a large 6-8 celled head as well as capitate trichomes with unicellular or multicellular stalk and unicellular or bicellular head (Valenti *et al.*, 1997).

Ascensao *et al.* (1998) conducted a study to view the glandular secretory structures of *Plectranthus madagascariensis*. Besides non-glandular trichomes, peltate and capitate glandular trichomes were reported. Segmented nature of the peltate trichomes was noted. Five distinct types of glandular hairs - one peltate and 4 capitate - with different localization, secretory modes and secretions were identified in *Salvia officinalis* (Corsi and Bottega, 1999). The peltate hairs comprise the secretory head composed of 12 cells arranged in a shield. The non-glandular hairs were multicellular, unbranched and consisted of 3-4 elongated cells. Morphological studies of epidermal hairs of *Salvia blepharophylla* unearthed three types of glandular trichomes, in addition to non-glandular hairs. The glandular hairs included peltate and two types of capitate trichomes (Bisio *et al.*, 1999).

The types of glandular trichomes and their distribution on leaves and flowers of *Plectranthus ornatus* were investigated at different stages of their development. Five morphological types of glandular trichomes - peltate, long stalked capitate, short stalked capitate, digitiform and conoidal trichomes on the reproductive organs - were described (Ascensao *et al.*, 1999). The essential oil secretory tissues of *Prostanthera ovalifolia* included glandular trichomes of the peltate type with a 16-celled secretory head (Gersbach, 2002). No capitate trichomes were noticed.

The leaves of medicinal plants used in controlling infectious diseases were studied for their ash values and mineral contents. Medicinal properties and Cobalt and Manganese concentrations in the underground and aerial parts of some herbal drugs were presented in the study carried out by Rai *et al.* (2001). Variation in heavy metal concentration was observed in roots of *Coleus forskohlii* procured from different geographical zones of India (Srivastava *et al*., 2002). The highest content of Sodium and Potassium was reported in *Coleus aromaticus* (Udayakumar and Begum, 2004).

Antioxidant Activity

Phenolic antioxidants, a specific group of secondary metabolites, play the very important role of protecting organisms against harmful effects of oxygen

radicals and other highly reactive oxygen species. Using tissue culture techniques several high phenolics containing clonal lines have been isolated with high antioxidant activity.

Secondary metabolites in *Mentha spicata* - rosmarinic acid and related phenolics - are natural antioxidants. Tissue culture-based selection techniques was employed to isolate high rosmarinic acid and phenolic antioxidant-producing clonal lines from a heterogeneous bulk seed population of *M. spicata* (Al-Amier *et al*., 1999). Tissue culture selection for phenolics and rosmarinic acid in thyme (*Thymus vulgaris*) was performed by Al-Amier *et al*. (2001). The absence of linear correlation between total phenolics and antioxidant activities was noticed when *in vitro* studies were carried out using different vegetables, fruits and medicinal plants (Gazzani *et al*., 1998a, 1998b; Velioglu *et al*., 1998; Al-Mamary, 2002).

Concentrations of phenolics in shoot cultures of 9 *Scutellaria* species were determined and they were classified into four groups on the basis of the major phenolics (Nishikawa *et al*., 2000). Influence of phytohormones in the production of antioxidant phenolics were assessed in the *in vitro* proliferated shoots of *Salvia officinalis* (Gomes *et al*., 2002). Increased accumulation of phenolic compounds was observed in presence of Kinetin (1.5, 2 & 4 mg/ml) and 2, 4-D (0.05 mg/ml). Accumulation of total phenolic compounds was found to be increased during the first two weeks of callus culture of *Salvia officinalis*. The antioxidant activity *in vitro* was not correlated with the observed accumulation of phenolics (Kintzios *et al*., 2002). Highest specific accumulation of total phenolics was reported in *Salvia officinalis* callus cultures (Gomes *et al*., 2003).

In view of an enormous data from several studies showing a clear implication of oxidative stress in causation and progression of various diseases globally, it appeared worth to have more antioxidant agents. Indian medicinal plants with antioxidant and immunomodulatory activities have been identified and their effects were reviewed by Devasagayam and Sainis (2002). The antioxidant characteristics of plant derived materials can be attributed to their content of polyphenols (Lugasi *et al*., 2003). Evaluation of antioxidant activities of the

essential oil, water soluable (polar) and water insoluable (nonpolar) subfractions of the methanol extracts from aerial parts of *Satureja hortensis* plants, and methanol extract from calluses was performed by Gulluce *et al*. (2003). The strongest antioxidant effect was observed for the tissue culture extract, with an IC 50 value of 23.76 ± 0.80 μg/ml which could be compared with the synthetic antioxidant agent butylated hydroxytoluene (BHT).

In vitro manipulation of plant regeneration in the Chinese medicinal species *Scutellaria baicalensis* Georgi resulted in 26 chemically distinct germplasm lines. The selective markers used to identify elite lines included antioxidant potential, growth rate and concentration of particular components (Murch *et al*., 2004). Phenolic phytochemicals isolated from grape seeds show strong antioxidant activity *in vitro* and in low concentration afford significant protection against oxidative damage in the DNA of mice spleen cells (Fan and Lou, 2004). The green leafy vegetables provide antioxidant vitamins, minerals, phenolics and various other phytochemicals that increase their antioxidant capacity (Simopoulos, 2004). The antioxidative activity of essential oils from oil-rich plants including *Coleus aromaticus* were analysed and was found to be effective (Singh *et al*., 2004b).

Estimation of total phenolics of clonal oregano (*Origanum vulgare*) led to the conclusion of the highest phenolic concentration in 60% ethanol extracts of the clones (Chun *et al*., 2005). They evaluated the antioxidant activity of phenolic-enriched clonal oregano extracts and were compared to commercial oregano from heterogeneous sources. Antioxidant activity was found to be correlated to the amount of total phenolics. Stimulation of total phenolics in tissue cultures of *Mentha pulegium* in response to *Pseudomonas mucidolens* was demonstrated (Al-Amier *et al*., 2005).

Determination of total content of phenolic compounds and their antioxidant activity in vegetables by spectrophotometric method confirmed correlation between content of the phenolics and antioxidant activities (Stratil *et al*., 2006). A preliminary study was undertaken to elucidate *in vitro* free radical scavenging potential and inhibition of lipid peroxidation by *Coleus aromaticus* hydro-alcoholic

extract. The results established an efficient antioxidant, anticlastogenic and radioprotective potential of the extract (Rao *et al*., 2006).

In a comparative study of the antioxidant activity of *Plectranthus grandis* and *P. ornatus* essential oils, the oil of *P. ornatus* showed a higher antioxidant activity than that of *P. grandis*, probably due to its higher yield of the phenolic compounds eugenol and thymol (Albuquerque *et al*., 2006).

The extensive survey of literature confirms the added advantage of the application of *in vitro* method in the quality improvement of medicinal and aromatic plants. The beneficial aspects of variation originated in the artificial environment of *in vitro* culture of many plants inspire to aim on the probability of quality improvement in this plant too. The comparative evaluation of the *in vitro* plants with their parent in different aspects such as cytological, genetical, phytochemical, structural and biological activities tried in a wide spectrum of plant species that helps to confirm the beneficial outcome of tissue culture could be useful to this study also.

MATERIALS AND METHODS

The experiments performed in the present investigation included the following:-

- Micropropagation of *Plectranthus zeylanicus*
- Cytological analysis
- Genetic evaluation using RAPD fingerprinting
- Essential oil characterization using GC-MS
- SEM observation of trichomes on the leaves
- Elemental analysis of the leaf surface by EDS
- Estimation of phenolics
- Assays to test antioxidant properties.

Micropropagation

Micropropagation was attempted through direct multiplication as well as callus mediated regeneration using different explants. Plants were collected from the Botanical Garden, University of Calicut, Kerala and were maintained in the experimental garden attached to the Department of Botany, University of Calicut. Node, internode, shoot apex and leaf were used as explants to initiate cultures. Surface sterilization was effected in a step-by-step manner. At first explants were washed in running tap water, then with dilute detergent for 10 min. and later rinsed thoroughly with distilled water. The plant material was then immersed in a solution of 0.1% mercuric chloride for 5 min. with intermittent shaking, followed by thorough washing in sterile double distilled water 3 or 4 times. The surface sterilized explants were inoculated on to the nutrient medium.

Murashige and Skoog (1962) basal medium (Appendix I) augmented with varied concentrations and combinations of plant growth regulators was employed as the nutrient medium for initiation and multiplication of cultures. Agar (0.8%) as solidifying agent and sucrose (3%) as carbon source were used for the preparation of medium. The pH was adjusted to 5.6 - 5.8. The medium was then sterilised at 120°C for 20 min. The cultures were grown at 25 ± 3°C under fluorescent lights at 2000 lux with 16/8 h. light and dark photoperiod. Subculturing was carried out every 4-6 weeks. A minimum of 20 cultures was raised for each treatment and all the experiments were repeated 3 times. All the cultures were examined periodically and observations were recorded.

Establishment of plants in the soil

Plants with well developed roots were taken from culture vessels, washed gently to remove agar, transferred to paper cups and initially covered with polythene bags to maintain high relative humidity. They were gradually exposed to low humidity by removing polythene covers, transplanted to earthenware pots and were maintained in normal light and temperature.

Cytological Analysis

Mitotic squash preparation

Cytological preparations were made with the help of improved techniques (Sharma and Sharma, 1990). Young healthy root tips were collected from both the parent and field transplanted *in vitro* plants at the period showing peak mitotic activity (9-10 a.m.). The root tips collected were subjected to pre-treatment in cytostatic chemicals. Saturated solution of para-dichlorobenzene with a trace of aesculine was used for pre-treatment. The pre-treatment solution was initially chilled to 0-5°C for 4-5 min. and the root tips were dipped in it. This was kept at 12-15°C for 2 h. After pre-treatment root meristems were fixed in 1:3 acetic acid-ethanol mixture overnight.

The fixed root tips were hydrolyzed in 1 N HCl for 10 min. After hydrolysis, they were stained in 2% aceto-orcein for 3-4 h. and squashed in 45% acetic acid.

All the slides were scanned under Olympus microscope CX 21 and the photographs were taken with Olympus Camedia C-4000 Zoom digital compact camera attached to the microscope.

Karyomorphology

Karyograms were prepared from the photomicrographs with the aid of computer-based programs such as Adobe Photoshop, AutoCAD and data based analyzing system (Microsoft Excel). Photographs were scanned and stored as digital images. These digital images were converted to grayscale images using Photoshop program. Identification numbers were allotted to each chromosome and then loaded to AutoCAD for karyomorphometrical analysis. Centromeric position of each chromosome was determined, from which arm lengths of each were measured and centromeric indices were calculated. On the basis of arm ratio and centromeric indices, homologous chromosomes were identified and were classified according to Abraham and Prasad (1983). The images were reloaded to Photoshop and karyograms were generated.

Karyotypic formula was expressed depending upon the length of chromosome, position of the centromere and presence or absence of the secondary constriction.

Disparity index (DI) of the chromosomes was calculated with the method of Mohanty *et al*. (1991), by using the formula,

$$DI = \frac{\text{Longest chromosome - shortest chromosome}}{\text{Longest chromosome + shortest chromosome}} \times 100$$

The variation coefficient (VC) among the chromosome complements was determined after Verma (1980) as follows:

$$VC = \frac{\text{Standard Deviation}}{\text{Mean length of chromosomes}} \times 100$$

Table 1. Details of chromosome nomenclature in relation to centromere location based on arm ratios and centromeric indices (Abraham and Prasad, 1983).

Nomenclature	Notation	R_1 s/l	R_2 l/s	I_1 100 s/c	I_2 100 l/c
Median	M	1.000	1.00	50.00	50.00
Nearly median	nm	0.99 to 0.61	1.01 to 1.63	49.99 to 38.01	50.01 to 61.99
Nearly submedian	nsm (-)	0.60 to 0.34	1.64 to 2.99	38.00 to 25.01	62.00 to 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly submedian	nsm (+)	0.32 to 0.23	3.01 to 4.26	24.99 to 18.20	75.01 to 81.80
Nearly subterminal	nst (-)	0.22 to 0.15	4.27 to 6.99	18.19 to 12.51	81.81 to 87.49
Subterminal	ST	0.14	7.00	12.50	87.50
Nearly subterminal	nst (+)	0.13 to 0.07	7.01 to 14.38	12.49 to 5.01	87.51 to 94.99
Nearly terminal	nt	0.06 to 0.01	14.39 to 19.99	5.00 to 0.01	95.00 to 99.99
Terminal	T	0.00	∝	0.00	100.00

The Total Forma percentage (TF %) or mean centromeric index value was calculated after Huziwara (1962) by the formula:

$$TF\% = \frac{\text{Total sum of short arm length}}{\text{Total sum of chromosome length}} \times 100$$

Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD analysis based on Polymerase Chain Reaction (PCR) with arbitrary primers - that by amplifying a set of DNA segments randomly distributed throughout the genome - detects genetic polymorphisms. The plant genomic DNA can be extracted with any DNA isolation procedure appropriate for the sample. Plants selected for DNA fingerprinting were same as that tagged for cytological analysis.

Isolation and purification of genomic DNA

DNA was extracted from young leaf tissues of *Plectranthus zeylanicus (in vivo* and *in vitro)* following CTAB method of Ausubel *et al.* (1995). The steps involved are described below.

- Grind 100 mg of fresh young leaf tissue in liquid nitrogen with a mortar and pestle. Add 900 µl of pre-heated (60°C) CTAB buffer (Appendix II).
- Incubate the mixture at 60°C for 10 min. on a water bath. Transfer the tubes to refrigerator (4°C) for 5 min.
- Add an equal volume (900 µl) of ice cold chloroform : isoamyl alcohol (24:1) mixture. Mix well and spin at 10,000 rpm for 10 min. at room temperature.
- Draw the clear supernatant, add 2/3rd volume of ice cold isopropanol and mix gently.
- Incubate the mixture at –20°C for 30 min. for better precipitation of DNA. Centrifuge at 14,000 rpm for 10 min. at 4°C.
- Discard the supernatant. Add 50 µl 70% ethanol and rinse the pellet by spinning at 5000 rpm for 5 min.
- Discard the supernatant and dissolve the pellet in 200 µl of TE buffer (Appendix III).
- Add an equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) mixture. Mix well and spin at 10,000 rpm for 10 min. at room temperature.
- To the clear supernatant, add 2/3rd volume of ice-cold isopropanol and incubate the mixture at –20°C for 30 min.
- Centrifuge at 14,000 rpm for 10 min. at 4°C. Discard the supernatant.
- Add 500 µl 70% ethanol and rinse the pellet by spinning at 5,000 rpm for 5 min.
- Discard the supernatant, air dry the pellet and dissolve the pellet in 200 µl TE buffer.
- Add 1 µl of RNase stock (1 mg/ml). Incubate at 37°C for 30 min.

- Add 1/10th volume of sodium acetate and 2.5 volume of ice-cold absolute alcohol. Allow for precipitation at –20^{o}C for 1 h.
- Centrifuge at 14,000 rpm for 10 min. at 4^{o}C. Discard the supernatant and wash the pellet in 70% ethanol.
- Air dry the pellet and dissolve in 60 µl of TE buffer and estimate the yield.

Quantification of DNA

The isolated DNA was quantified using UV scanning Shimadzu Spectrophotometer. 10 µl of isolated DNA was diluted to 2 ml using TE buffer. The optical density of the DNA samples at 260 nm and 280 nm was recorded. The concentration of DNA was calculated using the relation:-

DNA concentration (in micrograms) = 50 x A260 x Dilution factor.

where, an OD of 1 corresponds to ~50 µg/ml of double stranded DNA, A260 is the OD of the sample at 260 nm and

$$\text{Dilution Factor} = \frac{\text{Total volume of the sample}}{\text{Volume of DNA used}}$$

The genomic DNA extracted was visualized on agarose gel (0.8%) for its quality and stored at –20^{o}C.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction of the extracted DNA samples was carried out with 10 random oligonucleotide primers [OPAB series (Qiagen operon) and A series (Sigma Aldrich)] in a MJ Research PTC 200 Thermal Cycler.

The PCR was performed in 25 µl reaction mixture containing 2.5 µl (50-200 ng) genomic DNA, 1 µl (2.5 mM each dNTP) of dNTPs, 1 µl (5 -10 picomoles) of primer, 0.4 µl (1U) of Taq polymerase and 2.5 µl of 10x PCR buffer (Appendix IV). This mixture was made upto 25 µl using 17.6 µl double

distilled water. Amplification was carried out in a PTC 200 Thermal cycler under programmed cycling conditions. The cycling parameters for denaturation, annealing and extension were as follows: 1 cycle of 3 min. at 94°C, 38 cycles of 1 min. at 92°C, 2 min. at 36°C and 2 min. at 72°C. One last cycle of 6 min. at 72°C was performed to complete the reaction.

The products of the amplification was subjected to electrophoresis on a 1.2% agarose gel containing 10 µl ethidium bromide (0.5 µg/ml) in 1x TAE buffer (Appendix V) after mixing with 5 µl of gel loading dye (Appendix VI). The gels were then visualized and photographed on a UV transilluminator. 1 kb DNA ladder was used as the molecular weight standard.

Band pattern analysis

Polymorphism was scored on the basis of presence or absence of bands for each DNA sample. The differences in the intensity of the bands were also noted.

Essential oil Analysis

Essential oil extraction

The fresh leaves of both *in vitro* and *in vivo* plants were collected separately and dried in shade. They were cut into small pieces and subjected to hydro-distillation in Clevenger apparatus at 100°C for 4 h. The distillate was extracted with diethyl ether, dried with anhydrous sodium sulphate and ether was removed by evaporation at room temperature. The quantity of the essential oil was measured and it was transferred into small bottles covered with black paper and stored at 4°C. The percentage of oil was calculated on a dry weight basis to avoid faulty estimation that may arise due to different water content of the tissues analyzed each time (Von Rudoff, 1972).

Gas chromatography- Mass spectrometry (GC-MS)

The volatiles of the leaf essential oil were analyzed by the hyphenated system, GC-MS. This was performed on HP 6890 GC/HP 5973 MSD at 70 eV and

250ºC. The GC column used was: H5-5 (DB5) fused silica capillary – 0.32 mm x 30 m with film thickness 0.25 μ. The carrier gas was helium with a flow rate of 1.4 ml/min. The column temperature programme was initial temperature of 60ºC for 1 min., followed by an increase of 3ºC/min. to 250ºC. Run time was 62 min. The components were analyzed and ascertained with the help of Wiley Library 275 combined with the analyser.

Chemotaxonomic Evaluation

The data obtained from the qualitative analysis of both *in vitro* and *in vivo* plants were subjected to numerical analysis to understand the chemical affinity of both by arriving at a numerical constant, the coefficient of similitude (CS) using the following formula proposed by Sokall and Sneath (1963).

$$CS = \frac{\text{Number of similar components}}{\text{Total number of components}} x100$$

Trichome Observations by SEM

The abaxial and adaxial surfaces of the leaves of *in vivo* and *in vitro* plants of *P. zeylanicus* were examined under scanning electron microscope for identification of the types and distribution of foliar glands.

