# CHROMOSOME IMAGING, RAPD AND GC-MS ASSAYS ON *IN VITRO* AND *IN VIVO* PLANTS OF *TRACHYSPERMUM ROXBURGHIANUM* (DC.) CRAIB (APIACEAE)

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Doctor of Philosophy

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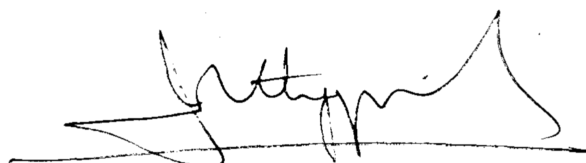
# CERTIFICATE

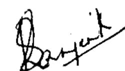
This is to certify that the thesis entitled "Chromosome Imaging, RAPD and GC-MS Assays on *In Vitro* and *In Vivo* Plants of *Trachyspermum roxburghianum* (DC.) Craib (Apiaceae)" is an authentic research work carried out by Ms. Sreeranjini K., in the Department of Botany, University of Calicut, during 2000-2004, under my supervision and guidance and that no part thereof has been presented earlier for any other Degree or Diploma.



**Dr. John E. Thoppil**

# DECLARATION

I hereby declare that the thesis entitled "Chromosome Imaging, RAPD and GC-MS Assays on *In Vitro* and *In Vivo* Plants of *Trachyspermum roxburghianum* (DC.) Craib (Apiaceae)" submitted for the Ph. D. degree of the University of Calicut, has not been submitted for the award for any other Degree or Diploma and that it represents the original work carried out by me.



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## ABBREVIATIONS USED

| | | |
|---|---|---|
| 2,4-D | : | 2,4- Dichlorophenoxy Acetic Acid |
| AP-PCR | : | Arbitrarily Primed Polymerase Chain Reaction |
| BA | : | Benzyl amino purine |
| CHIAS | : | Chromosome Image Analysis System |
| CS | : | Coefficient of Similitude |
| CTAB method | : | Cetyl Trimethyl Ammonium Bromide Method |
| DI | : | Disparity Index |
| dNTP | : | dinucleotide tri phosphate |
| EDTA | : | Ethylene Diamine Tetra Acetic acid |
| GC | : | Gas Chromatography |
| GC-MS | : | Gas Chromatography-Mass Spectrometry |
| IAA | : | Indole 3-Acetic Acid |
| KIN | : | Kinetin |
| MS Medium | : | Murashige & Skoog Medium |
| NAA | : | Napthyl Acetic acid |
| PCR | : | Polymerase Chain Reaction |
| PMCs | : | Pollen Mother Cells |
| RAPD | : | Random Amplified Polymorphic DNA |
| RFLP | : | Restriction Fragment Length Polymorphism |
| UI | : | Unidentified component |
| VC | : | Variation Coefficient |

# INTRODUCTION

Sreeranjini K. “Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)” Thesis. Department of Botany, University of Calicut, 2004

# INTRODUCTION

*"Exciting prospects lie in the possibility that plant cells, freed from the restraints of being part of a multicellular, multifunctional organism will, in effect be a new group of microorganisms with all the capabilities which this encompasses."*

(Nickell & Tulecke)

Plants form the lifeline of the spectrum of fauna residing on our mother earth. Besides providing the basic amenities to man, nature also keep in her fold luxuriant herbal cover in deep ravines, nooks, corners, rock ledges etc., which possess medicinal properties. This confirms an old saying, " For every disease that arises on this planet, plants or herbs gives a cure." The primitive human societies largely depended on natural forest wealth for food, medicine, shelter etc. Remedy through herbs is a unique but genuine and scientific healing system. Even now in an age dominated by scientific and technological marvels, the plants account for majority of prescription and nonprescription medicines. Today, about 25% of all prescription drugs are derived from higher flowering plants and 12% from lower plant forms (Anonymous, 1990 a). Herbal medicine can be viewed as a precursor of modern pharmacology; indeed many of today's powerful drugs are derived from plants. Herbs in the diet and herbal remedies are making a comeback as people rediscover the value of natural ingredients and natural cures and questions the side effects of pharmaceutical drugs. In America, there seems to be an unparalleled green wave of lay interest in herbs and natural plant medicines (Tyler, 1986). Plant products worth about £ 5000 million are sold annually in developed countries (Parikh, 1991). The medicinal properties of plant parts are due to the presence of certain chemicals with some definite physiological action on human body system. Hence, in recent years, the significance and awareness about medicinal plants has increased beyond mere anthropological curiosity. The research in the area of medicinal plants in the recent past has been accelerated after realization of their potential therapeutical and chemical uses in future. The growing awareness about the side effects of synthetic drugs has provided the necessary impetus to hasten the pace of research in medicinal plant biotechnology (Ajita *et al.*, 2001).

India has one of the richest plant based ethno medicinal traditions in the world. The global market for medicinal plants and herbal medicines is

estimated to be worth US $ 800 billions a year. The consequent rise in the value of these medicinal plants made them unaffordable for the poor in developing countries who largely depended on them for health care needs. As a result of increasing awareness of side effects of hazardous synthetic drugs and evolution of new strains resistant to antibiotics, the western pharmaceutical industry is turning to plant based system of Indian and Chinese medicine (Rajasekharan & Ganeshan, 2002). The increased demand for medicinal and aromatic plants has led to a situation of over-exploitation of these natural resources leading to habitat loss, genetic erosion and species loss perhaps leading to a crisis situation in future (Farooqui *et al.*, 2001). The depletion of natural resources and deterioration of the environment are issues of concern in progressing towards a sustainable future. Keeping the changing conditions in view, it is absolutely essential, to work out new modus-operandi (Kumar & Sharma, 2000).

With the development of technologies as tissue culture, the ability of humans to mitigate global damage to flora has increased considerably (Durzan, 1985). Tissue culture is the foundation on which all biotechnology rests (Maheswari, 1996). As the natural resources of useful genetic variation is nearing exhaustion, reforms in the conventional breeding techniques are imperative. The application of tissue culture techniques for propagation and improvement of plant species holds great promise, which is slowly being realized.

*In vitro* techniques as tissue and organ culture offer new openings for clonal propagation, genetic manipulation and production of inbred lines. Using *in vitro* methods, a million fold increase per year in clonal multiplication over conventional methods is possible. The advances in plant cell and tissue culture have been demonstrated to have considerable potential for the improvement of many agronomic crops of temperate, developed

countries (Litz, 1985). The application of tissue culture methods of propagation has dramatically altered the way many plants are routinely propagated. This technology provides the opportunity to produce uniform, disease free plants. Tissue culture techniques have made it possible to vegetatively propagate several plant species (Murashige, 1974; Hussey, 1978).

The inherent advantage of tissue culture over field propagation is the greatest plant production potential from a single plant (De Fossard, 1976). The micropropagation has emerged from theoretical confines to establish itself a multidollar industry. Variation has been a ubiquitous phenomenon associated with tissue culture of single plants (Carlson & Polacco, 1975; Green, 1977). Hence, cell and tissue culture offer ample scope of generating desired heritable variations in the regenerants (Batra, 1995).

Tissue culture technology presents a complement to methods usually applied in *ex situ* conservation and broadens the scope of ex *situ* conservation (Krogstrup *et al.*, 1992). Medicinal plants are sources of various alkaloids and other chemical substances quite essential to mankind; these chemicals are mostly organ and tissue specific (Tran Tranh Van, 1977). The exploration of tissue culture techniques in medicinal plants for the extraction of chemical compounds is indeed desirable (Tabata, 1977). Plant cell culture remains an untapped reserve for the production of pharmaceuticals, fragrances, flavours, pigments and fine chemicals (Panda *et al.*, 1989). Plant tissue culture therefore appears as an attractive method for the constant supply of homogenous products. Further more, by *in vitro* cultures it may be possible to produce new active compounds or to convert low value substrates to high value compounds by biotransformation (Laksmanan *et al.*, 2001).

The Apiaceae (Umbelliferae) is a large and widely distributed family and was the first to be recognized by taxonomists because of the characteristic

umbel inflorescence and cremocarp fruits (Iyengar *et al.*, 1997). Because of their distinctive chemistry, reflected in odour, flavour, esculence or toxicity, members of Apiaceae were familiar prehistorically to many people (Constance, 1971). This family is commonly called "Carrot family", " Parsnip family", or " Parsley family" (Benson, 1957; Chopra *et al.*, 1965; Lawrence, 1974). The family is distributed mostly in the Northern Hemisphere, widely dispersed throughout the temperate and subtropical regions but rare in tropics except in mountains (Joshi, 1968). They are abundant in Mediterranean region and Central Asia (Hooker, 1872). The family consists of approximately 455 genera and 3600 – 3751 species in the world (Pimenov & Leonov, 1993). Nearly 240 species of apiaceous plants have been reported from this country (Mukherjee & Constance, 1993) and in South India there are 16 genera and about 38 species much widespread in the plains as well as in the hills (Gamble, 1967). From an economic point of view, it is one of the most significant families because it yields vegetables, drugs, flavouring materials and even grown as ornamental plants (Benson, 1957; Lawrence, 1974). The members find their place in history as they have been used therapeutically from the time of Hippocrates to the present. Most of the culinary herbs belong to the family Apiaceae (Krishnamoorthy *et al.*, 2002). Chemically, the family is blessed with the abundance of aromatic compounds (Anonymous, 1948, 1952, 1956; Guenther, 1949). Apiaceae drugs are usually considered as grandmother's remedies (Iyengar *et al.*, 1997). The different properties of the family are due to the chemical principles, which exist in various proportions either in leaves, roots or fruits. Consequently, chemical investigations of this family are mainly concentrated on essential oils (Karlson *et al.*, 1994). The large family Apiaceae is rich in secondary metabolites and embodies numerous genera of high economic and medicinal value. It is well known that occurrence of essential oils and oleoresins is a characteristic feature of this family but only 10% of the known species have been investigated for these

constituents (Kubezcka, 1982). Isolated works have been done on the essential oil constituents of a few members of Indian Apiaceae.

The plants of the family Apiaceae are mostly herbs in habit. Other characteristic features are compound leaves rarely simple; alternate, sometimes in basal rosettes; leaflets entire or variously lobed, penni-/ palmi-nerved; petioles sheathing at leaf base; stipules occasional; umbels usually compound with several flowers in each; involucral bracts usually distinct or absent, entire, rarely lobed; involucel of bracteoles present or absent. Flowers pentamerous, sometimes polygamous. Calyx lobes five, sometimes obscure or absent; petals five, valvate, alternate with calyx lobes, equal, radiant sometimes unequal in the outer flowers with an emarginate / two lobed inflexed apex. Disc two lobed, free from styles, confluent style-base forming stylopodium. Stamens five alternate with petals, filaments often inflexed, inserted on the disc. Ovary inferior, two celled, ovule one, pendulous, style two, and stigma capitate. Fruits of two indehiscent laterally/dorsally compressed mericarps separated by a narrow commissure attached to and often pendulous from an entire/ forked carpophore, primary ribs usually five, often alternate with secondary ones, vittae usually present, rarely absent, seed one per mericarp (Saldanha & Nicolson, 1978; Mathew, 1995).

A medicinal plant, *Trachyspermum roxburghianum* (DC.) Craib is the subject of the present study.

The common names are:

| | |
|---|---|
| English | Bishop's weed |
| Hindi | ajmud |
| Sanskrit | ajmuda |
| Malayalam | ayamodakam |
| Tamil | ashamtagam |

The plant from India is also called Wild celery ([8]http). The plant is native to Asia Minor and Africa. It is believed to have originated in the Western Ghats of Peninsular India (Nadkarni, 1976). It is cultivated as a medicinal herb and spice in India, Afghanistan, Seychelles, Montsterrat, Pakistan and Indonesia (Uphof, 1968; Mukherjee & Constance, 1993; [6]http). *Trachyspermum stictocarpum* (Clarke) H. Wolff, found wild from lower Himalayas to South India has been assumed to be the ancestor of *T. roxburghianum* (DC.) Craib ([2]http). The plant is an erect annual herb with striate stems. The leaves are triangular-ovate, bipinnate or ternate pinnate. The inflorescence is an umbel and flowers are pure white in colour. The fruits are oval, shining, yellow and aromatic with faint ridges (Nadkarni, 1976; [10]http).

The plant is grown mainly for its seeds, which are highly valued as spices. The word spice is derived from the Latin word, 'species aromatacea', which means fruits of Earth (Balakrishnan & Puspakumari, 2001).

The plant is used as a culinary herb. The fruits are employed for flavouring curries, pickles, chutneys and as carminative, cardiotonic and emmenagogue ([2]http). The plant is used as a food additive for flavouring and as a folklore medicine ([3]http). The plant is one of the most used herbs for aushadi chikitsa in panchakarma ([4]http). The plant is used extensively in weight reduction drugs. Various mechanism of action like suppression of appetite, regulation of seratonin, hepatic functions, etc. are attributed to the rind. It is also useful in artherosclerosis and arthritis. It is used for joint, muscular and spinal pain, rheumatism due to inflammation of the colon in conjunction with *T. ammi, Zingiber officinale, Piper longum* etc. ([1]http). The methanolic extract is found to have inhibitory effect on the mutagenicity of Trp-P-1, a mutagenic heterocyclic amine, 3-amino-1, 4-dimethyl-5H-pyrido [4,3-b] indole ([5]http).

The aromatic seeds are used as a preservative for canned foods. The leaves are substituted for parsley (Singh *et al.*, 1990). The oil extracted from seeds and plant is used for flavouring purposes, although the seeds are most likely to be used for medicinal purposes ([6]http). The fruits are bitter, thermogenic, antispasmodic, stimulant, tonic, digestive, and antiflatulent. They are known to possess antibacterial properties. It is rich in calcium and iron ([12]http). It is used to cure indigestion, flatulence, bronchitis, cough, cold and diarrhoea ([7]http). Seeds are used as a condiment, stimulant and carminative (Uphof, 1968). This plant was regarded as a promising local control measure (Chander & Ahmed, 1983). The plant is said to have emetic and purgative effects ([9]http). The plant possesses cardiotonic, stimulant, and carminative properties and is also effective against dyspepsia ([11]http). In ayurveda, for pyrexia, drops of distillate from the plant are administered ([12]http). It is used to cure indigestion. The plant owns anthelmintic, digestive and antiseptic properties. The oil is used for cholera, stomach pain, dyspepsia and diarrhoea (Mahindru, 1994). The seeds are carminative, stimulant, stomachic, and useful in dyspepsia, hiccough, vomiting and bladder pain. Fruits are a remedy for colic, flatulence and stomach trouble (Chopra *et al.*, 1956; Kirtikar & Basu, 1975; Das & Agarwal, 1991; Agarwal, 1997 a). The leaves are used as carminative, given in bronchitis, asthma and dyspepsia (Singh *et al.*, 1990) and possess antibacterial activity (Agarwal, 1997 b).

The plant subsides vitiated vata and kapha (ayurveda), cures piles and useful in flatulence, colic, dyspepsia, diarrhoea, cholera, hysteria, spasmodic affections of the bowels, skin diseases and bronchitis. It is used in conjunction with asafoetida and rock salt to relieve rheumatic and neuralgic pain. With cumin, it is beneficial in piles. For stomachache, cough and indigestion the seeds are masticated and swallowed followed by a glass of hot water. The seeds with old jaggery taken internally for seven days are useful in urticarea. In tonsillitis, the seeds are kept in mouth day and night. The leaves are used as

a vermicide, roots diuretic and carminative (Thakral *et al.*, 2002; Rajput & Jakhar, 2003). The plant is used in skin diseases and also checks chronic discharges such as profuse expectoration from bronchitis (Thakral *et al.*, 2002).

The plant is hot and dry in the third degree, of a bitter taste and somewhat sharp withal; it provokes lust for purpose. It is said to digest humours, provokes women's courses and urine, being taken in wine it eases pains and griping in the bowels and is good against the biting of serpents; it is used to good effect in those which are given to hinder the poisonous operation of the Cantharides, upon the passage of urine. Mixed with honey and applied to black and blue marks, coming of blows and bruise, it takes them away and being drank or outwardly applied, it abates a high colour and makes it pale and the fumes thereof taken with rosins and raisins cleanses the mother (Culpeper, 1999).

The plant is used in eye diseases, insomnia, kidney troubles, rheumatism and stereochlorosis. The seed powder along with a little black salt in warm water before bed relaxes stomach and expels rectum enterobius (Agarwal, 1991). The seeds are used as a laxative, appetizer, anthelmintic, aphrodisiac, abortifacient, good in ophthalmia, scabies, scorpion and other stings; cures asthma, diseases of heart and spleen, amenorrhoea, urinary discharges, fever with cough, rheumatism, chest pain, inflammations and catarrh of the nose in the Yunani system of medicine (Kirtikar & Basu, 1975). The plant with mustard oil is used for curing stomachache (Sinha *et al.*, 1999).

*Carum carvi* is found to be effective against non-ulcer dyspepsia (Coon & Ernst, 2002). Biswas *et al.* (2001) reported that the herbal eye drop made up of a mixture of *C. copticum, Terminalia bellerica, Emblica*

*officinalis* etc. has a useful role in a variety of infective, inflammatory and degenerative ophthalmic disorders.

The plant can be propagated only by seeds and no vegetative propagation is possible. Obtaining the plant in between the year is not possible since the seed germination rate is very poor in the off-season. An alternative way is to perform the micropropagation of the plant through tissue culture and thereby obtain new plants continuously. Moreover, this method is advantageous as it can produce variants.

The formation of shoot meristem in callus cultures enhance the chance of recovering variants because of the high incidence of cytological changes in callus tissue (Vasil, 1985). Plants derived from the culture of somatic cells often differ from each other. The resulting variants termed "Calliclones" (Skirvin, 1978), "Protoclones" (Shepard *et al.*, 1980) and most widely "Somaclones" (Larkin & Scowcroft, 1981) is a rule rather than an exception when plants originate from callus cultures maintained for long duration. Research conducted in the 1970s proved that plant cell cultures derived from somatic tissues could serve as a source of somaclonal variation. In the case of plants, a new individual i.e., a somaclone can be regenerated from cultured somatic cells, during which process variability of cellular level can be raised to plant level. The study of their phenotypes in the eighties actually revealed variability. Somaclonal variation, in yield and quality characters, which are mostly under quantitative genetic control, would be of utmost importance in crop improvement.

The early detection of genetic and epigenetic variations would make a valuable contribution to the development of protocols for new cultivars and for quality control in micropropagation, with the *proviso* that the methods be economic and practical. To understand the molecular basis of elite traits, an

increased sophistication in research and innovation of new, arcane technologies like RAPD is needed.

The Random Amplified Polymorphic DNA (RAPD) is a novel type of DNA marker based DNA amplification and requires no knowledge of target sequence (Williams *et al.*, 1990). RAPD markers are generated by amplification of target DNA with a single primer of arbitrary sequence. RAPD markers have been very useful especially in constructing genetic maps (Carlson *et al.*, 1991; Reiter *et al.*, 1992; Williams *et al.*, 1993; Hemmat *et al.*, 1994) as well as for taxonomic and phylogenetic studies (Bachmann, 1992). Each primer, which is a short sequence of oligonucleotide can amplify one to several DNA bands based on the presence or absence of annealing sites. RAPD markers are detected as present/ absent, thus are dominant as compared to the codominant RFLP and isozyme markers (Rafalski & Tingey, 1993; Williams *et al.*, 1993). The use of PCR amplification to detect target DNA sequences has many applications in plant genotyping, gene mapping, diagnostics and diversity assessment. PCR itself is simple to set up and requires little hands-on time (Kreader *et al.*, 2001). PCR based DNA markers such as RAPDs and AFLPs, both dominant markers, scored either as present/absent have great advantage in terms of cost and time for development.

DNA polymorphism appears to be a particularly useful tool for distinguishing cultivars because 1) the results directly reflect the genotype, 2) the results are independent of environment, 3) a large number of potential polymorphic sequences or markers are available and 4) DNA can usually be extracted from nearly every tissue. DNA polymorphisms generated by RAPD technique (Welsh & Mc Clelland, 1990; Williams *et al.*, 1990) are of great interest because this technique requires minute amount of template DNA, it is

simple and is capable of detecting a high level of genetic variation (Ye *et al.*, 1998).

Tissue culture induced variations is defined as the variation that arises *de novo* during the period of dedifferentiated cell proliferation that takes place between the culture of an explant and production of regenerants (Munthali *et al.*, 1996). Tissue culture cells spontaneously accumulate many changes in both the number and structure of chromosomes. The morphogenetic ability of a callus depends upon the chromosome constitution of the cells. Tissue culture may play an important role in restructuring the chromosome after interspecific and intergeneric hybridization. Chromosomal constitution of plants can be highly stable *in vitro* (Sheridan, 1974) or it can fluctuate tremendously (Larkin, 1987; Taliaferro *et al.*, 1989; Webb & Watson, 1991; Lauzer *et al.*, 1992). Cytological techniques determine the chromosome constitution of the organism and facilitate recognition of individual chromosomes. Chromosome number is the raw material on which evolutionary forces have been acting, leading to the origin and evolution of entire biological diversity (Stebbins, 1950; Grant, 1971; Lewis, 1980). In recent years, majority of chromosome studies have been based on visible characteristics of chromosomes. Karyotype analysis, a well-established method is based on the morphological characteristics of the chromosomes and is widely used in cytological analysis. Karyological studies can bring to light the variations in chromosome number and size and suggest the direction of chromosomal evolution in specific taxa (Jones, 1978). Since chromosomes have a direct relationship to the genetic system, the various cytological parameters are effectively used as classificatory criteria (Den Hartog *et al.*, 1979). The remarkable constancy of chromosome morphology and number observed in plants has been invaluable in the study of plant systematics. Cytology is believed to be a dependable tool for solving taxonomic problems and for elucidating systematic relationships, phylogeny and evolution of

related plant groups. The information like chromosome number, structure, morphology and behaviour during mitotic and meiotic division is of considerable value in understanding interrelationships and delimitation of taxa during plant classification.

Several indices measuring karyotypic differences are available if homologies of chromosomes and chromosome arms are accurately ascertained (Duncan & Smith, 1978). A better objective method for assessing karyotype differences is needed. A numerical method is used for describing karyotype differences or similarities. Karyotypes are established using numerical parameters describing chromosome length, area, perimeter, visual apparent three-dimensional volume, uniformity coefficient, variation coefficient, disparity index of chromosomes, total forma percentage (mean centromeric index value or TF%) and number of discernible satellites.

Chromosome identification and mapping are indispensable in cytology and genome analysis. There are limitations for conventional measuring and characterization of chromosome complement by visual evaluation especially for very small chromosomes. The ordinary karyotype analysis has provided only limited success from the viewpoint of chromosome identification, not only in plants with small chromosomes but in many other plant species as well (Fukui & Mukai, 1988). Because of the similarity of chromosomes at mitotic metaphase, it has been difficult to identify chromosomes. Therefore, image analysis of chromosome (Fukui, 1985, 1986) was employed to obtain data, which are quantitatively more accurate. Semi-automatic karyotyping including numerical data acquisition, pairing and arrangement of chromosomes by digital manipulation of image using computer devices results in a detailed construct of descriptive data (Fukui, 1988; Fukui & Iijima, 1992).

The kinetochore is a very useful landmark in the morphological identification and nomenclature of chromosomes (Battaglia, 1955; Naranjo *et al.*, 1983). The relative length (r) of long arm (l) and short arm (s) is shown by the arm ratio (r = l/s). Based on this, Levan *et al.* (1964) grouped chromosomes into six categories, a modified version of which was proposed by Naranjo *et al.* (1983).

In this study, CHIAS (Computer aided chromosome image analysis system) is employed for karyomorphological analysis. Since the conventional methods are subjective in nature, CHIAS provides quantitative assessment of each chromosome and thereby knowledge of their cytogenetic constitution (Fukui & Kakeda, 1990, 1994; Fukui & Iijima, 1992; Kamisugi *et al.*, 1993; Fukui & Kamisugi, 1995; Verona & Galaseo, 1995).

Meiosis, a complex process that occurs in all sexually reproducing organisms, helps to maintain chromosome number constant from generation to generation and ensures the operation of Mendel's laws of heredity. Meiotic mutants are identified chiefly on the basis of cytological observations, genetic evidence and pollen or ovule abortion. Sometimes they exhibit changes in the general habit of the plants. Meiotic mutants arise by spontaneous origin in natural populations, may be induced by mutagenesis or may result from interspecific hybridization. The analysis of meiotic cells in the *in vivo* and *in vitro* conditions helps much in determining meiotic anomalies if any, that has arisen as a result of tissue culture stress, which in turn, may result in the development of variants.

'Essential oils' or 'essences' or 'volatile oils' are the complex mixtures of steam volatile and odoriferous liquid components of aromatic plants, trees and grasses. The word 'essential' is derived from quintessence, which literally means 'An extract of a substance containing its principle in its most concentrated form'. In ancient philosophical or alchemical terms,

quintessence was related to the fifth element and was thought to be the spiritual aspect of matter. Essential oils are also called 'ethereal oils' a German term, which aptly describes their otherworldly nature.

Essential oils are deposited by plants in the subcuticular space of glandular hairs, in cell organelles, in idioblasts, in excretory cavities and canals or exceptionally in heartwood. They are usually captured by steam distillation, a process whose origin can be traced back to the Ancient Mesopotamia. Complex nature of essential oils stimulated analytical and structural chemistry ever since man became involved in the fragrance of plants (Atal & Kapur, 1982). Chemically and biogenetically, the natural product class "essential oil" is heterogenous. Mono, sesqui and diterpenoids, phenyl propanoids and alkane derivatives are by far the most ubiquitous essential oil components. Usually, the bulk of an essential oil is formed of members of one, two or all of these biochemical families of compounds. Most essential oils have between 50 - 500 chemical constituents. The terpenes are synthesized via the mevalonic acid pathway and aromatic polypropanoids via the shikimic acid pathway. The bulk of essential oil bearing plants belong to seed plants. In Umbelliferae, essential oil bearing taxa with schizogenous ducts and cavities are present. In this family, chemical races within the species are rather common.

Essential oils from different plant species are known to exhibit various kinds of biological activities, which endow them with industrial, agricultural and medicinal importance. The various components of essential oil generally boil between 150-300$^{0}$C (Hicks, 1993). Due to the tiny structure of molecules which are usually less than 20 carbon atoms in length, essential oils applied to skin will be absorbed to blood stream. They also reach the blood as a result of aromatic molecules being inhaled. Once in the blood stream, the aromatic molecules interact with body's chemistry. Quite apart from medicinal

properties, just smelling the essential oil can uplift the spirits and make us feel better. This is because the sense of smell is an interrelated aspect of limbic system, an area of the brain primarily concerned with emotion and memory. This influence of aroma on the psyche has led to practice 'psycho-aromatherapy' whereby oils are used solely as mood enhancing substances. Aromatherapy has the means to fully exploit anti-infectious properties of essential oils based on serious criteria. Knowledge of antibacterial components of essential oil is of prime importance (Baudoux, 2001).

More aesthetic than tannins, more romantic than dyes, essential oils have been among the most coveted of herbal products. In plant metabolism, oils appear useful as moderately reactive intermediates capable of being oxidized, reduced, esterified, or compounded in large molecules. Frequently they are considered to be hydrolysis products of complex glucosides. By and large, they can be thought of as byproducts of carbohydrate and fat metabolism - rather readily accumulated – yet once formed, not so ready in the mainstream of metabolic activity. Uses of essential oils are varied. Most notable is their use for perfuming soaps, deodorants, toilet preparations, for flavouring various food and beverage products and tobacco. There is no dearth of essential oil in the World; they can be found in many species and many plant parts (Schery, 1972).

Massage with essential oils is especially helpful for women's problems, including premenstrual syndrome and menopausal distress. It can also soothe away moderate anxiety and depression, sleeping problems, emotionally induced sexual difficulties, digestive disorders, headaches, muscular aches and pains. Many essential oils are superb skin care agents. They help to balance the sebum and to tone the complexion by supporting capillary function. Plant essences can also be used in hair and scalp formulas to improve the circulation in the scalp, prevent dandruff and promote healthy

hair growth. Applied without massage, essential oils can heal skin problems such as athlete's foot, cold sores, ringworm and scabies. In steam inhalations, they are used to alleviate cold and flu symptoms. They are also efficacious for problems such as coughs, tonsillitis, sore throat, sinusitis and acute bronchitis (Baudoux, 2001).

Pharmacological and clinical tests and experience of traditional medicine show that essential oils have antiseptic, disinfectant, anthelmintic, diuretic, antispasmodic, stomachic, carminative, bactericidal, fungicidal and anti-inflammatory activities. It is used as local anaesthetic and useful in bronchial disorders (Guenther, 1949; Hocking, 1969). In medicine, it is used in the synthesis of syrups. Due to their antiseptic and rubifacient action, they play an important role in the manufacture of ointments and lotions. The use of essential oil in dentistry is based on antiseptic, obtunding and stimulating properties (Schroff, 1958). In France, in the perfume laboratories the workers never develop lung or bronchial disorders, a fact which bears out Pliny and Hippocrates, who classified perfumes among medicinals (Schery, 1954). Aromatherapy uses essential oils as natural rejuvenating and antiwrinkle agents (Varshney, 1991). Despite, the continuing inventions of synthetic aromatics, essential oils still remain the most important part of fragrances (Ranade, 1993). Since it is a multipurpose agent, there is great scope for utilization of essential oil plant resources (Thomas *et al.*, 1998).

The chemistry of plants differs fundamentally from that of animals. Among plants, the primary constituents i.e.; carbohydrates, fats, proteins, etc. are common, but secondary plant constituents, having no direct relation to active metabolism or function of the organism varies from plant to plant. When these substances, exert an influence on the structure or function of the body, they are known as active principles. At present, there are about 30,000 natural chemicals and they fall into discrete groups as terpenoids, saponins,

flavonoids, neoflavonoids, alkaloids etc. which are formed from various precursors through intervention of enzymes, characteristic of living organisms (Jain, 1989). The principal components of essential oils are terpenes and their components or derivatives (Baudoux, 2001). Terpenoids are much easier to handle compared with alkaloids, but much care is required to isolate low molecular compounds (Saleh, 1977).

Terpenoids are essential oil components that range from – the volatile mono and sesquiterpenes, through the less volatile diterpenes to the nonvolatile triterpenes and sterols. Although considerable data has been accumulated on difference in mono and sesquiterpenes level, and their variation between and within plant species, little is known about genetic basis of such variations. The empirical formula of terpenes is $C_{10}$ $H_{16}$ (De Mayo, 1959). Essential oils are aromatic principles, responsible for odour of the plant in which they occur (Chopra *et al.*, 1965). They are widely distributed in about sixty families especially Apiaceae, Asteraceae, Myrtaceae, Rutaceae, Geraniaceae, Poaceae, Fabaceae and Lamiaceae (Schery,1954; Samba Murthy & Subramanian, 1989). Secondary metabolites are involved in the physiology and ecology of the plants (Guenther, 1949; Schery, 1954). Some sesquiterpenes protect plants from insect attacks because of their insecticidal properties.

Chemically, terpenoids are lipid soluble, and are located in cytoplasm of plant cell. Their growth regulating properties are well documented, two of the major classes of growth are the sesquiterpenoid abscisins and diterpenoid based gibberellins. Terpenoids are important in pharmacological activities. The monoterpene, limonene is a cancer preventive, bactericide, insecticide and sedative (Rajan, 1997). The sesquiterpenes accompany the monoterpenes in plant essential oils, although because of their molecular weight, they are in

less volatile fraction. A number of sesquiterpenes are active against experimental tumours and are used as antibiotics (Hansen, 1972).

It is imperative in this scenario, to discover novel biological activities of utility through the use of cellular and molecular screens. This approach can produce drugs, agrochemicals and food industries input in a holistic manner. The judicious use of essential oil and their components offer enormous scope in developing plant derived biosafe products of direct utility to humans and plants (Khanuja, 2000). The production of these often very costly products by means of plant cell cultures has consequently been the subject of numerous studies (Kreiger *et al.*, 1988).

The versatile use of several aromatic plants in food and cosmetic industries demands an extensive screening of essential oil and their components. Individual chemicals isolated from the essential oil are much useful than oils as a whole (Brud & Gora, 1989). Therefore identification of trace compounds is very helpful to reveal the quality of the oil. The capacity of the chemists to create new structures with therapeutic properties has been found to be limited. Plants offer thousands of new molecules (Evans *et al.*, 1982; Gottleib, 1982) and therefore, an extensive study of naturally occurring molecules identified as, therapeutically active, is a desideratum. A number of plant-based drugs are still a part of standard therapy. The role of plants in standard therapy will be increased several fold in future, provided we make the base needed for it. Phytochemicals are a major source of dyes, flavours, sweeteners, aromas, perfumes, insecticides, antiparasitic drugs and many other substances. Further research on plants will provide apart from drugs, additional sources of these industrial raw materials. All this potential justifies the broadest and most exhaustive phytochemical research.

Analysis of the oil can be easily done using the technique of Gas Chromatography-Mass Spectrometry (GC-MS). GC is a technique for

separating the volatile components while analysis depends upon the retention characteristics under standard conditions. The mass spectrometer can be used as a detector for a gas chromatograph in which case the high degree of specificity of the mass spectrometer is an aid to the identification of the sample. The large number of spectra obtained in a short time from GC-MS technique and routine nature of much of the data obtained makes computer a very useful accessory to the GC-MS unit. With the help of GC-MS unit, it has been possible to analyse essential oil directly by the use of head-space analysis (Thappa *et al.*, 1982). MS differs from other type of spectral analysis in that the sample does not absorb radiation from the electromagnetic spectrum. MS is a destructive analysis, i.e., the sample cannot be recovered after MS analysis. It is highly sensitive and only a small quantity is required. When coupled with the separation techniques, like GC or HPLC, it is a highly specific way to identify organic compounds (Smith & Busch, 1999).

Most of the cytological works in Apiaceae are confined to the level of determination of chromosome numbers. Even where chromosome numbers have been determined, the knowledge concerning the details of karyotype pertaining to chromosome measurements, which are vital to the understanding of karyological affinities, is still meagre. The detailed studies of chromosomes of members of this family can elucidate structure and behaviour of karyotype and their interrelationships. Further, no reports are available on the comparison of *in vivo* and *in vitro* plant chromosomes in an important medicinal plant like *Trachyspermum roxburghianum*, and hence the present study.

Clonal or micropropagation of elite plants is a viable technology in tissue culture. However, no serious attempts have been made to clone medicinal plants on a large scale. Phytochemical variability is a common event in the medicinal and aromatic plants. By standardizing protocols of

selected elite plants, it is possible to achieve an increase of 8-10 fold in the product per unit area of cultivation (Heble, 1996). So far, no work on the tissue culture of *T. roxburghianum* is reported except an earlier work by Roy & Gupta (1977). Taking this into consideration, micropropagation of the plant gains relevance.

The essential oils and perfumery is an important industry manufacturing fragrant oils and isolates, not only for internal demand but also for export as well. The import statistics of Indian economy reveal that the essential oil worth rupees 178.7 million is still imported costing foreign exchange to the country. Considering the economic importance and the export potential of vast expanding essential oil industry, measures are to be taken to increase the production technology of essential oil plants (Tewari & Mishra, 1998). Since the development by area expansion is limited, only way is to increase the number of plants by tissue culture methods. Beholding this, the aim of the present work is to study the karyomorphometrics with the help of chromosome image analyses and phytochemical variations of the essential oil of the parent plant and the variant developed by tissue culture methods, their underlying genetic principles and thereby developing new varieties with better qualities.

# REVIEW OF LITERATURE

Sreeranjini K. “Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)” Thesis. Department of Botany, University of Calicut, 2004

# REVIEW OF LITERATURE

## Micropropagation

Plant tissue culture techniques have become a powerful tool for studying and solving basic and applied problems in plant biotechnology. In recent years, these techniques have gained greater momentum on commercial application in the field of plant propagation. A considerable amount of work had been undertaken internationally and nationally in agricultural and horticultural crops. The methods of micropropagation are now well developed (Paranjothy *et al.*, 1990; Machey *et al.,* 1995).

Rapid clonal propagation can be obtained through bud or shoot proliferation (Pierik, 1990), induction of bulbs or corms (Ziv, 1990), or somatic embryogenesis (Ammirato, 1989). A fairly general phenomenon of tissue culture is variation on the ability to produce embryoids, organ, tissues. One manifestation is the ability to form embryoids (De Bouchaud, 1972). Only certain cells are capable of normal embryo development (Barba & Nitchell, 1969). Somatic embryogenesis has tremendous potential for large-scale production of plant material (Ammirato & Styer, 1985; Durzan & Durzan, 1991). Murashige (1974) mentioned plant regeneration accomplishment from various explants namely, leaves, stem, cotyledons, microsporocytes as well as shoot tips. Gamborg *et al.* (1974) as well as Mukhopadyay & Bhojwani (1978) implied that most important determinant of plant multiplication and quality of regenerated plants is the initial explant. Cronauer & Krikorian (1983) were the first to develop a sustainable liquid cell culture for producing somatic embryos in bananas and plantains. Reports are also available which favour use of diverse explants such as nodal segment (Laksmisita, 1986), hypocotyls (Meiners *et al.*, 1991; Schroeder & Stimart, 1997), cotyledon (Knittell *et al.*, 1991, Chraibel *et al.*, 1992) and cotyledonary node (Meiners *et al.*, 1991; Distabanjong & Geneve, 1997; Sharma, 1999) in various plant species.

It took more than a decade after visualizing the utility of tissue culture to demonstrate that plant cells in culture can produce metabolites in reasonable concentrations. Kaul & Staba (1967) reported the production of visnagin by callus cultures of *Ammi visnaga*. Heble *et al.* (1968) reported the production of diosgenin by callus cultures of *Solanum xanthocarpum*.

Normal looking tissue cultured plants are quite often anatomically and physiologically aberrant due to ambient conditions *in vitro* (Debergh *et al.*, 1992). Old cultures of *Petroselinum hortense* exhibited variation in the development of embryoids (Vasil & Hildebrandt, 1966). Ibrahim (1969) obtained variants in carrot after high KIN treatment *in vitro*. The effect of growth regulators on polyamine levels of tissues during somatic embryogenesis in *Foeniculum vulgare* was analysed by Maatar & Hunault (1997). The analysis of cell types in somatic embryo stage of carrot was studied by Tsukahara & Komamine (1997). Localization and purification of p-hydroxy phenyl pyruvate dioxygenase from cultured cells and characterization of corresponding C-DNA was done by Garcia *et al.* (1997). Morphogenetic effects of brefeldin-A on embryogenic cell cultures of *Daucas carota* were investigated by Capitanio *et al.* (1997).

Carrot has been extensively studied as a model species for plant somatic embryogenesis and an embryonic phase was frequently involved in the production of transgenic carrot plants (Thomas *et al.*, 1989; Pawlicki *et al.*, 1992). The characters of cold preserved embryogenic suspension cells in fennel was reported by Umetsu *et al.* (1995). The morphological variations induced in *Apium graveolens* was studied by Jamwal & Kaul (1995). Anzidei *et al.* (1996) worked out the callus characteristics of *Foeniculum vulgare* in relation to morphogenesis. A set of genes from carrot somatic embryos were isolated and characterised by Lin *et al.* (1996). Production of petroselinic acid from suspension cultures of *Coriandrum sativum* was studied by Kim *et al.*

(1996 a) and the regeneration of *C. sativum* from somatic embryos from all cell suspension cultures were also conducted by Kim *et al.* (1996 b). The characteristics of the callus in relation to morphogenesis was analysed. Cytotoxic activity of *Trachyspermum ammi* was studied by Dubey *et al.* (1997). The various promotive and inhibitory effects of arabinogalactan proteins on somatic embryogenesis of *Daucas carota* were studied by Toomen *et al.* (1997).

Transgenic plants had also been obtained in celery (Catlin *et al.*, 1988). Callus formation and regeneration of adventitious embryos from carrot was analysed by Matsubara *et al.* (1995). *Carum carvi* (Caraway) had been mainly used in research either in embryogenesis (Ammirato, 1977; Furmanowa *et al.*, 1984) or on terpenoid biosynthesis (Toxopeus & Boumeester, 1993). The study conducted by Krens *et al.* (1997) suggested caraway as a model plant species for genetic engineering by genetic modification.

Plant tissue culture is an essential tool in the application of molecular genetics for crop improvement. Regeneration may be achieved through organogenesis or somatic embryogenesis and progress has been made in defining the conditions required for, and physiological and biochemical changes accompanying these types of development (Ammirato, 1987; Christianson, 1987; Thorpe, 1990). The effect of variations in culture conditions was analysed by Dougall & Verma (1978). They reported that wild carrot (*Daucas carota*) suspension cultures grew and produced embryos on ammonium ion as a sole source of nitrogen in the absence of any exogenous Kreb's cycle acid when $p^H$ of the medium was controlled. They also explained the differences in growth characteristics and chemical composition of carrot cells grown in the presence and absence of external supply of 2,4-D and described the time course changes in DNA, RNA and protein content of highly selected populations of cells, obtained by screening fourteen day old

stock suspensions of wild carrot. Halperin & Wetherell (1964) investigated adventive embryony in tissue cultures of *Daucas carota*. The ultrastructural changes during growth and embryogenesis in carrot cell cultures was analysed by Halperin & Jensen (1967). The cultured carrot tissue was investigated for phytochromes by Wetherell (1969). Gregor *et al.* (1974) studied the changes in chromosomal proteins from embryo induced carrot cells. Okamura *et al.* (1975) studied the physiological changes in carrot cell suspension culture during growth and senescence. Brown *et al.* (1976) reported the requirement of potassium for growth and embryogenesis in wild carrot suspension cultures. The effect of carbohydrate on quantitative aspects of growth and embryo formation in carrot suspension cultures was analysed by Verma & Wetherell (1977). The suitability of sucrose as a carbon source for the uptake and utilization of plantlets was analysed by Khuri & Moorby (1995) and they observed that sucrose is appropriate. Numerous observations on plant cell suspension cultures suggest that before entry into the cell, sucrose is first hydrolysed into its constituent hexoses, (Fowler, 1978; Fowler & Sarkissan, 1985; Bender *et al.*, 1987) glucose and fructose. However, total hydrolysis of sucrose does not seem to be necessary for the *in vitro* growth of plant cells (Fowler & Sarkissan, 1985) and the degree of hydrolysis varies between plant species (Cho & Komar, 1985; Kanabus *et al.,* 1986). Sucrose uptake without prior hydrolysis had shown to occur in some intact plant tissues, solid callus (Edelman & Hanson, 1971) and protoplast (Fowler & Sarkissan, 1985). In plant tissue, sucrose functions as a protected form to be released as glucose in actively growing cells (Giaquinta, 1980). Dijkema *et al.* (1988) analysed the uptake and utilization of sucrose by embryogenic suspension cultures of carrot growing in the presence of 2,4-D and by somatic embryos derived from these cultures using $^{13}C$ nuclear magnetic resonance.

A number of physiological and morphological changes had been reported in unorganized callus tissue including habituation, changes in

biochemical sensitivity and requirements, alteration of growth habit and modification of cellular constituents. Drastic colour changes were observed with growth habit changes in cultures of carrot (Sugano *et al.,* 1971; Nishi *et al.*, 1974; Mok *et al.*, 1976).

Vitrification of the apical shoots inducing poor growth of abnormal shoots, low percentage of rooting and poor survival, limits the commercial application of micropropagation methods. There are reports that during successive subcultures the number of abnormal plantlets increases (Ziv *et al.*, 1983; Leshem *et al.,* 1988; Safrazbekyam *et al.*, 1990). Great significance was given to factors like excess growth regulators present in the medium (Borkowska & Opiloswska, 1988), total water potential and relatively high humidity of culture vessel (Botcher *et al.*, 1988; Pasqualett *et al.*, 1988) and large quantities of $NH_4^+$ and $Cl^-$ ions in the MS medium (Vietez *et al.,* 1985; Daguin & Letouze, 1986) as promoting vitrification. Kataeva *et al.* (1991) examined some factors promoting vitrification and determined the level of internal hormones in normal and abnormal shoots. He observed high cytokinin promoting the vitrified shoots, supporting the hypothesis of high cytokinin in greater humidity responsible for vitrification.

Skoog & Miller (1957) documented a delicate and quantitative interaction between auxin and cytokinin in the control of bud and root formation from pith explants. The effect of sequential exposure to auxin and cytokinin on primordial development of sugar beet was investigated by Gurel & Wren (1995). The development of primordia and roots were disrupted by the procedure.

In some experimental system, cell division and differentiation occurs with auxin alone, the addition of cytokinin is stimulatory (Dalessandro, 1973; Minocha & Halperin, 1974). The interaction of gibberellic acid with either auxin or kinetin influenced growth stimulation *in vivo* (Wareing, 1958) and *in*

*vitro* (Dalessandro & Roberts, 1971). Hervey & Robbins (1978) suggested the effect of auxin, cytokinin and vitamin mixture as a cause for the development of plants with no chlorosis from chlorotic leaf discs.

The effect of growth hormones cannot be generalized because different plants may have different requirements for morphogenesis. Cytokinins are very effective for direct and indirect shoot bud initiation. Most commonly used cytokinin is BA (Moreno *et al.*, 1985; Bonabdallah & Branchard, 1986; Misra & Bhatnagar, 1995). Bennicii & Ceonini (1979) reported an increase of *in vitro* growth in *Phaseolus* embryos by the addition of zeatin or zeatin riboside. Fujimura & Komamine (1975) presented the promotive effect of zeatin on the embryo development in carrot cell culture. Investigations in wild carrot (Halperin & Wetherell, 1964) revealed that 2,4-D prevented embryo development if it was added to the regeneration media. In carrot, a suppressive effect of 2,4-D on the quality of somatic embryos was reported (Kamada *et al.*, 1989). Endogenous cytokinin is known to occur in plant tissue culture and to exhibit a change in concentration during cell growth (Mackenzie & Street, 1972; Nishinari & Syano, 1980; Ernst *et al.*, 1984). A change in the cytokinin content during somatic embryogenesis in an anise cell culture was demonstrated by Ernst *et al.* (1984). Ernst & Oesterhalt (1984) concluded from their work that cytokinins are not directly involved in embryo development, but by cumulating cell development, promote the development of more embryos in a culture. Fujimura & Komamine (1980) reported the action mode of 2,4-D and zeatin in somatic embryo development in carrot cell suspension culture.

Clonal multiplication and *in vitro* studies have been done on certain medicinal and economically valuable genera belonging to Umbelliferae. Micropropagation, callus regeneration and somatic embryogenesis was reported in fennel (Du Manior *et al.*, 1985; Miura *et al.*, 1987; Hunault *et al.*,

1989; Song *et al.*, 1991 a,b,c; Theiler-Hedrich & Kagi, 1991; Hunault & Du Manior, 1992; Lourdes & Alfermann, 1994; Hunault & Maatar, 1995). Tissue culture studies were reported in anise (Ernst, 1989), celery (Gosal & Grewal, 1991; Kim & Janick, 1991; Sakamoto *et al.*, 1991; Toth & Lacy, 1992), cumin (Dave & Batra, 1994; Shukla *et al.*, 1997 b) and fenugreek (Provorou *et al.*, 1996; Settu *et al.*, 1997). In parsley, multiple shoots could be produced in MS medium with BA (1.0 mg/l) and IBA (0.5 mg/l) in 70% of the cultures. In anise, multiple shoots (80%) and plant regeneration from callus (60%) was achieved in MS medium supplemented with 1.0 mg/l BA and 0.5 mg/l IBA. In fennel, MS medium supplemented with BA (1.0 mg/l) and IBA (0.5 mg/l) was suitable for both multiple shoot formation and callus regeneration. In coriander, *in vitro* flowering as well as seed formation was reported by Stephen & Jaybalan (1998). Donovan *et al.* (1994) studied the potential sources of variation in tissue-cultured plants of celery. Regeneration of plantlets from celery callus using a fermenter was reported by Okamoto *et al.* (1994). Somatic embryogenesis in some species of Apiaceae has been discussed by Lourdes & Alfermann (1994), in cumin by Dave & Batra (1995) and in caraway by Ammirato (1983). Effect of growth regulators on polyamine level in somatic embryogenic cell suspension had been polyploidized using colchicine (Hunault, 1997). Variation in essential oil composition of plants regenerated from protoplasts of peppermint were reported (Okuyama *et al.*, 1995). Plant regeneration was successfully induced from callus cultures of coriander, fennel, lavender, anise, parsley, poppy, origano and sage (Chand & Roy, 1981; Erdelsky *et al.*, 1990; Neena kumari & Saradhy, 1992; Sagina *et al.*, 1997; Sastri *et al.*, 1997; Shukla *et al.*, 1997 a). Callus formation followed by regeneration of adventitious embryos from microspores by anther and microspore cultures had been reported in fennel (Matsubara *et al.*, 1995). Kataeva & Popowich (1993) regenerated *Coriandrum sativum* plantlets *in vitro* using 4.6 $\mu$ M of KIN in basal medium,

which produced, shoots and on further transfer to the basal medium supplemented with 1.1 μ M IAA, roots were developed. Successful somatic embryogenesis of celery was reported by Chen (1976) and Williams & Collins (1976). The same procedure with few alterations had been used for isolation of cell lines resistant to *Fusarium oxysporum* by Rappaport & Pagliuso (1984) and for producing encapsulated embryos for fluid drilling (Steuart *et al.,* 1985; Rowe, 1986). In their work petiole was suggested as the best explant. Callus was induced in 2,4-D and on removing 2,4-D from the medium somatic embryogenesis was induced. Somatic embryogenesis in celery is similar to that reported in carrot and other species (Halperin & Wetherell, 1964). Roustan *et al.* (1989) used carrot cell suspension cultures to examine the effect of $CoCl_2$ and $NaCl_2$ on ethylene production and somatic embryo formation. Ethylene is involved in growth and differentiation of tissues. This gaseous plant hormone is known to promote callus formation from cultured citrus buds (Goren *et al.*, 1979) and to maintain tobacco callus in the undifferentiated state (Huxter *et al.*, 1981).

Embryogenesis from hypocotyl as explants in the Apiaceae is well documented in *Ammi majus* (Grewal *et al.*, 1976), *Anethum graveolens* (Johri & Sehgal, 1965) and *Daucas carota* (Reinert, 1967). Regeneration of plantlets from somatic embryos developed from hypocotyl explants of *T. ammi* was carried out by Jasrai *et al.* (1992). Callus was induced in hypocotyls on using MS medium supplemented with 9 μ M 2,4-D and 2.3 μ M KIN. Transferring callus to medium containing 2.25 μ M 2,4-D and 2.3 μ M KIN induced embryogenic callus. Culturing the somatic embryos on medium containing low KIN (1 μ M) for 2 weeks and then on growth regulator free MS medium promoted further growth and hardening of callus. Hypocotyl segments of *T. ammi* was induced for callus formation on MS medium with 2,4-D (9 μ M) and KIN (2.3 μ M). Embryoids were generated by transferring 2,4-D and KIN induced callus to MS basal liquid medium (Palmer *et al.*, 1997).

Prabha *et al.* (1991) reported 2,4-D induced inorganized callus proliferation while IAA and NAA promoted chlorophyll formation and semiorganisation and BA triggered plantlet formation in *in vitro* cultures of *Carum copticum.* Maheswari & Gupta (1965) reported embryoids from callus developed from nodal explant of *Foeniculum vulgare.* The variability of chromosome numbers in the callus cultures and *in vitro* celery plants was discussed by Orton (1983 b).

The production technology of *Carum copticum, Cuminum cyminum* and *Carum carvi* was reviewed by Singh *et al.* (1992). Sharma *et al.* (1997) developed a single step protocol for *in vitro* regeneration of *T. ammi.* Complete plantlet formation was achieved using shoot twigs as explants on MS + NAA (3 mg/l) or IAA (3 mg/l), thus eliminating need for different growth regulator combinations and concentrations to obtain shoots and roots. Protocol for *in vitro* regeneration of *T. ammi* was obtained in solid and liquid media by Sardana *et al.* (1998). Complete plantlets via shoot tips from seedlings developed on MS + NAA and BA. On solidified medium, multiple shoots were produced on MS + BA (8 mg/l) and IAA (3 mg/l) and rooted in IBA (5 mg/l). In liquid medium, shoots were produced with BA (0.5 mg/l) and IAA (7 mg/l) and rooted in IAA (3 mg/l). The effect of growth regulators on flavour production in cell cultures of *Apium graveolens* was studied by Watts *et al.* (1984). Large number of shoots were obtained by Ebrahimie *et al.* (2003) within a short period without sub culturing from imbibed embryos of *Cuminum cyminum.*

For the plant species yielding spices from fruits and belonging to family Apiaceae viz., ammi, anise, celery, coriander, cumin, dill, fennel and ajowan, embryogenesis had been reported in all except cumin. Jha *et al.* (1982) reported organogenesis from cumin. After carrot root became a model for studies on embryogenesis in cultures (Steward *et al.,* 1958; Reinert, 1959)

investigations were extended to several other umbellifers like *Anethum graveolens* (Johri & Sehgal, 1966), *Apium graveolens* (Collin & Isaac, 1991) and *Carum carvi* (Furmanowa *et al.*, 1991). Johri & Sehgal (1966) cultured post-fertilized ovaries of *T. ammi* and induced polyembryony. Based upon the comprehensive works of Lorz & Brown (1986), Semal (1986), Larkin *et al.* (1989), Dudits & Heszky (1990) and the work of others it is already an established fact that somaclonal variability can be traced back partly to the somatic mosaicism of intact plants and partly to the genetic instability of cultured cells.

Somaclonal variants for qualitative and quantitative characters had been reported in more than 60 species of higher plants (Bajaj, 1990 b). Plant tissue cultures proved to be of increasing value as systems for the study of phytoalexin elicitation. Extensive investigation had been done on the induction of flavonoid phytoalexins in *Petroselinum hortense* (Hahlbrock *et al.*, 1981; Chappel *et al.*, 1984).

Plant cells cultured *in vitro* produced wide range of primary and secondary metabolites of economic value. Partially, differentiated callus or suspension cultures of *Ruta graveolens*, chamomile, coriandrum and peppermint showed synthesis of flavour compounds. Callus cultures of saffron had been established, which produces flavour and pigments (Ravishankar & Venkataraman, 1990).

A pharmacological study of fresh decoction and chloroform extracted fraction of fruits of *Carum roxburghianum* was made by Gujral *et al.* (1954). The differentiated organs of *in vitro* grown callus of *T. roxburghianum* was subjected to morphological and histological studies by Roy & Gupta (1977). Ajita *et al.* (2001) analysed the various medicinal properties of *Trachyspermum ammi* and devised methods for the *in vitro* regeneration of the plant. Regenerants were obtained from shoot tip explants on MS medium

supplemented with auxin, IBA. Another medium devised by them, for the purpose include MS medium supplemented with IAA and BA inducing multiple shoots which when transferred to IBA induced rooting. Fennel seeds (*Foeniculum vulgare* Mill.) germinated *in vitro* in MS supplemented with KIN (0.5 $mgl^{-1}$), developed multiple shoots and roots in MS with 1 $mgl^{-1}$ BA and 0.5 $mgl^{-1}$ IBA. Plants could be successfully regenerated from callus cultures of fennel on the medium of same composition ([13]http).

The phytohormones at proper concentration and combination affect the growth of plants by influencing physiological and biochemical parameters (Rojina, 1991). Agarwal *et al.* (1987) succeeded in inducing callus formation using 2,4-D; the absence of 2,4-D and the presence of IAA resulted in chlorophyllous callus. Addition of NAA and KIN to IAA containing medium resulted in initiation of organogenesis and formation of semidifferentiated callus. Torrey & Shigemura (1957) had also showed that high concentration of yeast extract relative to 2,4-D concentration induce friability in pea callus. Blakeley & Steward (1961) had shown that friable and compact callus derived from *Haplopappus gracilis* were interconvertible. The change from one form to another could be achieved by changing the level of coconut milk and NAA in the culture medium. Grant & Fuller (1968) also showed that friable and compact forms of callus from the root of *Vicia faba* exist and could be interconverted. Grant & Fuller (1968) had also compared the chemical composition of friable with non-friable callus. The differences showed that the non-friable callus had a greater total amount of cell wall polysaccharides, more of each particular cell wall fraction per unit dry weight but a decreased percentage of cellulose compared with pectic substances and hemicelluloses. *Pisum* cultures had been used extensively to study the effects of various components of the culture medium such as KIN, auxin and yeast extract on chromosomal instability (Torrey, 1959, 1961, 1965), and also in studies of the influence of polyploidization on rhizogenesis (Torrey, 1967). *Vicia* cultures

had also been used for investigating mutational effects of the medium, in this instance, of compounds such as nucleic acid derivatives that were likely to accumulate during the culture process (Venketaswaran & Spiess, 1963, 1964). Ethylene accumulation in sealed containers where cells, tissues or plants were grown had been shown to affect growth and development of cultured cells or plants (Hussey & Stacey, 1981; Pua & Lee, 1995). There were substantial evidences which favoured that ethylene could increase hypocotyl diameter (Nejulbov, 1911; Crocper *et al.*, 1913; Apelbaum & Burgh, 1971), inhibit leaf expansion (Apelbaum & Burgh, 1971; Abeles, 1985), flower bud emergence (Abeles, 1967) and induce flower bud abortion (Abeles, 1967; Sisler & Yang, 1984). Earlier reports showed aminoacids to be stimulatory as well as inhibitory for growth in various systems (Fukunaga & King, 1982; Ronchi *et al.*, 1984; Tube *et al.*, 1984). The interaction with ABA and osmotic stress is interesting as both had been described useful in increasing embryogenesis and enhancing regeneration in cultures (Armstrong & Green, 1985; Kavikishore & Reddy, 1986; Renjen, 1986; Sethi *et al.*, 1990).

Establishment of *in vitro* cultures of several plant species was greatly hampered by the lethal browning of the explant and culture medium. Browning is considered to result from oxidation of phenol compounds, by polyphenol oxidases (Mayer & Harel, 1979), peroxidases (Vaughn & Duke, 1984) or air (Robinson, 1983). Several strategies were employed to overcome harmful effects of browning like use of juvenile explants, culture in darkness, culture in liquid medium, use of adsorbing agents etc. (Ziv & Halevy, 1983; Bon *et al.*, 1988; Hildebrandt & Harney, 1988). Bhat & Chandel (1991) devised a novel technique to overcome browning in tissue culture. The technique involved the sealing of the cut ends with paraffin wax.

The rapid decline of morphogenetic potential with culture time (Oono, 1975; Henke *et al.*, 1978; Inoune & Maeda, 1980; Chon *et al.*, 1983; Heszky

& Li, 1984) severely limits the usefulness of *in vitro* techniques for a wide range of applications. Recently, some attempts to maintain stable morphogenic potential of callus during continuous subculture have been successful. Visual selection and subculture of embryogenic callus (Nabors *et al.*, 1983; Siriwardhana & Nabors, 1983; Raghava & Nabors, 1984) had made it possible to regenerate plants in long term subcultures of rice. The stimulating effect of NaCl (Heyser *et al.*, 1985) and gamma radiation on the embryogenic callus proliferation and the high frequency of plant regeneration from callus sub cultured on medium containing NaCl (Yoshida *et al.*, 1983; Li & Heszky, 1984) indicate possibility of controlling and maintaining morphogenic potential during long term subcultures. Methods adopted for callus induction, subculture, plant regeneration and cytological examinations were described by Heszky *et al.* (1986) and Li & Heszky (1986).