Air dried leaf samples were mounted on specimen stubs using Scotch 3M double adhesive tapes. Samples were coated with gold in a JEOL JFC-1600 AUTO FINE COATER to a thickness of 200 Aº. Coated samples were viewed in a JEOL JSM-5610 LV Scanning Electron Microscope and photographed at different magnifications.

Elemental Analysis by EDS

Both surfaces of the leaves of parent and *in vitro* derived plants were analyzed qualitatively and quantitatively to characterise the elemental composition. Each element yields a characteristic spectral fingerprint that may be used to identify the presence of that element within the sample. The relative intensities of the

spectral peaks help to determine the relative concentration of each element in the specimen.

Energy Dispersive Spectrometer installed in Scanning Electron Microscope (JEOL JSM - 5610 LV) was used for chemical characterization. The results of elemental analysis and the proportion of weight and atomic percentage of those on the analysed volume were recorded.

Estimation of Total Phenolics

Preparation of Ethanolic extract

5 g of leaf tissue was homogenised using a mortar and pestle. 100 ml of 80% ethanol was added to it. The mixture was transferred into a round bottomed flask and refluxed for 2 h. using a water condenser. Cooled the mixture, centrifuged and collected the supernatant. Final volume of the extract was noted.

Test for Phenolics

Total phenol estimation was carried out according to Folin-Denis method (1915). The samples were diluted to an appropriate volume. Added 2 ml of 0.25 N Folin-Denis reagent (Appendix VII) to 2 ml of the diluted sample. Mixed well and after 3 min. 2 ml of 1 N Na_2CO_3 was added. After mixing, the tubes were kept for 1 h. Absorbance of the samples was measured at 700 nm using colorimeter. Tannic acid was used as the standard. The test results were expressed as mean ± standard error.

Antioxidant Assays

Preparation of methanolic extract

Leaves of the parent and *in vitro* plants of *P. zeylanicus* were cut into small pieces and were dried at 40°C. 10 g of the dried, powdered sample in 100 ml of 70% methanol was stirred overnight using a magnetic stirrer. The suspension thus obtained was centrifuged at 10,000 rpm for 15 min. at 4°C using a refrigerated centrifuge. From the supernatant, methanol and water traces were removed by

heating it at 40°C in a hot air oven. Dried extract was stored in a glass bottle and kept under refrigeration.

Superoxide scavenging activity

It was determined by the Nitro Blue Tetrazolium (NBT) reduction method (McCord and Fridovich, 1969). In this method superoxides generated by photoreduction of riboflavin reduces NBT salt that gives a blue colour which was measured at 530 nm. The assay mixture contained different concentrations (200 µg -1000 µg) of plant extracts, EDTA (6 µM), NaCN (3 µg), NBT (50 µM), riboflavin (2 µM) and phosphate buffer (67 mM, pH 7.8) to give a total volume of 3 ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 min. and the optical density was measured at 530 nm before and after illumination. The percentage inhibition of superoxide production by the plant extracts was evaluated by comparing the absorbance values of the control and experimental tubes.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \text{x100}$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances (TBARS) formation (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbic acid 0.1 mM), KH_2PO_4 - KOH buffer (20 mM, pH 7.4) and various concentrations (200 µg - 1000 µg) of the extract in the final volume of 1 ml was incubated for 1 h. at 37°C. At the end of the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS (8.1%), 1.5 ml thiobarbituric acid (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made upto 4 ml by adding distilled water and kept in a water bath

at 100°C for 1 h. After cooling, 1 ml distilled water and 5 ml butanol-pyridine mixture (15:1 v/v) was added and shaken vigorously. The tubes were centrifuged and absorbance of upper layer was read. The radical scavenging activity was determined by comparing the absorbance of control with that of the treatments. The percentage inhibition produced by different concentrations of the sample was calculated.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \text{x}100$$

Lipid peroxidation activity

Lipid peroxidation activity was induced in rat liver homogenate by the method described by Bishayee and Balasubramanian (1971). Different concentrations of the plant extracts (200 µg – 1000 µg) were incubated for 1 h. at 37°C with rat liver homogenate (0.1 ml, 25% w/v) in Tris HCl buffer (40 mM, pH 7), KCl (30 mM), ascorbic acid (0.06 mM) and ferrous iron (0.16 mM) in a total volume of 0.5 ml. The lipid peroxide formed was measured by TBARS formation (Ohkawa *et al.*, 1979). From the incubation mixture, 0.4 ml was treated with sodium dodecyl sulphate (SDS - 8.1%, 0.2 ml), thiobarbituric acid (TBA - 0.8%, 1.5 ml) and acetic acid (20%, 1.5 ml, pH 3.5). The total volume was then made upto 4 ml by adding distilled water and kept in a water bath at 100°C for 1 h. After the mixture had been cooled, 1 ml distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) was added and mixed thoroughly on a vortex mixture. After vigorous shaking, the tubes were centrifuged at 3000 rpm for 15 min. and the absorbance of the upper layer was measured at 532 nm. The percentage inhibition was calculated using the formula.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \text{x}100$$

The percentage inhibition values are expressed as mean ± standard error.

RESULTS

Micropropagation

In the present investigation, MS medium augmented with varied hormonal combinations resulted in the establishment of *in vitro* multiplication of *Plectranthus zeylanicus*. Among the entire explants tested, nodal cuttings and shoot apices showed better response. The varying concentrations of auxins and cytokinins influenced the growth and regeneration of explants in different levels.

Direct regeneration of multiple shoots from nodal and shoot tip explants occurred on MS medium supplemented with different concentrations of NAA (1-3 mg/l) along with BAP (0.5-3.0 mg/l) [Figs. 1, 2, 5 & 9]. Maximum number of shoots was obtained from nodal segments on MS medium fortified with BAP (3 mg/l) and NAA (1 mg/l) [Fig. 5]. 90% of the cultures produced 5 - 10 shoots from each explant. About 10% of cultures produced 1 - 2 shoots per explant. The hormonal combination of IAA (1-2 mg/l) and BAP (1 mg/l) as well as NAA (1 mg/l) and KIN (2 mg/l) showed multiple shoot induction (Figs. 6, 7 & 8). But the frequency of shoots and percentage of initiation was lower than the combination of NAA and BAP. When KIN and BAP were applied singly, it resulted in the development of single shoots from nodal explants (Fig. 3). For shoot apex, multiple shooting was obtained with BAP and NAA at an average proportion (Fig. 4).

When nodal cuttings and shoot apices were cultured on MS medium supplemented with NAA and BAP, callus growth was initiated in about 10 days and rapid growth followed for majority of cultures (Figs. 11, 13, 14, 15, 16, 18, 19, 20 & 21). The maximum response was observed on MS medium with NAA 1.5 mg/l and BAP 0.5 mg/l, where callus production was 90% (Fig. 13). Upon subculturing in the same medium profuse callusing was resulted. Nodal segments exhibited the highest (up to 90%) callusing response after 4 weeks in incubation media. The callus growth

from shoot apex was also significant (up to 80%). Of the 2 auxins tested, NAA proved better than 2,4-D in inducing callus and upon supplementing BAP, the percentage and amount of callus formation was increased. The explants produced white friable callus in medium with NAA 1 mg/l and BAP 0.5 mg/l and greenish soft callus with 2,4-D 2 mg/l after a period of 4-5 weeks in culture (Figs. 12 & 16). NAA when used singly (1 mg/l) formed roots along with small amount of callus. Medium with IAA and BAP produced very little callus at the cut end of the nodal explants and resulted in the axillary bud elongation.

Leaf explants inoculated on media with 2,4-D (1.5 mg/l) and BAP (0.5 mg/l) produced callus but the intensity was less (Fig. 17). With NAA (1-1.5 mg/l) and BAP (0.5 mg/l), white friable callus was obtained (Fig. 16). On subculturing they failed to regenerate with different hormonal combinations. Direct regeneration of plantlet with roots from leaf explants was noticed in the hormonal proportion of 2 mg/l BAP and 1.5 mg/l NAA (Fig. 10). The other hormonal combinations produced only swelling and crumpling of the tissue in the case of leaf explants. Negative response was noticed in all hormonal combinations when internodes were used as explant.

For plant regeneration organogenic calli were transferred to the development media. Shoot proliferation and multiplication was observed from the totipotent calli, in the suitable hormone supplemented medium within 3 weeks of culture. The optimum growth regulator combination for shoot regeneration among all the treatments was MS medium with 1.5 mg/l NAA and 1-2 mg/l BAP (Fig. 22). The age of the callus influenced the regeneration capability. Younger calli (4 weeks old calli) showed better morphogenic performance than the old calli (6-7 weeks old calli).

The shoots measuring about 5 cm in height were separated and inoculated for rooting on different concentration of auxins - NAA, IAA and IBA. IBA was clearly more effective in promoting root induction than NAA or IAA. The optimum medium for rooting was found to be having 1 mg/l IBA on which 95% of the regenerated shoots developed roots with an average number of 10-15 roots per shoots within 10 days (Figs. 23, 25 & 26). The varied morphogenic responses

obtained with different combinations and concentrations of cytokinins with auxins are summarized in Table 2.

Plantlets thus obtained were transferred to cups with 1:2 mixtures of soil and sand and acclimatized for a week under humid conditions and was found to be with 90% of survival efficiency. They were later successfully adapted to field conditions (Figs. 27, 28 & 29). The regenerated plants were free of any noticeable phenotypic variability.

Table 2. Effect of Phytohormones on multiple shoot and callus induction in *Plectranthus zeylanicus*

Explant	Auxins (mg/l)			Cytokinins (mg/l)		Effect on explants	Percentage of response	
	IAA	NAA	2,4-D	BAP	KIN		Shoot-ing	Callus-ing
Node	1	-	-	-	-	Rooting of the explant	40	-
Node	-	2	-	-	-	Rooting of the explant and little callus	30	20
Node	-	-	2	-	-	Greenish soft callus	-	50
Node	-	-	-	1	-	Axillary bud elongation	55	-
Node	1	-	-	-	1	2-3 shoots	60	-
Node	1	-	-	2	-	Multiple shoots with roots	65	-
Node	2	-	-	1	-	Very little callus at cut ends	-	40
Node	-	0.5	-	1.5	-	Yellow hard callus	-	70
Node	-	1	-	1.5	-	Subculturing of callus	-	
Node	-	1	-	0.5	-	Insignificant callus and axillary shoot development	30	10
Node	-	1	-	-	2	Multiple shooting	75	-
Node	-	1	-	2	-	Axillary	50	-

Explant	Auxins (mg/l)			Cytokinins (mg/l)		Effect on explants	Percentage of response	
	IAA	NAA	2,4-D	BAP	KIN		Shoot-ing	Callus-ing
						multiplication		
Node	-	1	-	2	-	Plant regeneration from callus on subculture	50	-
Node	-	1	-	3	-	Multiple shooting	85	-
Node	-	2	-	3	-	Multiple shooting and rooting	90	-
Node	-	1.5	-	0.5	-	Yellowish white friable callus	-	90
Node	-	1.5	-	2	-	Plant regeneration from callus on subculture	90	-
Node	-	1.5	-	1	-	Plant regeneration from callus on subculture	90	-
Node	-	3	-	1	-	Axillary bud elongation	50	-
Node	-	3	-	2	-	White callus	-	40
Node	-	3	-	-	2	Axillary growth	40	-
Node	-	-	1	-	0.5	Insignificant amount of callus	-	10
Node	-	-	3	-	1	Swelling and callusing at cut ends	-	50
Shoot apex	1	-	-	-	-	Profuse rooting	50	-
Shoot apex	-	0.5	-	-	-	Axillary and apical bud growth	55	-
Shoot apex	-	-	2	-	-	Little callus	-	40
Shoot apex	-	-	3	-	-	Little callus	-	30
Shoot apex	-	-	-	2	-	Multiple shooting	50	-
Shoot	-	0.5	-	1.5	-	Yellow hard callus	-	80

Explant	Auxins (mg/l)			Cytokinins (mg/l)		Effect on explants	Percentage of response	
	IAA	NAA	2,4-D	BAP	KIN		Shoot-ing	Callus-ing
apex								
Shoot apex	-	1	-	1.5	-	Yellow callus	-	50
Shoot apex	-	-	2	1	-	Little callus	-	40
Shoot apex	-	-	3	1	-	Little callus	-	30
Leaf	-	1.5	-	2	-	Shoot emergence with rooting	50	-
Leaf	-	2	-	-	-	Swelling and crumpling	-	30
Leaf	-	-	2	-	-	Little callus	-	40
Leaf	-	-	1.5	0.5	-	Greenish callus	-	60
Leaf	-	1	-	0.5	-	White callus	-	30
Leaf	-	1.5	-	0.5	-	White friable callus	-	80

Explant	Auxins (mg/l)			Cytokinins (mg/l)	Effect on explants	Percentage of response (Rooting)
	IAA	NAA	IBA	BAP		
Multiple shoots	1	-	-	-	Little rooting	40
Multiple shoots	-	1	-	-	Little rooting	50
Multiple shoots	-	-	1	-	Profuse rooting	95
Single shoot	-	-	1	-	Profuse rooting	95
Multiple shoots	-	2	-	3	Rooting	85
Multiple shoots	-	1.5	-	2	Rooting	70

Cytological Analysis

Analysis of cytological integrity of the *in vitro* developed plants was performed by mitotic squash preparations. The root tips from the parent plant and five callus regenerated plants were examined for this purpose.

Chromosome counts showed mosaicism with cells at 2n = 32, 2n = 34 and 2n = 17 in the same tissue of the parent plant. The diploid chromosome number 2n = 17 was observed in only ~2% of the screened cell population. Majority of the cells exhibited the chromosome number 2n = 34 (~60%). Cells having 2n = 32 comprised ~38%.

In the cell mass of the *in vitro* root meristem both 34-chromosome numbered as well as 32-numbered cells were identified. Here also the ratio of cells with karyotype 34 to 32 was approximately 3 : 2.

Observation of the callus tissue revealed 2n = 34 and 2n = 32 as the most frequent karyotypic occurrences. Along with these, some polyploid cells were also noticed in fewer incidences (2n = 64).

The range of chromosome length and other karyotypic features among the analysed parent plant cells, callus cells and *in vitro* plant cells showed divergence. The detailed karyotypic descriptions of them are shown in Tables 3 - 10. Summarized data of karyomorphometric features is tabulated in Table 11.

Based on chromosome metrics and the position of centromere, a general description of the common chromosome types found in the present study is given below:-

Type A: Chromosomes with 2 constrictions ranging from size 2.0047 μm to 1.6692 μm with nearly median to nearly submedian primary constriction.

Type B: Chromosomes ranging from size 2.5946 μm to 2.0047 μm with nearly median to nearly submedian primary constriction.

Type C: Chromosomes ranging from size 1.9381 μm to 1.0175 μm with nearly median to nearly submedian primary constriction.

Type D: Chromosomes ranging from size 0.9916 μm to 0.6536 μm with nearly median to nearly submedian primary constriction.

Photomicrographs, karyograms and idiograms of mitotic metaphase stages of the parent, callus and *in vitro* cells are described (Plates 7 - 14).

Plectranthus zeylanicus parent plant normal cell with 2n = 34 chromosomes

Normal somatic chromosome number	: 2n = 34
Karyotype formula	: $A_2B_6C_{26}$
Range of Chromosome Length (RCL)	: 2.3597 µm – 1.3026 µm
Average Chromosome Length (ACL)	: 1.8088 µm
Total Chromosome Length (TCL)	: 61.5008 µm
Disparity Index (DI)	: 28.86
Variation Coefficient (VC)	: 15.89
TF value (%)	: 40.66

Table 3. Detailed karyomorphometric data in *Plectranthus zeylanicus* parent plant with 2n = 34 (normal)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
B	2	2.3597	0.9520	1.4077	0.6763	1.4787	40.34	59.66	Nm
A*	2	2.2855	0.9437	1.3418	0.7033	1.4219	41.29	58.71	Nm
B	2	2.2011	0.9127	1.2884	0.7084	1.4116	41.47	58.53	Nm
B	2	2.0070	0.7380	1.2690	0.5816	1.7195	36.77	63.23	Nsm(-)
C	2	1.9381	0.7450	1.1931	0.6244	1.6015	38.44	61.56	Nm
C	2	1.8682	0.7463	1.1219	0.6652	1.5033	39.95	60.05	Nm
C	2	1.8586	0.7526	1.1060	0.6805	1.4696	40.49	59.51	Nm
C	2	1.8239	0.7511	1.0728	0.7001	1.4283	41.18	58.82	Nm
C	2	1.7916	0.7262	1.0654	0.6816	1.4671	40.53	59.47	Nm
C	2	1.7631	0.7583	1.0048	0.7547	1.3251	43.01	56.99	Nm
C	2	1.6877	0.6995	0.9882	0.7079	1.4127	41.45	58.55	Nm
C	2	1.6407	0.6672	0.9735	0.6854	1.4591	40.67	59.33	Nm
C	2	1.6288	0.6659	0.9629	0.6916	1.4460	40.88	59.12	Nm
C	2	1.5956	0.6625	0.9331	0.7100	1.4085	41.52	58.48	Nm
C	2	1.5408	0.6328	0.9080	0.6969	1.4349	41.07	58.93	Nm
C	2	1.4574	0.5827	0.8747	0.6662	1.5011	39.98	60.02	Nm
C	2	1.3026	0.5672	0.7354	0.7713	1.2965	43.54	56.46	Nm

Chr. – chromosome
c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,
I_1 – centromeric index 1, I_2 – centromeric index 2
PC – primary constriction
* chromosome pair with secondary constriction