Plants raised through florets produced more variation than those raised through leaf and stem explants (Khalid *et al.*, 1989). Suryanarayanan & Pai (1998) reported *in vitro* propagation of *Coleus forskohlii* using flowers as explants and were of opinion that flowers are a better alternative to regeneration from callus. Khalid *et al.* (1989) reported florets of *Chrysanthemum* as ideal explants for inducing somaclonal variations. Plants produced from florets showed more variations. Flowers on plants originating from petal segments showed a much wider range of abnormalities both in flower form and colouration. Singh *et al.* (1996) attempted the development of plants from florets of *Chrysanthemum morifolium* opening at different stages. In his study, he obtained variation in regeneration response in relation to the opening of flowers. *In vitro* flowering of regenerated plantlets of ginseng from somatic embryos was demonstrated (Chang & Ysing, 1980; Lee *et al.*, 1990). The flowering plantlets have practical value in hybridization and *in vitro* fertilization studies since flowering of regenerated plants from organogenesis can be induced at any time of the year (Tang, 2000).

As a means for studying the biological molecular mechanism, *in vitro* flowering provides an ideal experimental system (Mc Daniel *et al.,* 1991). *In vitro* flowering was observed in the shoot tips of bitter melon (*Momordica charantia* L.) cultured in MS medium by Wang *et al.* (2001). The transition in plants from vegetative state to reproductive development is of great interest to botanists but is poorly understood (Koonneef et *al*., 1998). Plant regeneration from cultured immature inflorescence of *Curcuma longa* was obtained by direct shoot development on MS medium supplemented with BA in combination with 2,4-D by Salvi *et al.* (2000). Regeneration of plants from inflorescence had been reported in many plants (Eapen & Rao, 1985; Babu *et al*., 1992; Wannakrairoj, 1997). Chen & Chang (2000) reported formation of somatic embryos and shoot buds directly from wound surfaces as well as through nodular mass proliferation from flower stalk of *Oncidium* (sweet sugar). Zhang & Leung (2000) obtained *in vitro* flowering from nodal explants of *Gentiana triflora* and comparison of *in vivo* and *in vitro* plants were made. The size, opening condition (full or partial open), colour, stigma development and pollen viability were compared. *In vitro* flowers were smaller and paler but the stigma development and pollen viability were similar in both the flowers. Flowering was reported in entire plants from seeds, preformed meristems, on stem internodes, leaf discs, hypocotyls, petiole and tendril segments and epidermal and sub epidermal sections. *In vitro* flowering was reported recently in somatic embryos and shoots of *Bambusa oldhamii* by Chang *et al.* (1997) from Taiwan. Gielis (1999) reported *in vitro* flowering of temperate and tropical woody bamboos. *In vitro* flowering in seedlings (Nadgauda *et al.,* 1990) and somatic embryo (Rao & Rao, 1990) of tropical bamboos was demonstrated.

All tissue culture systems exhibit some degree of somaclonal variation viz., genetic variation generated during culture (Larkin & Scowcroft, 1981). A study on the use of tissue culture as *ex situ* genetic conservation mode was

conducted by Krogstrup *et al.* (1992). The work reveals the relevance of tissue culture in broadening the scope of *ex situ* conservation. The use of machine vision in evaluating callus growth directly in petridishes is reported for carrot cell suspension growing in presence of increasing concentrations of various herbicides (Olofsdotter, 1993). Cassulino *et al.* (1991) used machine vision for estimating the number of carrot somatic embryos in suspension culture. Esnnon *et al.* (1988) demonstrated the ability of machine vision to recognize torpedo shaped embryos in sweet potato suspension culture. Smith & Spomer (1987) and Smith *et al.* (1989) used image analysis to quantify the development of callus in the whole plant micro culture grown in solidified medium. Tissue culture plants are different from the normal plants because of the environmental conditions in tissue culture container. The water retention capacity (WRC) of the headspace in the container and gas composition is responsible for the divergent physiological behaviour. Debergh *et al.* (1992) showed that by controlling the WRC of the headspace, the physiology and anatomy of tissue cultured plants can be improved to resemble normal plants. The rapidly dividing and differentiated cells fail to age and therefore clones obtained by vegetative propagation could be viable for an unlimited period of time (Kazaryan, 1969; Libbert, 1974). However, along with evidence indicating rejuvenation occurring in tissue culture during micropropagation (Mullins *et al.*, 1979; Lyrene, 1981), there are data showing that micropropagated plants can also mature and bloom at a very early age (Chang & Ysing, 1980; Greenwood, 1987; Nadgauda *et al.*, 1990). Studies on the coexistence of maturation and rejuvenation of shoot clones of *Coriandrum sativum* was done by Kataeva & Popowich (1993). The paper provided evidence of clone ageing phenomenon during prolonged subculture *in vitro*.

Mutation induced by plant tissue culture had been the subject of numerous scientific inquiries (D'Amato, 1977; Larkin & Scowcroft, 1983; Benzion *et al.*, 1986; Sun & Zheng, 1990; Kaeppler & Philips, 1993 a).

Phenotypic changes found in regenerated plants and their progeny was most strikingly observed as quantitative mutants, which have major phenotypic effects. Quantitative trait variation had also been observed by several researchers (Lee *et al.*, 1988; Dahleen *et al.*, 1991). Specific genetic changes associated with particular tissue culture induced phenotypic mutants was elucidated by Brettell *et al.* (1986) and Dennis *et al.* (1987). A review showing a glimpse at specific kinds of genetic changes encountered among regenerated plants and their progeny was presented by Phillips *et al.* (1994).

## Cytology

### Mitosis

The perpetuation of a genotype during growth and development depends upon the regular and ordered processes of chromosomal replication and division; imperfections and occasional irregularities in these processes provide basis for variation and evolution. Plant tissues and cells like their animal counter parts display more than the usual degree of nuclear irregularity when they were removed from the stabilizing environment of the intact organism and plunged into the alien environment of culture vessel. The simplest change was a repeated doubling of the basic set of chromosomes, 2x, 4x, 8x, 16x and so on (Polyploidy). The phenomenon as observed in early culture studies had been discussed in detail by Partanen (1963 b, 1965). A change in chromosome number through breakage almost invariably results from the formation of ring, dicentric or tricentric chromosomes. The formation of such chromosomes in tissue and cell cultures was not a rare event and had been reported for several species (Torrey, 1959; Mitra *et al.*, 1960; Mitra & Steward, 1961; Norstog *et al.*, 1969). Plant cells growing in an artificial culture may have numerous genetic changes such as increased frequencies of single gene mutations, chromosome breakages, transposable

element activation, quantitative trait variation and variation of normal DNA methylation patterns (Kaeppler & Phillips, 1993 b; Do *et al.*, 1999).

It has been reported frequently that plant tissues and cells display a high degree of instability under *in vitro* conditions resulting in the formation of mixoploid tissues (Sunderland, 1977). Several literature reviews dealing with ploidy, instability and related phenomena in *in vitro* cultured cells are available (D'Amato, 1952, 1978; Skirvin, 1978; Constantin, 1981). Somaclonal variation is mainly correlated with numerical or structural chromosome changes. Many authors (Pring *et al.*, 1981; Lee & Philips, 1987; Fluminhan *et al.*, 1996) had documented polyploidy, aneuploidy, translocations, inversions, deletions, fragmentation, heteromorphic pairs, ring chromosomes and single gene recessive mutations in regenerants. The frequency of mutation was affected by different aspects of tissue culture such as species (Bayliss, 1973; Kirti *et al.*, 1991; Kidwell & Osborn, 1993), genotype (Li & Stelly, 1989; Thorn, 1992; Puolimatka & Karp, 1993), culture medium (Wilkinson & Thompson, 1987; Amberger *et al.*, 1992; Ghaemi *et al.*, 1993), genotype-culture medium interaction (Wilkinson & Thompson, 1987), the origin of meristematic organized growth from the disorganized growth and the time spent in this state (Rhodes *et al.*, 1986; Karp, 1995) and the concentration and type of plant growth regulators (Bai & Knott, 1993). Variation in morphology and chromosome numbers of callus derived medicinal plants is a common observation (Bajaj *et al.*, 1978). Euploids and polyploids were observed in the callus cells of carrot (Mitra *et al.*, 1960), *Haplopappus* (Kao *et al.*, 1970), alfalfa (Clement, 1964) and *Brachyome lineariloba* (Gould, 1982). Karyotype modification such as aneuploidy or changes in the ploidy level will be directly detected by chromosome countings in the cells of the strains or the root tip cells of the regenerated plants. This type of variability has been widely described in many review articles (D'Amato, 1952, 1978, 1985; Reisch, 1983; Karp & Bright, 1985;

Ahloowalia, 1986). Plant cell and tissue cultures undergo genetic erosions and show changes of various types especially in chromosome number and ploidy level (Bajaj, 1990 a; Choi *et al.*, 2000; Melo, 2002). Karyotype analysis of metaphase chromosomes was used to determine rearrangements or numerical variation in the chromosomes of somaclones by many workers (Bhojwani *et al.*, 1986). Chromosomal abnormalities especially, chromosome doubling is a common feature associated with tissue culture (Morel, 1971). Chromosomal instability of callus and cell suspension cultures had been reported (D'Amato, 1977). Chromosomal instability in cell culture involved numerical variations, changes in chromosome structure and basic karyotype (Sunderland, 1977). Variation in callus culture was evaluated in regenerants of several plants (Sacristan, 1971; Orton, 1980). Numerical changes had been examined most often among flowering plants during micropropagation (Evans & Reed, 1981; Mohanty, 1990). Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture was assessed by Edallo *et al.* (1981).

*In vitro* cultured protocorms of *Spathoglottis plicata*, were treated with different mutagens viz., gamma rays, EMS and colchicine (Mazumder & Bhowmick, 1999). Different types of abnormalities were observed which were solely due to the effect of mutagens and not *in vitro* conditions. Three callus lines of *Allium fistulosum* culture and their regenerated plantlets were examined by Lee & Ono (1999) to elucidate the relationship between chromosome aberration and morphogenetic capacity. In addition to the normal diploid karyotype, which was identical to the mother plant, nine karyotypes altered numerically and structurally. In regenerated plantlets, from callus lines, three structurally and/or numerically altered karyotypes were observed in addition to the normal karyotype. The mitotic chromosome number in cultured tissues was determined by Furmanowa *et al.* (1991). The chromosomal aberrations of both callus lines and regenerated plantlets of *Scilla scilloides* was studied and their morphogenetic capacity relevant to

chromosomal aberrations was discussed by Ono *et al.* (1994). Joachimiak *et al.* (1993 a, b, 1995) recently reported chromosome alterations in callus cells of *Allium fistulosum.*

The androgenic haploids, produced *in vitro* were used to study the karyotype of *Nicotiana tabacum* by Dimitrov *et al.* (1982). Greisbach (1987) pointed out that tissue cultured cells spontaneously accumulate many changes in both the number and structure of chromosomes. Shamina (1966) showed that in *Haplopappus gracilis* callus cultures started from a diploid plant, 13% of the cells were triploid after only four months of culture. There were several examples of 2,4-D being a potent agent for increasing the chromosome number (Sunderland, 1977). In one year old tobacco callus 26% of the cells were tetraploid, 20% were octaploid and 54% were aneuploid (Murashige & Nakano, 1967). Undifferentiated cultured cells appeared to tolerate aneuploidy more than differentiated cells (Murashige, 1974). Cells that had been in a culture for a long time can accumulate a wide range of chromosomal structural changes. Among the regenerants derived from immature embryos of oat plants, Mc Coy *et al.* (1982) reported a number of cytogenetically abnormal plants by detailed meiotic analysis. The most common cytogenetic alteration was chromosome breakage, which resulted in partial chromosome loss. About 50% of the plants regenerated from 12 month old callus cultures of *Avena sativa* were cytogenetically abnormal. In diploid *Nicotiana tabacum* callus, a wide range in DNA content was detected (Berlyn, 1983). The growth regulator 2,4-D was known to induce somatic crossing over (Turkula & Jalal, 1985).

The karyological examination of *in vivo* and *in vitro* plants of *T. ammi* was conducted by Sehgal & Abbas (1994). The chromosome studies and amount of nuclear DNA in two varieties of *Carum copticum* was estimated by Chattopadhyay & Sharma (1990). Constancy in the amount of DNA in

different varieties of the same species was observed. Direct genetic changes was very common in cultured plant cells and there were many well documented examples of increased ploidy levels (Renfroe & Berlyn, 1985) as well as structural rearrangement of chromosomes (Bayliss, 1980). The incidence of epigenic change is very high although not so readily recognised as chromosomal modifications (Meins, 1983). Certain species produce aneuploids in tissue culture which was associated with phenotypic changes in the subsequent callus derived plants. Marketzi & Nickell (1973) had produced aberrant clones of sugarcane which were aneuploids. The callus cultures on periodical subculturing , undergo a variety of nuclear changes and show chromosomal abberations, mutations and increase in level of ploidy (Bajaj, 1981).

Variation in chromosome number within plants obtained through tissue culture had been reported by many workers (Tlaskal *et al.*, 1970; Niizeki & Oono, 1971). The morphogenesis of tissue cultures mainly depend on the specific genotype of the plant and a proper balance in the proportion of hormones in the growth medium. Analysis of karyotype and changes in the 4C DNA content of fifteen members of tribe Ammineae were investigated. Members of subtribe Carinae (*Carum, Cuminum, Petroselinum* species) and those of subtribe Seselinae (*Anethum, Foeniculum and Selinum* species) showed numerical and structural variations in their chromosomes and changes in their nuclear content (Das & Mallick, 1993). Data on the amount of 4C DNA and several chromosome characteristics was tabulated for *Carum copticum, C. carvi, Petroselinum crispum* and three varieties of *Cuminum cyminum* (Das, 1991). Six varieties of *Apium graveolens* were subjected to karyomorphological studies by Hore (1977). Bivalent formation was observed to be universal in all varieties indicating homozygosity. Structural changes had been attained in these varieties through selection in cultivation.

There had been many studies on chromosomal aberrations in cultured plant cells and their regenerants using various plant materials (Singh, 1993). Subramanian (1986) conducted studies on 21 species coming under 11 genera of Umbelliferae. The study included *Carum copticum* and resulted in the conclusion that aneuploidy and euploidy together with karyotypic asymmetry of chromosomes play an important role in the evolution of species studied. A study was conducted by Hore (1979) to determine the role of structure and behaviour of chromosomes in the evolution of different genera and species of the family Umbelliferae. The study concentrated on members of tribe Apieae and Saniculeae of Umbelliferae family. The cytology of *in vitro* produced calli of fenugreek was analysed by Gupta (1973). Multinucleate cells, apparent nuclear fission or fusion, bridge formation at anaphase and telophase were observed in the study. A comparative data of cytological and palynological salient features of chemically induced autoploids and translocation heterozygotes of certain members of Umbelliferae was prepared by Joshi (1968). A comparative idea of cytological anomalies exhibited by plants under study was also provided. Darlington & Wylie (1955), Garde & Garde (1949) had published chromosome counts of members of Umbelliferae. Baijal & Kaul (1973) discussed the karyotypes of *Cuminum cyminum* and *Coriandrum sativum* and their interrelationships. Notable contributions in the field of cytology of Indian Umbelliferae have been made by Shah (1953), Sharma & Ghosh (1954), Sharma & Bhattacharya (1959) and Datta & Maiti (1968). Similar other contributions in the family had been made by Bell & Constance (1957, 1960, 1966), Engstrand (1970), Constance *et al.* (1971) and Crawford & Hartman (1972). Previous reports on the chromosome numbers of *Trachyspermum roxburghianum* is given in Table A. Through the use of tissue culture (Larkin & Scowcroft, 1981), it may be possible to induce mitotic pairing in somatic cells of tissues. In cytology, image techniques were

used both for whole cell analysis and for more detailed analysis of organelles (Price & Osborne, 1990).

Number of papers had appeared pointing out that cells adapted to growth *in vitro* were not identical to the parent ones growing *in vivo*. Great strides had been made in the nutrition and physiology of tissue culture but cytology is still lagging behind (Partanen, 1963 a; White, 1967). It was only recently that the cytology of plant tissue culture had begun to attract attention. A survey of literature in the cytology of plant tissue culture had revealed that attention had been directed to only a few species. It is imperative therefore that wider survey of the subject be made for better understanding of the phenomenon. Most of the reports showed polyploidy and aberrations in callus cultures as a common phenomena. The tissue growing *in vitro* constitutes an unorganised stem. Their varying manifestation was sporadic and reflects perhaps the capacity of the genotype to respond to local changes in the environment. The assessment of the karyological state of plant cell in culture helped to study the responses of cells or tissues to alteration of media and added factors. The general observation was that chromosome number in plant cell or tissue culture was not stable at diploid level, but was subjected to variation, usually through the process of endoreduplication (Partanen, 1963 a). Karyological aberrations play a major role in the evolutionary sequences and their significance was said to be mainly connected with their effect on the genetic system. Knowledge of the karyotype of species ie, the number of chromosomes, their size and morphology is necessary for full understanding in plant genetic studies and plant improvement. The application of *in vitro* somatic cell genetic methods which include a combination of approaches seem very promising and had been successfully employed in inducing stable and heritable 'somaclonal variations' in widely divergent plant species (Mathur *et al*., 1988). The relative paucity on how other cell processes may be related to the level of ploidy suggested that the karyological state of the

system being studied as one of the parameters that needed to be defined in any careful study (Kruse & Patterson, 1973).

## Meiosis

Rees (1961) advocated that meiosis as well as karyotypic asymmetry had played an indispensable role in the evolution of genic system and chiasma frequency which manifests that the genic recombination is under genotypic control. The degree to which chromosomal aberrations persists at meiosis affect the viability of gametes and therefore the fertility of the plant. Genetic consequences are to be expected in the form of gene mutations, and expression of these will be associated with the chromosomal aberration persisting at this stage. The analysis of chromosomal associations in the taxa provided sufficient information on the mechanism of evolution and the potentialities of genetic recombinations present in the species or population (Sinha & Sinha, 1977). There are few earlier reports which suggest that severe fluctuations in some environmental factors like temperature shocks (Sax, 1937), cultural practices (Oehlkers, 1937) and nutritional conditions (Gregory, 1940; Straub, 1941) affect and modify the normal process of meiosis which may range from reduction of chiasma frequency to complete asynapsis of chromosomes.

Cytological analysis with respect to either meiotic or mitotic behaviour was considered to be one of the dependable indices to estimate the degree of variation. Sharma & Ghosh (1954) quoted Ogawa as the first man who worked with a limited number of umbellifers. His work was confined to meiosis. Wanscher (1932) studied meiosis and mitosis of several plants. The meiotic behaviour of different Brazilian populations of *Centella asiatica* was studied by Consolaro *et al.* (1996). High incidence of chromosome irregularities like irregular chromosome segregation, abnormal spindle, chromosome transfer among microsporocyte and sticky chromosomes were

observed. To characterize the reproductive biology of *Dasypyrum villosum* and to establish its tolerance to temperature variability, the duration and regularity of meiotic process and microspore growth in natural and controlled conditions had been examined by Stefani & Colonna (1996). Meiotic behaviour in temperature range of 5-35°C was studied. It was found that high temperature reduce meiotic duration while low temperature increase its duration. Sterility and abnormal pollen grain types were observed in tobacco plants regenerated from old cultures (Syono & Furuya, 1972). Sheidai *et al.* (1996) worked on the meiotic analysis of three genus viz., *Bunium persicum, Carum carvi* and *Cuminum cyminum* of Umbelliferae. The meiotic analysis with regard to chiasma showed lack of heterogeneity among the different populations and between the species studied. The somatic chromosome number determined were 2n = 14, 14 and 12 respectively.

## RAPD

The RAPD analysis has proved to be useful in determining genetic relationships and variation in many plant species (Yu & Pauls, 1993; Liu *et al.*, 1994; Swobodha & Bhalla, 1997; De Bustos *et al.*, 1999). RAPD has been used for the genetic analysis of micropropagated plants (Rani *et al.*, 1995; Shoyama *et al.*, 1997; Goto *et al.*, 1998; Watanabe *et al.*, 1998). Genetic molecular markers are considered to be reliable in monitoring variability in DNA sequences of plants. Several workers have applied the RAPD technique to detect somaclonal variation (Bohm & Zyprian, 1998; Al-Zahim *et al.*, 1999; De Verno *et al.*, 1999) and to identify micropropagated plants and cultivars (Ho *et al.*, 1997). Variation in DNA of double haploid plants was studied using RAPD technique (Hang *et al.*, 1997). Polymerase chain reaction was developed in the late 1980s (Saiki *et al.*, 1985) and had been applied widely in the genetic identification of biological samples. RAPD markers were used for distinguishing plant species and cultivars, such as the cereals

(Ko *et al.*,1994) and *Festuca* (Valles *et al.*, 1993), for genetic mapping (Williams *et al.*, 1990; Yu & Pauls, 1993) and for analysing genetic stability of tissue cultured plants (Valles *et al.*, 1993). RAPD technique had been extensively used for DNA polymorphisms and marker assisted breeding programmes. RAPD markers are more useful in discriminating populations and also for creating linkage maps, locating disease resistance genes and identifying chromosome specific markers. This technique had been found to be technically easier with low statistical errors (Gallego & Martinez,1997).

Genetic relationships and diversity in carrot and other *Daucas* taxa was studied by Vivek & Simon (1998) based on nuclear restriction fragment length polymorphisms (nRFLPs). Li & Quiros (2000) used amplified fragment length polymorphism markers for celery identification. Random amplified polymorphic DNA (RAPD) markers were used to measure genetic diversity of *Changium smyrnioides* (Apiaceae), a genus endemic to eastern China and an endangered medicinal plant by Fu *et al.* (2003). A total of 92 amplified bands were scored from the 13 RAPD primers, and a mean of 7.1 amplified bands per primer and 69% (64 bands) of polymorphic bands was found. Arnoldt-Schimtt *et al.* (1987) analysed the genome of carrot lines and their tissues *in vivo* and callus *in vitro* by restriction enzyme analysis and observed a great variation between plants and tissues. These results clearly indicate that DNA amplification and related events contribute to 'phylogenetic' and 'endogenetic' diversity of plants and tissues *in vitro* and *in vivo*. Kumar *et al.* (1999) reported the seed surface architecture and RAPD profiles of *Paulownia fortunii, P. tomentosa* and their hybrid, where five random primers were used. Despite the polymorphisms exhibited between parents and hybrids, monomorphic bands were also observed which might explain the success of hybridization between the two species. Some distinct parental bands were also observed in the hybrid, which indicates RAPD polymorphic bands were useful as genetic markers for different taxa under

study. The genetic closeness of various species of *Vanda* was determined by the study of Lim *et al.* (1999) using 8 different primers. The potential of bulk analysis of RAPD and ISSR-PCR markers for fingerprinting processes was evaluated using ten RAPD and ISSR primers by Fernandez *et al.* (2002). The nuclear c-DNA values of many angiosperms including *Carum copticm* was reported by Bennett & Leitch (1995). They revealed the 4C DNA content of the plant as ranging from 11.4, 11.7 and 18.8 in different varieties. It may be essential to carry out molecular analysis based on the detection of DNA markers to verify the genetic identity between donor plants and the *in vitro* proliferated plant. RAPD markers can be used to obtain rapid information about genetic similarities in micropropagules. Variation in clonally propagated *Angelica* plants was reported by Watanabe *et al.* (1998). The genetic variation in the mitochondrial and nuclear genomes in carrot were screened by Nakajima *et al.* (1997) using RAPD analysis. Attempts were made by Mohan *et al.* (1995) to study the relationship between growth and differentiation of the cultured tissues and the expression of isozyme patterns. Twenty one celery cultivars were screened for polymorphic RAPD markers with 28 arbitrary, 10–mer primers (Yang & Quiros, 1993). In celery, several morphologic isozymes and RFLP markers had been developed (Quiros *et al.*, 1987 b; Heustis *et al.*, 1998). Four isozyme and six stem protein markers were used to classify 17 celery cultivars into 11 subgroups by Quiros *et al.* (1987 a). Molecular markers were utilized in earlier studies for detecting genetic basis and breeding applications of somaclonal variations (Evans, 1989). Bousquet *et al.* (1990) obtained DNA amplification from somatic and gametic tissues by PCR. Wallner *et al.* (1996) applied PCR technique to compare the field grown and *in vitro* micropropagated plants. Simultaneously, Parani *et al.* (1997) used RAPD approach to compare the mother plant and the micropropagated progenies to maintain the fidelity of elite genotypes that were to be conserved. Molecular characterization of several other plants had

been done by RAPD technique (Ayana *et al.,* 2000; Patra *et al.*, 2001; Pradeepkumar *et al.*, 2001).

The genetic diversity in 47 individual genotypes of *Stellaria longipes* was analysed using RAPD by Million & Chinnappa (2000). The DNA variation developed in tissue culture derived plants of rice was studied by Muller *et al.* (1990). They used RFLP analysis to determine the occurrence and extent of somaclonal variation. RFLP analysis showed that both callus cultures and regenerated plants of *Zea mays* exhibit high levels of DNA polymorphisms (Brown, 1989). Taylor *et al.* (1995) worked on the sensitivity of RAPD analysis in analysing the genetic change in sugarcane by tissue culture. The work efficiently differentiated sugarcane cultures and proved suitable for detecting gross genetic change such as that which can occur in sugarcane subjected to prolonged tissue culture like in protoplast derived callus. Chowdhury (1996) investigated both RAPD and RFLP methods for molecular characterization of tissue culture inducing variation in oil palm clones. Prakash *et al.* (2002) used RAPD markers to estimate molecular diversity of 41genotypes of guava. Mneney *et al.* (2001) obtained polymorphisms between and within geographically diverse lines of cashew. The RAPD study of cultivars of mango by Kumar *et al.* (2001) unveiled maximum diversity among South Indian varieties which can be utilised for crop improvement in this genus. Gutman *et al.* (2001) carried out RAPD analysis fingerprint in 29 genotypes of *Cereus* species to examine their genetic diversity. A dissimilarity matrix and dendrogram were constructed to help visualize genetic relations between different genotypes. RAPD based assessment of diversity in Indica rice genotypes were conducted by Rana *et al.* (1999). 12 rice genotypes were subjected to RAPD analysis by using random sequence decamer primers. Genetic diversity studies using RAPD technique were carried out in a set of 103 olive cultivars by Belaj *et al.* (2002). A total of 126 polymorphisms were obtained from 21 primers used

and results were consistent with the predominantly allogamous nature of *Olea europea*. For genomes of *Lolium* and *Festuca,* molecular markers had been used to distinguish between species and cultivars or to study population dynamics (Ostergaard *et al.*, 1985; Hayward *et al.*, 1990; Murphy *et al.*, 1990; Livesey & Norrington-Davies, 1991; Danielli & Bonetti, 1992; Charmet *et al.*, 1993; Moller & Spoor, 1993). Molecular markers had also been used in taxonomic studies of the *Lolium-Festuca* complex (Darbyshire & Warwick, 1992). Isozymes had been used to distinguish embryogenic from non embryogenic tissues in *Zea mays* (Everett *et al.*, 1985; Franze *et al.*, 1989), *Hordeum vulgare* (Coppens & Gillis, 1987), *Daucas carota* (Chibbar *et al.*, 1988; Joersbo *et al.*, 1989; Rambaud & Rambour, 1989). The RAPD technique had already been successfully applied in the identification of species from *Lolium–Festuca* complex (Stammers *et al.* 1995; Weisner *et al.*, 1995). Hybrid genomes of *Festuca mairei* and *Lolium perenne* were successfully monitored using RFLP technology to evaluate distant genomic interrelationships (Chen *et al.*, 1984). Protein and isozyme electrophoretic profiles had been successfully used for identification of either somatic or sexual hybrid genomes (Eizenga & Buckner, 1986; Humphreys, 1989). Isozyme techniques had been investigated for the identification of grape vines (Wolfe, 1976; Bachman & Blaich, 1988; Weeden *et al.*, 1988; Parfitt & Arulsekar, 1989; Walters *et al.*, 1989) and had been successful at distinguishing closely related cultivars derived from sexual process. RFLP analysis had been used to detect DNA polymorphism within *Vitis* using heterologous probes (Striem *et al.*, 1990; Yamamoto *et al.*, 1991) and homologous probes (Borquin *et al.*, 1992, 1993; Mauro *et al.*, 1992; Bowers *et al.*, 1993; Bowers & Meredith, 1996). There had been numerous reports on the use of RAPD markers for cultivar identification or for detection of genetic variation among cultivars (Hu & Quiros, 1991; Castilione *et al.*, 1993; Mailer *et al.*, 1994; Yu & Nguyen, 1994). RAPD and microsatellite polymorphisms

had also shown to be useful in grape cultivar identification (Gogorcena *et al.*, 1993; Thomas & Schott, 1993, 1994; Cipraini *et al.*, 1994; Tschammer & Zyprian, 1994; Mulcahey *et al.*, 1995; Ye *et al.*, 1996). Studies on the chromosome numbers, karyotypes (Jones, 1983) and restriction patterns (Cheah *et al.*, 1989) of tissue cultured oil palms were reported.

DNA markers were advantageous over other morphological and biochemical markers in that these are never influenced by environmental factors. Extensive investigation had been carried out on molecular markers of important medicinal plants both in relation to linkage among genotypes and chemotypes and also biosynthesis of pharmaceutically valuable compounds.

## GC-MS

Essential oil of aromatic plants is a mixture of a variety of terpenes synthesized by the action of a large number of enzymes in a multibranched pathway. An important group of natural products, with industrial interest are the essential oils as they are used in perfumery as aroma product, as flavouring agent in food and beverages, in cosmetic products and drugs. By means of modern analytical techniques it had been possible to identify hundreds of different components of essential oil mainly terpenes, phenylpropanes, their derivatives and aliphatic compounds. Most oils contain one or a couple of main components, but the minor components are also important. GC has become one of the most important tools in the analysis of essential oils and it plays today a central role in the study of volatiles. The production of these often very costly products by means of plant cell cultures has consequently been the subject of numerous studies. GC-MS analyses of essential oils of various plants were done for identifying their components (Choudhury & Leclercq, 1995; Choudhury *et al.*, 1996, 1997 a, 1997 b, 1998 a, 1998 b).

Reports are available where the tissue cultures are having more essential oil content as compared to donor plant (Violon *et al.*, 1984; Becker & Schrall, 1990). The effect of a range of nutritional and hormonal conditions in particular on the synthesis and accumulation of secondary compounds *in vitro* have been the subject of intense investigation and had been well reviewed (Mantell & Smith, 1983). There had been many works on medicinal plants regarding the production of active principles by *in vitro* cultures raised from any plant part and optimization of the product yield by manipulating the medium composition and the physical factors (Rideau, 1987). Aggregated or temporarily stressed cells contained increased amounts of pthalides and other terpenoids. The role of monoterpene glycosides in the process of accumulation of essential oils in Lamiaceae species had been discussed by Skopp & Horster (1976).

Pharmacological screening of total oil, essential oil and glycosidal fraction of *Carum copticum* was done by Mukherjee *et al.* (1967). The essential oil composition of *Trachyspermum roxburghianum* was reported by Ashraf *et al.* (1978). The oil of *C. copticum* was reported to be very effective against eight pathogenic bacteria, causing infections in the human body (Singh *et al.*, 2002). Ghannadi and Sadeh (2003) isolated the essential oil of *Coriandrum sativum* by steam distillation and analyzed the components by TLC, GC, GC-MS, $^{1}$H-NMR. Eight compounds representing 95.3% of the total components were characterized. Linalool (56.2%), γ- terpinene (12.0%) and δ-3-carene (9.7%) were the major constituents of the oil. The somaclonal variation for total seed oil content, fatty acid composition of seed lipids, besides certain quantitative and qualitative characters was assessed by Seeta *et al.* (2000). Increase in oil content and fatty acids like linoleic acid, oleic acid and palmitic acid were analyzed in the somaclones.

Potential cancer chemopreventive agents were isolated from caraway oil by Zheng *et al.* (1991). The three monoterpenes isolated were anethofuran, limonene and carvone. Ishikawa *et al.* (2001) reported that 25 components were isolated, including five new monoterpenoid glucosides, a new monoterpenoid, two new aromatic compound glucosides, and two new glucides from the water-soluble portion of the methanol extract of the fruit of *Carum ajowan* (ajowan), which has been used as a spice and medicine. Their structures were clarified by spectral investigation. From the water-soluble portion of the methanolic extract of caraway (fruit of *Carum carvi*), an aromatic compound, an aromatic compound glucoside and a glucide were isolated together with 16 known compounds. Their structures were clarified as 2-methoxy-2- (4'-hydroxyphenyl) ethanol, junipediol 2-O-beta-D-glucopyranoside and L-fucitol, respectively (Matsumura *et al.*, 2002). Gersbach & Reddy (2002) applied magnetic resonance imaging to locate the position of essential oil accumulation in fruit of *Carum copticum*. Selective imaging of whole *C. copticum* fruits showed localization to the secretory canals of the fruit wall. GC-MS analysis of essential oil of *Carum carvi* was reported by Chaudhary (2002). The main components were carvone (81.5%), citronellyl acetate, dehydrocarvone, eugenol, iso limonene and limonene oxide. Trace components detected were δ-3-carene, camphene, caryophyllene, carvol, p-cymene, dihydrocarvol, linalool, p-mentha-2,8-dien-1-ol, myrcene, α-pinene, β-pinene, phellandrene, sabinene, α-terpinene and terpinolene. By comparing the constituents in the essential oil of coriander, Antitescu *et al.* (1997) studied the difference in steam distillation and super critical $CO_2$ extraction. Studies on the essential oil content of *Micromeria dolichodontha* was conducted by Baser *et al.* (1997).

The constituents of leaf essential oil of *Eryngium foetidum* was analysed by Pino *et al.* (1997). Phytochemical studies of *Foeniculum vulgare* were done by Muckenstrum *et al.* (1997). Studies on the detection of various

aroma compounds in essential oil of different varieties of carrot were done by Habegger *et al.* (1996). The carvone productivity of dill and annual caraway plants were compared and studies on carvone production in the umbellate seeds was done by Mheen (1996 a, b). Studies on the chemical constituents of *Centella asiatica* was conducted by Srivastava & Shukla (1996). The oil content of wild and cultivated forms of caraway was analysed by Galambosi & Peura (1996). The increase in seed yield and essential oil of coriander by application of nitrogen and phosphorus was proved by Tiwari & Banafar (1995). Agarwal (1996 a) analysed the essential oil constituent of *Cuminum cyminum.* The effect of volatile oil of *Foeniculum vulgare* was studied by Saleh *et al.* (1996). Different entries of fennel fruits were analysed for their volatile oil constituents by Agarwal (1996 b). Baser *et al.* (1996) worked on the essential oil of *Pimpinella.* The content of essential oil of carrot and its antibacterial activity was studied by Kilbarda *et al.* (1996). Chemical investigations of the family Apiaceae are mainly concentrated on essential oils (Karlson *et al.* 1994).

The large family Apiaceae is rich in secondary metabolites, but only 10% of the well known plants have been investigated for their constituents (Kubezcka, 1982). Mehta *et al.* (1994) worked on ajowan and concluded that the methanolic extract of ajowan exhibits good antioxidative activity and can be recommended as a potential source of natural antioxidants to decrease the rate of lipid oxidation. Nilov (1934, 1936) demonstrated that the formation of essential oils occurs in different ways depending on the nature of the life cycle using *Carum copticum* as an example. Qualitative alterations during the development of constituents in fruit organs were later proved by Nilov (1937, 1938). In coriander, changes in the components of essential oil caused by individual development have been reported (Tetenyi, 1991). The physiological and biochemical aspects of carvone synthesis during plant development were reported by Bouwmeester *et al.* (1995 a, b).

Joshi (1961) tested six varieties of ajowan at IARI, New Delhi and found that varieties viz., NP (D) 30 and 79, NP (I) 8 and 66, NP (P) 151 and NP (K) 15 had given 25-60% more yield than the local variety in small scale trials. NP (P) 151 gave 9.78% essential oil content as compared to 2.52% of the commercial material. This variety also gave 40% more yield than local ones. Studies conducted by Singh & Singh (1971) revealed that the application of nitrogen, potassium and sulphur had no significant effect on seed yield and oil content of ajowan. In Egypt, Balbaa *et al.* (1973) studied the volatile oil from the plant and fruits of *C. copticum* at different stages of plant growth and maturity. The percentage of oil in the plant reached 2.8% and that in the fruit varied from 4.4 - 8.9% on a dry weight basis. However, in Pakistan, Ashraf & Bhatty (1975) studied the essential oil of fresh large and small seeds of ajowan. They found that the respective yields of oil were 3.5 % and 5.2%.

Many members of the family Umbelliferae contained valuable secondary metabolites i.e., terpenoids (Krens *et al.* 1997). Kamisako *et al.* (1984) carried out studies in the changing triterpenoid content during the growth cycle of cultured plant cells. They observed that the contents of triterpenoids decreased for several days after callus inoculation, then increased rapidly during the mid and late exponential phases of growth. Krishnamoorthy & Madalageri (2000) reported increase in essential oil content with increased addition of nitrogen and phosphorous. The results obtained were in agreement with those obtained in dill (Randhawa & Singh, 1998), caraway (Munshi *et al.* 1990) and coriander (Bhat & Sulikeri, 1992). De (2000) reported that the oil content of *T. ammi* as 2-4%, while Chadha (1989) opined oil yield to vary from 2-3.5%. Datta *et al.* (2001) reported that the oil yield ranges from 2.48% to 4.53%. The phenols in the oil of ajowan were studied by Sobti & Singh (1923). They could identify thymol and carvacrol in the oil. Guenther (1953) reported the oil yield of *C. copticum* as

3-4% from the seeds whereas the Indian varieties examined by Tandon & Gupta (1955) was found to contain 2.5-4% essential oil.

Bhargava & Haksar (1959) reported an exhaustive chemical examination of essential oil of *T. ammi*. Tripathi *et al.* (1986) reported the fungitoxic properties of the oil of *T. ammi* and antifungal principles of the oil had been isolated and identified as thymol and p-cymene. The physico-chemical properties of the oil were also monitored. There are many reports on the fungitoxicity of essential oils (Garg, 1974; Avadhoot & Verma, 1978; Banerjee & Nigam, 1978; Dikshit *et al.*, 1979). There are enough references to believe that higher plants emit volatile substances, which keep the air remarkably free of pathogenic organisms. Chialva *et al.* (1993) analysed the essential oil constituents of *T. copticum* fruits by GC and GC-MS. 27 compounds were identified of which thymol, p-cymene and $\gamma$-terpinene are the main components. Choudhury *et al.* (1998 c) analysed the seed oil composition of *T. ammi* by GC and GC-MS. The yield of oil was 1.2%. Georgiev & Khadzhiiski (1969) discussed the variation in the yield and composition of oil according to storage period of seeds. Most workers identified thymol as the major constituent of oil from various plant parts of *T. ammi* (Tamhane & Rao, 1972; Kalsi *et al.*, 1979; Mudgal & Thampi, 1989; Kumar *et al.*, 1992; Aftab *et al.*, 1995; Asghari & Lockwood, 1996).

The essential oil of *T. roxburghianum* was analysed by Choudhury *et al.* (2000) using GC and GC-MS. Previous reports on the essential oil composition of *Trachyspermum roxburghianum* is given in Table B and the biological properties of some of these components are cited in Table C. Qadry (1976) reported the composition of oil of *T. roxburghianum*. According to Chopra & Mukherjee (1932) the seeds of *C. copticum* from different parts of the country yielded varying proportions of oil ranging from 2-3.5%. They reported that the percentage of thymol in the Indian species was not more than

33-37%. Varghese *et al.* (1949) discussed the thymol production from ajowan seeds. Palmer *et al.* (1997) reported the presence of thymol in *in vitro* cultures of *T. ammi.* The benzene extract from various sources viz., seeds, embryogenic callus and nonembryogenic callus were subjected to TLC. The standard thymol band was obtained at an Rf value of 0.75. The fruits yield 2-4% of colorless to brownish essential oil in which thymol is present up to 35-60%. Banthorpe *et al.* (1986) recorded the ability of the callus cultures of plants to synthesize and accumulate terpenoids. The work included essential oil yielding plants like *Anethum graveolens, Lavandula anguistifolia* etc.

Sharma & Sharma (1979) analysed the various chemical components of *T. roxburghianum.* Oil of ajowan was analysed for trace constituents by column and gas liquid chromatography. Camphene, α-pinene, δ-3-carene and myrcene were detected in the thymene fraction and a hitherto unreported alcohol was detected in the phenol eluate (Nigam *et al.,* 1963). A detailed account of thymol was made by Lakhani *et al.* (1921). The various sources of thymol and the necessity of bringing about progress in increasing the oil yield in the plant was discussed, unless which India will be pushed back in the international market in the field of thymol trade. The oil of *Carum carvi* was surveyed using GC-MS method and about 38 components were confirmed, with carvone and limonene as principal constituents (Gorunovic *et al.*, 1991). The change in the oil composition of roots of some members of Apiaceae in relation to the development of oil duct in the system was presented by Stahl & Wichtmann (1991). The work revealed that the oil content of the seedlings mainly contained sesquiterpene hydrocarbons, which undergoes major changes when the secondary oil ducts appear.

Prabha *et al.* (1991) evaluated the thymol formation in *in vitro* cultures of *Trachyspermum ammi.* Correlation between secondary metabolite formation and primary metabolism was compared in the cultures using $^{14}C$

labeled Kreb's cycle intermediates. The work revealed that the thymol content increased several fold upon chlorophyll formation. A comparative study of oil glands in the pericarp of 15 Indian Umbelliferae was surveyed by Dave *et al.* (1992). The work reported oil gland as schizogenous and the cavity at maturity gets filled with secretion. Aroma compounds of fresh freeze-dried and dried dill (*Anethum graveolens*) were isolated and combined fused silica capillary gas chromatography – mass spectrometry was employed for the identification of aroma compounds. 70 volatile components were detected of which 13 were identified as α-pinene, α-phellandrene, limonene, β-phellandrene etc. (Huopalahti & Kesalahti, 1985). The preliminary studies on effect of different processes on the aroma constituents of dill were performed by Drawert *et al.* (1981) and Schreier *et al.* (1981).

Ramachandraiah *et al.* (1986) surveyed the essential and fatty oil content of the umbelliferous seeds. Thymol production of *T. ammi* was studied by Agarwal *et al.* (1987). They explained thymol production increased with time in undifferentiated and semidifferentiated callus, but the content was greater in the latter when compared to the former. Agarwal & Patwardhan (1993) found that the addition of monoterpenes as precursors in the medium, adversely affected the thymol production in *in vitro* cultures of *T. ammi*. The seeds of *T.ammi* were subjected to chemical and technological studies (Nagalakshmi *et al.*, 2000), which revealed the yield of oleoresin as 24.66% containing 12.15% volatile oil and 87.85% nonvolatile oil. The oleoresin samples retained their quality at low as well as ambient temperature. The GC-MS analysis of essential oil of *C. copticum* was done by Srivastava *et al.* (1999 b) and he identified 11 components in the oil. The oil exhibited antimicrobial activity against the gram positive and negative bacteria tested. Callus cultures of *Coriandrum sativum* produced geraniol, but other monoterpenes associated with the flavouring principle of coriander oil had not been found (Sardesai & Tipnis, 1969). Undifferentiated callus cultures of

*Foeniculum vulgare* did not accumulate anethol (Paupardin, 1976). The enantiomers of linalool were isolated as the major monoterpene alcohols from the essential oils of coriander and sweet basil, respectively (Ravid & Putievsky, 1985).

Accumulation of monoterpenes resulting from *de novo* synthesis had been demonstrated in tissue cultures of several oil-bearing plants like *Gardenia* species (Ueda *et al.,* 1981). Spectacular synthesis of certain sesquiterpenes had been demonstrated in several culture lines (Anastrasis *et al.,* 1982; Witte *et al.,* 1983). Major differences were reported between the terpene content of cell cultures and leaves of whole plant of *Thuja occidentalis* (Witte *et al.,* 1983). Callus cultures of *Tanacetum vulgare* were able to synthesize monoterpenoids and carotenoids, and the quantitative composition differed from that of intact plants (Banthorpe & Justice, 1972). Charlwood & Charlwood (1983) reported the preliminary results concerning the production of monoterpenes in callus and suspension cultures of *Pelargonium* variants. The monoterpenes accumulated in these cultures are not normally associated with the genus. It had been shown that organogenesis was necessary for the induction of volatile substances comparable to those of intact plants in various tissue cultures of Labiates. Becker (1970) reported that no volatiles were accumulated in undifferentiated callus cultures of *Origanum vulgare, Rosmarinus officinalis* and *Salvia officinalis.* Likewise, no monoterpenes could be detected in undifferentiated tissue cultures of *R. officinalis* (Webb *et al.*, 1984) grown under a variety of conditions. However, shoots regenerated from these cultures accumulated monoterpenes. The essential oil production in relation to organogenesis was studied in tissue cultures of *Eucalyptus citridora* by Gupta & Mascarenhas (1983). Here undifferentiated callus did not contain monoterpenes whereas organogenesis stimulated oil production. There are many reports on the production of active principles by tissue cultures, but only a few on the biosynthesis of essential oil

*in vitro.* GC-MS analysis of essential oils of heartwood and resin of *Shorea robusta* was done by Kaur *et al.* (2001) and germacrene-D was found to be the chief constituents of both the oils. GLC was used by Saxena & Sharma (1998) to identify the essential oil constituents of *Commiphora mukul* of family Burseraceae. The essential oil of *Achillea eriophora* was analysed for the constituents by Weyerstahl *et al.* (1997).

*In vitro* plants are now concentrated on for altering the secondary metabolite pathways and their synthesis. Ellis (1984) made a study on the probing of secondary metabolism in plant cell cultures. Variation in the secondary metabolites produced by plant cell cultures had been reported in many plants (Wolters & Eilert, 1982; Tietjen *et al.*, 1983).

Table A

Previous Reports on the Chromosome Numbers of *Trachyspermum roxburghianum*

| Chromosome Number | Authors | Year |
|---|---|---|
| 18 | Sharma & Ghosh | 1954 |
| 20, 24, 42, 44 | Hore | 1971 |
| 18 | Subramanian | 1986 |
| 42 | Krishnappa & Basappa | 1988 |

Table B

## Previous reports on the chemical components identified from the essential oil of *Trachyspermum roxburghianum*

| Plant part used | Components | Authors | Year |
|---|---|---|---|
| Plant | α-pinene<br>β-pinene<br>Sabinene<br>Terpinolene<br>α-phellandrene<br>β-phellandrene<br>α-terpinene<br>γ-terpinene<br>Limonene<br>p-cymol | Qadry | 1976 |
| Plant | Limonene<br>Sabinene<br>Terpinen-4-ol<br>(z)-ligustilide<br>γ-terpinene | Choudhury *et al.* | 2000 |
| Seeds | d-limonene<br>α-terpinene<br>Dipentene<br>d-linalool<br>Terpineol<br>dl-piperitone<br>Thymol<br>Ketonic acid | Anonymous | 1943 |
| Seeds | d-limonene<br>α-terpinene<br>Dipentene<br>d-linalool<br>Terpineol<br>dl-piperitone<br>Thymoquinol<br>Thymol | Chopra *et al.* | 1956 |

| | | | |
|---|---|---|---|
| Seeds (1.8-2%) | Fixed oil (4.4%)<br>Ketonic crystalline compound (0.09%) | Chopra *et al.* | 1969 |
| Seeds | Bergapten | Anonymous | 1973 |
| Seeds | α-pinene<br>β-pinene<br>Sabinene<br>Terpinene<br>α-phellandrene<br>β-phellandrene<br>Linalool<br>α-terpineol<br>Thymol<br>Carvacrol | Anonymous | 1976 |
| Seeds | l-cadinene (23.28%)<br>Limonene (15.06%)<br>Seselin (13.1%)<br>Thymol (2%)<br>γ-terpinene (1.92%)<br>p-cymene (1.92%)<br>Fenchone (1.8%)<br>α-terpineol (1.4%)<br>Linalool (1.3%)<br>dl-piperitone (1.2%)<br>Isopimpinellin (0.8%)<br>α-pinene (0.66%)<br>Myrcene (0.56%)<br>Bergaptene (0.5%)<br>δ-3-carene (0.33%)<br>Piperitol (trace) | Muhammed *et al.* | 1977 |
| Seeds | α-pinene<br>Myrcene<br>Car-3-ene<br>Limonene<br>γ-terpinene | Anonymous | 1977, 1979 |

| Seeds | p-cymene<br>(-)cadinene<br>Fenchone<br>Thymol<br>Linalool<br>Piperitone<br>Seselin<br>α-terpineol<br>Bergapten<br>Isopimpinellin | Anonymous | 1977,1979 |
|---|---|---|---|
| Seeds | β-cyclolavandulic acid<br>Seselin | Anonymous | 1978 |
| Seeds | 6-methylcoumarin<br>Angelicin<br>Psoralen<br>Bergapten<br>Umbelliferone | Sharma & Sharma | 1979 |
| Seeds | α-limonene<br>Terpinene<br>Dipentene<br>d-linalool<br>Terpineol<br>dl-piperitone<br>Thymoquinol<br>Thymol | Dey | 1980 |
| Seeds | 7-methoxy-6-methyl coumarin<br>7-hydroxy-6-methyl-coumarin<br>Angelicin<br>Psoralen<br>Bergapten<br>β-sitosterol<br>Umbelliferone | Rastogi & Mehrotra | 1993 |

Table C

## Previous Reports on biological activities of some phytochemicals detected in the essential oil analysis of present study

| Component | Biological Activity | Authority | Year |
|---|---|---|---|
| Thujene | Bioactive principle | Paramonov *et al.* | 2000 |
| | | Usubillaga *et al.* | 2001 |
| | | Jirovetz *et al.* | 2001 |
| | Allergenic | Mitchell & Rook | 1923 |
| β-myrcene | Analgesic<br>Antimutagenic | Kauderer *et al.* | 1991 |
| | Fungicidal | Keeler & Tu | 1991 |
| | Antioxidant | Sternberg & Duke;<br>Chaudhari *et al.* | 1996;<br>1989 |
| γ-terpinene | Antioxidant<br>Insectifuge<br>Perfumery | Sternberg & Duke | 1996 |
| | ACE Inhibitor<br>Antiacetylcholineesterase<br>Antioxidant | Duke | 2003 |
| | Aldose-Reductase-Inhibitor | Okamura *et al.* | 1992 |
| | Irritant | Zebovitz | 1989 |
| | Pesticide | Duke | 1992 |

| β-terpineol | Insectifuge | Jacobson | 1990 |
|---|---|---|---|
| | Perfumery | Sternberg & Duke | 1996 |
| | Antitumourous | Setzer *et al.* | 1999 |
| Germacrene-D | Pheromonal | Sternberg & Duke | 1996 |
| Cedrene | Cancer Preventive | Stitt | 1990 |
| β-caryophyllene | Antinemic | Srivastava *et al.* | 2000 |
| | Antibacterial | Cobos *et al.* | 2001 |
| α-caryophyllene | Anticarcinogenic Bactericide | Muroi & Kubo | 1993 |
| | Antiedemic Antifeedant Antiinflammatory | Shimizu | 1990 |
| | Antitumour | Zheng *et al.* | 1992 |
| | Spasmolytic, Termitifuge Insectifuge | Jacobson | 1990 |
| | Perfumery | Anonymous | 1996 a |

| | | | |
|---|---|---|---|
| Aromadendrene | Antiseptic | Harborne & Baxter | 1983 |
| | Cancer preventive | Stitt | 1990 |
| δ-Cadinene | Anticarcinogenic<br>Bactericide | Muroi & Kubo | 1993 |
| Caryophyllene oxide | Antiedemic<br>Antiinflammatory | Shimizu | 1990 |
| | Antifeedant<br>Insecticidal | Bettarini & Borgonovi | 1991 |
| | Antitumourous | Zheng *et al.* | 1992 |
| | Perfumery<br>Flavour | Chowdhury & Kapoor | 2000 |
| n-Hexadecanoic Acid | Acidulant | Harborne & Baxter | 1983 |
| | Flavour | Anonymous | 1996a |
| Ocimene | Perfumery | Anonymous | 1996b |
| α -Farnasene | Antimicrobial<br>Antifungal | Arambewela *et al.* | 1999 |
| | Pheromonal | Harborne & Baxter | 1983 |
| β-Farnasene | Antimicrobial | Arambewela *et al.* | 1999 |
| | Antifungal<br>Insect control | Croteau *et al.* | 2001 |

| Limonene | Anticancer<br>Antiflu<br>Antiviral<br>Enterocontractant<br>Nematicide<br>Spasmolytic<br>Irritant<br>Fungiphilic<br>Bactericide<br>Antilithic<br>Antimutagenic | Sternberg & Duke | 1996 |
|---|---|---|---|
| | Sedative | Wagner & Wolffe | 1977 |
| | Bioactive principle | Usubillaga *et al.* | 2001 |
| | Antifungal | Jirovetz *et al.* | 2001 |
| | Antimicrobial | Rao *et al.* | 2000 |
| | Antitumourous | Rao *et al.*; Setzer *et al.* | 2000;1999 |
| | Larvicidal<br>Insecticidal<br>Ovicidal | Singh *et al.* | 2000 |
| | Allergic<br>Antialzhemeran | Mitchell & Rook | 1923 |
| | Expectorant | Harborne & Baxter | 1983 |
| | ACE Inhibitor<br>Insecticide | Grundy & Still | 1985 |

| | | | |
|---|---|---|---|
| Limonene | Cancer Preventive | Stitt | 1990 |
| | Insectifuge | Jacobson | 1990 |
| | Candidistat<br>Fungistat | Kang *et al.* | 1992 |
| | Nematicide | Nigg & Seigler | 1992 |
| | Antitumour | Yu *et al.* | 1995 |
| | Antibacterial<br>Antifungal | Aggarwal *et al.* | 2002 |
| | Allelochemic<br>Antiacetylcholinesterase<br>Antifeedant<br>Antiseptic<br>Chemopreventive<br>Ornithine-Decarboxylase-Inhibitor | Duke | 2003 |
| | Pesticide | Duke | 1992 |
| | Antispasmodic<br>Photosensitizer | Rinzler | 1990 |
| δ-3-carene | Antifeedant<br>Irritant<br>Pesticide<br>Allergenic<br>Dermatitigenic | Grundy & Still | 1985 |
| | Antiinflammatory | Anonymous | 1990 |

| δ-3-carene | Bactericide | Anonymous | 1994 |
|---|---|---|---|
| | Insectifuge | Jacobson | 1990 |
| | Irritant<br>Pesticide | Duke | 2003 |

# MATERIALS AND METHODS

Sreeranjini K. “Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)” Thesis. Department of Botany, University of Calicut, 2004

# MATERIALS AND METHODS

## *In vitro* Multiplication

Seeds of *Trachyspermum roxburghianum* (DC.) Craib were collected from the Herbal Garden, Kottakkal in Kerala, South India. The collected seeds were sown in the net house of Botany Department and voucher plant specimens were authenticated at Calicut University Herbarium (CALI 51311). Nodal segments (1-1.5 cm) of plants just before flowering were used as explants to initiate cultures. Small cut twigs were brought to the laboratory in water. Expanded leaves were removed and stems were washed in labolene detergent for ten minutes and later washed thoroughly under running water. Surface sterilization was carried out with 0.1% mercuric chloride for 30 min. These explants were then rinsed in double distilled sterile water and implanted on to the nutrient medium.

Murashige & Skoog (1962) basal medium (Table 1) with 3% sucrose, 100 mg/l myoinositol and 0.8% agar was used. The induction media consist of basal MS medium supplemented with different concentrations and combinations of auxin and cytokinins. The $p^H$ of the medium was adjusted to 5.7 – 5.8 before dispensing into culture tubes. Media was sterilized at $122^0$ C for 15 minutes. Each experiment was set up with 10 –12 replicates and repeated twice. The cultures were grown at 25 $\pm$ $3^0$ C with humidity of 50 – 60% under fluorescent daylight tubes emitting 2000 lux for 16/8 h. light/ dark period and were sub cultured every 4-6 week.

## Establishment of Plants in Soil

Two to four weeks old regenerated plants were sub cultured to MS medium with different auxin concentrations for rooting and standardized. The rooted plants were removed from the tube and were potted in a sterilized mixture of soil and sand (1:1). They were initially irrigated with ½ MS solution for one week. Established plants were transplanted to the field in

earthen pots and watered regularly. These plants were then utilized for further analysis.

Table 1

Murashige and Skoog (1962) Basal Medium

| Stock Chemicals | Mg/l | Stock Concentration | Stock |
|---|---|---|---|
| I $NH_4NO_3$ | 1650.00 | | 82.50 g/l |
| $KNO_3$ | 1900.00 | | 95.00 g/l |
| $KH_2PO_4$ | 170.00 | 50X | 8.50 g/l |
| $MgSO_4.7H_2O$ | 370.00 | | 18.50 g/l |
| II $CaCl_2.2H_2O$ | 440.00 | 50X | 22.00 g/l |
| III $Na_2EDTA$ | 37.30 | | 3.70 g/l |
| $FeSO_4.7H_2O$ | 27.80 | 100X | 2.80 g/l |
| IV $MnSO_4.4H_2O$ | 22.30 | | 2.23 g/l |
| $ZnSO_4.7H_2O$ | 8.60 | | 860 mg/l |
| $H_3BO_3$ | 6.20 | | 620 mg/l |
| KI | 0.83 | 100X | 83 mg/l |
| $Na_2MoO_4.2H_2O$ | 0.25 | | 25 mg/l |
| $CoCl_2.6H_2O$ | 0.025 | | 2.5 mg/l |
| $CuSO_4.5H_20$ | 0.025 | | 2.5 mg/l |
| V Vitamins | | | |
| Glycine | 2.00 | | 200 mg/l |
| Nicotinic acid | 0.50 | 100X | 50 mg/l |
| Pyridoxine - HCl | 0.50 | | 50 mg/l |
| Thiamine - HCl | 0.10 | | 10 mg/l |

## Cytological Analysis

### Mitotic Squash Preparation

Somatic chromosome spreads were prepared with the help of improved techniques (Sharma & Sharma, 1990). Young root tips were collected from plants grown in pots at the period showing peak mitotic activity (9-10 a.m.). The root tips were thoroughly washed in distilled water and treated in pretreatment chemicals. Saturated solution of paradichlorobenzene with a trace of aesculin was used for pretreatment. Small quantity of saponin was also added to remove oil particles from the cells. The pretreatment solution is initially chilled to 0-5° C for 4-5 min. and the root tips were treated at 12-15° C for 2½ - 3 hours. Then the treated root tips were washed thoroughly with distilled water and fixed in 1: 3 acetic acid-ethanol mixture overnight.