Plectranthus zeylanicus parent plant variant cell with 32 chromosomes

Variant somatic chromosome number : 32

Karyotype formula : $A_2B_6C_{22}\ D_2$

Range of Chromosome length (RCL) : 2.1951 µm – 0.9253 µm

Average Chromosome Length (ACL) : 1.6912 µm

Total Chromosome Length (TCL) : 54.1176 µm

Disparity Index (DI) : 40.69

Variation Coefficient (VC) : 21.06

TF value (%) : 42.26

Table 4. Detailed karyomorphometric data in *Plectranthus zeylanicus* parent plant with 32 chromosomes (variant)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	2	2.1951	0.7443	1.4508	0.5130	1.9492	33.91	66.09	Nsm(-)
B	2	2.1312	0.7590	1.3722	0.5531	1.8079	35.61	64.39	Nsm(-)
B	2	2.0482	0.7696	1.2786	0.6019	1.6614	37.57	62.43	Nsm(-)
B	2	2.0482	0.9222	1.1260	0.8190	1.2210	45.02	54.98	Nm
C	2	1.9134	0.8657	1.0477	0.8263	1.2102	45.24	54.76	Nm
C	2	1.8214	0.8009	1.0205	0.7848	1.2742	43.97	56.03	Nm
C	2	1.7976	0.8077	0.9899	0.8159	1.2256	44.93	55.07	Nm
C	2	1.7366	0.7518	0.9848	0.7634	1.3099	43.29	56.71	Nm
C	2	1.6947	0.7541	0.9406	0.8017	1.2473	44.50	55.50	Nm
C	2	1.6587	0.7445	0.9142	0.8144	1.2279	44.88	55.12	Nm
C	2	1.5890	0.6888	0.9002	0.7652	1.3069	43.35	56.65	Nm
C	2	1.5669	0.7076	0.8593	0.8235	1.2144	45.16	54.84	Nm
C	2	1.4889	0.6755	0.8134	0.8305	1.2042	45.37	54.63	Nm
C	2	1.3349	0.5453	0.7896	0.6906	1.4480	40.85	59.15	Nm
C	2	1.1087	0.4356	0.6731	0.6472	1.5452	39.29	60.71	Nm
D	2	0.9253	0.4614	0.4639	0.9946	1.0054	49.86	50.14	Nm

Chr. – chromosome

c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,

I_1 – centromeric index 1, I_2 – centromeric index 2

PC – primary constriction

* chromosome pair with secondary constriction

Plectranthus zeylanicus parent plant variant cell with 17 chromosomes

Variant somatic chromosome number	: 17
Karyotype formula	: A_1C_{16}
Range of Chromosome Length (RCL)	: 1.8981 µm – 1.1872 µm
Average Chromosome Length (ACL)	: 1.5140 µm
Total Chromosome Length (TCL)	: 25.7372 µm
Disparity Index (DI)	: 23.04
Variation Coefficient (VC)	: 13.97
TF value (%)	: 42.50

Table 5. Detailed karyomorphometric data in *Plectranthus zeylanicus* parent plant with 17 chromosomes (variant)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	1	1.8981	0.5693	1.3288	0.4284	2.3341	29.99	70.01	Nsm(-)
C	1	1.8301	0.8189	1.0112	0.8098	1.2348	44.75	55.25	Nm
C	1	1.7974	0.8084	0.9890	0.8174	1.2234	44.98	55.02	Nm
C	1	1.7361	0.7632	0.9729	0.7845	1.2748	43.96	56.04	Nm
C	1	1.6196	0.6624	0.9572	0.6920	1.4451	40.90	59.10	Nm
C	1	1.5975	0.6442	0.9533	0.6758	1.4798	40.33	59.67	Nm
C	1	1.5401	0.6548	0.8853	0.7396	1.3520	42.52	57.48	Nm
C	1	1.5142	0.6442	0.8700	0.7405	1.3505	42.54	57.46	Nm
C	1	1.4870	0.6170	0.8700	0.7092	1.4101	41.49	58.51	Nm
C	1	1.4589	0.5910	0.8679	0.6810	1.4685	40.51	59.49	Nm
C	1	1.4546	0.6646	0.7900	0.8413	1.1887	45.69	54.31	Nm
C	1	1.3909	0.6119	0.7790	0.7855	1.2731	43.99	56.01	Nm
C	1	1.3475	0.5779	0.7696	0.7509	1.3317	42.89	57.11	Nm
C	1	1.3471	0.6196	0.7275	0.8517	1.1741	46.00	54.00	Nm
C	1	1.3420	0.6408	0.7012	0.9139	1.0943	47.75	52.25	Nm
C	1	1.1889	0.5256	0.6633	0.7924	1.2620	44.21	55.79	Nm
C	1	1.1872	0.5239	0.6633	0.7898	1.2661	44.13	55.87	Nm

Chr. – chromosome
c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,
I_1 – centromeric index 1, I_2 – centromeric index 2
PC – primary constriction
* chromosome with secondary constriction

Plectranthus zeylanicus callus normal cell with 2n = 34 chromosomes

Normal somatic chromosome number : 2n = 34

Karyotype formula : $A_2C_{28}D_4$

Range of Chromosome Length (RCL) : 1.9139 µm – 0.8143 µm

Average Chromosome Length (ACL) : 1.3947 µm

Total Chromosome Length (TCL) : 47.4202 µm

Disparity Index (DI) : 59.76

Variation Coefficient (VC) : 22.86

TF value (%) : 41.77

Table 6. Detailed karyomorphometric data in *Plectranthus zeylanicus* callus cell with 2n = 34 (normal)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
C	2	1.9139	0.8734	1.0405	0.8394	1.1913	45.63	54.37	Nm
A*	2	1.8297	0.8479	0.9818	0.8636	1.1579	46.34	53.66	Nm
C	2	1.7302	0.7616	0.9686	0.7863	1.2718	44.02	55.98	Nm
C	2	1.7017	0.7420	0.9597	0.7732	1.2934	43.60	56.40	Nm
C	2	1.6617	0.7216	0.9401	0.7676	1.3028	43.43	56.57	Nm
C	2	1.6129	0.7012	0.9117	0.7691	1.3002	43.47	56.52	Nm
C	2	1.5164	0.6149	0.9015	0.6821	1.4661	40.55	59.45	Nm
C	2	1.4542	0.5664	0.8878	0.5121	1.5674	33.86	66.14	Nsm(-)
C	2	1.3832	0.5349	0.8483	0.6677	1.5859	40.04	59.96	Nm
C	2	1.3250	0.5154	0.8096	0.6607	1.5708	39.78	60.22	Nm
C	2	1.2884	0.5018	0.7866	0.6552	1.5676	39.59	60.41	Nm
C	2	1.2242	0.4788	0.7454	0.6732	1.5568	40.23	59.77	Nm
C	2	1.1596	0.4546	0.7050	0.6792	1.5508	40.45	59.55	Nm
C	2	1.0928	0.4426	0.6502	0.6807	1.4690	40.50	59.50	Nm
C	2	1.0669	0.4265	0.6404	0.6660	1.5015	39.98	60.02	Nm
D	2	0.9350	0.3835	0.5515	0.6954	1.4381	41.02	59.98	Nm
D	2	0.8143	0.3355	0.4788	0.7007	1.4271	41.20	58.80	Nm

Chr. – chromosome

c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,

I_1 – centromeric index 1, I_2 – centromeric index 2

PC – primary constriction

* chromosome pair with secondary constriction

Plectranthus zeylanicus callus variant cell with 32 chromosomes

Variant somatic chromosome number : 32

Karyotype formula : $A_2C_{28}D_2$

Range of Chromosome Length (RCL) : 1.7391 µm – 0.9289 µm

Average Chromosome Length (ACL) : 1.3450 µm

Total Chromosome Length (TCL) : 43.0386 µm

Disparity Index (DI) : 30.37

Variation Coefficient (VC) : 16.10

TF value (%) : 44.08

Table 7. Detailed karyomorphometric data in *Plectranthus zeylanicus* callus cell with 32 chromosomes (variant)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	2	1.7391	0.7960	0.9431	0.8440	1.1848	45.77	54.23	Nm
C	2	1.6165	0.7203	0.8962	0.8037	1.2442	44.56	55.44	Nm
C	2	1.5810	0.7031	0.8779	0.8009	1.2486	44.47	55.53	Nm
C	2	1.5165	0.6720	0.8445	0.7957	1.2567	44.31	55.69	Nm
C	2	1.4836	0.6510	0.8326	0.7819	1.2790	43.88	56.12	Nm
C	2	1.4151	0.6325	0.7826	0.8082	1.2373	44.70	55.30	Nm
C	2	1.3776	0.6098	0.7678	0.7942	1.2591	44.27	55.73	Nm
C	2	1.3556	0.6025	0.7531	0.8000	1.2500	44.45	55.55	Nm
C	2	1.3335	0.5902	0.7433	0.7940	1.2594	44.26	55.74	Nm
C	2	1.3118	0.5789	0.7329	0.7899	1.2660	44.13	55.87	Nm
C	2	1.2916	0.5666	0.7250	0.7815	1.2796	43.87	56.13	Nm
C	2	1.2382	0.5430	0.6952	0.7811	1.2803	43.85	56.15	Nm
C	2	1.1736	0.5305	0.6431	0.8249	1.2123	45.20	54.80	Nm
C	2	1.1204	0.4847	0.6357	0.7625	1.3115	43.26	56.74	Nm
C	2	1.0363	0.4337	0.6026	0.7197	1.3894	41.85	58.15	Nm
D	2	0.9289	0.3699	0.5590	0.6617	1.5112	39.82	60.18	Nm

Chr. – chromosome

c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,

I_1 – centromeric index 1, I_2 – centromeric index 2

PC – primary constriction

* chromosome pair with secondary constriction

Plectranthus zeylanicus callus variant cell with 64 chromosomes

Variant somatic chromosome number	: 64
Karyotype formula	: $A_4B_2C_{42}D_{16}$
Range of Chromosome Length (RCL)	: 2.5946 µm – 0.6536 µm
Average Chromosome Length (ACL)	: 1.3894 µm
Total Chromosome Length (TCL)	: 88.9206 µm
Disparity Index (DI)	: 40.30
Variation Coefficient (VC)	: 33.28
TF value (%)	: 46.08

Table 8. Detailed karyomorphometric data in *Plectranthus zeylanicus* callus cell with 64 chromosomes (variant)

Chr. type	No. of Chr.	c (μm)	s (μm)	l (μm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	2	2.5946	1.2424	1.3522	0.9189	1.0883	47.89	52.12	Nm
A*	2	2.2434	1.0537	1.1897	0.8857	1.1291	46.97	53.03	Nm
B	2	2.0423	0.9525	1.0898	0.8740	1.1442	46.64	53.36	Nm
C	2	1.9313	0.9023	1.0290	0.8769	1.1404	46.72	53.28	Nm
C	2	1.8884	0.8861	1.0022	0.8842	1.1310	46.92	53.07	Nm
C	2	1.8373	0.8785	0.9589	0.9162	1.0915	47.81	52.19	Nm
C	2	1.7940	0.8589	0.9350	0.9186	1.0886	47.88	52.12	Nm
C	2	1.7527	0.8394	0.9134	0.9190	1.0882	47.89	52.11	Nm
C	2	1.6766	0.7943	0.8823	0.9003	1.1108	47.38	52.62	Nm
C	2	1.6035	0.7497	0.8538	0.8781	1.1389	46.75	53.25	Nm
C	2	1.5729	0.7356	0.8372	0.8786	1.1381	46.77	53.23	Nm
C	2	1.5303	0.7203	0.8100	0.8893	1.1245	47.07	52.93	Nm
C	2	1.4632	0.6820	0.7811	0.8731	1.1453	46.61	53.38	Nm
C	2	1.4321	0.6697	0.7624	0.8784	1.1384	46.76	53.24	Nm
C	2	1.4070	0.6595	0.7475	0.8823	1.1334	46.87	53.13	Nm
C	2	1.3726	0.6314	0.7411	0.8520	1.1737	46.00	53.99	Nm
C	2	1.3377	0.6021	0.7356	0.8185	1.2217	45.01	54.99	Nm
C	2	1.3216	0.5953	0.7263	0.8196	1.2201	45.04	54.96	Nm
C	2	1.2374	0.5421	0.6952	0.7798	1.2824	43.81	56.18	Nm
C	2	1.2068	0.5281	0.6786	0.7782	1.2850	43.76	56.23	Nm
C	2	1.1587	0.5009	0.6578	0.7615	1.3132	43.23	56.77	Nm
C	2	1.1068	0.4754	0.6314	0.7529	1.3281	42.95	57.05	Nm
C	2	1.0609	0.4588	0.6021	0.7620	1.3123	43.25	56.75	Nm
C	2	1.0175	0.4524	0.5651	0.8006	1.2491	44.46	55.54	Nm
D	2	0.9916	0.4380	0.5536	0.7912	1.2639	44.17	55.83	Nm
D	2	0.9699	0.4286	0.5413	0.7918	1.2630	44.19	55.81	Nm
D	2	0.9367	0.4159	0.5209	0.7984	1.2525	44.40	55.61	Nm
D	2	0.9032	0.3980	0.5052	0.7878	1.2694	44.07	55.93	Nm
D	2	0.8530	0.3763	0.4767	0.7894	1.2668	44.11	55.89	Nm
D	2	0.8173	0.3623	0.4550	0.7963	1.2559	44.33	55.67	Nm
D	2	0.7454	0.3491	0.3963	0.8809	1.1352	46.83	53.17	Nm
D	2	0.6536	0.3079	0.3457	0.8907	1.1228	47.11	52.89	Nm

Chr. – chromosome
c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,
I_1 – centromeric index 1, I_2 – centromeric index 2
PC – primary constriction
* chromosome pair with secondary constriction

Plectranthus zeylanicus in vitro plant normal cell with 2n = 34 chromosomes

Normal somatic chromosome number : 2n = 34

Karyotype formula : $A_2C_{30}D_2$

Range of Chromosome Length (RCL) : 1.6692 µm – 0.9287 µm

Average Chromosome Length (ACL) : 1.3098 µm

Total Chromosome Length (TCL) : 44.5328 µm

Disparity Index (DI) : 28.50

Variation Coefficient (VC) : 13.60

TF value (%) : 40.85

Table 9. Detailed karyomorphometric data in *Plectranthus zeylanicus in vitro* plant with 2n = 34 (normal)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	2	1.6692	0.6200	1.0492	0.5909	1.6923	37.14	62.86	Nsm(-)
C	2	1.4982	0.5043	0.9939	0.5074	1.9709	33.66	66.34	Nsm(-)
C	2	1.4757	0.5777	0.8980	0.6433	1.5544	39.15	60.85	Nm
C	2	1.4676	0.6148	0.8528	0.7209	1.3871	41.89	58.11	Nm
C	2	1.4327	0.6120	0.8207	0.7457	1.3410	42.72	57.28	Nm
C	2	1.3983	0.5934	0.8049	0.7372	1.3564	42.44	57.56	Nm
C	2	1.3619	0.5740	0.7879	0.7285	1.3726	42.15	57.85	Nm
C	2	1.3298	0.5557	0.7741	0.7179	1.3930	41.79	58.21	Nm
C	2	1.2954	0.5373	0.7581	0.7087	1.4109	41.48	58.52	Nm
C	2	1.2739	0.5351	0.7388	0.7243	1.3807	42.00	58.00	Nm
C	2	1.2665	0.5345	0.7320	0.7302	1.3695	42.20	57.79	Nm
C	2	1.2365	0.5136	0.7229	0.7105	1.4075	41.54	58.46	Nm
C	2	1.2229	0.5234	0.6995	0.7482	1.3365	42.80	57.20	Nm
C	2	1.2074	0.5283	0.6791	0.7779	1.2854	43.76	56.24	Nm
C	2	1.1536	0.5162	0.6374	0.8099	1.2348	44.75	55.25	Nm
C	2	1.0481	0.4237	0.6244	0.6788	1.4737	40.43	59.57	Nm
D	2	0.9287	0.3328	0.5959	0.5585	1.7906	35.84	64.16	Nsm(-)

Chr. – chromosome

c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,

I_1 – centromeric index 1, I_2 – centromeric index 2

PC – primary constriction

* chromosome pair with secondary constriction

Plectranthus zeylanicus in vitro plant variant cell with 32 chromosomes

Variant somatic chromosome number : 32

Karyotype formula : A_2C_{30}

Range of Chromosome Length (RCL) : 2.0047 µm – 1.0875 µm

Average Chromosome Length (ACL) : 1.4432 µm

Total Chromosome Length (TCL) : 46.181 µm

Disparity Index (DI) : 29.66

Variation Coefficient (VC) : 16.20

TF value (%) : 39.74

Table 10. Detailed karyomorphometric data in *Plectranthus zeylanicus in vitro* plant with 32 chromosomes (variant)

Chr. type	No. of Chr.	c (µm)	s (µm)	l (µm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of PC
A*	2	2.0047	0.6204	1.3843	0.4482	2.2313	30.95	69.05	Nsm(-)
C	2	1.7193	0.6378	1.0815	0.5897	1.6957	37.10	62.90	Nsm(-)
C	2	1.6294	0.6114	1.0180	0.6006	1.6650	37.52	62.47	Nsm(-)
C	2	1.5603	0.6055	0.9548	0.6342	1.5769	38.81	61.19	Nm
C	2	1.5497	0.6406	0.9091	0.7047	1.4191	41.34	58.66	Nm
C	2	1.5386	0.6512	0.8874	0.7338	1.3627	42.32	57.68	Nm
C	2	1.5129	0.6504	0.8625	0.7541	1.3261	42.99	57.01	Nm
C	2	1.4668	0.6121	0.8547	0.7162	1.3963	41.73	58.27	Nm
C	2	1.4398	0.5985	0.8413	0.7114	1.4057	41.57	58.43	Nm
C	2	1.3834	0.5574	0.8260	0.6748	1.4819	40.29	59.71	Nm
C	2	1.3316	0.5388	0.7928	0.6796	1.4714	40.46	59.54	Nm
C	2	1.2699	0.4890	0.7809	0.6262	1.5969	38.51	61.49	Nm
C	2	1.2380	0.4877	0.7503	0.6500	1.5385	39.39	60.61	Nm
C	2	1.1908	0.4724	0.7184	0.6576	1.5208	39.67	60.33	Nm
C	2	1.1678	0.4964	0.6714	0.7394	1.3525	42.51	57.49	Nm
C	2	1.0875	0.5073	0.5802	0.8744	1.1437	46.65	53.35	Nm

Chr. – chromosome

c – total length, l – long arm, s – short arm, R_1– arm ratio1, R_2 – arm ratio 2,

I_1 – centromeric index 1, I_2 – centromeric index 2

PC – primary constriction

* chromosome pair with secondary constriction

Table 11. Summary of karyomorphometric features of *in vivo*, callus and *in vitro* plants of *Plectranthus zeylanicus*

Karyotype observed	Type of cells	TCL (µm)	ACL (µm)	RCL (µm)	DI	VC	TF%	Karyotypic formula
Parent cell with 34 chromosomes	Normal	61.5008	1.8088	2.3597 - 1.3026	28.86	15.89	40.66	$A_2B_6C_{26}$
Parent cell with 32 chromosomes	Variant	54.1176	1.6912	2.1951 - 0.9253	40.69	21.06	42.26	$A_2B_6C_{22}D_2$
Parent cell with 17 chromosomes	Variant	25.7372	1.5140	1.8981 - 1.1872	23.04	13.97	42.50	A_1C_{16}
Callus cell with 34 chromosomes	Normal	47.4202	1.3947	1.9139 - 0.8143	59.76	22.86	41.77	$A_2B_{28}D_4$
Callus cell with 32 chromosomes	Variant	43.0386	1.3450	1.7391 - 0.9289	30.37	16.10	44.08	$A_2C_{28}D_2$
Callus cell with 64 chromosomes	Variant	88.9206	1.3894	2.5946 - 0.6536	40.30	33.28	46.08	$A_4B_2C_{42}D_{16}$
In vitro plant cell with 34 chromosomes	Normal	44.5328	1.3098	1.6692 - 0.9287	28.50	13.60	40.85	$A_2C_{30}D_2$
In vitro plant cell with 32 chromosomes	Variant	46.1810	1.4432	2.0047 - 1.0875	29.66	16.20	39.74	A_2C_{30}

Random Amplified Polymorphic DNA (RAPD) Analysis

DNA was isolated from the parent plant and 5 micropropagated plants (T1, T2, T3, T4 and T5). The amount of DNA present in the samples, quantified by spectrophotometer is shown in Table12.