The fixed root tips were washed in distilled water followed by treatment in 1N HCl for 7 min. at room temperature. The root tips were again thoroughly washed to remove last traces of acid. After acid treatment, root tips were stained in 2% aceto orcein for 3-4 hours at room temperature. Stained root tips were washed in 45% acetic acid to remove excess stain and then squashed in 45% acetic acid. The microphotographs of well spread mitotic plates were taken using LEICA–GALEN III Research Microscope attached with a PENTAX photosystem.

In *in vitro* plants also root tip was used for mitotic preparation with the same procedure, as given above. The callus was also subjected to the same method except that the treatment in 1N HCl was for 10 min. at room temperature.

## Meiotic Smear Preparation

For meiotic studies, inflorescence of both *in vivo* and *in vitro* plants were collected. Floral buds of appropriate size were removed from the inflorescence and fixed in 1:3:6 (Acetic acid: chloroform: ethyl alcohol) fixative for 1½ h. and stored in 70% ethyl alcohol. The anthers of flower buds were removed individually and dissected. They were cut transversely and the content of pollen sacs were extracted on a drop of 2% acetocarmine (Belling, 1926; Darlington & La Cour, 1976) by pressing the anther halves. After placing the cover slip the slide was warmed over an alcohol lamp to improve staining contrast between chromosomes and cytoplasm. Microphotographs of suitable stages were taken.

## Karyomorphological Studies

### Chromosome Image Analysis System

Karyograms were generated with the aid of computer-based programs such as Adobe Photoshop, AutoCAD and a data based analyzing system (Microsoft Excel). Photographs were scanned and stored as digital images. These digital images were converted into gray scale images using Photoshop program. Identification numbers were allotted to each chromosome and then loaded to AutoCAD for karyomorphometric analysis. After determining the centromeric position, the arm length of each chromosome was measured and the centromeric indices were calculated. On the basis of arm ratio and centromeric indices, homologous chromosomes were identified and classified (Table 2), according to Abraham & Prasad (1983). The images were reloaded to Photoshop and karyograms were generated.

Karyotype formula was calculated depending upon the length of chromosome, position of centromere and the length of chromosome with secondary constriction.

Disparity index of the chromosomes were calculated after Mohanty *et al.* (1991) by using the formula

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

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

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

Table 2

Details of the chromosome nomenclature in relation to centromere location based on arm ratios and centromeric index (Abraham & Prasad, 1983).

| Nomenclature | Notation | $R_1$ s/l | $R_2$ l/s | $I_1$ 100s/c | $I_2$ 100l/c |
|---|---|---|---|---|---|
| Median | M | 1.00 | 1.00 | 50.00 | 50.00 |
| Nearly median | nm | 0.99-0.61 | 1.01-1.63 | 49.99-38.01 | 50.01-61.99 |
| Nearly submedian | nsm(-) | 0.60-0.34 | 1.64-2.99 | 38.00-25.01 | 62.00-74.99 |
| Submedian | SM | 0.33 | 3.00 | 25.00 | 75.00 |
| Nearly submedian | nsm(+) | 0.32-0.23 | 3.01-4.26 | 24.99-18.20 | 75.01-81.80 |
| Nearly subterminal | nst(-) | 0.22-0.15 | 4.27-6.99 | 18.19-12.51 | 81.81-87.49 |
| Subterminal | ST | 0.14 | 7.00 | 12.50 | 87.50 |
| Nearly subterminal | nst(+) | 0.13-0.07 | 7.01-14.38 | 12.49-5.01 | 87.51-94.99 |
| Nearly terminal | nt | 0.06-0.01 | 14.39-19.99 | 5-0.01 | 95.00-99.99 |
| Terminal | T | 0 | ∞ | 0 | 100 |

The total forma percentage (TF%) or mean centromeric index value was calculated after Huziwara (1963) 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 method reveals sequence polymorphism between template DNAs based on selective amplification of DNA sequence. The template DNA can be prepared with any DNA purification protocol appropriate for the biological sample under study.

**DNA Extraction and RNAase Treatment**

Total DNA was extracted from young leaf tissues of *Trachyspermum roxburghianum* (DC.) Craib (*in vivo* and *in vitro* plant) following CTAB method of Doyle & Doyle (1987) with minor modifications (Ausubel *et al.*, 1995) as described below.

1. Grind the young leaves (5 g) in liquid nitrogen with mortar and pestle and mix with preheated (65° C) 25 ml of CTAB buffer (100mM Tris-HCl ($p^H$ 8), 1.4M NaCl, 20 mM EDTA ($p^H$ 8), 2% CTAB, 0.2% Mercaptoethanol, 10 µl RNA ase).

2. Incubate the mixture at 60° C for 30-60 min. in a water bath. Then add to it an equal volume of chloroform: isoamyl alcohol (24:1). Mix thoroughly and centrifuge at 10000 rpm for 10 min. at 4°C in a refrigerated centrifuge (Hitachi, Himac CR21, Japan).

3. Transfer the supernatent (aqueous phase) from top of the tube to a new centrifuge tube and then add two-third volume of ice cold isopropyl alcohol and mix by gentle inversions.

4. After incubating the mixture at –20° C; for 20 min. to enhance the precipitation of DNA, centrifuge at 10000 rpm for 15 min. at 10° C.

5. Discard the supernatent and invert the tube on a paper towel for 2 min. Then rehydrate the pellet in 15 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, $p^H$ 8).

6. Add 10 µl of RNA ase and incubate at 37° C for 30 min.

7. Centrifuge at 10000 rpm for 10 min. at 10° C after thorough but gentle mixing with equal volume of phenol ($p^H$ 7).

8. Add equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) to the supernatent, mix gently and centrifuge at the same conditions as in step 7.

9. Take the aqueous phase and add equal volume of chloroform: isoamyl alcohol (24:1) and centrifuge at 10000 rpm for 10 min.

10. To the supernatent, add one-tenth volume of 3M sodium acetate ($p^H$ 5.2), add 2.5 ml of absolute ethanol and incubate at –20° C for 1 h. or at –70° C for 30 min.

11. Centrifuge and wash the pellets in 70% ethanol. Air dry the pellets and dissolve in double distilled water and estimate the yield.

DNA was quantified and estimated spectrometrically (Shimadzu, UV 160A, Japan). 15 µl of isolated DNA is diluted in 3 ml of water and its optical density at wavelength 260 nm was noted.

From the data obtained, the quantity of DNA is calculated using the formula:

Quantity of DNA (in micrograms) = $A_{260}$ x quantity of DNA which corresponds to optical density one, where $A_{260}$ is the optical density of the given sample at 260 nm UV.

## Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

The arbitrarily primed PCR (AP-PCR) is a modification of the PCR that generates informative genomic fingerprinting. AP-PCR combines PCR and primers of arbitrary sequence to amplify genomic DNA and produce a fingerprint. For the present reaction, 27 different oligonucleotide primers of OPA series and seven random primers of OPB, OPC and OPD series, Operon Technologies Inc., Almeda, USA was used. The sequences of primers are given in Table 11. The PCR was performed in 25 μ l reaction mixture containing 3 μ l of genomic DNA, 3.75 μ l of the four dNTPs, 2 μ l of each primer, 0.33 μ l units of Taq polymerase, 2.5 μl of 10x reaction buffer (10mM 3-Tris (hydroxymethyl) aminopropane sulphonic acid ($p^H$ 8), 1.5 μ l $MgCl_2$, 50 mM KCl) and double distilled water (11.92 μ l). The reaction mixtures were overlaid with 15 μl of mineral oil. The PCR used was MJ Research PTC-100. Amplification was performed in an omnigene thermal cycler under programmed cycling conditions as follows: 1 cycle of 5 min. at 94° C, 33 cycles of 1 min. at 94° C, 1 min. at 40° C and 1 min. at 72° C, 1 cycle at 72° C for 15 min. and at $15^0$ C for 1 h. 10 μl of each reaction product was subjected to electrophoresis (99V for 2 hours) in a 1.5% agarose gel in 1 x TBE (89 mM Trisborate, 10 mM EDTA $p^H$ 8) and the DNA bands were stained by 6 μl of ethidium bromide. The gels were then photographed on an UV transilluminator. 100 bp ladder (Genei, Bangalore), EcoR I + Hind III double digest of the λ phage DNA (New England, Biolab) were used as the molecular weight standard.

## Essential Oil Analysis

### A. Essential Oil Extraction

The fresh aerial plant parts of *T. roxburghianum* (DC.) Craib (both *in vivo* and *in vitro*) were collected at the time of flowering separately. Flaked

and homogenized plant materials were hydro distilled separately in a Clevenger apparatus for 4 h. at 100° C. The quantity of the essential oil was measured and the isolated oil was dried over anhydrous sodium sulphate and stored in small amber coloured bottles at 4-6° C.

## B. Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS of the essential oil was carried out on a Shimadzu QP-5050 instrument at 700V at 250° C. GC Column: ULBON HR-1, fused silica capillary 0.25 mm x 50 m with film thickness 0.25 μ; carrier gas; Helium; flow rate 1.5 ml/min., Temperature programme: Initial temperature was 80° C for 1 min. and then heated at the rate of 5° C per min. to 250° C. Mass spectral identification was based on the associated computerized data.

## C. Chemotaxonomic Evaluation

The data obtained from the qualitative analysis of both *in vitro* and *in vivo* developed 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 & Sneath (1963).

$$CS = \frac{\text{Number of similar components}}{\text{Total number of components}} \times 100$$

# RESULTS

Sreeranjini K. “Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)” Thesis. Department of Botany, University of Calicut, 2004

# RESULTS

## Micropropagation

The MS medium was used with varied hormonal combinations for initiating multiple shoot cultures. Among the different explants used (leaves, nodes, internodes, inflorescence axis, flower, root) positive response was exhibited by nodes, flower and axis of inflorescence.

For the induction of multiple shoot regeneration, explants were inoculated in the medium with combinations of auxins and cytokinins. The nodal segments (Fig. 1) were cultured on MS medium (Table 1) supplemented with different concentrations of BA with IAA, BA with 2,4-D, KIN with NAA, and 2,4-D alone (Table 3). The multiple shoot initiation was noticed in the medium with 1 mg/l BA and 7 mg/l IAA (Figs. 5, 6, 15 & 17). About 75% of the cultures produced 4-5 shoots from each explant. The frequency of shoots and percentage of initiation was lower in medium containing 2 mg/l BA and 8 mg/l IAA. About 20% of cultures produced 1-2 shoots per explant (Fig. 18). Lower concentrations of BA along with IAA were also used [BA (0.1 mg/l) & IAA (0.1 mg/l), BA (1 mg/l) & IAA (0.2 mg/l), BA (1.5 mg/l) & IAA (0.5 mg/l), BA (2 mg/l) & IAA (0.5 mg/l), BA (1 mg/l) & IAA (2 mg/l)]. Positive response was observed only with higher concentration of IAA and lower concentration of BA. In the combination of BA with IAA, culture establishment were in the form of clustered shoots from proliferated callus (Figs. 2-4 & 7) as well as shoot elongation from axillary buds of nodal segments (Fig. 25). The use of KIN (1 mg/l) and NAA (6 mg/l) had little effect since only insignificant callus growth and occasional shoot development was noticed. The nodal cultures obtained from callus were used for further analysis. 2,4-D (2 mg/l) was used in MS medium for callus proliferation in nodal explants. Profuse callusing was observed (Fig. 11) in this medium. These calli on transferring to MS medium fortified with BA (1 mg/l) and IAA (7 mg/l) showed development of multiple shoots. 75% of

cultures showed 2-3 shoots. Using NAA (2 mg/l) fortified in MS medium, callus proliferation was observed (Fig. 10), but comparatively lesser than that with 2,4-D (2 mg/l). About 80% of cultures responded positively. 2,4-D (1 mg/l) with BA (2 mg/l) in MS medium had no effect on nodal explant. Callus proliferation on using BA (1 mg/l) and IAA (7 mg/l) was observed in 80% of the cultures. The callus obtained varied in colour from green (Fig. 2-4), pale yellow (Fig. 12) and also with a violet tinge (Fig. 13) and was nonfriable, whereas callus proliferated in 2,4-D was green (Fig. 11) and nonfriable in nature. The callus initiation from nodal explant showed decreased rate of growth in darkness (Fig. 20). *In vitro* flowering was also observed in the plants regenerated from nodal explants in MS medium containing BA (1 mg/l) and IAA (7 mg/l), both in plants regenerated from axillary buds (Figs. 29 & 30) as well as that from callus (Figs. 8 & 9) (Table 5). The flowers were smaller in size when compared to flowers from the parent plant. These flowers were used for meiotic analysis.

Flowers were used as explants in various combinations of hormones. The callus growth started within two weeks from the basal portion of the floret. Later, green patches were observed all over the florets. Flower explant in BA (2 mg/l) and IAA (8 mg/l) showed callusing but shoot development was rarely observed. The use of KIN (1 mg/l) and NAA (6 mg/l) resulted in little or no response in the *in vitro* development of the flower explant. Flower explant in BA (1 mg/l) and IAA (7 mg/l) showed callusing and shoot development in 60% of cultures (Figs. 36-39).

The inflorescence axis was inoculated in different hormonal combinations. The use of BA (1 mg/l) and IAA (7 mg/l) resulted in profuse callusing in 70% of cultures (Fig. 32). The callusing and shoot initiation was low in IAA (8 mg/l) and BA (2 mg/l) combination. 80% of cultures showed this result. The calli obtained in IAA (7 mg/l) and BA (1 mg/l) produced

multiple shoot initiation later in the same medium (Fig. 33), which also showed *in vitro* flowering (Figs. 34 & 35). Multiple shoot development (Figs. 16, 17, 19) and single shoot (Figs. 21-23) development from proliferated callus of nodal explant, axillary shoot development from nodal explant (Figs. 25-28), callus development from inflorescence axis (Figs. 31 & 32) as well as shoot development from single flower (Figs. 36-39), was observed in MS medium fortified with BA (1 mg/l) and IAA (7 mg/l). Single shoot development from calli of nodal explant showed *in vitro* flowering (Fig. 24). The effect of phytohormones on *in vitro* flowering of various explants are shown in Table 5.

The use of leaf, internode and root in MS medium fortified with BA (1 mg/l) and IAA (7 mg/l) showed negative response in the case of development *in vitro*. There was an inverse relationship between callus age and differentiation. The younger the calli, the better the morphogenic performance. Maximum response was elicited from 2-week old calli, while regeneration was poor from 6 to 7 week old calli. Those remaining in the culture for more than 8 weeks were unable to form shoot buds.

Some of the calli developed from nodes in medium containing IAA (7 mg/l) and BA (1 mg/l), showed rhizogenesis (Fig. 14) after 5 weeks of inoculation but they failed to produce shoots. Rhizogenesis was also observed in calli developed from nodal explants in 2,4-D (2 mg/l) when retained in the medium for about 5 weeks. Rhizogenesis in conjunction with callus formation was observed on MS medium supplemented with NAA (1 mg/l) when retained in the same medium for about 4 weeks.

The clumps of multiple shoots were separated and sub cultured for multiplication (Figs. 40-42). These were further inoculated for rooting on different concentrations of auxin, IAA and IBA. Profuse rooting was observed in MS medium containing IBA (3 mg/l) (Fig. 44). 70% of cultures showed rooting profusely. The rate of cultures showing rooting and the extent of rooting were reduced in cultures containing IBA (5 mg/l) (Fig. 43). Rooting was also observed in medium containing IAA (3 mg/l). The root induction percentage of different auxins are depicted in Table 4. The cultures were inoculated mainly in IBA (3 mg/l) for rooting (Fig. 45), which were later transferred to the sterilized soil: sand mixture (Fig. 46). About 70% field survival was obtained in pots (Fig. 47). The regenerants and the parent plant were subjected to further molecular and cytological analysis for determining the variations that might have developed internally in the cells.

Table 3

Effect of phytohormones on multiple shoot induction

| Material used for inoculation | Auxins (mg/l) | | | Cytokinins (mg/l) | | Effect on explants | % of response |
|---|---|---|---|---|---|---|---|
| | IAA | NAA | 2,4-D | BA | KIN | | |
| Node | - | - | 2 | - | - | Callus | 89 |
| Node | - | 2 | - | - | - | Little callus proliferation | 80 |
| Node | - | - | 1 | 2 | - | No regeneration | - |
| Node | 7 | - | - | 1 | - | Callus and further plant regeneration | 75 |
| Node | 8 | - | - | 2 | - | Callus and further plant regeneration | 20 |
| Node | - | 6 | - | - | 1 | Insignificant callus growth and occasional shooting | 50 |
| Callus | 7 | - | - | 1 | - | Shooting | 75 |

| Material used for inoculation | IAA | NAA | 2,4-D | BA | KIN | Effect on explants | % of response |
|---|---|---|---|---|---|---|---|
| Flower | 7 | - | - | 1 | - | Profuse callus and plant regeneration | 60 |
| Flower | 8 | - | - | 2 | - | Profuse callus, but no plant regeneration | 65 |
| Flower | - | 6 | - | - | 1 | No effect | - |
| Peduncle | 7 | - | - | 1 | - | Callus and Plant regeneration | 70 |
| Peduncle | 8 | - | - | 2 | - | Low callus proliferation and scarce shoot development | 80 |
| Leaf | 7 | - | - | 1 | - | No response | - |
| Internode | 7 | - | - | 1 | - | No response | - |
| Root | 7 | - | - | 1 | - | No response | - |

Table 4

Effect of phytohormones on root induction

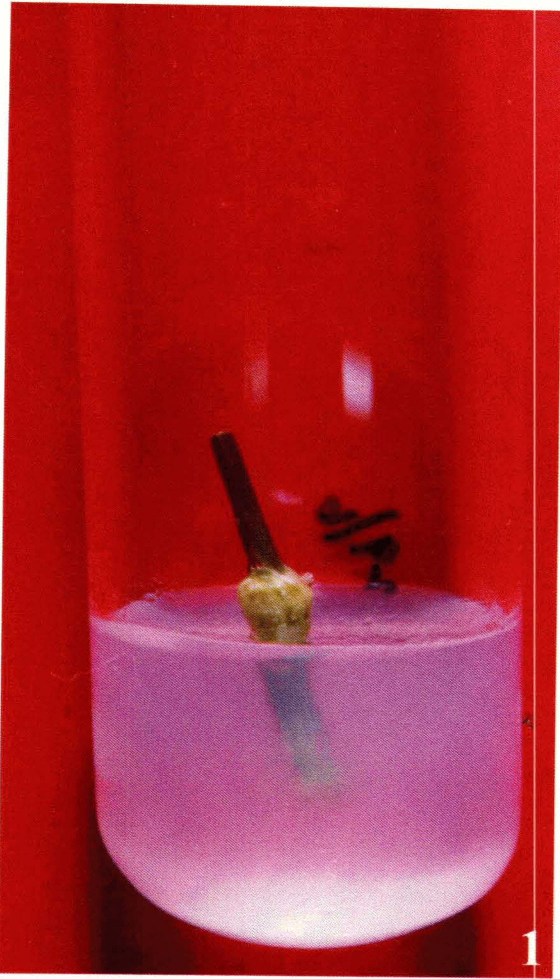
| Material used for inoculation | Auxins (mg/l) | | | | Cytokinins (mg/l) | Effect | % of rooting |
|---|---|---|---|---|---|---|---|
| | NAA | IBA | 2,4-D | IAA | BA | | |
| Multiple shoots | - | 3 | - | - | - | Profuse rooting | 70 |
| Multiple shoots | - | 5 | - | - | - | Reduced rooting | 60 |
| Multiple shoots | - | - | - | 3 | - | Little rooting | 45 |
| Callus | - | - | - | 8 | 2 | Little rooting | 40 |
| Callus | - | - | 2 | - | - | Little rooting | 50 |
| Callus | 1 | - | - | - | - | Little rooting | 55 |

Fig. 1 Nodal explant of *Trachyspermum roxburghianum* (DC.) Craib

Fig. 2 Shoot initiation from callus of nodal explant (1 mg/l BA + 7 mg/l IAA)

Fig. 3 Profuse callusing (1 mg/l BA + 7 mg/l IAA)

Fig. 4 Later developmental stage of profuse callus (1 mg/l BA + 7 mg/l IAA)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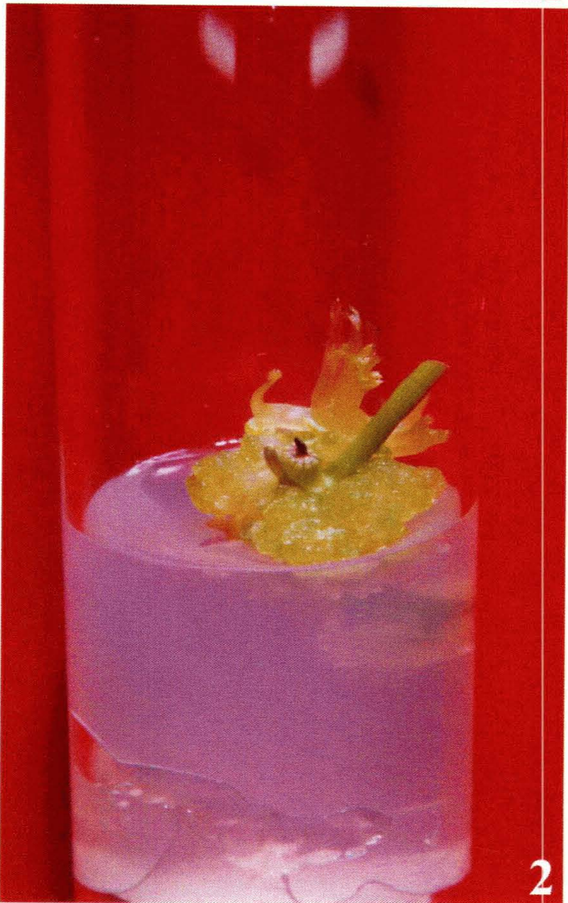
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Figs. 5 & 6 Multiple shoots developed from callus (1 mg/l BA + 7 mg/l IAA)

Fig. 7 Later stages of multiple shoot development in callus (1 mg/l BA + 7 mg/l IAA)

Figs. 8 & 9 Multiple shoots showing initial stages of inflorescence development (1 mg/l BA + 7 mg/l IAA)

5

6

7

8

9

Fig. 10 Callus development in NAA (2 mg/l)

Fig. 11 Callus development in 2,4-D (2 mg/l)

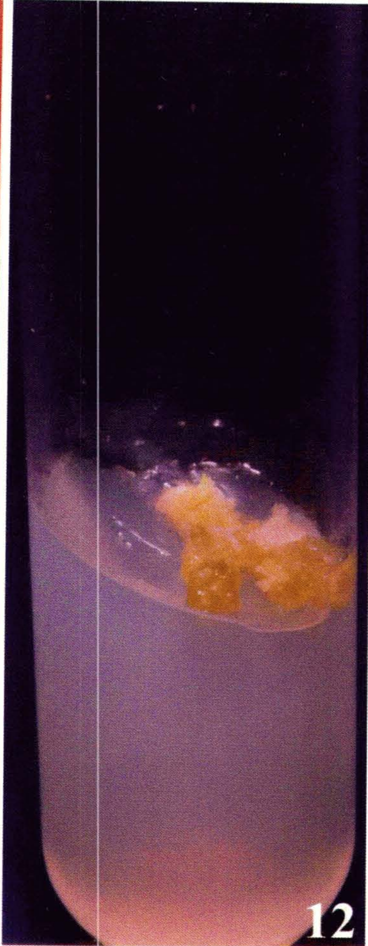
Fig. 12 Callus with a pale yellow colour (1 mg/l BA + 7 mg/l IAA)

Fig. 13 Callus with a violet tinge (1 mg/l BA + 7 mg/l IAA)

Fig. 14 Callus showing root development

Fig. 15 Callus with initiation of multiple shoots (1 mg/l BA + 7 mg/l IAA)

10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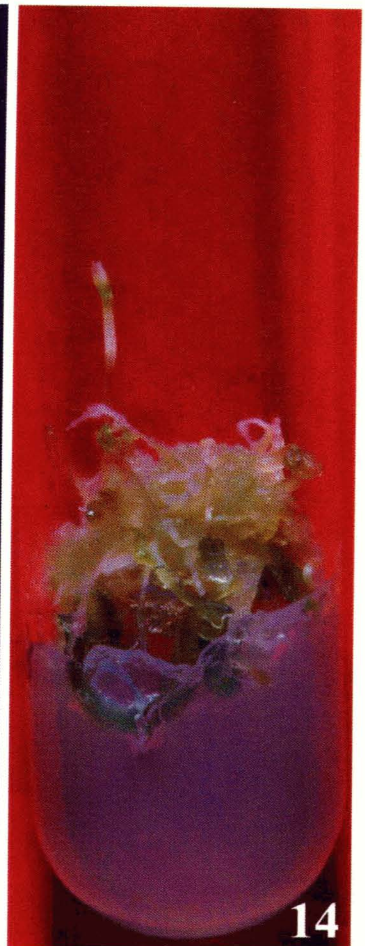
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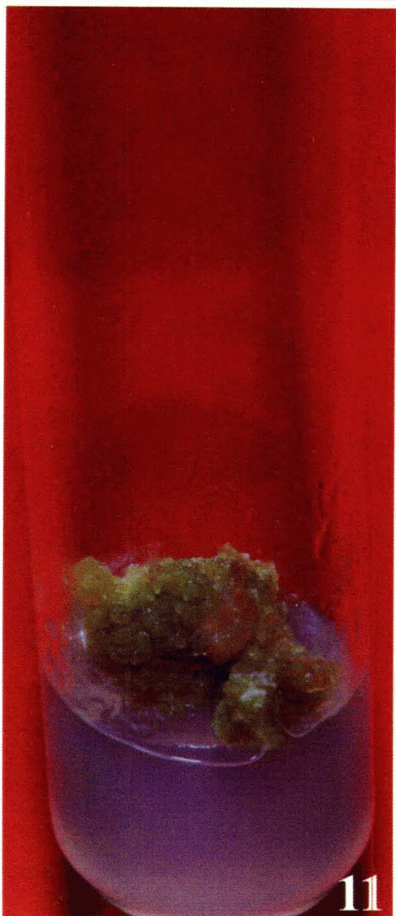
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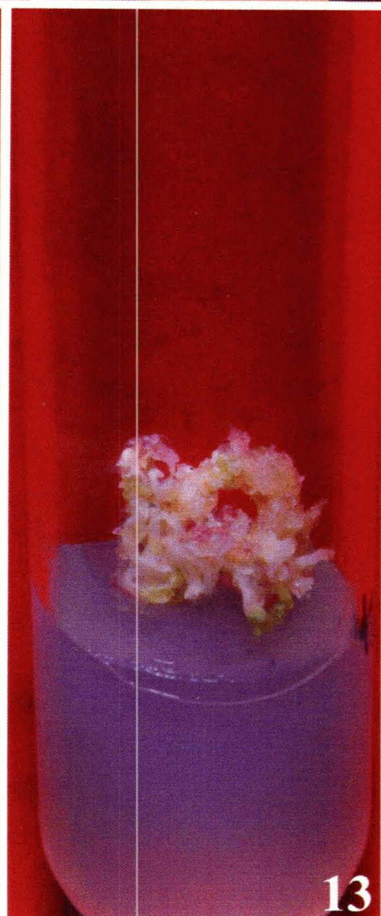

13

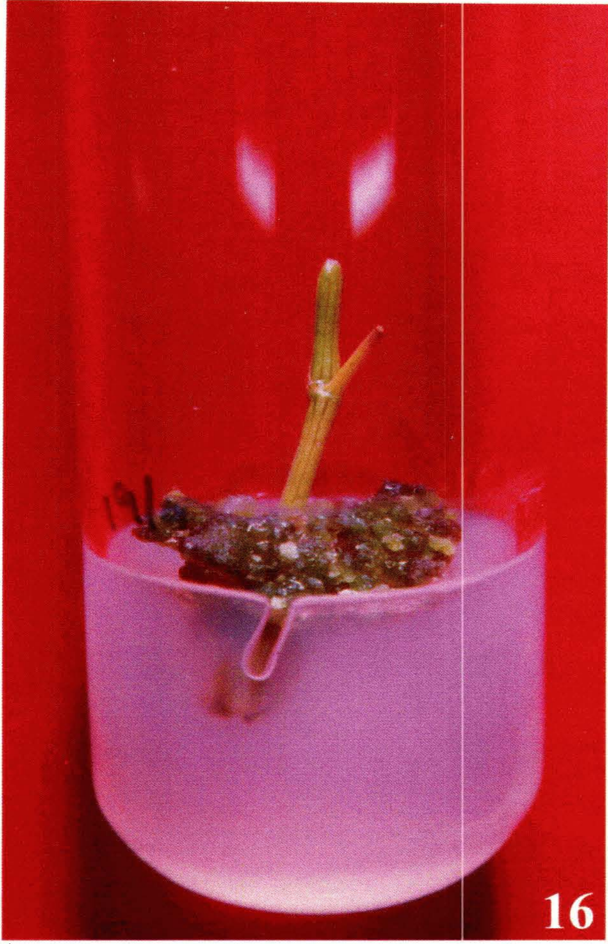
15

Fig. 16 Callus proliferation in nodal explant (1 mg/l BA + 7 mg/l IAA)

Fig. 17 Axillary bud development along with multiple shoots from callus (1 mg/l BA + 7 mg/l IAA)

Fig. 18 Multiple shoot development from callus of nodal explant (2 mg/l BA + 8 mg/l IAA)