Table 12. Amount of DNA present in the leaf samples of *Plectranthus zeylanicus*

Sample	Amount of DNA present (µg/µl)
Parent plant	1.10
T 1	0.90
T 2	0.96
T 3	0.92
T 4	0.99
T 5	0.89

T - Tissue cultured plant

RAPD analysis was carried out with 16 primers of arbitrary sequences. Of them, the results of those which successfully amplified the extracted DNA with consistent reproducible bands are shown in Plates 16 - 18. The number of bands resolved per primer ranged from a minimum of 1 to a maximum of 6. The size of the amplification products also differed and ranged from approximately 750 bp to 10000 bp. Most of these bands were monomorphic among the individuals. The primers and the characterization of consistent bands are listed in Table 13.

The RAPD fingerprint of T3 differed from the parent with 5 primers (OPAB 02, OPAB 06, OPAB 08, OPAB 11 and OPAB 17). Few parental bands were found to be absent in T3 when these primers are used. Amplification products with OPAB 02 generated no bands at 2000 bp length and 5000 bp length in the T3 but bands were clearly visible in the parent plant, T1, T2, T4 and T5 in these regions. When the primer OPAB 06 was used for amplification, a fragment with nearly 6000 bp was found missing in T3 which was clearly present in the

fingerprints of other plants. With OPAB 11, two bands were found to be absent in T3, one nearly at 5000 bp and other at 10000 bp. One fragment of DNA nearly at 8000 bp was missing with primer OPAB 08 in T3. When primer OPAB 17 was tested, 2 bands, one nearly at 8000 bp and the other at 4000 bp were found to be absent in T3. No additional bands could be detected by this marker screening.

The other primers used could not generate any polymorphism, but certain intensity differences in the bands were noticed in the amplification products of some primers tested.

Table 13. Primers and characterization of bands in parent and 5 tissue cultured plants of *Plectranthus zeylanicus.*

Primer	Sequence	Number of bands					
		Parent	T1	T2	T3	T4	T5
OPAB 01	CCGTCGGTAG	6	6	6	6	6	6
OPAB 02	GGAAACCCCT	6	6	6	3	6	6
OPAB 06	GTGGCTTGGA	6	6	6	4	6	6
OPAB 08	GTTACGGACC	5	5	5	4	5	5
OPAB 10	GGGCGACTAC	3	2	0	2	2	2
OPAB 11	GTGCGCAATG	5	5	5	3	5	4
OPAB 17	TCGCATCCAG	6	6	6	3	4	4
OPAB18	CTGGCGTTGT	1	1	1	1	1	1
Primer A 10	GACCGCTTGT	3	3	3	3	3	3
Primer A 17	GTGATCGCAG	3	3	3	3	3	3

In the case of T1, T2, T4 and T5, certain band differences were noticed with certain primers, but they showed minor differences. The major difference was noticed in the case of T3 and the reproducibility of the bands was consistent in the case of T3 alone. The representative profile of the *in vitro* raised plants and the control with 10 primers is shown in plates 16 - 18.

Essential oil Analysis

The cytological and genetic variability may or may not be visible in the qualitative characteristics of the regenerants. Screening of the plants for desirable qualities like essential oil yield and composition becomes significant in this aspect. Since significant molecular differences were shown by the tissue cultured plant T3, qualitative and quantitative screening of essential oil was done only in the parent plant and the tissue cultured plant T3. The essential oil isolated by hydro-distillation of leaves of *in vivo* and *in vitro* plants of *P. zeylanicus* showed a yield of 0.8% based on dry weight of the sample. The GC-MS pattern of the *in vitro* plant oil was distinctly different when compared with that of the parent plant oil (Plates 19 & 20). In the parent plant 18 compounds were identified which included monoterpenes and sesquiterpenes. Only 13 compounds could be identified in the *in vitro* derived plants. The components identified by GC-MS analysis with their percentage composition are listed in Table 14.

Though the composition of the oil varies, the colour remained the same as orange yellow in both the samples. The analysis of the oil samples revealed a range of variation in their constituents. The major components identified from the essential oils of *in vivo* and *in vitro* plants were similar (geraniol, geranyl acetate and nerol), even though, there is marked variation in their percentage of occurrence. Considerable increase in the quantity of geraniol (31.4 and 40.8) and nerol (8.8 and 11.4) and decrease in the percentage of geranyl acetate (35.0 and 28.0) was recorded in the *in vitro* plant oil. In the *in vitro* (T3) plant oil five minor compounds – 1octen 3-yl-acetate, α-terpineol, germacrene-D and cadina-1,4-diene – were found to be absent. The mass spectra of the compounds identified in the GC-MS analysis are shown in Plates 21 - 26.

The total number of chemical components detected by GC-MS in both the *in vivo* and *in vitro* grown plants was found to be 18. However, the number of similar components which occur both in the parent and the *in vitro* plant was 13. The coefficient of similitude between the parent plant and callus regenerated plant was found to be 72.2.

Table 14. Chemical composition of the leaf essential oil of *Plectranthus zeylanicus*

No.	Retention Time	Chemical compounds	Class of compounds	Percentage	
				Parent plant	*In vitro* plant (T3)
1	5.27	1 Octen-3-ol	Alcohol	0.6	0.4
2	8.85	Linalool	Monoterpenoid	2.3	1.7
3	9.30	1 Octen-3yl-acetate	Alcohol-derivative	0.7	-
4	12.17	α-Terpineol	Monoterpenoid	0.3	-
5	13.70	Nerol	Monoterpenoid	8.8	11.4
6	14.18	Z-Citral	Monoterpenoid	2.5	1.1
7	14.79	Geraniol	Monoterpenoid	31.4	40.8
8	15.39	Geranial	Monoterpenoid	3.5	2.3
9	19.24	Neryl acetate	Monoterpenoid	3.1	3.4
10	19.50	α-Copaene	Sesquiterpenoid	0.4	0.4
11	20.05	Geranyl acetate	Monoterpenoid	35.0	28.0
12	24.97	Germacrene-D	Sesquiterpenoid	0.4	-
13	25.36	δ-Cadinene	Sesquiterpenoid	2.1	2.5
14	28.34	β-Cubebene	Sesquiterpenoid	2.2	2.6
15	28.76	Cadina-1,4-diene	Sesquiterpenoid	0.3	-
16	29.72	α-Cadinol	Sesquiterpenoid	2.7	2.4
17	30.26	Valencene	Sesquiterpenoid	2.7	3.0
18	32.79	Farnesol	Sesquiterpenoid	1.0	-

Trichome Observations by SEM

As in plants of most Lamiaceae species, the surface of *Plectranthus zeylanicus* leaves possess glandular and non-glandular trichomes on both abaxial and adaxial sides. The glandular trichomes included peltate and capitate types. They are present on both leaf sides, being predominant on the abaxial surface. The distribution and morphology of the glandular trichomes showed no significant variations in the comparative analysis of the *in vivo* and *in vitro* (T3) plants.

Non-glandular trichomes are seen on both leaf surfaces scarcely. They are abundant on the veins. Each of them is simple, uniseriate and unbranched. The Scanning Electron microscopic observations of the glandular and non-glandular trichomes are shown in Plates 27 & 28.

Peltate trichomes consist of a short stalk cell and a large head with 4-8 secretory cells arranged in a single disc as noticed in other lamiaceous members. The surface of the mature peltate glands appeared to have a more or less spherical shape because the accumulation of secretions in the subcuticular space distended the cuticle at the onset of secretory phase. A mature peltate trichome is about 60 μm ($\pm$10) in diameter at the head.

Capitate trichomes are composed of a long stalk and a globoid or ovoid glandular head with slightly raised apex. The secretory product accumulates inside the apical cells and in a very small subcuticular space, probably being exuded through cuticle micropores. At maturity the capitate trichomes are about 100 μm ($\pm$10) in height and the horizontal diameter of the head is 35 μm ($\pm$10).

Elemental Analysis by EDS

The elements identified using EDS and their weight and atomic percentages are shown in Table 15. They exist as ingredients of diverse structural and biochemical compounds present in the plant tissues. The adaxial and abaxial sides of the leaves show almost similar elements except cadmium and aluminium. Cadmium is present on the abaxial surface and aluminium on the adaxial surface only. The absence of sodium and the presence of aluminium are noticed only in *in vitro* derived plants. The concentration of the elements showed slight variations in the abaxial and adaxial surfaces and also among *in vivo* and *in vitro* (T3) plants. Potassium is found to occur on the leaf surfaces in considerable quantity followed by calcium. In both the parent and *in vitro* leaf surfaces the major element potassium was found to be abundant on the abaxial surface and calcium was seen to be abundant on the adaxial side. The distribution of potassium was higher on the leaf surfaces of the parent, but the distribution of calcium was more on the *in vitro* leaf surface. Comparatively higher concentration of Na, Mg, Si, Cl and K was noticed on the abaxial surface of both the parent and *in vitro* (T3) plants, while Ca, Cu and Zn showed higher composition on the adaxial surface of the leaves of both plants.

The comparative observation of the elemental composition on the leaf surfaces of the parent and *in vitro* (T3) plants revealed differences in the distribution of elements. The *in vitro* leaf surface showed higher percentage of Mg, Ca, Cu and Zn. But Cl and K were seen more on the leaf surface of the parent. The spectra of the elements are shown in plates 29 - 30.

Table 15. Composition of elements on the epidermal surfaces of the leaves of *Plectranthus zeylanicus*

Element	Adaxial surface				Abaxial surface			
	Weight %		Atomic %		Weight %		Atomic %	
	Parent	***In vitro***	**Parent**	***In vitro***	**Parent**	***In vitro***	**Parent**	***In vitro***
Na	3.54	-	6.10	-	4.97	-	8.28	-
Mg	5.65	6.63	9.21	10.99	8.17	11.41	12.86	18.19
Al	-	2.36	-	3.53	-	-	-	-
Si	2.25	2.19	3.17	3.14	-	3.11	-	4.29
Cl	9.44	5.53	10.54	6.29	10.90	8.92	11.78	9.75
K	40.30	36.33	40.82	37.48	46.65	37.88	45.70	37.56
Ca	16.77	23.97	16.57	24.12	14.01	18.84	13.39	18.22
Cu	12.87	13.92	8.02	8.84	5.63	12.49	3.39	7.62
Zn	9.19	9.08	5.57	5.60	5.35	7.36	3.13	4.36
Cd	-	-	-	-	4.32	-	1.47	-

Total Phenolics

The leaves of *Plectranthus zeylanicus* were found to be rich in phenolics as evident from the high value (3.037mg/g) of fresh leaf tissue when measured by Folin-Denis method. The distribution of phenolics on leaves of the *in vitro* (T3) and *in vivo* plants did not show considerable variation (Table 16).

Table 16. Total Phenolics in *Plectranthus zeylanicus* leaves

Concentration of phenolics mg/g of tissue	
In vivo plant	*In vitro* (T3) plant
3.037±0.017	3.017±0.015

Antioxidant Activity

The methanolic extract of both *in vivo* and *in vitro* (T3) plants of *Plectranthus zeylanicus* was found to scavenge the superoxide generated by riboflavin photoreduction method. 1000 µg/ml of the extract showed 92.21% inhibition indicating remarkable effect in scavenging of superoxide radical. The percentage inhibition of the *in vitro* (T3) plant extract was lesser (77.31%) when compared to the parent plant (Plate 31).

Degradation of deoxyribose by hydroxyl radical generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system was found to be inhibited by the extracts of the parent and the *in vitro* (T3) plants. The effect of varied concentrations of the drug of *Plectranthus zeylanicus* parent and the regenerated (T3) plants on scavenging of Fenton reaction mediated OH radical as determined by inhibition of 2-deoxyribose degradation has been depicted in Plate 32. It rendered a dose dependent inhibition of 2-deoxyribose degradation. The extract scavenged the generated hydroxyl radical by 75.91% at 1000 µg/ml for the parent and 73% for the regenerant (Plate 32). This indicated the significant hydroxyl radical scavenging activity of the extract.

Methanolic extract of the plant was effective in inhibiting the lipid peroxidation in the *in vitro* system. Lipid peroxidation induced by Fe2+/ascorbate in rat liver homogenate was found to be inhibited by 66.67% for the *in vivo* and 57.86% for the *in vitro* plant extract at a concentration of 1000 µg/ml of the extract (Plate 33). The percentage inhibition in different concentrations of the plant extracts are shown in Tables 17 - 19.

Table 17. Concentration dependent superoxide radical scavenging activity of *Plectranthus zeylanicus* extracts

Concentration of drug (µg/ml)	Percentage of inhibition	
	***In vivo* plant**	***In vitro* (T3) plant**
100	40.69 ± 1.493	36.11 ± 0.875
200	60.65 ± 0.987	54.62 ± 0.699
400	84.26 ± 0.045	58.50 ± 0.984
600	89.01 ± 0.569	71.88 ± 1.185
800	90.42 ± 0.455	73.37 ± 0.816
1000	92.21 ± 1.400	77.31 ± 1.384

Table 18. Concentration dependent hydroxyl radical scavenging activity of *Plectranthus zeylanicus* extracts

Concentration of drug (µg/ml)	Percentage of inhibition	
	***In vivo* plant**	***In vitro* (T3) plant**
100	48.33 ± 0.923	38.13 ± 1.224
200	69.37 ± 0.282	51.89 ± 0.875
400	72.21 ± 1.092	61.51 ± 1.120
600	74.34 ± 0.676	67.37 ± 1.788
800	75.26 ± 0.641	71.15 ± 0.397
1000	75.91 ± 1.074	73.00 ± 1.542

Table 19. Concentration dependent lipid peroxidation inhibition of *Plectranthus zeylanicus* extracts

Concentration of drug (µg/ml)	Percentage of inhibition	
	***In vivo* plant**	***In vitro* (T3) plant**
200	43.55 ± 0.910	10.40 ± 0.401
400	50.47 ± 0.408	19.90 ± 0.625
600	53.56 ± 1.286	34.59 ± 0.838
800	64.09 ± 0.629	48.30 ± 0.821
1000	66.67 ± 0.546	57.86 ± 0.538

Values are expressed as mean ± standard error

DISCUSSION

Micropropagation

Regeneration of plants by plant tissue culture techniques have become a powerful method of plant propagation and more speculatively for the selection of specific genetic modifications leading to crop improvement. Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin and Scowcroft, 1981) which is often heritable. There are many biological routes through which variation arises when plant tissues are passed through tissue culture. The variations can be analysed at the level of the phenotype or the genotype. Phenotypic variations can be the result of a modification of the genome itself or only of the expression of the genome. Genotypic variations can be genomic, chromosomic or genic. Genomic mutations affect the number of chromosomes (the ploidy) and can be detected by chromosome counting. Chromosomic mutations like inversion, deletion or translocations and genic mutations could be detected by genetic molecular markers like RFLPs and RAPDs which reveals DNA sequence modifications (Fourre *et al*., 1997).

Plants regenerated from relatively undifferentiated callus cultures possess a vast array of genetic changes. Such variations can contribute to the beneficial aspects of the plant in culture. The amount of variation caused by tissue culture differs in different systems and for different genotypes. Even if complete genetic fidelity is guaranteed, no *in vitro* propagation technique or condition can promise exact true-to-type or genotypic performances (Chatterjee and Prakash, 1993). According to McClintock (1984), no two of the callus-derived plants are exactly alike, and none is just like the plant that donated the cell or cells for tissue culture.

In the present investigation, a new protocol was attempted for the micropropagation of *Plectranthus zeylanicus*. Nodal segments and shoot apices were subjected to callus development and shoot initiation. The clonal multiplication of the

plant becomes significant in the light of the medicinal virtues of it. The cultured explants regenerate shoots directly or through callus phase (Figs. 1 - 29). Emergence of shoots directly from cultured explants will be useful in the propagation of true-to-type plants and that from the callus will induce variations.

The morphogenic response of explants was dependent on the type and concentration of growth hormones (Table 2). Tissue culture studies on a number of medicinal plants (Irawati and Nyman, 1986; Kumar, 1992; Babu *et al*., 1992) suggest that a fine balance of exogenous auxin and cytokinin are necessary for successful regeneration of plants. The role of cytokinin in shoot organogenesis is well established (Evans *et al*., 1983). The sugar, potassium and phosphate concentration in the medium, nitrogen sources, pH of the cultures and addition of organic acids of buffer media have been found to affect the concentration of the propagules in plant tissue culture (Dougall, 1981). The present observation indicates that a combination of NAA and BAP was practically effective in inducing multiple shoots in *P. zeylanicus* (Figs. 5 & 9). Maximum multiplication was occurred in the medium with 2 mg/l NAA and 3 mg/l BAP (Fig. 9). Though shooting was induced in the medium with KIN, the results were not much satisfactory as in BAP (Fig. 7). The superiority of BAP over other cytokinins for multiple shoot formation has been reported in many fruit plants (Lundergan and Janic, 1980; Rahman and Blake, 1988; Sen and Sharma, 1991). Two different auxins *viz*., NAA and IAA were used with KIN and BAP for shoot proliferation. Significant synergistic effect of auxin treatment was observed in the combinations of NAA with BAP. Maximum multiple shoot generation was occurred on particular growth regulator concentrations. This is in agreement with the hypothesis that the balance of growth regulators as well as their concentration is critical in determining the direction of morphogenesis (Sharief and Jagadishchandra, 1999). Generally, a cytokinin or a combination of cytokinins and auxins is required for *in vitro* shoot proliferation (Thorpe and Patel, 1984). Of the two cytokinins tested, BAP was found to be comparatively more effective than KIN in inducing multiple shoots (Table 2). Skoog and Miller (1957) reported that shoot bud regeneration depends on quantitative interaction between various growth regulators, *viz*., auxins and cytokinins.

In callus culture, the success of regeneration depends upon the type of medium used in each phase of culture as callus initiation, maintenance and regeneration. According to Hakman and Fowke (1987) and Von and Woodward (1988) presence of auxin together with cytokinin promotes the induction and formation of organogenic callus. Heszky *et al*. (1991) identified the influence of an optimum combination of auxin and cytokinin in the expression of morphogenic potential. Here in the present study also, simultaneous inclusion of auxin and cytokinin was essential for callus proliferation and shoot regeneration. Variations in endogenous hormone levels in the plant also affect callus formation. The younger tissues are known to contain high auxin levels (Sheldrake, 1973) that could promote callus formation.