Fig. 19 Shoot elongation (1 mg/l BA + 7 mg/l IAA)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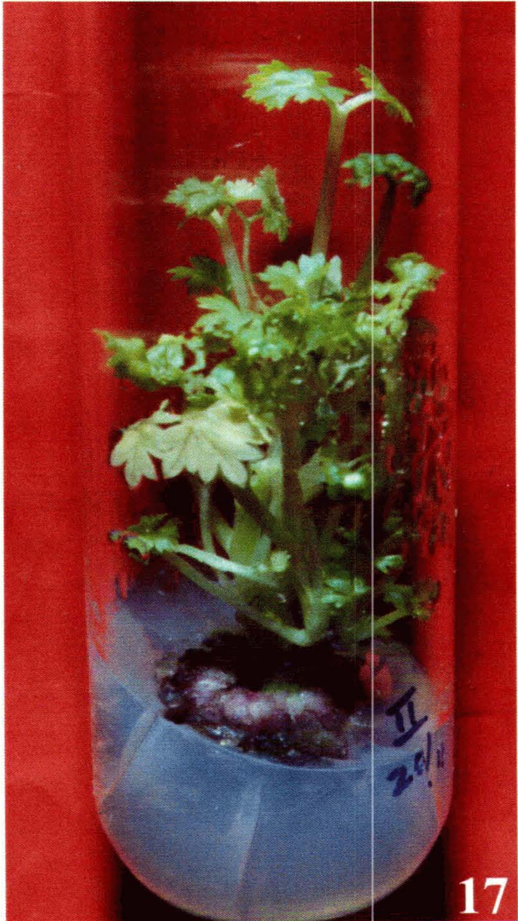
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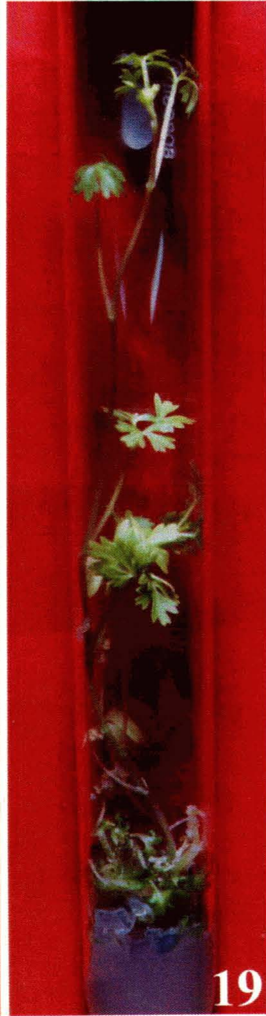
19

Fig. 20 Decreased callusing under darkness (1 mg/l BA + 7 mg/l IAA)

Figs. 21 & 22 Stages of single shoot development (1 mg/l BA + 7 mg/l IAA)

Fig. 23 Single shoot from callus of nodal explant (1 mg/l BA + 7 mg/l IAA)

Fig. 24 Single shoot from callus of nodal explant showing *in vitro* flowering (1 mg/l BA + 7 mg/l IAA)

20

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Fig. 25 Axillary buds developing from nodal explant (1 mg/l BA + 7 mg/l IAA)

Figs. 26 – 28 Developmental stages of axillary buds from nodal explant (1 mg/l BA + 7 mg/l IAA)

Figs. 29 & 30 Axillary bud showing *in vitro* flowering (1 mg/l BA + 7 mg/l IAA)

25

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71 P

Fig. 31 Callus initiation from inflorescence axis (1 mg/l BA + 7 mg/l IAA)

Fig. 32 Callus at developing stages from inflorescence axis (1 mg/l BA + 7 mg/l IAA)

Fig. 33 Shoot initiation from callus of inflorescence axis – later stage (1 mg/l BA + 7 mg/l IAA)

Figs. 34 & 35 Multiple shoots from callus of inflorescence axis showing *in vitro* flowering (1 mg/l BA + 7 mg/l IAA)

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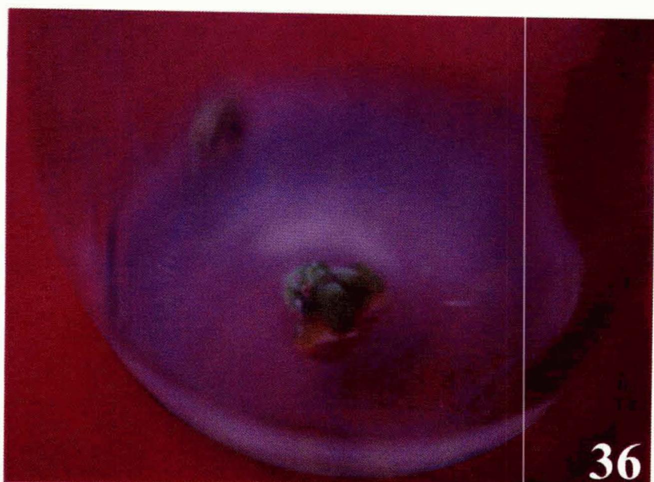
Fig. 36 Callus development from single flower as initial explant
(1 mg/l BA + 7 mg/l IAA)

Figs. 37 & 38 Stages of shoot development from callus of single flower
(1 mg/l BA + 7 mg/l IAA)

Fig. 39 Shoot developed from callus of single flower (1 mg/l BA + 7 mg/l IAA)


36

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39

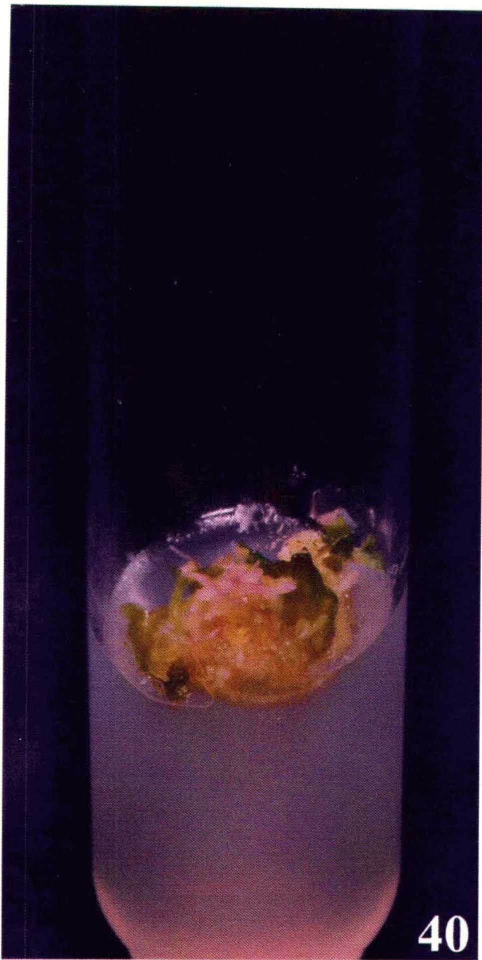
Fig. 40 Initial stages of shoot development from callus of nodal explant separated by sub culture (1 mg/l BA + 7 mg/l IAA)

Fig. 41 Shoots developing from, sub cultured callus with multiple shoots (1 mg/l BA + 7 mg/l IAA)

Fig. 42 Multiple shoots developed in sub culture (1 mg/l BA + 7 mg/l IAA)

Fig. 43 Rooting of separated shoots (5 mg/l IBA)

Fig. 44 Rooting of separated shoots (3 mg/l IBA)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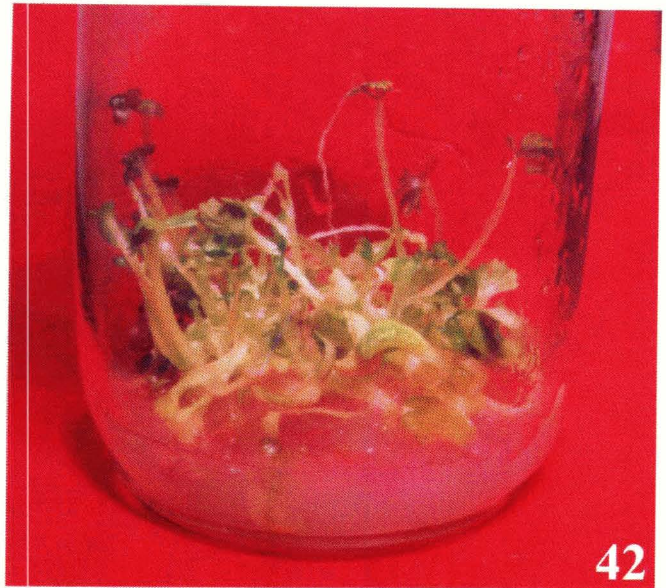
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43

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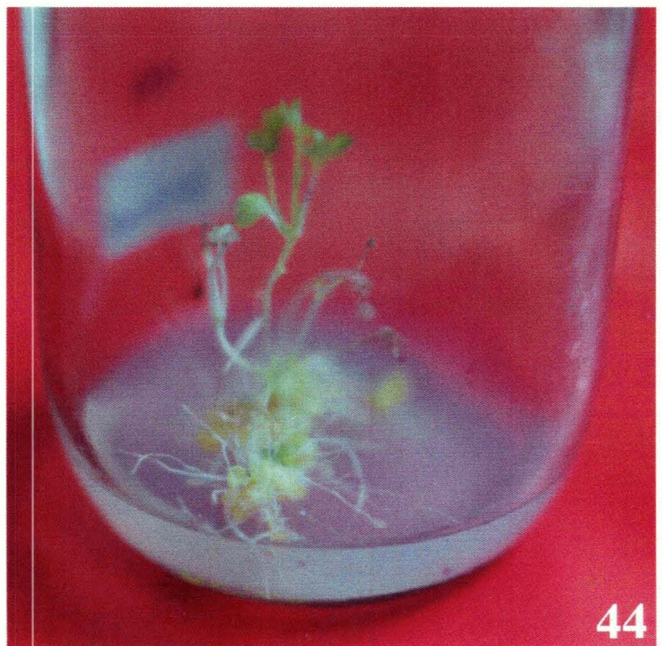
44

Fig. 45 Single plantlet with developed roots (3 mg/l IBA)

Fig. 46 Plantlet at hardening stage

Fig. 47 Single plant developed

45

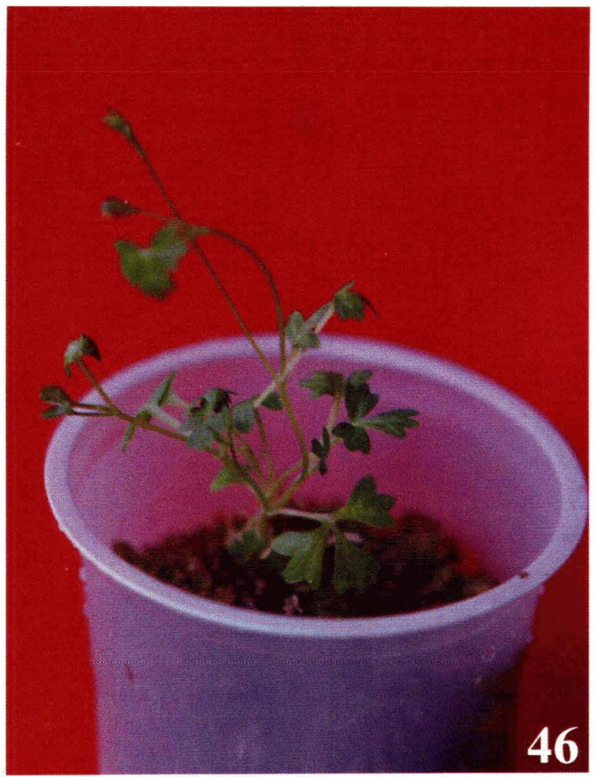
46

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Table 5

Effect of phytohormones on *in vitro* flowering

| Material used for inoculation | Auxin (IAA) (mg/l) | Cytokinin (BA) (mg/l) | Effect on explant | % of response |
|---|---|---|---|---|
| Callus developed from node | 7 | 1 | *In vitro* flowering from regenerated shoots | 87 |
| Axillary bud from node | 7 | 1 | *In vitro* flowering from regenerated shoots | 80 |
| Peduncle | 7 | 1 | *In vitro* flowering from regenerated shoots | 65 |

## Cytological Analysis

### Mitosis

The present study is aimed to examine the karyomorphological changes in the chromosome complement of the *in vivo* and *in vitro* plants of *T. roxburghianum* (DC.) Craib and to know the cytological basis of these variations. The ploidy levels of both the donor plant and the variant were invariably tetraploid ( $2n = 4x = 44$). Octaploid and diploid cells were observed in the callus amidst tetraploid cells. Chromosome sets of the parent plant, callus cells showing the most frequent tetraploid condition, and the less frequent diploid and octaploid conditions, the *in vitro* plant are given in Figs. 1 .1-5 .1. The chromosome number of the calli observed were $2n = 2x = 22$ and $2n = 8x = 88$ amidst the normal chromosome number,

i.e., 2n = 4x = 44. The cells containing the tetraploid number was more when compared to the other two types of cells observed in the calli. The number of secondary constrictions also showed variations. The parent plant, tetraploid callus and the variant plant showed 4 chromosomes with secondary constriction, whereas the number of chromosomes with secondary constriction was found to be 2 in the diploid callus and 8 in the octaploid callus.

Changes in chromosome length, disparity index, variation coefficient and total forma percentage was noticed. The total chromosome length of the parent plant was noticed as 43.0762 μ m, that of the variant was 28.8582 μ m. The total chromosome lengths of diploid, tetraploid and octaploid calli were 16.6340 μ m, 28.0124 μ m and 54.1692 μ m respectively. The disparity indices observed in the parent and variant were 43.0986 and 30.4527 respectively. The diploid, tetraploid and octaploid calli recorded disparity indices of 29.6822, 38.7633 and 64.9929 respectively. The total forma percentages of the parent, variant, diploid, tetraploid and octaploid calli were estimated as 43.0924, 40.6484, 45.2218, 39.4654 and 45.5318 respectively. The total length, disparity index, variation coefficient and total forma percentage were observed to be greater in the parent plant when compared to the *in vitro* plant. The average chromosome length of the parent was greater than the variant. The average length of the chromosomes in the diploid callus was greater than tetraploid and octaploid calli. The total chromosome length, disparity indices etc. were found to be decreasing in the order octaploid, tetraploid and diploid callus. Total forma percentage was found to be decreasing in the order octaploid, diploid and tetraploid respectively.

Karyotype formulae deduced for the karyotypes of the parent, *in vitro* plant, calli with diploid, tetraploid and octaploid conditions showed variation in the type of chromosomes.

**Mitotic metaphase stages of *in vivo* and *in vitro* plants of *Trachyspermum roxburghianum***

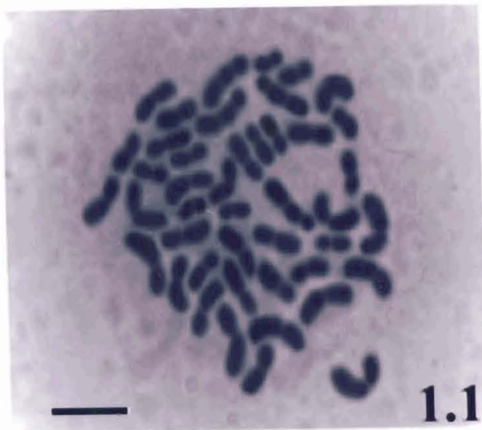
Fig. 1.1 *In vivo* plant (bar = 1.5 μm)

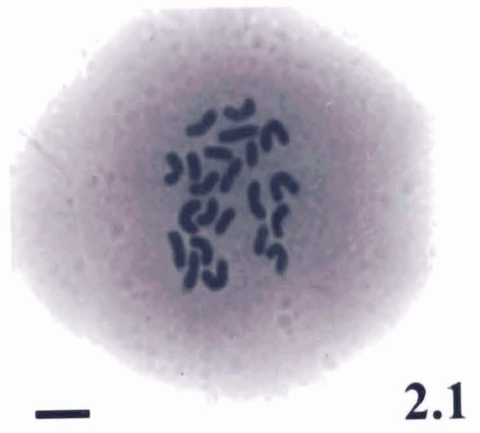
Fig. 2.1 Diploid callus (bar = 1.5 μm)

Fig. 3.1 Tetraploid callus (bar = 1.5 μm)

Fig. 4.1 Octaploid callus (bar = 1.5 μm)

Fig. 5.1 *In vitro* plant (bar = 1.5 μm)


1.1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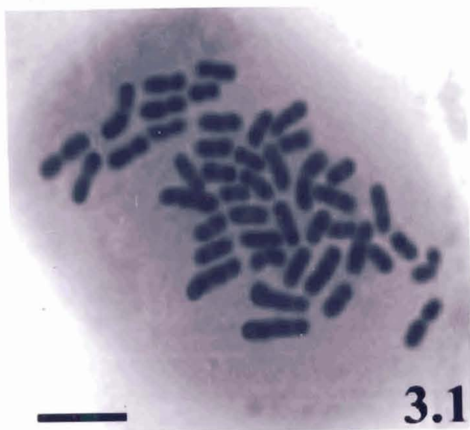
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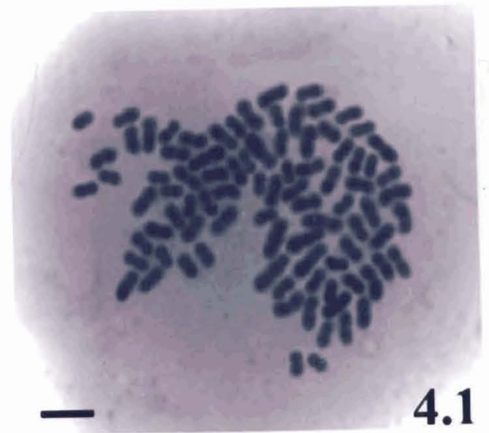
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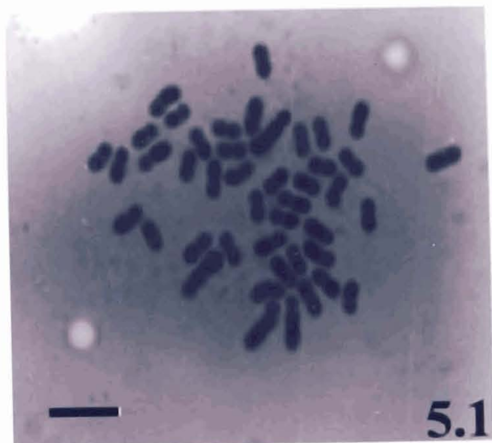

5.1

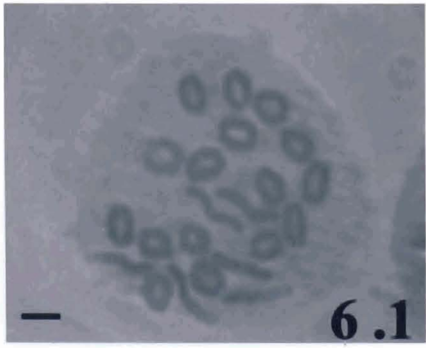
**Meiotic stages of *in vivo* and *in vitro* plants of *Trachyspermum roxburghianum***

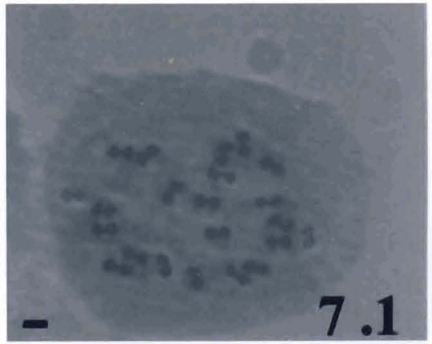
Fig. 6 .1 Diakinesis stage of *in vivo* PMC (bar = 1.5 μm)

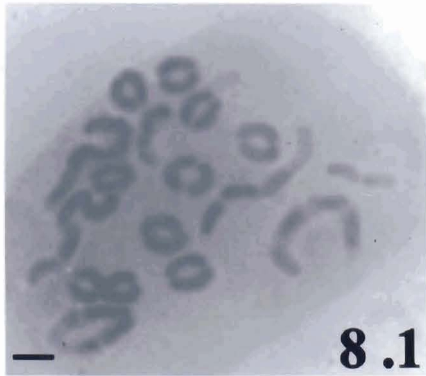
Fig. 7 .1 Metaphase I of *in vivo* PMC (bar = 1 μm)

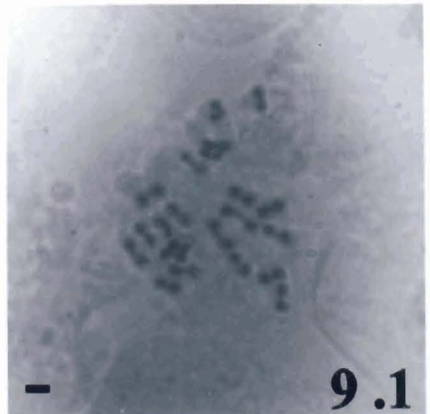
Fig. 8 .1 Diakinesis stage of *in vitro* PMC (bar = 1.5 μm)

Fig. 9 .1 Metaphase I of *in vitro* PMC (bar = 1 μm)









*Trachyspermum roxburghianum*

**Karyotype Images of Parent Plant (2n = 4x = 44)**

**1 .1 D**

**Fig. 1 .1 A Computer Scanned Original Image (Bar = 1 .5 μ m)**
**Fig. 1 .1 B Inverted Image**
**Fig. 1 .1 C Resolved Image**
**Fig. 1 .1 D Karyogram**

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*Trachyspermum roxburghianum*

**Karyotype Images of Callus - Diploid Cell (2n = 2x = 22)**

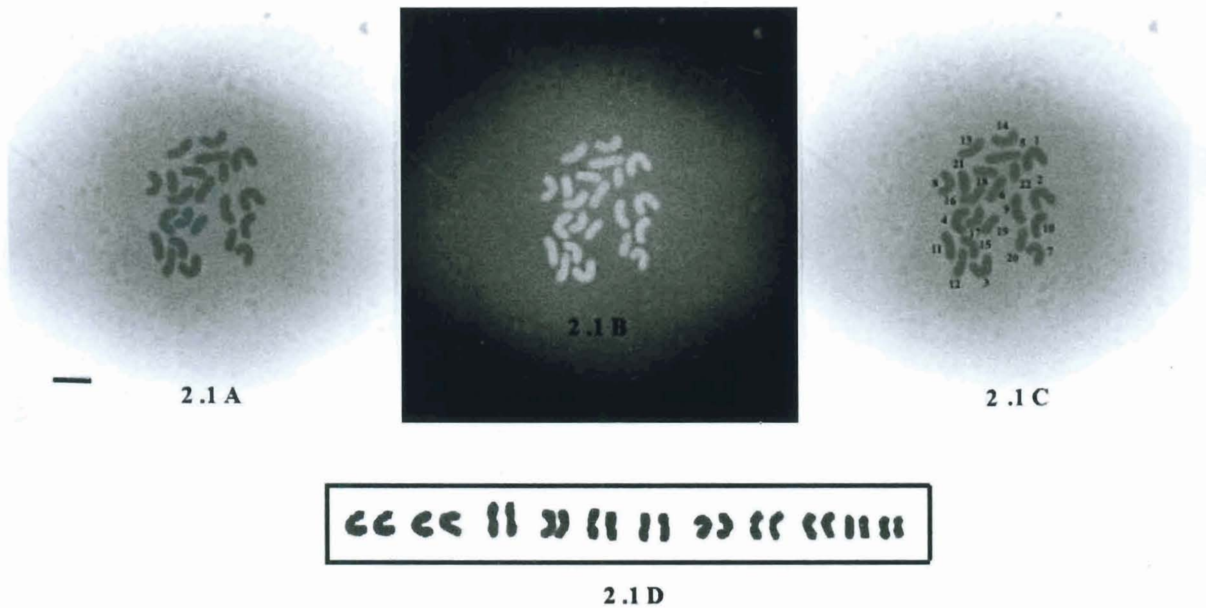
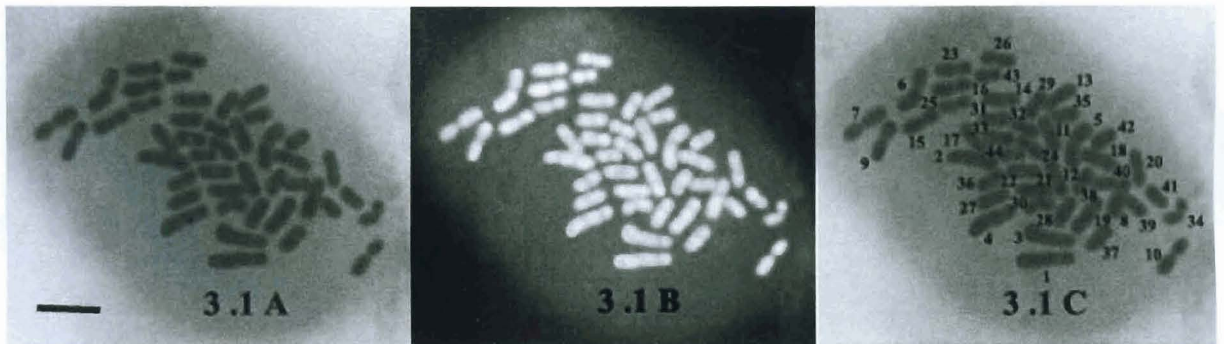


**Fig. 2 .1 A Computer Scanned Original Image (Bar = 1 μ m)**
**Fig. 2 .1 B Inverted Image**
**Fig. 2 .1 C Resolved Image**
**Fig. 2 .1 D Karyogram**

5

## *Trachyspermum roxburghianum*

**Karyotype Images of Callus - Tetraploid Cell (2n = 4x = 44)**

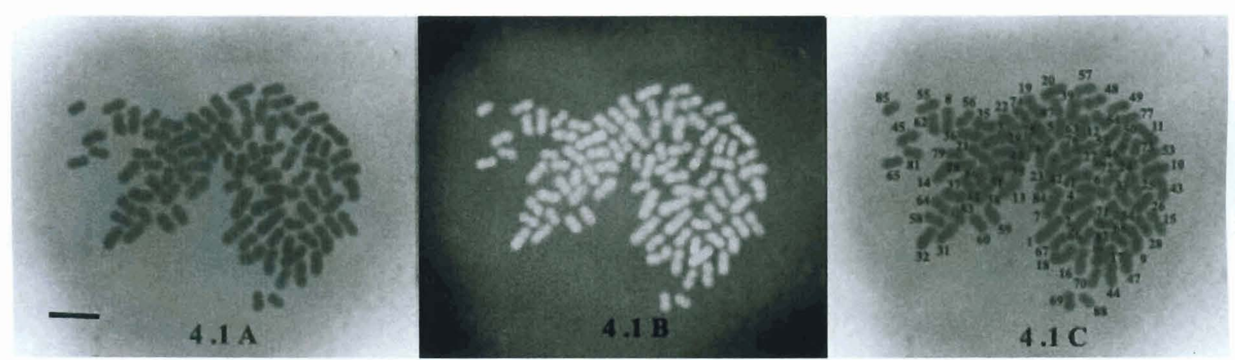


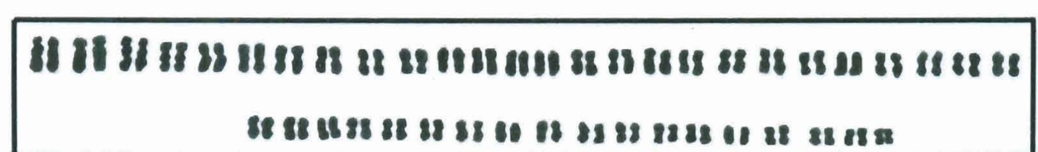
3 .1 D

**Fig. 3 .1 A Computer Scanned Original Image (Bar = 1 μ m)**
**Fig. 3 .1 B Inverted Image**
**Fig. 3 .1 C Resolved Image**
**Fig. 3 .1 D Karyogram**

## *Trachyspermum roxburghianum*

**Karyotype Images of Callus - Octaploid Cell (2n = 8x = 88)**



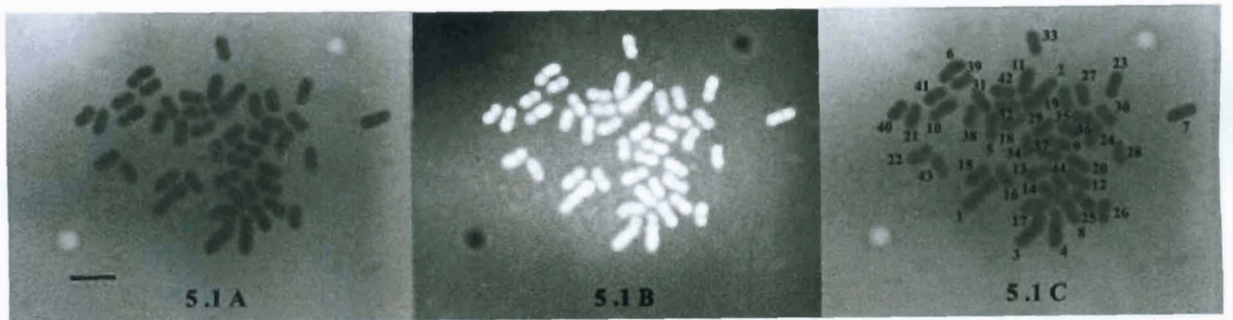



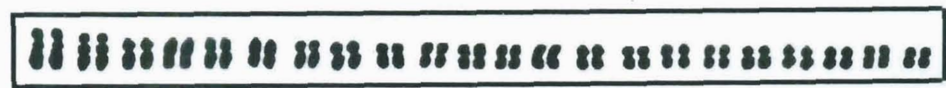
4 .1 D

**Fig. 4 .1 A Computer Scanned Original Image (Bar = 2 μ m)**
**Fig. 4 .1 B Inverted Image**
**Fig. 4 .1 C Resolved Image**
**Fig. 4 .1 D Karyogram**

*Trachyspermum roxburghianum*

Karyotype Images of *In Vitro* Plant (2n = 4x = 44)