Axillary shoot multiplication was observed in medium containing BAP (3 mg/l) and NAA (2 mg/l) [Fig. 9]. The hormonal combinations of KIN+NAA and IAA+BAP also contributed multiple shoot induction (Figs. 6, 7 & 8). The role of cytokinins in overcoming the apical dominance of the terminal shoot bud and enhancing the branching of the lateral buds from axils was observed. BAP is known as the most effective synthetic cytokinin for stimulating axillary shoot proliferation for different plant systems (Bhojwani, 1980; Hasegawa, 1980; Kitto and Young, 1981; Welander *et al*., 1989; Nadel *et al*., 1991; Devi *et al*., 1994; Gangopadyay *et al*., 1998). The stimulatory effect of BAP on multiple shoot formation was reported in *Trachyspermum ammi* (Ajita *et al*., 2001). The response of the present study confirmed the effectiveness of BAP in combination with auxin for axillary bud formation, which is in accordance with the findings of Mujib *et al*. (1995) in *Catheranthus roseus*.

The incorporation of an auxin in the medium generally promotes rooting (Gautheret, 1945). The relative levels of auxins have been known to greatly influence morphogenic responses like rooting (Sitborn *et al*., 1993). The observation of the performance of IBA in root induction than other auxins like IAA and 2,4-D (Amin and Rahman, 1994) emphasized the fact that auxin types differ in their morphogenic ability and organogenic effect on plant tissues in culture (Nagasawa

and Finer, 1988). The promotory role of IAA in rooting was noticed in a number of species (Thorpe, 1978). IAA and IBA were known to have root induction capacity in the experiments by Purohit *et al*. (1995a, 1995b). Though IAA induced rooting of the regenerants, IBA was more effective in rhizogenesis in the present study (Figs. 23, 25 & 26).

Tissue culture conditions are expected to lead to peculiar patterns of gene expression in plant cells, which may cause some transient changes in regenerated plants (Taylor *et al*., 1995b). The variability observed in these regenerants usually included morphological changes in plants such as size of leaves and flowers, cytological changes like euploid and aneuploid conditions and phytochemical changes such as composition of essential oil components. In the present study phenotypic evaluation of the regenerants confirmed the absence of variation in the external morphological features. The variations included the aspects as cytological, phytochemical and biological activity of the plant. Several types of genetic changes are known to occur in tissue cultured plant cells especially in those which pass through a callus stage of development. These include gross karyotypic changes, chromosomal re-arrangements, somatic crossing over with sister chromatid exchange, activation of transposable sites along certain regions of nuclear DNA and re-arrangements of nucleotide sequences in both nuclear and organellar DNA. According to Bajaj (1990) any genetic variability brought about by *in vitro* culture may be included in the term somaclonal variation. Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue cultures (Skirvin *et al*., 1993). Somaclonal variation is also called tissue or culture-induced variation (Kaeppler *et al*., 2000).

Many variables influence the amount of somaclonal variation in tissue culture. Generally, meristematic or shoot tip cultures where no callus is formed will have the least amount of variation, while the induction of callus and adventitous shoots produce the most variation. The induction of callus or adventitous shoots disrupts normal cell function and may activate transposable elements, stress-induced enzymes or other products. Factors such as genotype, ploidy, culture duration,

selection pressure, culture conditions, medium and proliferation rate can affect variation (Evans *et al*., 1984; Peschke and Phillips, 1992; Skirvin *et al*., 1994). Variation in plant genome can also occur in response to various internal and external influences like passage of plant cells through cycles of tissue culture and regeneration. The characterization of the genomic changes has indicated that they mainly occur in the highly repetitive fraction of the genome and are limited to specific subsets of these sequences. These subsets may be localized at particular chromosomal sites. These genomic variations lead to reorganization of the genome in response to stress in order to increase the pool of variability from which new types can be selected (Cullis and Creissen, 1987). Somaclonal variation can provide means for amplifying variability within the existing cultivar, thereby opening new opportunities to clonal selection.

According to Larkin (1987), somaclonal variation can be due to either the variation induced by mutagenic action of culture media or variation induced by stress (leading to the activation of mobile genetic elements). McClintock (1984) placed tissue culture as one of the stress factors that could cause widespread genomic restructuring facilitated by transcriptional transposon activation, transposition of mobile elements and chromosome breakage-fusion-bridges. The most common factors affecting somaclonal variation are genotype, explant source, *in vitro* period and cultivation conditions in which the culture is established (Evans and Sharp, 1988; Bordallo *et al*., 2004). Transposon activation and some examples of gene silencing indicate that epigenetic mechanisms contribute to the process of somaclonal variation. The observation of chromosome breakage events in culture supports the contention that tissue culture induces a genomic shock. Genomic shock has been shown to activate transposable elements (McClintock, 1984). Quiescent transposons and retrotransposons exist in the genomes of many plant species and their activation in tissue culture supports the notion that derepression of epigenetically silenced sequences is induced by the culture process. Stress induced by tissue culture process cause alterations in the DNA. These alterations could affect the expression of specific genes (Kaeppler and Philips, 1993). Conditions in the artificial environment of cell culture may enhance the spontaneous mutation rate.

Increasingly disorganized growth of cultured tissue may allow for more genetic rearrangement and variation in regenenerated plants (Reisch, 1983). Lee and Phillips (1988) have proposed a mechanism for somaclonal variation based on late replication of heterochromatin.

Qualitative mutation is frequent among tissue culture regenerants. Conditions in the artificial environment of cell culture may enhance the spontaneous mutation rate. Specific site mutations could be the result of the activity of certain types of transposons, infidelity of synthesis or repair mechanisms or base instability. Duplicate sequences in the genome, which peacefully coexisted under normal cellular conditions, begin to interact under the stress conditions of culture, inducing a mutagenic process.

Epigenetic variation is another important cause of somaclonal variation in plants. Gene silencing through DNA methylation has been implicated in epigenetic variation (Kaeppler *et al*., 2000). Methylation of both DNA and histone appears to be intimately involved in the maintenance or formation of heterochromatin. A change in the methylation background either by loss of DNA or histone methylation or a rearrangement of methyl groups within the genome can cause changes in gene transcription. And further, the loss of methylation is also correlated with transcriptional and transpositional activation of transposons, which in turn can cause gene mutations and phenotypic changes.

The incidence of somaclonal variation depends on the explant origin and regeneration method as well as the regenerant source (Ahuja, 1998). In addition, the duration of tissue culture also affects somaclonal variation. Somaclonal variation generally increases with the time that a culture has been maintained *in vitro*, especially for callus culture (Skirvin *et al*., 1994; Bouman and de Klerk, 1997). It is known that factors such as explant source, duration in culture and growth regulators influence the frequency of somaclonal variation (Karp, 1995). The genetic alterations due to the artificial culture environment are manifested as increased frequencies of single gene mutations, chromosome breakages, transposable element activations, quantitative trait variations and modifications of normal DNA

methylation patterns. Evidence is presented that indicates a high frequency of DNA hypomethlation as the result of the tissue culture process.

Somaclonal variation is likely to be a reflection of response to cellular stress. The stress during the *in vitro* culture activates transposable elements, which would be responsible for high frequency of chromosome breakage, recessive mutations and expression of silent genes (Lee and Phillips, 1987; D' Amato, 1991). The causes identified for somaclonal variations include: changes in the structure and/or chromosome number, noticeable point mutations, changes in the expression of a gene as a result of structural changes in the chromosomes or activation of transposable elements, chromatin loss, DNA amplification, somatic crossing over, somatic reduction and structural changes in the organellar DNA (Rao *et al*., 1992; Kaeppler *et al*., 2000). Correlation between the culture time-length and the accumulation of chromosome variations was first documented in *Daucus carota* (Smith and Street, 1974). According to George and Sherrington (1984) high concentration of growth regulators in the medium and long term culture are the main causes of variation in plants cultured *in vitro*. Addition of growth regulators to culture medium is known to have influence on the frequency of the karyotype alterations in cell cultures. Frequently, the auxin 2,4-D is considered to be responsible for the chromosome variation (Singh *et al*., 1975). Increasing amounts of 2,4-D enhances methylation levels in carrot cultures (Kaeppler *et al*., 2000). Larkin (1987) reported that longer period of *in vitro* cultivation seemed to increase somaclonal variations, whereas a shorter callus phase should reduce them.

Skirvin (1978) opined that variation is quite ubiquitously associated with *in vitro* propagated plants. Somaclonal variation can provide means for amplifying variability within the existing cultivar, thereby opening new opportunities to clonal selection.

Cytological analysis

In the present study, the chromosome numbers frequently observed in the meristematic cells of the parent, callus and the *in vitro* root meristem were

invariably the same as 2n = 34 and 2n = 32 (Plates 7, 8, 10, 11, 13 & 14). Some of the parent cells showed a haploid chromosome number of 17, amidst the cells with 32 and 34 chromosomes (Plate 9). Some callus cells exhibited a polyploid chromosome complement with 64 chromosomes along with other normal cells (Plate 12). The two reasons that can be predicted for the observed chromosomal variability in *P. zeylanicus* are :-

- Evolution at the basic chromosome level
- Aneuploidy (disomy) at the somatic chromosome level

Basic chromosome number forms one of the widely used characters in formulating phylogenetic speculations and hence can be considered as a dependable and stable marker of the direction of evolution (Jones, 1970, 1974, 1978). A great variability in the number of chromosomes in the basic set is a characteristic feature for the mint family (Leshukova, 1970). Both primary as well as secondary base numbers are involved in the evolution of Lamiaceae members. In Lamiaceae, the secondary base numbers might have evolved from the primary numbers, $x_1 = 6 - 9$.

In the present study the species seems to be dibasic with the primary base numbers 8 and 9. The chromosome numbers, 2n = 32 and 34 might have originated by three possible ways

- Doubling of the primary base number $x_1 = 8$ by protoautoploidy leads to the formation of the secondary base number $x_2 = 16$ which by autopolyploidy gives rise to cells having 2n = 32 chromosomes. This secondary base number of $x_2 = 16$ undergoes an ascending dysploidy leading to an increase in a chromosome in the secondary basic set forming $x_2 = 17$ from which the other chromosome number of 2n = 34 might have originated.
- Doubling of the primary base number $x_1 = 9$ by protoautoploidy leads to the formation of the secondary base number $x_2 = 18$. This secondary base number of $x_2 = 18$ undergoes a descending dysploidy leading to a decrease in a

chromosome in the secondary basic set forming $x_2 = 17$ from which the normal chromosome number of 2n = 34 might have originated.

- Joining of the 2 primary base numbers $x_1 = 8$ and $x_1 = 9$ leads to the formation of the secondary number $x_2 = 17$ (amphiploidy), doubling of which forms cells with 34 chromosomes. From the secondary base number of $x_2 = 17$, one chromosome might have lost by descending dysploidy to form $x_{2 =} 16$, from which 2n = 32 might have originated (Plate 15).

These three mechanisms either singly or together might have contributed to the chromosomal variability observed during the study. Thus both auto and amphiploidy contributed to the rise of the secondary base numbers in *Plectranthus zeylanicus.*

The mosaicism noticed in the parent meristematic cells are maintained in the callus as well as in the *in vitro* regenerated calliclones. In the callus cells the occurrence of polyploidy was the peculiarity observed, when compared with the parent plant and the regenerated plants.

The reduction in the number of chromosomes that was observed in *Plectranthus zeylanicus* parent plant seems to be of the aneuploid type. The basic characteristics of aneuploid plants are structural rearrangement and variation in chromosome number (Choi *et al*., 2000). The aneuploidy found occasionally in *Plectranthus zeylanicus* was of disomic (2n – 2) type. Analysis of the data revealed that the normal chromosome number occurs in greater frequency than the variant number *i. e.*, 60% of the cells exhibit a normal diploid number, 2n = 34 and 38% shows the variant aneuploid number, 2n = 32. Monoploidy was shown by 2% of the root tip meristematic cells. Plants that show chromosome numbers lower than that of the normal chromosome number may be termed as break-down diploids. These break-down diploids were found to be occurring occasionally among the normal cells and may originate from the aberrant conditions of physiological stress that may occur during various stages of the cell cycle (Rajhathy, 1963). The probable causes of aneuploid variations are non-disjuction of chromosomes, non-orientation of

centromeres and chromosome elimination, centromere breakage and chromosome fragmentation, chromosome degradation, spindle abnormalities, complement fractionation, cytomixis and semigamy. Monoploid cells may originate from a diploid cell through a tripolar mitosis (1:2:1) (Pera, 1970). Another mechanism for the origin of monoploid from diploid cells is homologous segregation (Huskins and Cheng, 1950).

Plant cells growing in an artificial culture environment make numerous genetic mistakes. These alterations are manifested as increased frequencies of single gene mutations, chromosome breakages, transposable element activations, quantitative trait variations and modifications of normal DNA methylation patterns (Kaeppler and Phillips, 1993).

Chromosomal and sequence changes are prevalent in culture, consistent with the high frequency of phenotypic variations. Cytogenetic abnormalities including ploidy changes and chromosome rearrangements have been found among tissue culture regenerants. Hang and Bregitzer (1993) found ploidy changes to be the most prevalent cytological change among barley regenerants, although chromosome breakage events also occurred. A comparative analysis in oat and maize showed that chromosome breakage occurred more frequently than ploidy changes (Benzion *et al*., 1986; Kaeppler *et al*., 1998). Translocations seem to be the most frequent chromosomal abnormality observed along with inversions and insertions/deletions. Late replication of heterochromatin in tissue culture leads to chromosome bridges and breakage events (Johnson *et al*., 1987; Hang and Bregitzer, 1993).

Genetic variation in culture is primarily embodied as cytological changes, including ploidy changes and chromosome breakages. Single gene mutations are most likely due to base changes and gene activation/inactivation including transposons (Kaeppler *et al*., 1998)

The primary cytological changes observed among regenerated plants and their progeny include chromosome rearrangements and changes in chromosome number. Data from oat and maize indicate that chromosome rearrangements are primarily due to chromosome breakage events (Kaeppler and Phillips, 1993).

Ploidy changes also occur in culture with polyploidy generally occurring more frequently than aneuploidy. Polyploidy is more likely due to an endoreduplication event. Endoreduplication would be unlikely to be caused by late replication (Kaeppler *et al*., 2000). Aneuploidy could be reasonably due to non-disjunction mechanism giving rise to breakage events or could result from neo-centrometric activity of heterochromatin. The primary cytological aberrations observed in tissue culture are caused by modification of the chromatin of repeats, or perhaps with non-canonical sequences having a centromere function under conditions of genome stress.

Somaclonal variation is caused by changes in chromosome numbers (polyploidy or aneuploidy), damage to chromosomes (insertions, deletions, translocations, mutations etc.) or changes in methylation of chromatin (Evans *et al*., 1984; Kaeppler and Phillips, 1993; Peschke and Phillips, 1992; Phillips *et al*., 1994). Endomitosis is one of the causes of polyploidy. Endomitosis differs from normal mitosis in the absence or abnormal functioning of a mitotic spindle leading to doubling of chromosome number at the end of nuclear division.

Polyploidy has direct relationship with the occurrence of chromosomal mosaicism. Intra-individual variation of chromosome number is an abnormality in the form of simultaneous occurrence of normal, euploid and aneuploid cells in the tissue of the same plant. Various causes cited for mosaicism are endopolyploidy, genome reduction, spindle abnormalities, chromosome irregularities, syncyte formation and semigamy which are not mutually exclusive. The useful outcome of these is non-disjunction, somatic reduction, chromosome elimination, multiple spindles, complement fractionation, genome doubling *etc*. leading to mixoploidy, polysomaty and aneusomaty. Mosaicism involving euploidy is much more common and is found in a wider variety of tissues than aneuploidy (Nirmala and Rao, 1996).

Although the chromosomal constitution of certain plants seems to be highly stable *in vitro*, there is a possibility of variability in tissue culture that can be directly or indirectly attributed to gross chromosomal changes and abnormalities (Skirvin, 1978). The genomic changes that have been observed to occur in tissue culture

include aneuploidy, chromosome rearrangements such as translocations, inversions, deletions, gene amplification and deamplification, activation of transposable elements, point mutations, cytoplasmic genome rearrangements and changes in ploidy level (Larkin and Scwcroft, 1983; Orton, 1984; Evans *et al*., 1984). The structural and numerical chromosome variations found in cell populations cultured *in vitro* occurs from endoreduplication producing polyploidy and mitotic irregularities resulting in aneuploidy and chromosomal breakage (Singh *et al*., 1982). Chromosomal abnormalities such as laggards, sticky bridges, anaphasic bridges, unequal distribution of chromosomes due to multipolarity, endomitosis and such abnormalities might be possible mechanisms for the origin of *in vitro* aneuploidy and polyploidy (Torrey, 1959; D' Amato *et al*., 1980).

The variations in the karyotype are expressed as changes in the centromeric position and total length of chromosomes. In the present study, the average chromosome length and total chromosome length was found to be slightly higher in the parent plant than that of the tissue cultured plants. Chromosomal breakage and deletion may lead to slight changes in the size of chromosomes. The differences in the chromosome length and volume may be attributed to differential spiralization and condensation of chromosomes along with the content of protein and DNA. Eventhough there are differences in the karyomorphometrical features such as total chromosome length (TCL), average chromosome length (ACL), karyotypic formula, range of chromosome length (RCL), the nature of the primary constriction of chromosomes, disparity index (DI), variation coefficient (VC) and total forma percentage (TF%), no definite evolutionary significance can be predicted (Table 11).

Normal cell behaviour is the result of a complex cascade of genetic programs that are sensitive to disruption by biotic and abiotic stresses. Under tissue culture conditions, these regulation processes can be so disturbed that cells behave abnormally. An example is the occurrence of some mitotic irregularities, which underlie some somatic variations in cultures (Larkin and Scowcroft, 1981; Peschke and Phillips, 1992; Phillips *et al*., 1994). Hao *et al*. (2004) concluded in their cytological examination of *Citrus* callus that chromosome number variations arose

spontaneously as a consequence of some mitotic irregularities such as lagging chromosomes, meiosis like division and asymmetrical chromosomal distribution. Culture conditions interfere with normal cell regulation and induce genomic instability in cells so that chromosome variations are produced. Differences in total chromosome length between the parent, callus and *in vitro* plant having the same chromosome number may indicate duplication or deletion of chromosome segments. Variation in karyotypic asymmetry may indicate segment translocation (Shan *et al*., 2003).

It is well documented that *in vitro* culture conditions induce a genomic stress that might result in chromosome breakage. Many studies have indicated that the break positions do not appear to be random but occur in a heterochromatic region and could lead to chromosomal translocations, inversions or deletions (Benzion and Phillips, 1988; Lapitan *et al*., 1988). During micropropagation, the regulating processes of plant regeneration are frequently interfered by *in vitro* processes, causing irregular cells with abnormal chromosomes (Hao and Deng, 2002). Consequently chromosomal aberrations give rise to phenotypic variation in regenerated plants. Chromosome breakage and rearrangement can generate somaclonal variation in one way or another, for example late replication of heterochromatin, insertion and excision. Mitotic crossing over can also account for some of the variation in regenerated plants. Any form of mitotic recombination could produce chromosome rearrangement in micropropagated plants. Chromosome based variations like changes in chromosome number, chromosome rearrangement, breakage and lagging is believed to be the fundamental cause of somaclonal variation (Bayliss, 1980; D' Amato, 1985; Phillips *et al*., 1994; Gupta, 1998).

There have been many studies of chromosomal aberrations in the cultured plant cells and their regenerants using various plant materials (Singh, 1993). It has been reported that plant tissues and cells display a high degree of instability under *in vitro* conditions resulting in the formation of mixoploid tissues (Sunderland, 1977; Bennici and D' Amato, 1978; Bennici, 1979). Several literature reviews dealing with ploidy, instability and related phenomena in the *in vitro* cultured cells are available

(D' Amato, 1952, 1977, 1978; Skirvin, 1978; Constantin, 1981). These reports are in agreement with the results obtained in the present study since various ploidy levels are observed among the callus cells.

For a species, any form of chromosome rearrangement can lead to many changes in the genome at structural and/or possibly at functional levels, which may play a central role in generating several forms of somaclonal variation. Chromosomal differences reflect the fundamental changes in morphological, physiological and biochemical characters that result from different gene action and expression (Sharma and Sen, 2002). Generally gross chromosomal aberrations have been a common phenomenon of micropropagated plants. Alteration in chromosome number may result in differences in gene duplication or deficiency. The alteration of a chromosome segment may affect several genes, and thereby several sets of phenotypic characteristics (Zhao *et al*., 2005).

Chromosomal rearrangements would also add to the diversity of the genomes. Structural rearrangements of chromosomes may have happened during karyotype change which keeps the chromosome number constant but induce variations in terms of karyotype organization, karyotype asymmetry, average chromosome length *etc*.

The decrease in the chromosome number from 34 to 32 may be due to processes including dysploid reduction, aneuploid decrease or Robertsonian fusion (Shan *et al*., 2003).

The regenerated plants from all the cultures did not show polyploidy or any recognisable chromosomal structural variations. They were diploids despite their origin from a callus with higher ploidy level cells, indicating that diploid cells have a higher ability for regeneration. Literature studies on the behaviour of chromosomes in tissue cultures has also shown that in many instances the regenerated plantlets contain a normal chromosome complement (Shimada *et al*., 1969; Swedlund and Vasil, 1985) suggesting that diploid cells are selectively favoured during plant regeneration. During shoot and root morphogenesis in diploid species, cells with unbalanced chromosome numbers cannot compete with balanced

cells which enhances the regeneration of a large number of plants with normal chromosome complements (Vasil, 1983; Larkin and Scowcroft, 1981; Nontaswatsri and Fukai, 2005). Even if a small number of cells have spontaneously changed their chromosome structure or number, due to competitive disadvantage, growth of chromosomal variant cells would be suppressed and organogenesis is observed only in normal cells (Chen and Goeden, 1979). The less range of variation of regenerated plants compared to the cultured tissues shows that the selection pressure of maintaining a certain karyotype might be operating during the course of regeneration (Oghihara, 1981). The above-cited reasons may be the probable reason for the development of diploid plants in *Plectranthus zeylanicus* after micropropagation in the present investigation.

Many factors are involved in chromosomal abnormalities in micropropagated plants. Plant growth regulators applied during micropropagation is an important factor associated with chromosome alteration. It has been known that growth regulator applied in culture medium can influence frequency of karyotypic alterations. Plant cell behaviour is the result of a complex process of genetic programming and is sensitive to hormonal changes, especially under the *in vitro* stressful conditions (Zhao *et al*., 2005). The karyotypic alterations observed in micropropagated plants of *Plectranthus zeylanicus* might be the aftereffect of hormonal stress during micropropagation. The factors known to affect chromosomal instability in plant tissues during *in vitro* growth are genotype, initial ploidy level, explant source, medium composition, growth regulators used and time in culture (Constantin, 1981; Karp, 1988). The chemical composition of the culture medium has been shown to affect the cytogenetic behaviour of plant cells *in vitro* (Bennici *et al*., 1970; Karp, 1992). It is known that a proper balance of auxins and cytokinins in the cuture medium is important, since their combination is essential for DNA synthesis and mitosis (D' Amato, 1978). The concentration and type of hormone in the culture medium also influence the pattern of methylation (LoSchiavo *et al*., 1989). Singh (1986) reported a few chromosomal variations in callus cultures of crops, which are produced due to the effect of media components. The effects of cytokinins and auxins in inducing chromosome changes were studied in tissue

culture studies of *Nicotiana* (Ronchi *et al*., 1976). The significance of nutrient medium in chromosomal behaviour of *Allium* and *Capsicum* cultures was studied by Nair *et al*. (1993) and Nair and Kumar (1998). The age of culture also influences the chromosomal stability. A prolonged culture can accumulate more chromosomal and genic abnormalities. Increase in length of subcultures or number of subcultures enhance the rate of variation.

The major changes in the chromosome complement may not be accompanied by corresponding changes in the phenotype of the plant. The gain or loss of chromosomes sometimes may not be sufficient to cause a large change in morphological characters and it is also possible that changes can also occur that are not visibly expressed (Liu and Chen, 1976). This may be the reason for the absence of morphological variation in the regenerated plants of *Plectranthus zeylanicus*.

Random Amplified Polymorphic DNA (RAPD) analysis

The significance of tissue culture variation or somaclonal variation in crop improvement depends upon establishing a genetic basis for this variation. RAPD-PCR markers (Welsh and McClelland, 1990; Williams *et al*., 1990) are widely used in genome analysis as they are easily obtained and possess the intrinsic potential to cover the genome widely. In the present study the total DNA content of the parent plant was found to be higher than that of the regenerants (Table 12). An increase in the chromosome length of the parent may be responsible for the increase in the DNA content. Somaclonal variation at DNA level is evident from the electrophoretic gels of the amplified fragments of DNA. A few bands were found to be missing in the regenerants mainly in the T3 when compared with the banding pattern of the parent (Table 13). DNA amplification products could result from changes in either the sequence of the primer binding site or changes which alter the size and prevent the successful amplification of target DNA (Rani *et al*., 1995; Rout *et al*., 1998).

The utility of RAPD markers as a means of molecular analysis of *in vitro* regenerated plants has been very well documented (Piccioni *et al*., 1997; Gupta and Varshney, 1999; Olhoft and Phillips, 1999; Kaeppler *et al*., 2000). Under stressful

conditions of micropropagation, the genome is abnormally reprogrammed and genome expression may be reset or may not follow the same orderly sequence that occurs under normal conditions (Jain, 2001). The plant regenerated under such stressful environment may involve in DNA resetting or restructuring which can give rise to variability at genomic and/or morphological levels (Zhao *et al*., 2005).

RAPD polymorphism originates from DNA base changes, base substitutions and deletions or insertions occurring within a primer binding site, hence repetitive elements are considered as an efficient molecular marker (Brown *et al*., 1993). Duplicate sequences in the genome, which peacefully coexisted under normal cellular conditions, begin to interact under the stress conditions of culture inducing a mutagenic process (Phillips *et al*., 1994). It has been suggested that DNA methylation may contribute to the generation of RAPD polymorphisms (Olmos *et al*., 2002). If epigenetic variation is caused by differences in methylation of genes, genotypes should have different banding patterns. Efforts to describe somaclonal variation at the molecular level have revealed the amplification of highly repetitive DNA sequences in cell cultures of *Nicotiana glauca* (Durante *et al*., 1983) and an increase in DNA methylation but a decrease in DNA content in regenerated pea plants (Cecchini *et al*., 1992). Assessment of somaclonal variation in rice at the DNA level showed that DNA methylation increases (Muller *et al*., 1990) and some of the variations at the DNA level might be in the highly repetitive sequences that have no phenotypic effects (Godwin *et al*., 1997).

The genotypic polymorphism detected was not expected based on phenotypic assessment. The possible explanation for not detecting the DNA alterations phenotypically is that a significant proportion of the variant RAPD loci found to vary were in the region of highly repeated sequence DNA that do not impart a phenotype. A significant proportion of RAPD variation detected may not represent any change in expressed sequences as the many RAPD bands are in fact high copy sequences and not transcribed DNA (Devos and Gale, 1992; McCouch *et al*., 1988). The present study demonstrated the sensitivity of RAPD for variation analysis in tissue cultured plants of *Plectranthus zeylanicus*. In the present

investigation among the five tissue cultured plants, the *in vitro* plant T3 showed remarkable variation at the molecular level, when compared with the *in vivo* plant (Plates 16, 17 & 18). Sequence variation arising through culture process has been detected in several plants using different ways including genome scanning with RAPDs (Kaeppler *et al*., 1998).

Essential oil analysis

Tissue culture generated plants may vary from the parent plant for its morphological characters as well as useful agronomic characters such as oil yield, oil content *etc*. These variations in yield and quality have utmost importance in crop improvement. In the present study the quantity of essential oil obtained was similar in the *in vivo* and *in vitro* (T3) plants. But the change in the essential oil quality of the tissue cultured plant (T3) from the parent plant of *P. zeylanicus* appeared to be significant.

Volatile oils are chemically complex mixtures often containing 100 or more individual components. *Plectranthus zeylanicus* is one of the oil-rich species having the main constituents as mono- and sesquiterpenoids. The major components impart characteristic odour and taste to the oil, but the minor products also play their part in the final product (Waterman, 1993). The economic properties of the essential oil are due to its components, which can be effectively exploited to produce fragrance (Tisserand, 1990) and flavouring agents (Heath, 1981).

In the present study GC-MS analyses revealed 18 components in the parent plant and 13 in the *in vitro* plant (Table 14). The gas chromatogram of the two plants showed variation in the pattern of peaks (Plates 19 & 20). The major components were geranyl acetate, geraniol and nerol in both the oils but the percentage composition was slightly variable in the micropropagated plant, T3. This is in accordance with a previous report (Jirovetz *et al*., 1998).

The parent plant was characterized by components like 1-octen-3-ol, linalool, 1-octen-3yl acetate, α-terpineol, nerol, Z-citral, geraniol, geranial, neryl acetate, α-copaene, geranyl acetate, germacrene-D, δ-cadinene, β-cubebene,

cadina-1,4-diene, α-cadinol, valencene and farnesol. Thirteen components were found to be common in both the oils. The results indicate the occurrence of variation in the micropropagated plant (T3) with respect to the essential oil composition (Table 14).

Essential oil yielding plants such as *Mentha piperita* (Nadaska *et al*., 1990), *M. arvensis* (Kukhreja *et al*., 1992), *Cymbopogon winterianus* (Mathur *et al*., 1988) and *C. martini* (Patnaik *et al*., 1999) and several other plants showed favourable variation in the oil content after *in vitro* development (Jain *et al*., 1989). Decrease in the essential oil yield and at the same time increase in the percentage of major components was reported in *Lavandula vera* (Tsuro *et al*., 2001).

The value of coefficient of similitude (72.2) obtained on comparing the essential oils of parent and *in vitro* plants show the more similar nature of essential oil composition. The dissimilarity arises due to lack of minor oil components, which may be probably due to variation in the biosynthetic pathways of essential oils that are genetically controlled (Heffendehl and Murray, 1973). Since the major components were the same in the *in vivo* and *in vitro* (T3) plants, the changes due to culture stresses did not affect their biosynthetic pathway.

The apparent increase in the percentage of the major components (geraniol and nerol) points out the suitability of plant tissue culture technique in enhancing the yield of a particular essential oil component. Production of phytochemicals in cultures largely depends on various factors like physiological, biochemical and environmental conditions of cell cultures. The stress induced by the culture conditions may cause genetic and biochemical changes leading to the altered expression in the essential oil composition of the *in vitro* (T3) plant. However, the highly stable genetic make up of the individual tries to avoid the induced changes and escape from the drastic alterations by preventing regeneration from the altered cell lines. And so the *in vitro* derived plants tend to have almost the same characteristics of the parent. In the present observation there is a marked difference in the minor components, which may be due to culture-induced variation in the biosynthetic pathways leading to their formation. The lack of production of some

components after the *in vitro* culture may have been due to either a loss in genetic ability or to a repression of the relevant genes under the culture conditions (Brown and Charlwood, 1986). The contribution of minor components cannot be neglected in the characteristic odour, appearance and potential of the essential oil (Waterman, 1993). Although the similar and enhanced occurrence of major components is an added advantage, the disappearance of a few minor ones in the culture-derived plant (T3) diminishes the effect.

The production of each component of the essential oil is affected by the genetical as well as environmental factors. The genetic basis of biosynthesis of mono- and sesquiterpenoids has already been reported (Lincoln *et al*., 1986). The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. Quantitative changes in the major monoterpene components and sesquiterpene contents of the essential oil were observed in response to the effect of varying growth regulator concentration in the culture medium (Sudria *et al*., 1999). Since there is a close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al*., 1989). Direct evidence for the involvement of auxin in enzyme activation was reported by Hino *et al*. (1982). The stress induced by the culture conditions may be one of the reasons for changes in the essential oil composition of the *in vitro* (T3) plant of *Plectranthus zeylanicus*.

Changes in the constituents of *in vivo* and *in vitro* plants are influenced by various nongenetic and genetic factors (Gerhardt, 1972; Franz, 1989). The phenomenon of production of a particular compound is fixed in the genome of a plant. Better understanding of genes, factors involved in regulation of biosynthetic pathways and biosynthetic enzymes provides a feasible method for extraction of medicinally important plant products from cells and tissues under culture that will be of great use to essential oil industry and pharmaceutical industry. In the present investigation the essential oil variation observed in the *in vitro* (T3) plant of

Plectranthus zeylanicus may be the after-effect of genetic changes revealed by chromosome studies and RAPD analysis.

Trichome observations by SEM

As in plants of most Lamiaceae species, glandular and non-glandular trichomes are widely distributed over the aerial vegetative organs especially over the leaves of *Plectranthus zeylanicus.* On the basis of external morphology, the glandular trichomes observed on the leaf surfaces of *in vitro* and *in vivo* plants of *Plectranthus zeylanicus* were similar to the two main types of trichomes occurring in the Lamiaceae: peltate hairs and capitate hairs (Plates 27 & 28). In this family, the morphology, distribution and frequency of glandular trichomes are used as discriminative characters at subfamilial level (El-Gazzar and Watson, 1970; Abu-Asab and Cantino, 1987; Cantino, 1990).

The peltate trichomes with 4-8 celled heads arranged in a single circle in the *in vitro* and *in vivo* plants of *Plectranthus zeylanicus* seems to be similar to those reported for *Leonotis leonurus* (Ascensao *et al*., 1995), *Plectranthus ornatus* (Ascensao *et al*., 1999), *Ocimum basilicum* (Werker *et al*., 1993), *Salvia aurea* (Valenti *et al*., 1997), *Salvia blepharophylla* (Bisio *et al*., 1999) *etc*. The secretory material produced by the head cells passes through the apical walls and accumulates within a space formed by the detachment of the cuticle together with a pectinic layer of the cell wall. The secretory material remains trapped between this relatively thick cuticular sheath and the head cells giving to each peltate trichome a spherical shape. The secretory products are exposed by cuticular rupture as in the peltate glands of other Lamiaceae species that have been explored.

The long stalked capitate trichomes with ovoid glandular head are widespread in the parent and micropropagated (T3) plants of *P. zeylanicus*, which also shows similarity to that in other members of Lamiaceae. The slightly raised apical region of the capitate trichome represents the cuticular pore through which the secretory product stored in the small subcuticular spaces is released out. Peltate and

capitate trichomes of leaves become active at the same time and should be considered as long term glandular hairs according to Werker's (1993) classification.

It has been generally assumed that peltate trichomes contain the bulk of the essential oils produced by Lamiaceae members (Maffei *et al*., 1989; Kokkini *et al*., 1994; Clark *et al*., 1997). But the study conducted by Ascensao *et al*. (1999) does not agree with this suggestion, since conoidal and long stalked capitate trichomes do seem to produce significant amounts of essential oils.

Differences in trichome densities are a probable factor associated with oil yield as observed earlier in the different cultivars of Patchouli (Sugimura *et al*., 1990). In the comparative study of the trichomes on the leaves of *P. zeylanicus* parent and *in vitro* cultured plant (T3), the morphology and distribution of glandular and non-glandular hairs was almost similar without significant differences. The trichome density, the type and the size of the glands remain more or less the same in both the plants. So it can be concluded that the quantitative and qualitative differences between the parent and *in vitro* plant are not due to any differences either in the trichome density or in the trichome morphology but due to genetic factors as evidenced by cytological and RAPD analyses. Regarding the functional significance of the glandular trichomes, it has been suggested that their secretions may be involved in the chemical defence of plants or may also act as floral attractants/rewards to pollinators.

Elemental analysis by EDS

In the present study, chemical analysis is performed to identify the mineral substances using energy dispersive spectroscopy in conjunction with a scanning electron microscope. In this technique, X-rays emitted from the sample by electron bombardment are used to characterize the elemental composition of the analyzed volume.

The elements identified exist as ingredients of diverse structural and biochemical compounds present in the plant tissues. They are grouped into different categories as invariable secondary elements (K, Ca, Mg, Na, Cl), invariable

microconstituents (Cu, Si) and variable microconstituents (Al, Cd, Zn). These macro and microelements are required for the normal growth of the plants.

Mineral elements play several important roles in plants as structural, catalytic and electrochemical functions that promote physiological health of the plant. Structural roles are played by elements that are incorporated into the chemical structure of biological molecules or are used in forming structural polymers. Catalytic roles are played by elements involved in the active sites of enzymes. Electrochemical roles include balancing of ionic concentrations, stabilization of macromolecules and colloids, charge neutralization and so forth. Sometimes these categories overlap, *i.e*., magnesium forms a part of the chlorophyll molecule and so could be said to play a structural role. However, chlorophyll is an important catalytic molecule.

In the comparative analysis of *in vivo* and *in vitro* (T3) plants, quantitative changes in the composition of elements were noticed (Plates 29 & 30). Potassium was found to occur in considerable quantity followed by calcium (Table 15). The absence of the secondary element, sodium was recorded in the *in vitro* (T3) plant. Though the microelements are required in very small quantities, they have important functions.