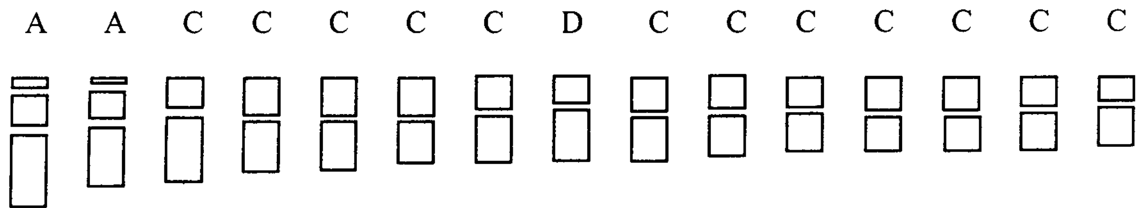



5.1 D

**Fig. 5.1 A Computer Scanned Original Image (Bar = 1 μ m)**
**Fig. 5.1 B Inverted Image**
**Fig. 5.1 C Resolved Image**
**Fig. 5.1 D Karyogram**

*Trachyspermum roxburghianum*

**Idiogram of Parent Plant ( n = 22)**



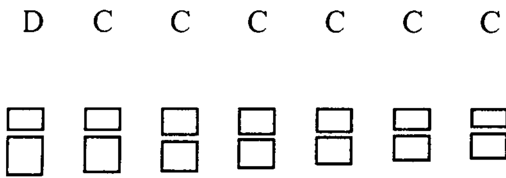




Fig. 1 .1 E

*Trachyspermum roxburghianum*

**Idiogram of Callus - Diploid Cell (n = 11)**

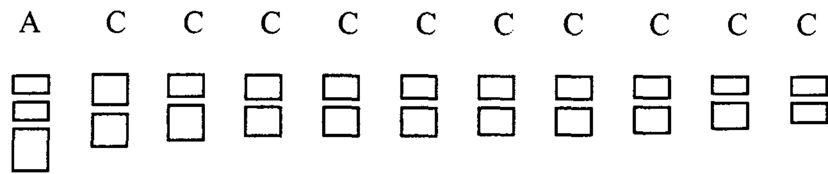



Fig. 2 .1 E

*Trachyspermum roxburghianum*

**Idiogram of Callus - Tetraploid Cell (n = 22)**

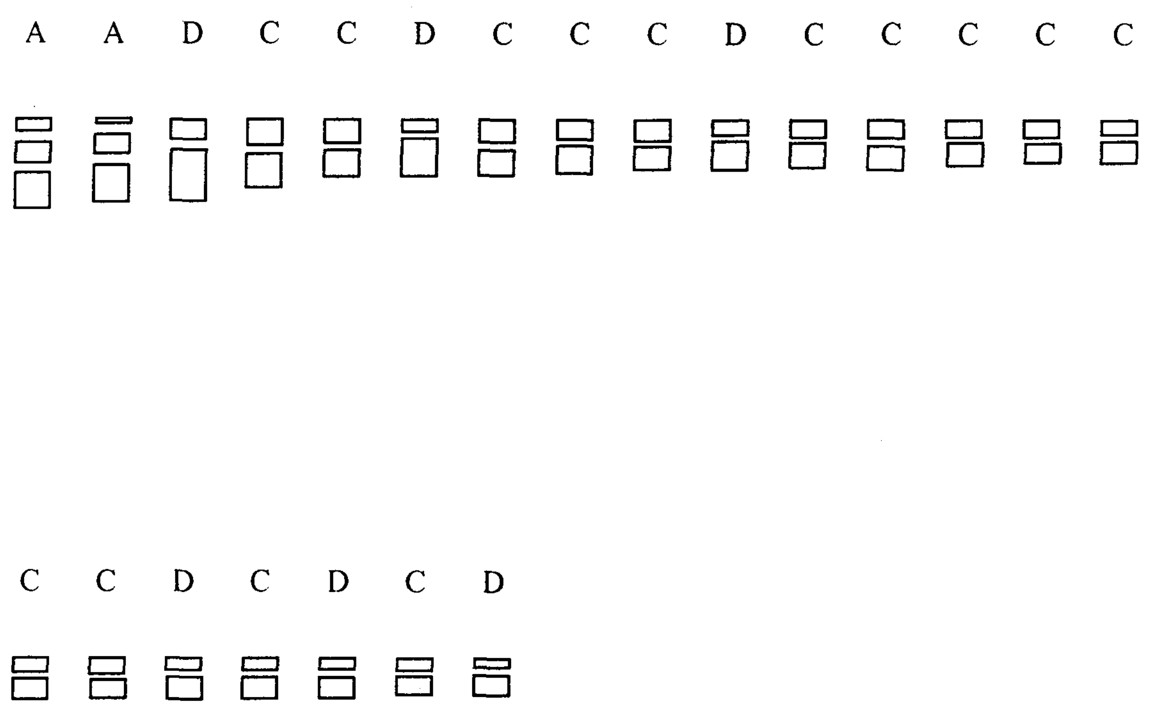



Fig. 3 .1 E

***Trachyspermum roxburghianum***

**Idiogram of Callus - Octaploid Cell (n = 44)**

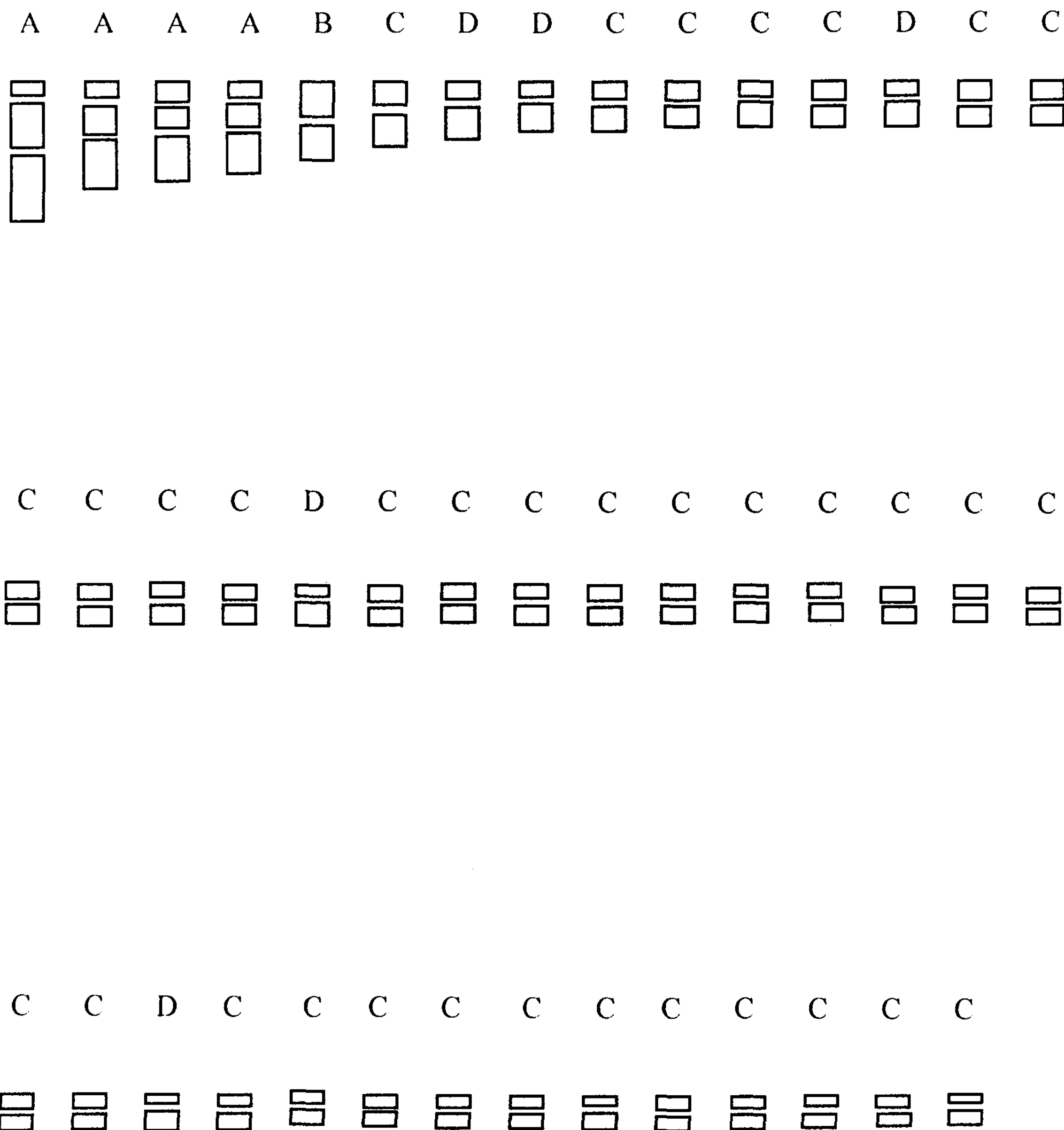
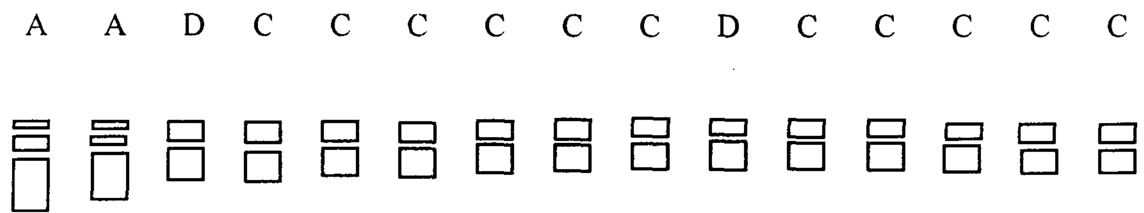



Fig. 4 .1 E

*Trachyspermum roxburghianum*

**Idiogram of *In Vitro* Plant (n = 22)**



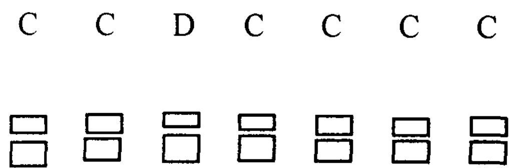




Fig. 5 .1 E

**Diagrammatic representation of the chromosome types observed in the present study obtained from parent plant, *in vitro* plant and three types of calli.**

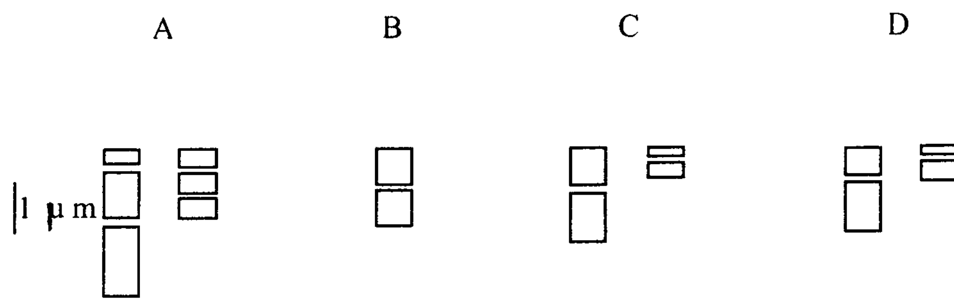



Fig. 6 .1E

The general description of the common chromosome types found in the parent plant, *in vitro* plant and different calli cells are given below.

Type A: Chromosome with secondary constriction ranging from 1.8089 μ m to 0.8947 μ m with nearly median / nearly submedian (-) / nearly submedian (+) primary constriction.

Type B: Chromosome with length of 1.0258 μ m with median primary constriction.

Type C: Chromosome with length ranging from 1.2178 μ m to 0.3838 μ m with nearly median primary constriction.

Type D: Chromosome with length ranging from 1.0833 μ m to 0.4402 μ m with nearly submedian (-) primary constriction.

The chromosomal nomenclatures were depicted according to the system followed Abraham & Prasad (Table 2).

The karyotype formula of the parent plant was $A_4C_{36}D_4$, of the variant was $A_4C_{34}D_6$, of the diploid callus was $A_2C_{20}$, of the tetraploid callus was $A_4C_{28}D_{12}$ and of the octaploid callus was $A_8B_2C_{68}D_{10}$. Diagrammatic representation of different chromosome types observed in the present investigation is shown in Fig. 6 .1E. Detailed karyotype description (Tables 6 -10), microphotographs of mitotic metaphase stages (Fig. 1. 1 – 5. 1), computer scanned images of karyotypes (Figs. 1 .1A, 1 .1B, 1 .1C – 5 .1A, 5 .1B, 5 .1C), karyograms (Figs. 1 .1D – 5 .1D) and idiograms (Figs. 1 .1E – 5 .1E) of the *in vivo* plant, diploid, tetraploid, octaploid calli and the *in vitro* plant are shown below.

*Trachyspermum roxburghianum* **(DC.) Craib (2n = 4x = 44 = $A_4C_{36}D_4$)**

**Parent Plant**

| | |
|---|---|
| Normal Somatic Chromosome Number | 44 |
| Chromosome Pair with Secondary Constriction | 2 |
| Total Chromosome Length | 43.0762 μ m |
| Range of Chromosome Length | 1.5323 μ m - 0.6093 μ m |
| Average Chromosome Length | 0.9790 μ m |
| Disparity Index | 43.0986 |
| Variation Coefficient | 23.0204 |
| TF Value | 43.0924 |

Table 6

Detailed Karyomorphometrical Data of *Trachyspermum roxburghianum* (Parent Plant)

| Chromos-ome Type | No.of Pairs | Total Length (μ m) | s (μ m) | l (μ m) | $R_1$ (s/l) | $R_2$ (l/s) | $I_1$ (s/c%) | $I_2$ (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.5323 | 0.5809 | 0.9514 | 0.6106 | 1.6378 | 37.9103 | 62.0897 | nm |
| A* | 1 | 1.2663 | 0.4777 | 0.7886 | 0.6058 | 1.6508 | 37.7241 | 62.2759 | nm |
| C | 1 | 1.2178 | 0.5187 | 0.6991 | 0.7420 | 1.3478 | 42.5932 | 57.4068 | nm |
| C | 1 | 1.2068 | 0.5241 | 0.6827 | 0.7677 | 1.3026 | 43.4289 | 56.5711 | nm |
| C | 1 | 1.1986 | 0.5339 | 0.6647 | 0.8032 | 1.2450 | 44.5436 | 55.4564 | nm |
| C | 1 | 1.0994 | 0.5296 | 0.5698 | 0.9294 | 1.0759 | 48.1717 | 51.8283 | nm |
| C | 1 | 1.0974 | 0.4698 | 0.6276 | 0.7486 | 1.3359 | 42.8103 | 57.1897 | nm |
| D | 1 | 1.0833 | 0.3942 | 0.6891 | 0.5721 | 1.7481 | 36.3888 | 63.6112 | nsm(-) |
| C | 1 | 1.0705 | 0.4685 | 0.6020 | 0.7782 | 1.2850 | 43.7646 | 56.2354 | nm |
| C | 1 | 1.0111 | 0.4567 | 0.5544 | 0.8238 | 1.2139 | 45.1686 | 54.8314 | nm |
| C | 1 | 0.9417 | 0.4225 | 0.5192 | 0.8138 | 1.2289 | 44.8657 | 55.1343 | nm |
| C | 1 | 0.9333 | 0.4610 | 0.4723 | 0.9761 | 1.0245 | 49.3946 | 50.6054 | nm |
| C | 1 | 0.9291 | 0.4572 | 0.4719 | 0.9688 | 1.0322 | 49.2089 | 50.7911 | nm |
| C | 1 | 0.9198 | 0.4133 | 0.5065 | 0.8160 | 1.2255 | 44.9337 | 55.0663 | nm |
| C | 1 | 0.8715 | 0.3381 | 0.5334 | 0.6339 | 1.5776 | 38.7952 | 61.2048 | nm |
| D | 1 | 0.8390 | 0.3133 | 0.5257 | 0.5960 | 1.6779 | 37.3421 | 62.6579 | nsm(-) |
| C | 1 | 0.7995 | 0.3072 | 0.4923 | 0.6240 | 1.6025 | 38.4240 | 61.5760 | nm |
| C | 1 | 0.7857 | 0.3700 | 0.4157 | 0.8901 | 1.1235 | 47.0918 | 52.9082 | nm |
| C | 1 | 0.7581 | 0.3572 | 0.4009 | 0.8910 | 1.1223 | 47.1178 | 52.8822 | nm |
| C | 1 | 0.7156 | 0.3323 | 0.3833 | 0.8669 | 1.1535 | 46.4366 | 53.5634 | nm |
| C | 1 | 0.6520 | 0.2979 | 0.3541 | 0.8413 | 1.1887 | 45.6902 | 54.3098 | nm |
| C | 1 | 0.6093 | 0.2572 | 0.3521 | 0.7305 | 1.3690 | 42.2124 | 57.7876 | nm |

S: Short Arm

L: Long Arm

$R_1$ & $R_2$: Arm Ratios

$I_1$ & $I_2$: Centromeric Indices

* Chromosome Pair with Secondary Constriction

*Trachyspermum roxburghianum* (DC.) Craib ($2n = 2x = 22 = A_2 C_{20}$)

**Callus - Diploid Condition**

| | |
|---|---|
| Somatic Chromosome Variant Number | 22 |
| Chromosome Pair with Secondary Constriction | 1 |
| Total Chromosome Length | 16.6340 μ m |
| Range of Chromosome Length | 1.0466 μ m - 0.5675 μ m |
| Average Chromosome Length | 0.7561 μ m |
| Disparity Index | 29.6822 |
| Variation Coefficient | 17.1408 |
| TF Value | 45.2218 |

Table 7

Detailed Karyomorphometrical Data of *Trachyspermum roxburghianum* (Callus - Diploid)

| Chromosome Type | No.of Pairs | Total Length (μ m) | s (μ m) | l (μ m) | $R_1$ (s/l) | $R_2$ (l/s) | $I_1$ (s/c%) | $I_2$ (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.0466 | 0.4597 | 0.5869 | 0.7833 | 1.2767 | 43.9232 | 56.0768 | nm |
| C | 1 | 0.9021 | 0.4447 | 0.4574 | 0.9722 | 1.0286 | 49.2961 | 50.7039 | nm |
| C | 1 | 0.8021 | 0.3164 | 0.4857 | 0.6514 | 1.5351 | 39.4465 | 60.5535 | nm |
| C | 1 | 0.7643 | 0.3494 | 0.4149 | 0.8421 | 1.1875 | 45.7150 | 54.2850 | nm |
| C | 1 | 0.7475 | 0.3402 | 0.4073 | 0.8353 | 1.1972 | 45.5117 | 54.4883 | nm |
| C | 1 | 0.7412 | 0.3363 | 0.4049 | 0.8306 | 1.2040 | 45.3724 | 54.6276 | nm |
| C | 1 | 0.7143 | 0.3332 | 0.3811 | 0.8743 | 1.1438 | 46.6471 | 53.3529 | nm |
| C | 1 | 0.7133 | 0.3331 | 0.3802 | 0.8761 | 1.1414 | 46.6984 | 53.3016 | nm |
| C | 1 | 0.6890 | 0.3192 | 0.3698 | 0.8632 | 1.1585 | 46.3280 | 53.6720 | nm |
| C | 1 | 0.6291 | 0.2562 | 0.3729 | 0.6870 | 1.4555 | 40.7248 | 59.2752 | nm |
| C | 1 | 0.5675 | 0.2727 | 0.2948 | 0.9250 | 1.0810 | 48.0529 | 51.9471 | nm |

S: Short Arm

L: Long Arm

$R_1$ & $R_2$: Arm Ratios

$I_1$ & $I_2$: Centromeric Indices

* Chromosome Pair with Secondary Constriction

## *Trachyspermum roxburghianum* (DC.) Craib ($2n = 4x = 44 = A_4C_{28}D_{12}$)

### Callus - Tetraploid Condition

| | |
|---|---|
| Normal Somatic Chromosome Number | 44 |
| Chromosome Pair with Secondary Constriction | 2 |
| Total Chromosome Length | 28.0124 µm |
| Range of Chromosome Length | 0.9975 µm - 0.4402 µm |
| Average Chromosome Length | 0.6366 µm |
| Disparity Index | 38.7633 |
| Variation Coefficient | 24.0699 |
| TF Value | 39.4654 |

Table 8

Detailed Karyomorphometrical Data of *Trachyspermum roxburghianum* (Callus - Tetraploid)

| Chromosome Type | No.of Pairs | Total Length (µm) | s (µm) | l (µm) | $R_1$ (s/l) | $R_2$ (l/s) | $I_1$ (s/c%) | $I_2$ (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 0.9975 | 0.1947 | 0.8028 | 0.2425 | 4.1233 | 19.5188 | 80.4812 | nsm(+) |
| A* | 1 | 0.8947 | 0.3842 | 0.5105 | 0.7526 | 1.3287 | 42.9418 | 57.0582 | nm |
| D | 1 | 0.8772 | 0.2723 | 0.6049 | 0.4502 | 2.2214 | 31.0420 | 68.9580 | nsm(-) |
| C | 1 | 0.8387 | 0.3678 | 0.4709 | 0.7811 | 1.2803 | 43.8536 | 56.1464 | nm |
| C | 1 | 0.7253 | 0.3436 | 0.3817 | 0.9002 | 1.1109 | 47.3735 | 52.6265 | nm |
| D | 1 | 0.7105 | 0.1866 | 0.5239 | 0.3562 | 2.8076 | 26.2632 | 73.7368 | nsm(-) |
| C | 1 | 0.6921 | 0.3329 | 0.3583 | 0.9291 | 1.0763 | 48.1000 | 51.7700 | nm |
| C | 1 | 0.6813 | 0.2896 | 0.3917 | 0.7393 | 1.3526 | 42.5070 | 57.4930 | nm |
| C | 1 | 0.6429 | 0.3132 | 0.3297 | 0.9500 | 1.0527 | 48.7168 | 51.2832 | nm |
| D | 1 | 0.6315 | 0.2342 | 0.3973 | 0.5895 | 1.6964 | 37.0863 | 62.9137 | nsm(-) |
| C | 1 | 0.6099 | 0.2619 | 0.3480 | 0.7526 | 1.3288 | 42.9415 | 57.0585 | nm |
| C | 1 | 0.6088 | 0.2372 | 0.3536 | 0.6708 | 1.4907 | 38.9619 | 58.0815 | nm |
| C | 1 | 0.5884 | 0.2599 | 0.3285 | 0.7912 | 1.2639 | 44.1706 | 55.8294 | nm |
| C | 1 | 0.5436 | 0.2597 | 0.2839 | 0.9148 | 1.0932 | 47.7741 | 52.2259 | nm |
| C | 1 | 0.5349 | 0.2261 | 0.3088 | 0.7322 | 1.3658 | 42.2696 | 57.7304 | nm |
| C | 1 | 0.5177 | 0.2154 | 0.3023 | 0.7125 | 1.4034 | 41.6071 | 58.3929 | nm |
| C | 1 | 0.5151 | 0.2432 | 0.2719 | 0.8944 | 1.1180 | 47.2141 | 52.7859 | nm |
| D | 1 | 0.5072 | 0.1910 | 0.3162 | 0.6040 | 1.6555 | 37.6577 | 62.3423 | nsm(-) |
| C | 1 | 0.5038 | 0.1910 | 0.3128 | 0.6106 | 1.6377 | 37.9119 | 62.0881 | nm |
| D | 1 | 0.4874 | 0.1831 | 0.3043 | 0.6017 | 1.6619 | 37.5667 | 62.4333 | nsm(-) |
| C | 1 | 0.4575 | 0.1924 | 0.2651 | 0.7258 | 1.3779 | 42.0546 | 57.9454 | nm |
| D | 1 | 0.4402 | 0.1476 | 0.2926 | 0.5044 | 1.9824 | 33.5302 | 66.4698 | nsm(-) |

S: Short Arm

L: Long Arm

$R_1$ & $R_2$: Arm Ratios

$I_1$ & $I_2$: Centromeric Indices

* Chromosome Pair with Secondary Constriction

## *Trachyspermum roxburghianum* (DC.) Craib ($2n = 8x = 88 = A_8B_2C_{68}D_{10}$)

**Callus - Octaploid Condition**

| | |
|---|---|
| Somatic Chromosome Variant Number | 88 |
| Chromosome Pair with Secondary Constriction | 4 |
| Total Chromosome Length | 54.1692 µ m |
| Range of Chromosome Length | 1.8089 µ m - 0.3838 µ m |
| Average Chromosome Length | 0.6156 µ m |
| Disparity Index | 64.9929 |
| Variation Coefficient | 47.5052 |
| TF Value | 45.5318 |

Table 9

Detailed Karyomorphometrical Data of *Trachyspermum roxburghianum* (Callus - Octaploid)

| Chromosome Type | No.of Pairs | Total Length (μ m) | s (μ m) | l (μ m) | $R_1$ (s/l) | $R_2$ (l/s) | $I_1$ (s/c%) | $I_2$ (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.8089 | 0.8610 | 0.9479 | 0.9083 | 1.1009 | 47.5980 | 52.4020 | nm |
| A* | 1 | 1.3930 | 0.6699 | 0.7231 | 0.9264 | 1.0794 | 48.0905 | 51.9095 | nm |
| A* | 1 | 1.2929 | 0.6300 | 0.6629 | 0.9504 | 1.0522 | 48.7277 | 51.2723 | nm |
| A* | 1 | 1.1985 | 0.5961 | 0.6024 | 0.9895 | 1.0106 | 49.7372 | 50.2628 | nm |
| B | 1 | 1.0258 | 0.5129 | 0.5129 | 1.0000 | 1.0000 | 50.0000 | 50.0000 | M |
| C | 1 | 0.8161 | 0.3530 | 0.4631 | 0.7623 | 1.3119 | 43.2545 | 56.7455 | nm |
| D | 1 | 0.7449 | 0.2734 | 0.4715 | 0.5799 | 1.7246 | 36.7029 | 63.2971 | nsm(-) |
| D | 1 | 0.6477 | 0.2403 | 0.4074 | 0.5898 | 1.6954 | 37.1005 | 62.8995 | nsm(-) |
| C | 1 | 0.6433 | 0.2746 | 0.3689 | 0.7444 | 1.3434 | 42.6861 | 57.3449 | nm |
| C | 1 | 0.6101 | 0.2921 | 0.3180 | 0.9186 | 1.0887 | 47.8774 | 52.1226 | nm |
| C | 1 | 0.6078 | 0.2373 | 0.3705 | 0.6405 | 1.5613 | 39.0424 | 60.9576 | nm |
| C | 1 | 0.6062 | 0.2882 | 0.3180 | 0.9063 | 1.1034 | 47.5421 | 52.4579 | nm |
| D | 1 | 0.5993 | 0.2263 | 0.3730 | 0.6067 | 1.6483 | 37.7607 | 62.2393 | nsm(-) |
| C | 1 | 0.5988 | 0.2981 | 0.3007 | 0.9914 | 1.0087 | 49.7829 | 50.2171 | nm |
| C | 1 | 0.5946 | 0.2950 | 0.2996 | 0.9846 | 1.0156 | 49.6132 | 50.3868 | nm |
| C | 1 | 0.5724 | 0.2579 | 0.3145 | 0.8200 | 1.2195 | 45.0559 | 54.9441 | nm |
| C | 1 | 0.5653 | 0.2664 | 0.2989 | 0.8913 | 1.1220 | 47.1254 | 52.8746 | nm |
| C | 1 | 0.5589 | 0.2533 | 0.3056 | 0.8289 | 1.2065 | 45.3212 | 54.6788 | nm |
| C | 1 | 0.5440 | 0.2400 | 0.3040 | 0.7895 | 1.2667 | 44.1176 | 55.8824 | nm |
| D | 1 | 0.5403 | 0.1852 | 0.3551 | 0.5215 | 1.9174 | 34.2773 | 65.7227 | nsm(-) |
| C | 1 | 0.5328 | 0.2644 | 0.2684 | 0.9851 | 1.0151 | 49.6246 | 50.3754 | nm |
| C | 1 | 0.5322 | 0.2510 | 0.2812 | 0.8926 | 1.1203 | 47.1627 | 52.8373 | nm |
| C | 1 | 0.5228 | 0.2278 | 0.2950 | 0.7722 | 1.2950 | 43.5731 | 56.4269 | nm |
| C | 1 | 0.5208 | 0.2545 | 0.2663 | 0.9557 | 1.0464 | 48.8671 | 51.1329 | nm |
| C | 1 | 0.5066 | 0.2396 | 0.2670 | 0.8974 | 1.1144 | 47.2957 | 52.7043 | nm |
| C | 1 | 0.5012 | 0.2293 | 0.2717 | 0.8439 | 1.1849 | 45.7502 | 54.2099 | nm |
| C | 1 | 0.5010 | 0.1981 | 0.3013 | 0.6575 | 1.5209 | 39.5409 | 60.1397 | nm |
| C | 1 | 0.4899 | 0.2442 | 0.2457 | 0.9939 | 1.0061 | 49.8469 | 50.1531 | nm |
| C | 1 | 0.4839 | 0.2134 | 0.2705 | 0.7889 | 1.2676 | 44.1000 | 55.9000 | nm |
| C | 1 | 0.4830 | 0.2371 | 0.2459 | 0.9642 | 1.0371 | 49.0890 | 50.9110 | nm |
| C | 1 | 0.4774 | 0.2373 | 0.2401 | 0.9883 | 1.0118 | 49.7067 | 50.2933 | nm |
| C | 1 | 0.4640 | 0.2268 | 0.2372 | 0.9562 | 1.0459 | 48.8793 | 51.1207 | nm |
| D | 1 | 0.4586 | 0.1627 | 0.2959 | 0.5498 | 1.8187 | 35.4775 | 64.5225 | nsm(-) |
| C | 1 | 0.4477 | 0.1893 | 0.2584 | 0.7326 | 1.3650 | 42.2828 | 57.7172 | nm |
| C | 1 | 0.4452 | 0.1981 | 0.2471 | 0.8017 | 1.2473 | 44.4969 | 55.5031 | nm |
| C | 1 | 0.4394 | 0.2130 | 0.2264 | 0.9408 | 1.0629 | 48.4752 | 51.5248 | nm |
| C | 1 | 0.4371 | 0.2024 | 0.2347 | 0.8624 | 1.1596 | 46.3052 | 53.6948 | nm |
| C | 1 | 0.4269 | 0.1982 | 0.2287 | 0.8666 | 1.1539 | 46.4277 | 53.5723 | nm |
| C | 1 | 0.4260 | 0.1622 | 0.2638 | 0.6149 | 1.6264 | 38.0751 | 61.9249 | nm |
| C | 1 | 0.4231 | 0.2214 | 0.2217 | 0.9986 | 1.0014 | 52.3281 | 52.3990 | nm |
| C | 1 | 0.4181 | 0.1883 | 0.2298 | 0.8194 | 1.2204 | 45.0371 | 54.9629 | nm |
| C | 1 | 0.4066 | 0.1849 | 0.2217 | 0.8340 | 1.1990 | 45.4747 | 54.5253 | nm |
| C | 1 | 0.3877 | 0.1862 | 0.2015 | 0.9241 | 1.0822 | 48.0268 | 51.9732 | nm |
| C | 1 | 0.3838 | 0.1509 | 0.2329 | 0.6479 | 1.5434 | 39.3174 | 60.6826 | nm |