The variations observed in the concentrations of elements reflect the specific physiological nature of the leaf surface. The differential distribution of elements on the abaxial and adaxial surfaces could be according to the various physiological roles or functions that they undertake. Minute differences exhibited by the *in vitro* (T3) plant when compared with the parent may be due to culture stress induced by the artificial *in vitro* environment. The increased occurrence of certain elements such as Mg, Ca, Cu and Zn on the *in vitro* leaf surface could be due to supplementing them as ingredients of the MS medium used for tissue culture.

Total Phenolics

The presence of a vast range of chemicals in plants leads them to be exploited for economic purposes. Many of the plant secondary metabolites are

characteristic of a particular or a group of species. Phenolic compounds are ubiquitous in plants. *Plectranthus zeylanicus* was found to be rich in phenolics as evident from the estimation using Folin Denis method. While the array of biological activity exhibited by phenolic compounds is very considerable, it is possible to relate some types of activity to readily quantifiable levels of phenolic compounds.

In the comparative estimation of total phenolics in *in vivo* and *in vitro* (T3) *plants* of *P. zeylanicus*, the concentration of phenolics was found to be 3 mg/g of leaf tissue (Table 16). The *in vitro* stress had no apparent positive or negative effect on the phenolic concentration of the leaf tissue. Secondary metabolic processes have their own specific complement of specialist enzymes, which are under strict genetic control, and have appreciable energetic and metabolic requirements. The biosynthesis of phenolic compounds are observed to be unaffected by the artificial tissue culture environment.

Antioxidant Activity

The principle function of antioxidants is in delaying the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reaction by free radicals and they may reduce oxidative damage to the human body (Namiki, 1990). Among the exogenous antioxidants, polyphenolic compounds especially flavanoids present only in plants, vegetables and herbs are potent phytochemicals protecting the body from oxidative damage. A number of phenolic compounds with strong antioxidant activity have been identified in the extracts of many leafy spices in Lamiaceae (Nakatani, 1997). The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans *et al*., 1995). Supplementation of the natural protective mechanisms with non-toxic antioxidants has a chemoprotective role in the human body.

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by

body's use of oxygen such as in respiration and some cell-mediated immune functions (Halliwell and Gutteridge, 1989). Reactive oxygen species (ROS) is a collective term, which includes not only the oxygen radicals but also some non-radical derivatives of oxygen. These include H_2O_2, HOCl and ozone (O_3). Due to its high reactivity, the reactive oxygen species readily combine and oxidize biomolecules such as carbohydrates, proteins, lipids and nucleic acids; making them inactive with subsequent damage to cells, tissues and organs (McCord and Fridovich, 1969). It can also generate DNA modification and chromosomal aberrations.

Despite the existence of endogenous defence mechanism against ROS, it has been observed that whenever either the level of the cellular antioxidant system goes down or when the ROS reach abnormally high levels, oxidative damage to the cells occur which leads to several pathological conditions. Lipids are major target for free radical attack in cell membrane because oxygen is more soluble in hydrophobic membrane. Peroxidation of the polyunsaturated fatty acids in lipid membranes severely damages the cell membrane and thereby produces the loss of fluidity and causes breakdown of the membrane secretory functions and transmembrane ionic gradient. Lipid peroxidation induced by Fe^{2+}-ascorbate system in the rat liver homogenate in the presence and absence of extracts form thiobarbituric acid reacting substance (TBARS). The TBARS when heated with TBA at acidic pH gives a colour, which was read at 532 nm (Ohkawa *et al*., 1979).

Deoxyribose is oxidised when exposed to hydroxyl radicals; heating the products in the presence of thiobarbituric acid under acidic conditions, which leads to development of a pink chromogen, and measuring absorbance at 532 nm against appropriate blanks can detect such degradation. Inhibition of deoxyribose degradation was expressed as percent decrease in absorbance, when compared to the control.

Superoxide assay is based on the ability of the extract to inhibit the reduction of nitroblue tetrazolium (NBT) to deep blue-coloured formazan. The riboflavin illuminated in the presence of either EDTA or of amino acid methionine causes a

reduction of flavin. It then reoxidises and simultaneously reduces oxygen to superoxide. The superoxide is allowed to react with a detector molecule NBT. The absorbance at 530 nm is measured before and after illumination (McCord and Fridovich, 1969).

The results of the antioxidant effects of the investigated methanolic extracts, obtained with different methods of assessment, point out very strong protective activities of the extracts as free radical scavengers.

The comparative analysis of the antioxidant activity of the *in vivo* and *in vitro* (T3) plants of *P. zeylanicus* exhibited differences. The higher scavenging activity was found to be exhibited by the parent plant in the three assays conducted as evident from the Tables 17 - 19 and Plates 31 - 33. The variation in terms of biological activity can be correlated to the differences of the *in vitro* (T3) plant from the parent in cytological, genetical and phytochemical aspects. Since there is a close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al*., 1989). It is clearly evident that the biosynthesis of secondary plant products is controlled by genetic factors (Franz, 1989). The use of aromatic plants and spices in phytotherapy is mostly due to the abundance of various secondary metabolites in them. The *in vitro* stressful condition causes the diminution of the free radical scavenging capability of the micropropagated (T3) plant extract.

The highlights of the present investigation are :

- Micropropagation of *Plectranthus zeylanicus* was acquired in MS medium supplemented with growth regulator combination of NAA (1-1.5 mg/l) and BAP (0.5-3 mg/ l).

- The predominant chromosome complements in the *in vivo*, callus and *in vitro* plants are 2n = 34 (normal) and 2n = 32 (variant). This together with euploid variants (17 and 64) points towards the existence of chromosomal mosaicism.

- The karyomorphometric features like total chromosome length, average chromosome length, range of chromosome length, nature of primary constriction, disparity index, variation coefficient and total forma percentage in the *in vivo*, callus and *in vitro* plant exhibit differences.

- Banding pattern differences noticed in the *in vitro* (T3) plant as a result of RAPD assays indicates the genotypic variation emerged due to *in vitro* culture conditions.

- Essential oil profiling of the *in vivo* and *in vitro* (T3) plants revealed resemblance in quantitative occurrence in both, but they showed some qualitative differences. The decrease in percentage or absence of a few components in the essential oil of the *in vitro* (T3) plant than the *in vivo* was a limitation. However enhancement in quantity of the major components and some minor components can be considered as a gain of the micropropagated (T3) plant.

- Two types of glandular trichomes as peltate and capitate glands were observed on the leaf surfaces of *P. zeylanicus*. The distribution and types of glands were similar in both *in vivo* and *in vitro* (T3) plants.

- The plant was found to be rich in phenolics. Noteworthy variation was lacking in the amount of phenolics of *in vivo* and *in vitro* (T3) plants.

- The plant extract was proved to have significant free radical scavenging ability. The comparative analysis unveils the declining effect of the *in vitro* (T3) plant extract in free radical scavenging.

As a conclusion of the present investigation, rapid micropropagation method was developed in *P. zeylanicus*. The comparative analysis of the *in vivo* and *in vitro* (T3) derived plants exhibited differences in chromosome architecture, genetic profile, phytochemical composition and biological activity. In the phytochemical evaluation, the increase in the percentage of two major components and some minor components that have proved to be valuable in the medicinal field substantiate the

use of modern techniques like tissue culture for obtaining quantitatively and qualitatively superior plants. Thus as far as individual components are concerned the method is adequate to improve or enhance the percentage of occurrence. But the absence of a few minor phytochemicals, that also may contribute to the quality of the plant as well as the reduced free radical scavenging capacity of the plant extract in tissue culture derived (T3) plant confirms the superior phytochemical and biological efficiency of plants grown in natural environment than the micropropagated plants developed under stressful *in vitro* conditions in *Plectranthus zeylanicus*.

SUMMARY

Lamiaceae is one of the largest and highly evolved angiosperm families having considerable pharmaceutical and culinary interest. The economic importance of the family stems chiefly from the copious presence of volatile oils among many of its genera.

The genus *Plectranthus* of the family Lamiaceae comprises a large number of herbaceous medicinal plants, which are particularly employed in home remedies for various ailments. *Plectranthus zeylanicus* is a medicinal herb commonly called Iriweriya or iruweli. The herb is identified as the source of an Ayurvedic drug Hribera (Iruweli) in Kerala. The drug is cooling, carminative and tonic and cures dyspepsia, indigestion, dysentery, vomiting, thirst, fever, dermatitis, ulcers and bleeding disorders.

On account of the medicinal value of the plant, clonal multiplication was attempted to produce large number of plants within a short time. Comparison of the *in vitro* derived plants with the parent was carried out to find out the improved or decreased performance of the micropropagated ones over the parent. Comparative features in different fields such as cytogenetical, phytochemical, structural and biological activity were used to test the efficiency of the micropropagation method.

Micropropagation

An efficient design was formulated for the production of clonal plantlets of the medicinally important aromatic plant, *Plectranthus zeylanicus*. The propagation regime has the capacity for producing many plantlets from one shoot after 3-4 week long subculture cycles, making it highly attractive for implementation as an *in vitro* conservation strategy. Direct regeneration of multiple shoots was effected in Murashige and Skooge (MS) medium supplemented with hormones BAP 3 mg/l and NAA 1 mg/l. The maximum callus growth was observed with hormones NAA

1.5 mg/l and BAP 0.5 mg/l that on subculturing with increased cytokinin (BAP 1-2 mg/l) resulted in callus regeneration. The parent and the micropropagated plants were used for further analysis.

Cytogenetic Assays

Mitotic observations and Random Amplified Polymorphic DNA (RAPD) analysis are the cytogenetic assays carried out in the present investigation.

Mitotic observations of the root tip cells of the parent and *in vitro* plants as well as the intervening callus cells were carried out. The normal karyotype of the plant contained 34 chromosomes. Variant cells with 32 chromosomes were also noticed with less frequency (38%). The frequency of monoploidy and polyploidy was not that much significant (2%). The karyomorphometrical characters revealed the structural alterations of the chromosomes but the variations could not be correlated or interpreted in a regular evolutionary order with respect to the parent, callus and *in vitro* plants.

The genetic characterization using RAPD markers employing 16 primers of arbitrary sequences resulted in the production of DNA bands. Most of these bands were monomorphic among the individuals showing the genetical similarity of the regenerated plants with the parent plant. Noticeable genetic polymorphism was observed in one tissue cultured plant (T3) due to the absence of some parental bands. Further investigations were carried out on the parent and *in vitro* (T3) plants.

Phytochemical Assays

This included GC-MS analysis of essential oil and total phenolic content estimation of the leaf extract.

The essential oils of *Plectranthus zeylanicus* parent and the *in vitro* (T3) plant was analysed quantitatively and qualitatively. The oil yield of the in vitro (T3) plant was similar to that of the donor plant (0.8%). Dissimilarity was evident in the essential oil quality with respect to the composition of oil components. Although 18 components (monoterpenes and sesquiterpenes) were identified in the *in vivo* plant

oil, the *in vitro* (T3) plant oil showed only 13 components. The enhanced occurrence of major components, geraniol and nerol supports the better performance of essential oil biosynthesis in the *in vitro* plants. But the absence of minor components that also contributes to the quality of oil is a limitation of the *in vitro* (T3) plant.

The phenolic content estimation of ethanolic extract of leaves of both *in vivo* and *in vitro* (T3) plants of *P. zeylanicus* by Folin Denis method showed almost identical values unaffected by *in vitro* stress (for *in vivo* 3.037 mg/g and for *in vitro* 3.017 mg/g of leaf tissue).

Structural Analysis

The structural analysis of oil secreting glandular trichomes and non-glandular trichomes using Scanning Electron Microscopy did not show any variation in both the *in vivo* and *in vitro* plants of *P. zeylanicus*. Both glandular and non-glandular trichomes were distributed on the leaf surface with similar frequency. Peltate and capitate types of glandular trichomes were clearly noticed. Peltate trichomes consisted of a short stalk cell and a large head with 4 - 8 secretory cells arranged in a single disc. Capitate trichomes were composed of a long stalk and ovoid glandular head with slightly raised apex.

Elemental analysis using Energy Dispersive Spectroscopy revealed the mineral element composition on the leaf surfaces, which occurs as ingredients of diverse structural and biochemical compounds present in the plant tissues. The composition of the micro-elements showed small variations among *in vivo* and *in vitro* (T3) plants, which is not much significant.

Biological Activity

The antioxidant activity of the methanolic extracts of leaves was tested using 3 assays *viz*., superoxide scavenging activity, hydroxyl scavenging activity and lipid peroxidation inhibition. The high inhibition values scored for 1 mg/ml of the methanolic extract revealed the rich antioxidant property of the plant extract. The percentage inhibition of superoxide radical production at 1000 µg/ml was 92% in

the method involving photoreduction of riboflavin and that of hydroxyl radical generation at 1000 μg/ml was 76% in deoxyribose degradation method. 67% inhibition of lipid peroxidation was noticed at 1000 μg/ml of the extract in Fe_2^+/ ascorbate system. The results confirm the effectiveness of the extract as scavenger of free radicals and thereby assure its role as a natural antioxidant. On comparison, the free radical scavenging ability was less in the *in vitro* (T3) plant extract, as obvious from the lesser values of inhibition (77% for superoxide, 73% for hydroxyl and 58% for lipid peroxidation).

Conclusion

Clonal multiplication of *Plectranthus zeylanicus* through artificial culture medium provides an opportunity to raise a number of plants within a short time, in a limited space to meet the ever-increasing demand of the natural products. The comparison of the *in vivo* and *in vitro* plants using different parameters reveals the changes due to *in vitro* environment that may affect the quality of the plant positively or negatively. Here both positive and negative effects were noticed. When we are achieving the micropropagation with improved performance, it will be a great benefit to the quality improvement of the plant. Here enhanced production of two major essential oil components and some minor components that have promising medicinal effect is an achievement. But the decrease in percentage of some minor essential oil components, the disappearance of certain minor oil components and the slight decrease in the antioxidant potential of the *in vitro* plant extract can be considered as an insufficiency in the field of the tissue culture mediated quality improvement of this medicinal herb.

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* Original publication not referred

Appendix I

Murashige and Skoog (1962) Basal Medium

Stock chemicals		mg/l	Stock concentration	Stock (g/l)
I.	NH_4NO_3	1650.00	50 X	82.50
	KNO_3	1900.00		95.00
	KH_2PO_4	170.00		8.50
	$MgSO_4.7H_2O$	370.00		18.50
II.	$CaCl_2.2H_2O$	440.00	50 X	22.00
III.	Na_2EDTA	37.30	100 X	3.70
	$FeSO_4.7H_2O$	27.80		2.80
IV.	$MnSO_4.4H_2O$	22.30	100 X	2.23
	$ZnSO_4.7H_2O$	8.60		0.860
	H_3BO_3	6.20		0.620
	KI	0.83		0.083
	$Na_2MoO_4.2H_2O$	0.25		0.025
	$CoCl_2.6H_2O$	0.025		0.0025
	$CuSO_4.5H_2O$	0.025		0.0025
V.	Vitamins		100 X	
	Glycine	2.00		0.200
	Nicotinic acid	0.50		0.050
	Pyridoxine – HCl	0.50		0.050
	Thiamine – HCl	0.10		0.010

CTAB buffer

Appendix II

CTAB	:	2 %
Tris HCl	:	0.1 M, pH 8
NaCl	:	1.4 M
EDTA	:	20 mM, pH 8
β-mercapto ethanol	:	1 %
PVP	:	1 %

TE buffer **Appendix III**

Tris HCl	:	10 mM, pH 8
EDTA	:	0.1 mM, pH 8

PCR buffer (10x) **Appendix IV**

Tris Hcl	:	10 mM, pH 8.8
KCl	:	500 mM
$MgCl_2$	:	15 mM
Gelatin	:	1 %
Tween – 20	:	0.05 %
NP 40	:	0.05 %

TAE buffer (50x) **Appendix V**

Tris base	:	12.1 g
Glacial acetic acid	:	2.855 ml
EDTA	:	5 ml, 0.5 M

(for 50 ml of solution)

Gel Loading buffer **Appendix VI**

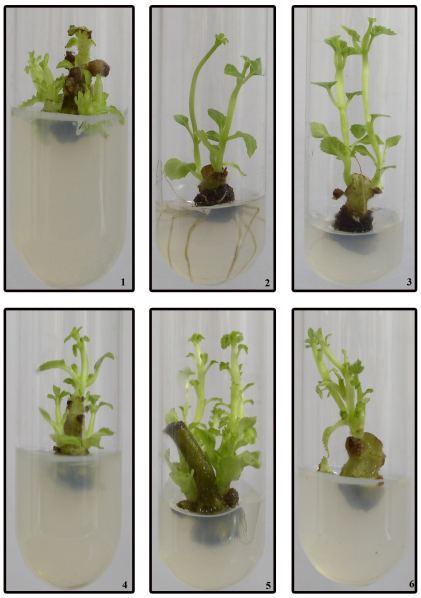
Sucrose	:	40 %
Bromophenol blue	:	0.25 %
Xylene cyanol	:	0.25 %

Folin – Denis Reagent **Appendix VII**

Sodium tungstate	:	25 g
Phosphomolybdic acid	:	5 g
Orthophosphoric acid	:	12.5 ml

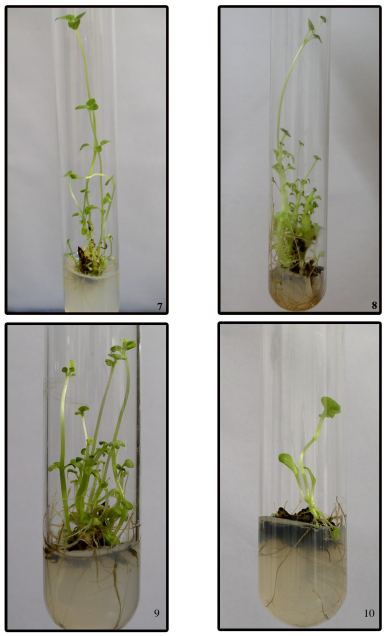
(for 250 ml of the reagent)

PLATE 1



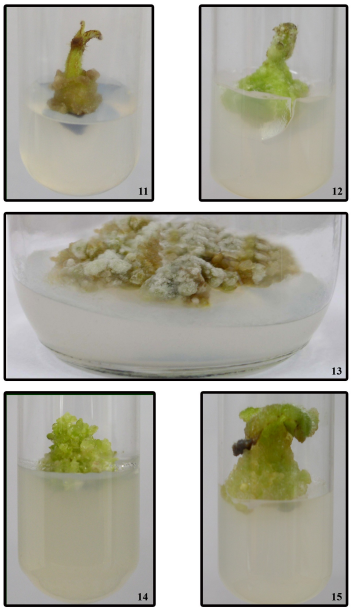
1. Axillary growth from nodal explant (NAA 1 mg/l and BAP 2 mg/l)
2. Shooting and rooting from the node (NAA 3 mg/l and BAP 1 mg/l)
3. Shoot emergence from 2 axillary buds of a node (BAP 1 mg/l)
4. Apical and axillary growth from shoot apex (NAA 0.5 mg/l)
5. Multiple shoots (6) from node (NAA 1 mg/l and BAP 3 mg/l)
6. 3 shoots from a node (IAA 1 mg/l and BAP 1 mg/l)

PLATE 2



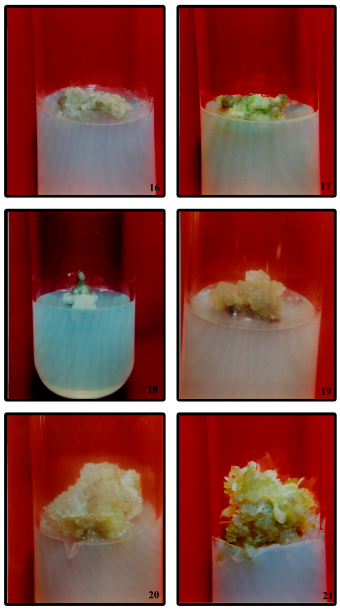
7. 5 shoots from a node with little rooting (NAA 1 mg/l and KIN 2 mg/l)
8. Nodal multiplication and profuse rooting of the explant (IAA 1 mg/l and BAP 2 mg/l)
9. Multiple shoots (9) from the nodal explant with rooting in the same medium (NAA 2 mg/l and BAP 3 mg/l)
10. Shooting and rooting from the leaf explant (NAA 1.5 mg/l and BAP 2 mg/l)

PLATE 3



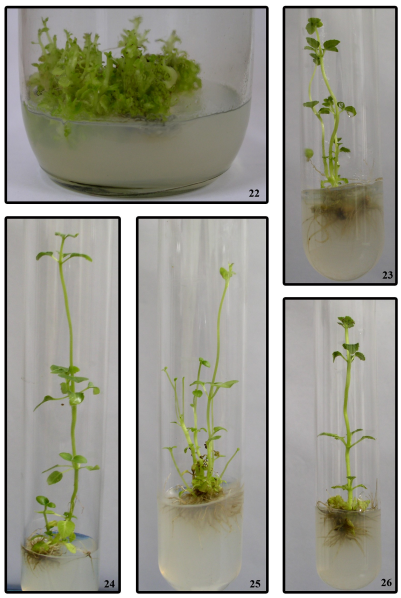
11. Yellow callus from the node of a shoot apex (NAA 0.5 mg/l and BAP 1.5 mg/l)
12. Green callus from node (2,4-D 2 mg/l)
13. Yellowish white callus (subcultured) (NAA 1.5 mg/l and BAP 0.5 mg/l)
14. Callus showing regeneration (NAA 1 mg/l and BAP 2 mg/l)
15. Yellow hard callus from shoot apex (NAA 0.5 mg/l and BAP 1.5 mg/l)

PLATE 4



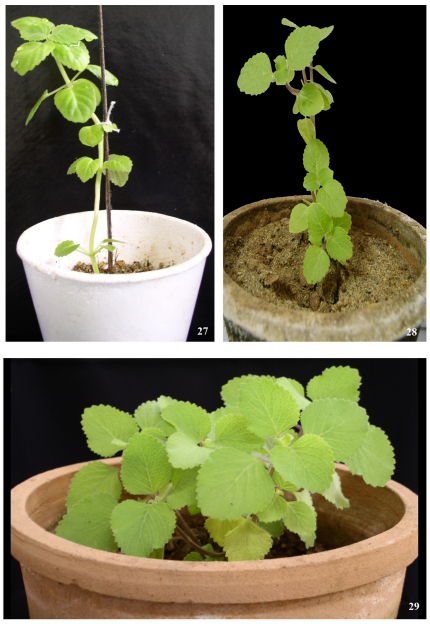
16. White friable callus from leaf (NAA 1 mg/l and BAP 0.5 mg/l)
17. Green callus from leaf (2,4-D 1.5 mg/l and BAP 0.5 mg/l)
18. White powdery callus from node (NAA 3 mg/l and BAP 2 mg/l)
19. Callus subcultured (NAA 0.5 mg/l and BAP 1.5 mg/l)
20. Callus subcultured (NAA 1 mg/l and BAP 1.5 mg/l)
21. Shoot regeneration from callus (NAA 1.5 mg/l and BAP 2 mg/l)

PLATE 5



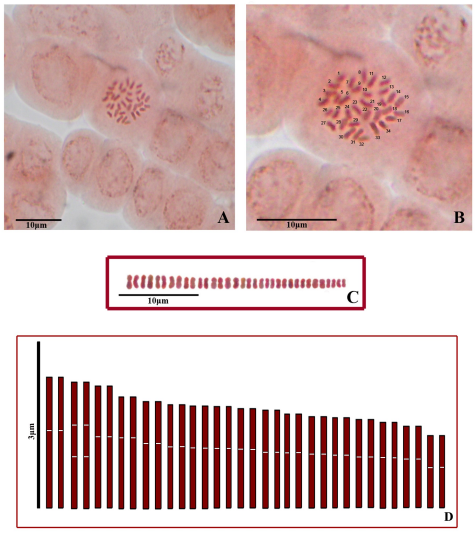
22. Shoot emergence from callus (NAA 1.5 mg/l and BAP 2 mg/l)
23. Rooting of the regenerated plant (IBA 1 mg/l)
24. Rooting of the regenerant (NAA 1 mg/l)
25. Rooting of the multiple shoots (IBA 1 mg/l)
26. Rooting of the single shoot (IBA 1 mg/l)

PLATE 6



27 – 29. Transplantation of rooted plant into soil

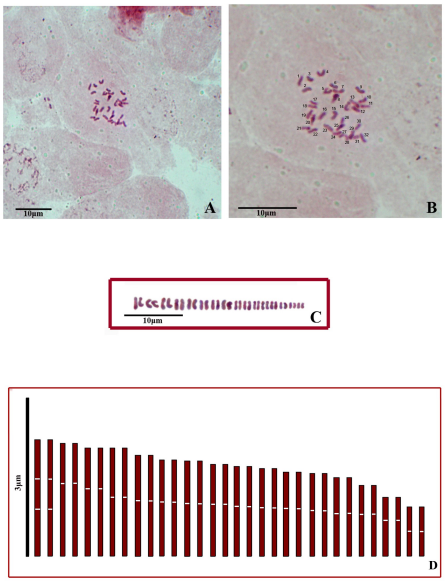
PLATE 7



Figs. A - D. Normal chromosome complement images of the *in vivo* plant of *Plectranthus zeylanicus* (2n = 34)

A - Cell at mitotic metaphase
B - Normal karyotype with 2n = 34 chromosomes
C - Karyogram
D - Idiogram

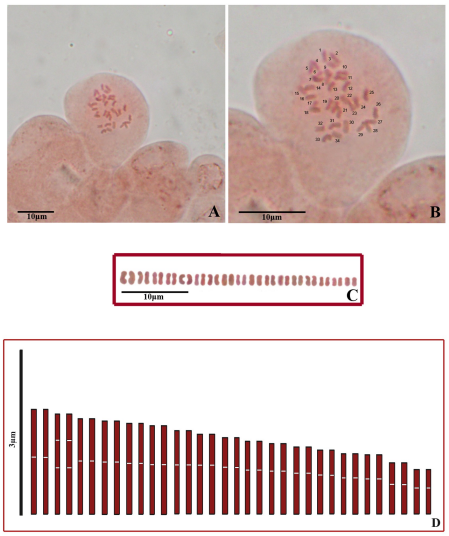
PLATE 8



Figs. A - D. Variant chromosome complement images of the *in vivo* plant of *Plectranthus zeylanicus* with 32 chromosomes

A - Cell at mitotic metaphase
B - Variant karyotype with 32 chromosomes
C - Karyogram
D - Idiogram

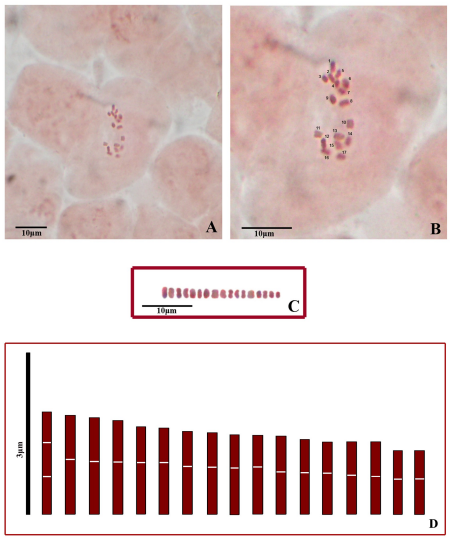
PLATE 10



Figs. A - D. Normal chromosome complement images of the callus cell of *Plectranthus zeylanicus* (2n = 34)

A - Cell at mitotic metaphase
B - Normal karyotype with 2n = 34 chromosomes
C - Karyogram
D - Idiogram

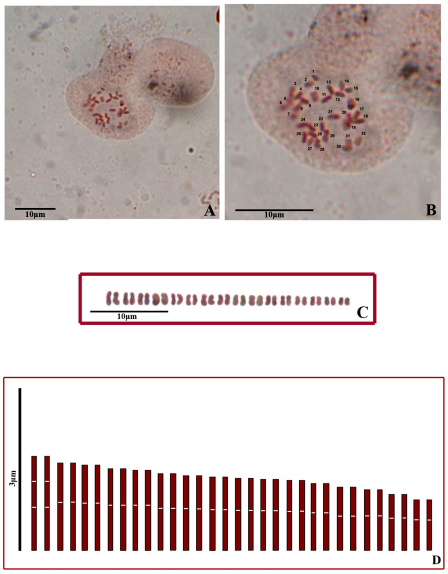
PLATE 9



Figs. A - D. Variant chromosome complement images of the *in vivo* plant of *Plectranthus zeylanicus* with 17 chromosomes

A - Cell at mitotic metaphase
B - Variant karyotype with 17 chromosomes
C - Karyogram
D - Idiogram

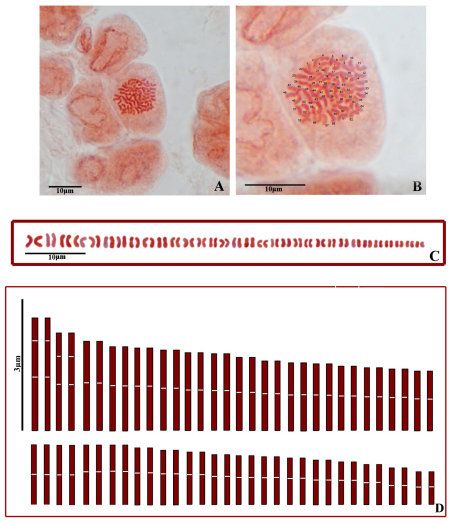
PLATE 11



Figs. A - D. Variant chromosome complement images of the callus cell of *Plectranthus zeylanicus* with 32 chromosomes

A - Cell at mitotic metaphase
B - Variant karyotype with 32 chromosomes
C - Karyogram
D - Idiogram

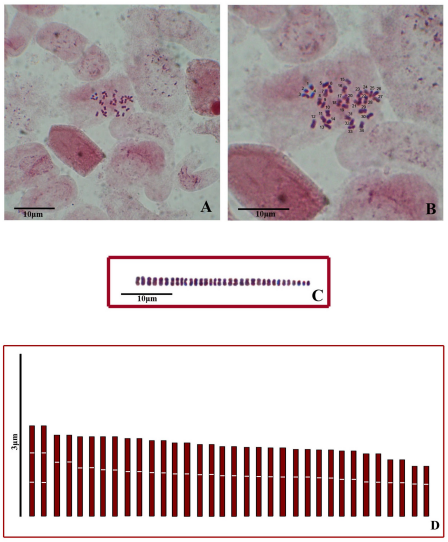
PLATE 12



Figs. A - D. Variant chromosome complement images of the callus cell of *Plectranthus zeylanicus* with 64 chromosomes

A - Cell at mitotic metaphase
B - Variant karyotype with 64 chromosomes
C - Karyogram
D - Idiogram

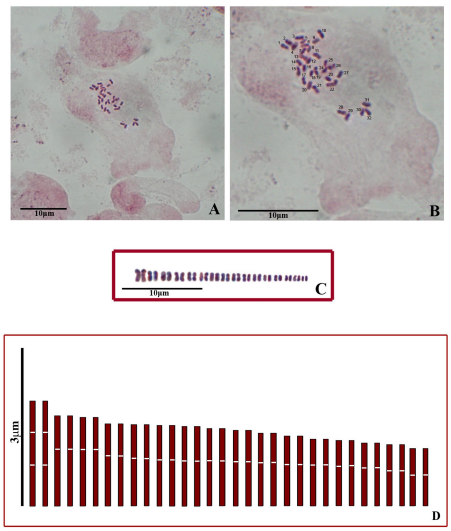
PLATE 13



Figs. A - D. Normal chromosome complement images of the *in vitro* plant of *Plectranthus zeylanicus* (2n = 34)

A - Cell at mitotic metaphase
B - Normal karyotype with 2n = 34 chromosomes
C - Karyogram
D - Idiogram

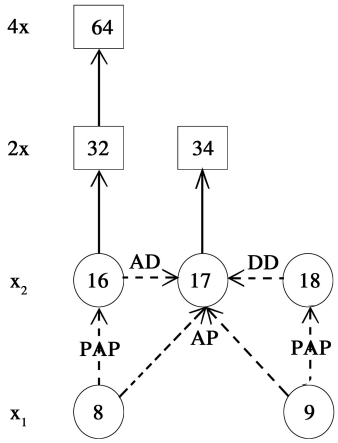
PLATE 14



Figs. A - D. Variant chromosome complement images of the *in vitro* plant of *Plectranthus zeylanicus* with 32 chromosomes

A - Cell at mitotic metaphase
B - Variant karyotype with 32 chromosomes
C - Karyogram
D - Idiogram

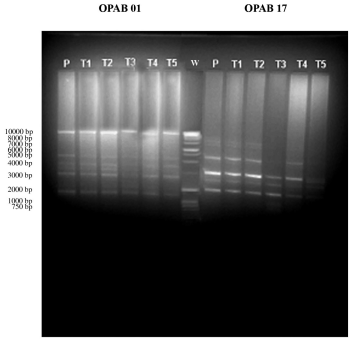
PLATE 15



Phylogenetical scheme showing the probable evolution of chromosome number in *Plectranthus zeylanicus* Benth.

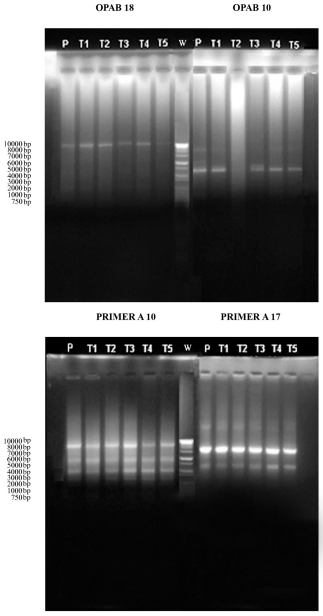
x_1 - Primary base number
x_2 - Secondary base number
AP - Amphiploidy
PAP - Proto auto ploidy
AD - Ascending dysploidy
DD - Descending dysploidy

PLATE 18



Agarose gel electrophoresis of amplified sequence from RAPD reaction using primers OPAB 01 and OPAB 17.
P – Parent plant
T1-T5 – Tissue cultured plants
W – Weight marker

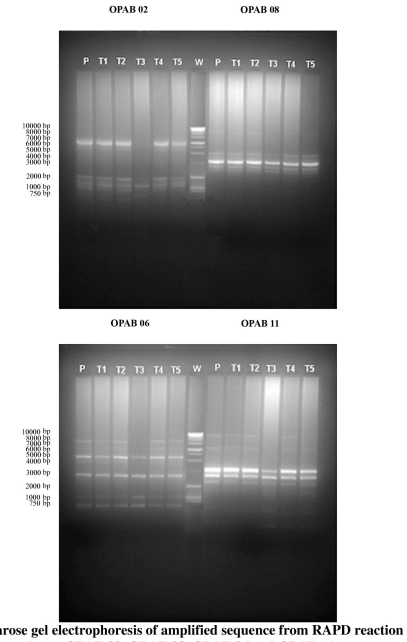
PLATE 16



Agarose gel electrophoresis of amplified sequence from RAPD reaction using primers OPAB 18, OPAB 10, Primer A10 and Primer A17.

P - Parent plant
T1-T5 - Tissue cultured plants
W - Weight marker

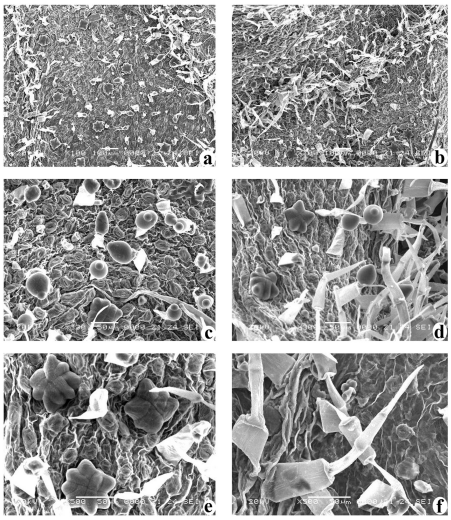
PLATE 17



Agarose gel electrophoresis of amplified sequence from RAPD reaction using primers OPAB 02, OPAB 08, OPAB 06 and OPAB 11.

P – Parent plant
T1-T5 – Tissue cultured plants
W – Weight marker

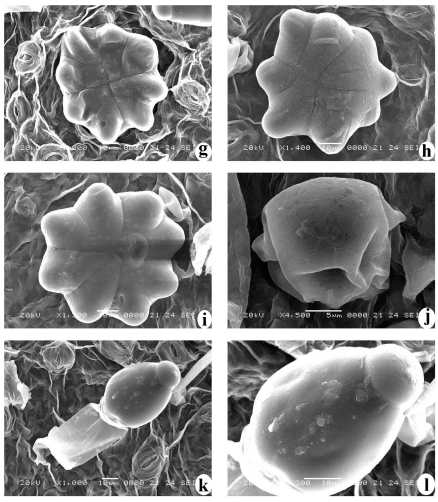
PLATE 27



Electron micrographs showing different types of glandular and non-glandular trichomes on the surface of both the *in vivo* and *in vitro* (T3) plants of *Plectranthus zeylanicus*

a. Glandular trichomes
b. Abundant non-glandular trichomes on the veins
c. Capitate glands
d. Peltate and capitate glands
e. Peltate heads at 4-8 celled stage
f. Non-glandular trichome

PLATE 28



Electron micrographs showing different types of glandular trichomes on the surface of both the *in vivo* and *in vitro* (T3) plants of *Plectranthus zeylanicus*

g-i. Peltate glands 8-celled stage
j. Mature peltate gland with swollen head due to accumulation of essential oil within the subcuticular space
k. Capitate gland
l. Capitate head with drops of secreted material