S: Short Arm

L: Long Arm

* Chromosome Pair with Secondary Constriction

$R_1$ & $R_2$: Arm Ratios

$I_1$ & $I_2$ : Centromeric Indices

## *Trachyspermum roxburghianum* (DC.) Craib (2n = 4x = 44 = $A_4C_{34}D_6$)

### *In Vitro* Plant

| | |
|---|---|
| Normal Somatic Chromosome Number | 44 |
| Chromosome Pair with Secondary Constriction | 2 |
| Total Chromosome Length | 28.8582 µ m |
| Range of Chromosome Length | 1.0431 µ m - 0.5561 µ m |
| Average Chromosome Length | 0.6559 µ m |
| Disparity Index | 30.4527 |
| Variation Coefficient | 17.7912 |
| TF Value | 40.6484 |

Table 10

Detailed Karyomorphometrical Data of *Trachyspermum roxburghianum* (*In Vitro* Plant)

| Chromos-ome Type | No.of Pairs | Total Length (µ m) | s (µ m) | l (µ m) | $R_1$ (s/l) | $R_2$ (l/s) | $I_1$ (s/c%) | $I_2$ (l/c%) | Nature of Primary Constriction |
|---|---|---|---|---|---|---|---|---|---|
| A* | 1 | 1.0431 | 0.3447 | 0.6984 | 0.4936 | 2.0261 | 33.0457 | 66.9543 | nsm(-) |
| A* | 1 | 0.9053 | 0.2716 | 0.6337 | 0.4286 | 2.3332 | 30.0011 | 69.9989 | nsm(-) |
| D | 1 | 0.7256 | 0.2843 | 0.4413 | 0.6442 | 1.5522 | 39.1814 | 60.8186 | nsm(-) |
| C | 1 | 0.7207 | 0.3078 | 0.4129 | 0.7455 | 1.3415 | 42.7085 | 57.2915 | nm |
| C | 1 | 0.6864 | 0.2928 | 0.3936 | 0.7439 | 1.3443 | 42.6573 | 57.3427 | nm |
| C | 1 | 0.6793 | 0.2751 | 0.4042 | 0.6806 | 1.4693 | 40.4976 | 59.5024 | nm |
| C | 1 | 0.6757 | 0.2745 | 0.4012 | 0.6842 | 1.4616 | 40.6245 | 59.3755 | nm |
| C | 1 | 0.6602 | 0.2895 | 0.3707 | 0.7810 | 1.2805 | 43.8503 | 56.1497 | nm |
| C | 1 | 0.6388 | 0.2744 | 0.3644 | 0.7530 | 1.3280 | 42.9555 | 57.0445 | nm |
| D | 1 | 0.6368 | 0.2292 | 0.4076 | 0.5623 | 1.7784 | 35.9925 | 64.0075 | nsm(-) |
| C | 1 | 0.6321 | 0.2589 | 0.3732 | 0.6937 | 1.4415 | 40.9587 | 59.0413 | nm |
| C | 1 | 0.6306 | 0.2471 | 0.3835 | 0.6443 | 1.5520 | 39.1849 | 60.8151 | nm |
| C | 1 | 0.6039 | 0.2316 | 0.3723 | 0.6221 | 1.6075 | 38.3507 | 61.6493 | nm |
| C | 1 | 0.6029 | 0.2729 | 0.3300 | 0.8270 | 1.2092 | 45.2646 | 54.7354 | nm |
| C | 1 | 0.5919 | 0.2747 | 0.3172 | 0.8660 | 1.1547 | 46.4099 | 53.5901 | nm |
| C | 1 | 0.5832 | 0.2457 | 0.3375 | 0.7280 | 1.3736 | 42.1296 | 57.8704 | nm |
| C | 1 | 0.5820 | 0.2719 | 0.3101 | 0.8768 | 1.1405 | 46.7182 | 53.2818 | nm |
| D | 1 | 0.5792 | 0.2141 | 0.3651 | 0.5864 | 1.7053 | 36.9648 | 63.0352 | nsm(-) |
| C | 1 | 0.5686 | 0.2232 | 0.3454 | 0.6462 | 1.5475 | 39.2543 | 60.7457 | nm |
| C | 1 | 0.5684 | 0.2693 | 0.2991 | 0.9004 | 1.1107 | 47.3786 | 52.6214 | nm |
| C | 1 | 0.5583 | 0.2539 | 0.3044 | 0.8341 | 1.1989 | 45.4773 | 54.5227 | nm |
| C | 1 | 0.5561 | 0.2580 | 0.2981 | 0.8655 | 1.1554 | 46.3945 | 53.6055 | nm |

S:Short Arm

L: Long Arm

$R_1$ & $R_2$: Arm Ratios

$I_1$ & $I_2$: Centromeric Indices

* Chromosome Pair with Secondary Constriction

## Meiosis

The meiotic studies of the *in vivo* as well as the *in vitro* flowers were conducted. The microphotographs of the stages obtained are shown in Figs. 6 .1 – 9 .1. The diakinesis of the prophase I, and metaphase I of the *in vivo* plant is shown in Figs. 6 .1 & 7 .1 and of the *in vitro* plant is shown in Figs. 8 .1 & 9 .1.

The meiotic chromosomes could best be studied at diakinesis and metaphase I. The perfect pairing of homologous chromosomes in the diakinesis stages of meiosis points towards the very balanced nature of karyotype. The *in vivo* plant showed no cytological abnormalities in meiotic cells and formed 22 bivalents at metaphase I of PMCs. On the other hand the metaphase I of the *in vitro* plant showed 18 bivalents and 2 quadrivalents.

The diakinesis stage of prophase I of *in vivo* flower showed normal number of chromosomes, i.e., 22 bivalents, hence the tetraploid chromosome number is 44. The diakinesis stage of *in vitro* flower consisted of 10 bivalents and 6 quadrivalents. The diakinesis and metaphase I of the *in vitro* flower was abnormal with quadrivalents among bivalents, which in turn may lead to partial sterility.

## Random Amplified Polymorphic DNA ( RAPD) Analysis

DNA was isolated from the parent plant and two cultured plants designated as TC 1 and TC 2. 20-30 ng of DNA is required to amplify a specific DNA segment by PCR. 3 : 1 of the template DNA was used for the preparation of reaction mixture. To detect the variation of the *in vitro* regenerated plant at the molecular level, RAPD analysis was carried out using 27 primers of arbitrary sequences. Of the twenty-seven primers used, only ten successfully amplified the extracted DNA with consistent reproducible bands (Figs. 10 .1 – 13 .1). The base sequence of primers leading to gene

amplification is given in Table 11. The number of bands resolved per primer ranged from a minimum of 2 to a maximum of 12. The size of amplification products also differed and ranged from approximately 100 bp to 900 bp in the 1000 bp ladder and approximately 300 bp to 3000 bp in the 4000 bp ladder.

The RAPD fingerprint of the variant (TC1) differed from the parent plant with seven primers (OPA 02, OPA 06, OPA 09, OPA 11, OPC 03, OPC 11 & OPD 01). A few bands were found to be missing in the variant when these primers were used. Additional bands in the variant were also detected by this marker screening (Figs. 10 .1 – 13 .1). Amplification products with OPA 02 generated no bands above 600 bp ladder in TC 1, but band was present above this length in the *in vivo* plant's amplification product as well as the fingerprint of TC 2. Simultaneously, TC 1 and TC 2 showed no bands below 400 bp length, which was different from the parent. The amplification product of TC 1 showed bands in the region of 400 and 600 bp length whereas, TC 2 showed 400, 500 and 700 bp amplification. When the primer OPA 09 was used, bands corresponding to 600, 700, 900 and 1000 bp length were missing in TC 1 and TC 2, which were detected in the parent. An additional band corresponding to 800 bp length appeared in the amplification product of both the samples, which was not detected in the parent. For the OPA 11 primer, no bands were detected for both TC 1 and TC 2 above length of 700 bp. The parent plant failed to show bands corresponding to lengths below 500 bp, which were shown clearly by TC 1 and TC 2. With this primer, TC1 showed 5 additional bands corresponding to 100, 200, 300, 400 and 700 bp length and six additional bands corresponding to 100, 200, 300, 400, 500 and 700 bp length in TC2, when compared to the parent. Also, the bands shown by the parent failed to appear in TC1, which were corresponding to 500, 800 and 1000 bp length. The bands corresponding to 800 and 1000 bp length in the parent was found to be absent in TC2.

On using the OPA 06 primer, 2 bands appeared close to the 1000 bp ladder, at approximately 750 bp in TC1 and TC2. These bands were absent in the parent. The intensity of the bands corresponding to 1000 bp length was greater in TC1 and TC2 when compared to the parent. Two bands appeared in the region between 1000 bp and 1500 bp in TC1 and TC2 but it was absent in the parent. The intensity of the band corresponding to the 1500 bp ladder was found to be increasing from parent through TC1 to TC2. Bands corresponding to 2500 bp appeared with greater intensity in TC1 and little less intensity in TC2, which was absent in the parent. Using OPB 15 primer, the intensity of the bands decreased from parent through TC1 to TC2. With OPB 18 primer, the band intensity decreased progressively from parent to TC2. With OPC 03 primer, single band just below approximately at 300 bp length was observed in the parent, which was not detected in TC1 and TC2. Band corresponding to approximately 2750 bp length in TC1 was absent in parent and TC2. Using OPC 10 primer, amplification of bands was not observed. Bands corresponding to 2000 bp length present in TC1 and scarcely in TC2, was not detected in parent using OPC 11 primer. Two bands appeared in the parent approximately at 400 bp and 500 bp, both of which were absent in TC1 and TC2. The band close to 1000 bp was present in low intensity in TC2, on using OPD 07 primer. The primer showed amplification only in the 1000 bp region of the three samples. Using OPD 01 primer, bands were observed only in the parent plant just above and below 1000 bp region. These were not detected in TC1 and TC2.

With the primer OPA 02, two bands were similar to the parent for TC 1 whereas three bands were identical with parent for TC 2. Also for the primer OPA 11, TC 2 showed an additional band than TC 1, which was similar to the parent plant. Since the amplification of TC 1 was showing more difference than that TC 2, the former was considered as the variant and used for further analysis.

## RAPD fingerprints of *Trachyspermum roxburghianum*

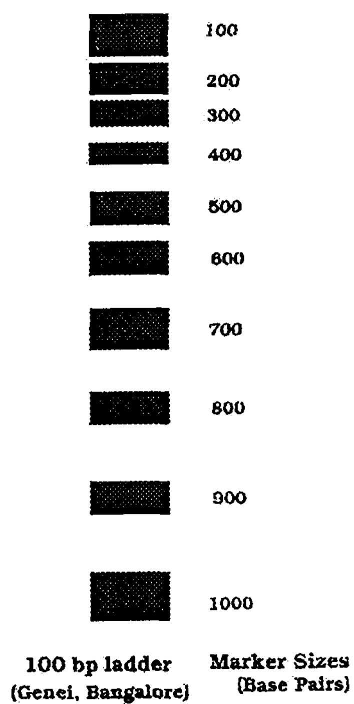
Fig. 10 .1 OPA 02
Fig. 11 .1 OPA 09
Fig. 12 .1 OPA 11

W Molecular Weight markers

P Parent plant

TC1 *In vitro* plant – sample 1

TC 2 *In vitro* plant – sample 2



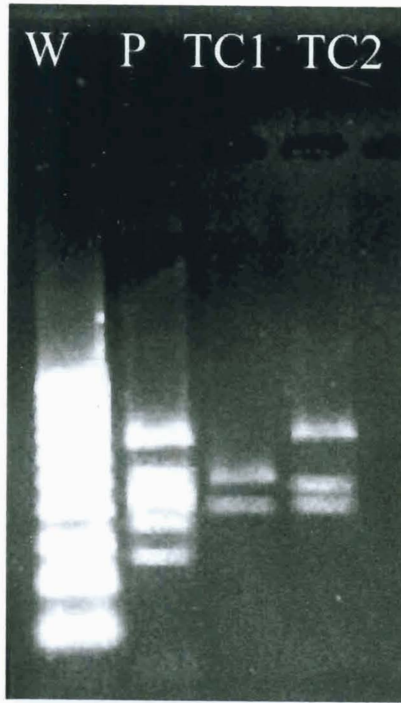



Fig. 10 .1

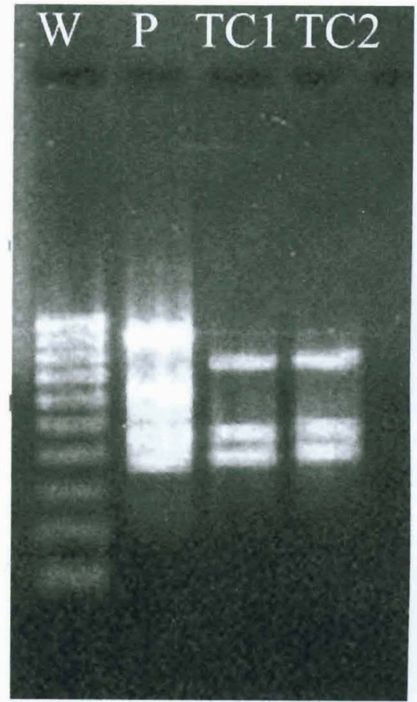



Fig. 11 .1

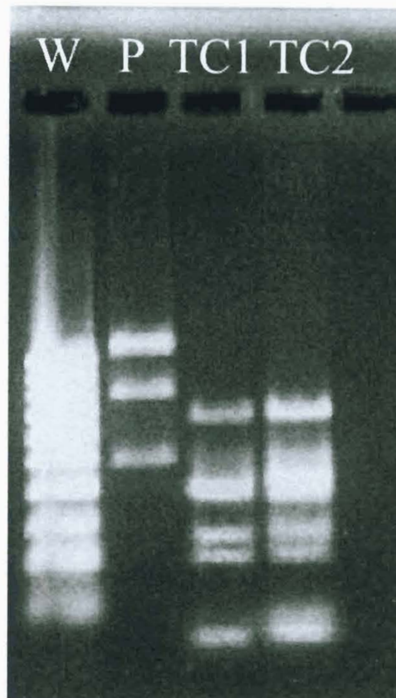



Fig. 12 .1

## RAPD fingerprints of *Trachyspermum roxburghianum*

Fig. 13 .1A -
- a - OPA 06
- b - OPB 15
- c - OPB 18
- d - OPC 03

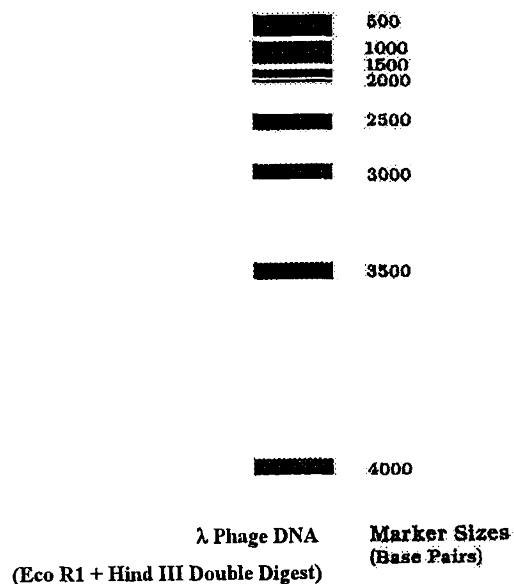
Fig. 13 .1B –
- a - OPC 10 Primer without amplification of any markers for P, TC1 & TC2
- b - OPC 11
- c - OPD 07
- d - OPD 01

W Molecular Weight markers

P Parent plant

TC1 *In vitro* plant – sample 1

TC2 *In vitro* plant – sample 2



77 D

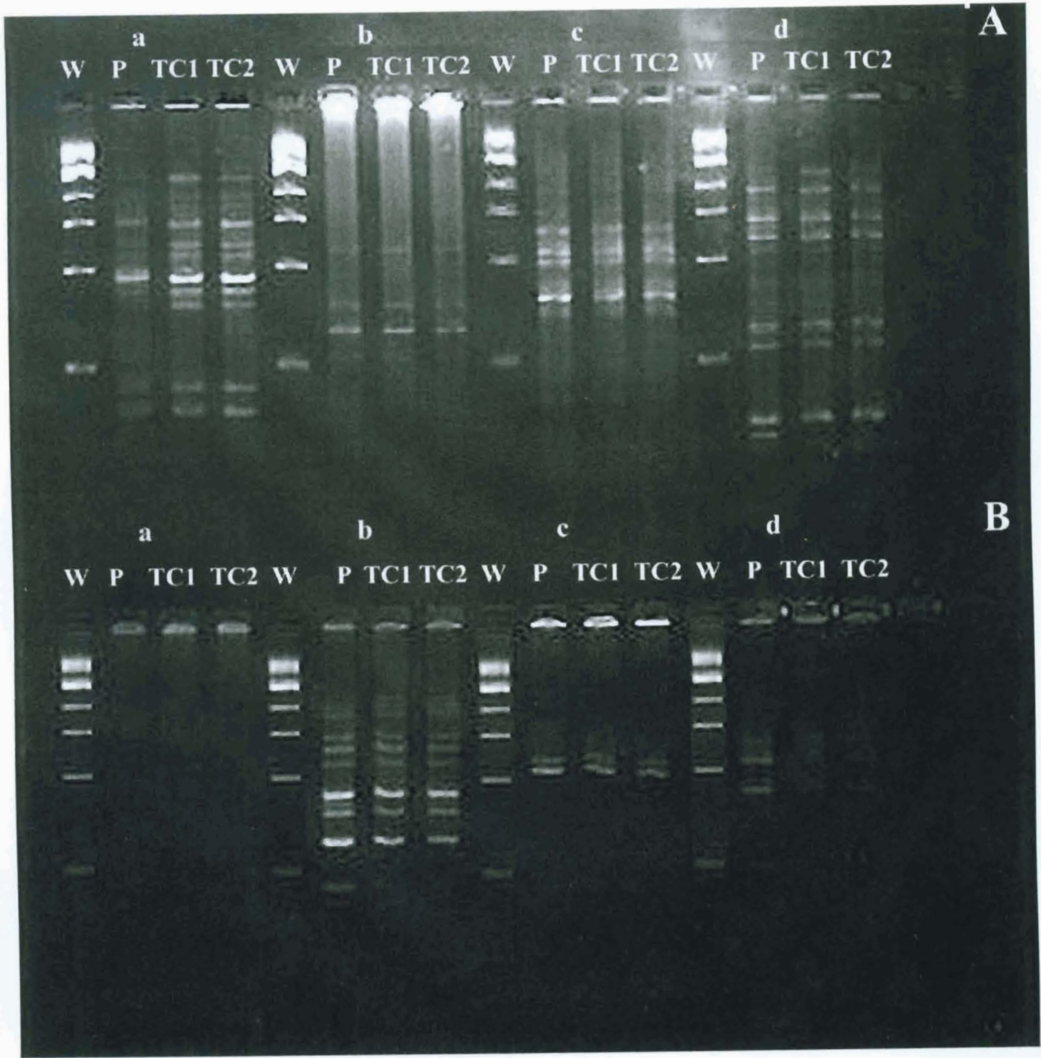



Fig. 13 .1

Altogether, four bands for the primer OPA 02 as well as OPA 11 and three bands for OPA 09 were absent in the variant (TC1) in comparison with the parent. The variant also showed one additional band for OPA 09 and five additional bands for OPA 11 than the parent. With OPA 06 the variant showed five additional bands than the parent. With OPC 03, one additional band was detected in variant, which was absent in the parent as well as TC2. Using OPC 11, one additional band was observed in the variant (TC1) and TC2, which again was not detected in the parent. The parent showed one additional band for OPC 03, two for OPC 11 and two for OPD 01 when compared to the variant and TC2. The reproducibilty of these genomic DNA bands were consistent in successive repetitions.

Table 11

Primers and characterization of consistent bands in parent and two tissue cultured plants of *T. roxburghianum* (DC.) Craib

| Primer | Sequence | Number of bands | | |
|---|---|---|---|---|
| | | Parent | *TC1 | TC2 |
| OPA 02 | 5'TGCCGAGCTG3' | 6 | 2 | 3 |
| OPA 06 | 5'GGTCCCTGAC3' | 4 | 9 | 9 |
| OPA 09 | 5'GGGTAACGCC3' | 6 | 3 | 3 |
| OPA 11 | 5'CAATCGCCGT3' | 4 | 5 | 7 |
| OPB 15 | 5'GGAGGGTGTT3' | 3 | 2 | 2 |
| OPB 18 | 5'CCACACCAGT3' | 4 | 4 | 4 |
| OPC 03 | 5'GGGGGTCTTT3' | 7 | 7 | 6 |
| OPC 11 | 5'AAAGCTGCGG3' | 8 | 7 | 7 |
| OPD 07 | 5'TTGGCACGGG3' | 2 | 2 | 2 |
| OPD 01 | 5'ACCGCGAAGG3' | 2 | 0 | 0 |

*Somaclonal variant hitherto mentioned.

## Essential Oil Analysis

### (i) Gas Chromatography-Mass Spectrometry

Plants obtained from *in vitro* generation should be examined since it is necessary for assessing the field performance of regenerants. The present study on *in vitro* and *in vivo* plants of *T. roxburghianum* (DC.) Craib was undertaken to screen desirable qualities like essential oil composition and yield.

The essential oils of *T. roxburghianum* (DC.) Craib and its micropropagated plants were analysed both quantitatively and qualitatively. The oil yield of the *in vitro* plant (1.2 %) was slightly greater than the normal plant (0.9 %). The essential oils were analysed by the GC-MS and the components detected included monoterpenes, sesquiterpenes, furan derivative, aryl alkyl aldehyde, alkanol, long chain acid with 16 carbon atoms, bicycloalkene, monoterpene alcohol and an epoxy compound. The GC-MS pattern of the *in vitro* essential oil was distinctly different when compared to that of the parent plant (Figs. 14 .1, 15 .1, 16 .1$A_1$-16 .1$A_{32}$). The qualitative and quantitative differences observed in the essential oil directly influenced the colour of the oil distilled. The oil from *in vivo* grown plant have light yellow colour whereas essential oil of *in vitro* grown plant showed deep yellow colour.

The results of GC-MS analysis of *in vivo* and *in vitro* plants of *T. roxburghianum* (DC.) Craib are listed in Table 12.

The analysis of the essential oil samples revealed variation in their constituents. The oil of *in vivo* and *in vitro* plants show similarities in the presence of some components, but show percentage variation. Some components present in the *in vivo* plant appeared to be absent in the *in vitro* plant and vice versa. The essential oil composition of the *in vivo* and *in vitro*

plants varied in the major component itself. The major component of the *in vitro* plant is isothujol (16.42%) whereas that of the *in vivo* plant is epiglobulol (19.55%). The *in vivo* plant is characterized by the presence of β-myrcene (0.35%), limonene (0.31%), γ-terpinene (0.52%), α-cubebene (1.55%), α-caryophyllene (1.69%), eudesma-4 [14], 11-diene (10.94%), aromadendrene (4.26%), δ-cadinene (1.74%), epiglobulol (19.55%), caryophyllene oxide (1.86%), aromadendrene oxide (0.45%), isobenzofuranone (1.35%), β-methyl benzene propanal (15.74%), tetradecanol (0.73%) and n-hexadecanoic acid (1.70%), which were found to be absent in the *in vitro* plant. The essential oil of the *in vitro* plant possess ocimene (1.10%), α-farnasene (1.96%), 1,R, 3Z-9S-4, 11,11-trimethyl 8-methylene bicyclo [7.2.0] undec-3-ene (3.81%), isothujol (16.42%), β-farnasene (1.35%), α-β-epoxycumene (14.72%) and α-bergamotene (3.37%) which were not detected in the *in vivo* plant. The components which were found to be common in *in vitro* and *in vivo* plants were thujene, δ-3-carene, β-terpineol, germacrene-D, cedrene, β-caryophyllene, α-neoclovene, α-bisabolene oxide and patchoulane, together with one unidentified component.

**(ii) Chemotaxonomic Studies**

The total number of chemical components detected by GC-MS in both *in vivo* and *in vitro* grown plants were found to be 32. However, the number of similar components which occur both in parent plant and the micropropagated plant was found to be 10. Coefficient of similitude between the *in vivo* and the *in vitro* plants was found to be 31. 2724.

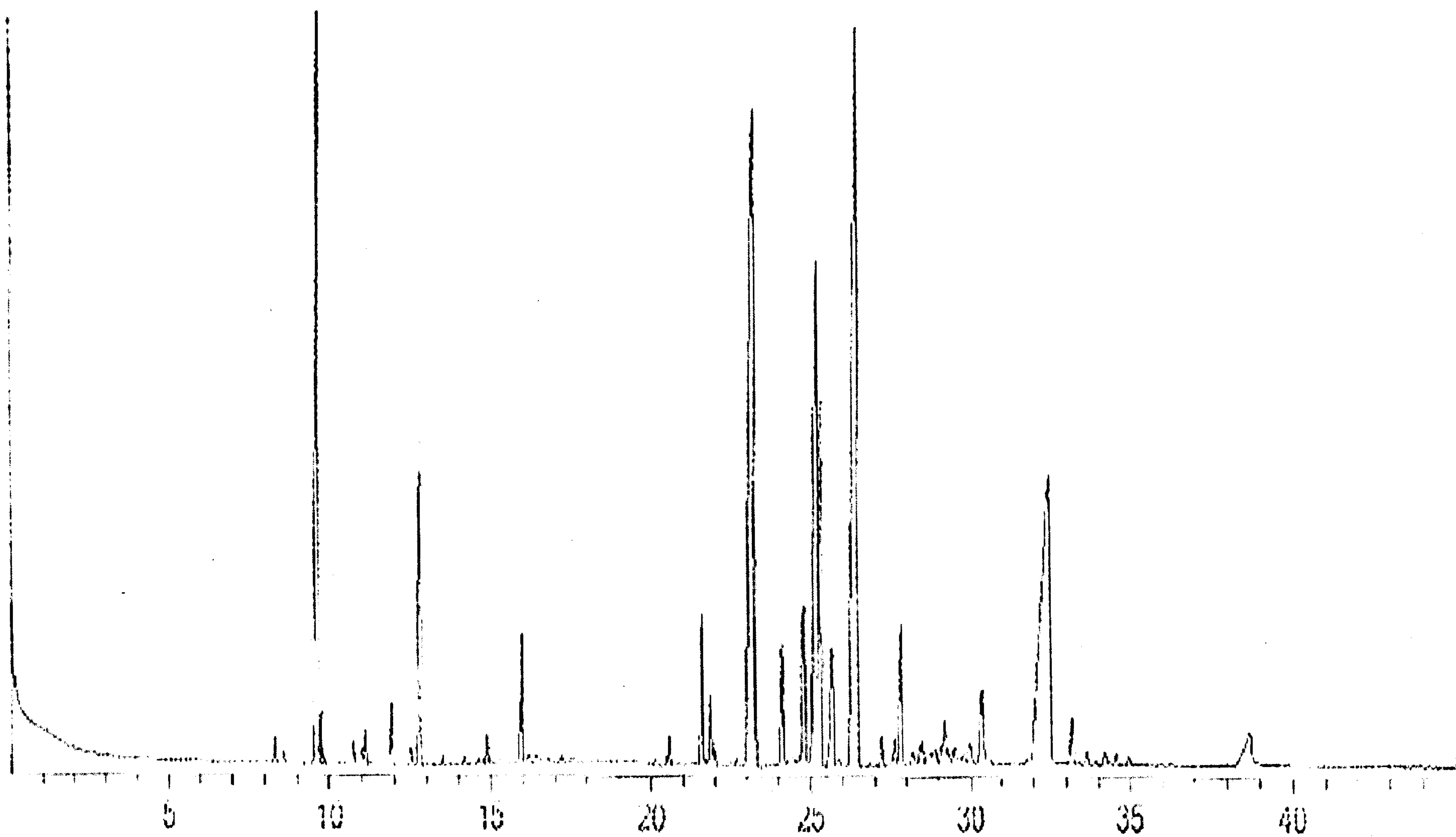



Fig. 14 .1 Gas Chromatogram of the *in vivo* plant

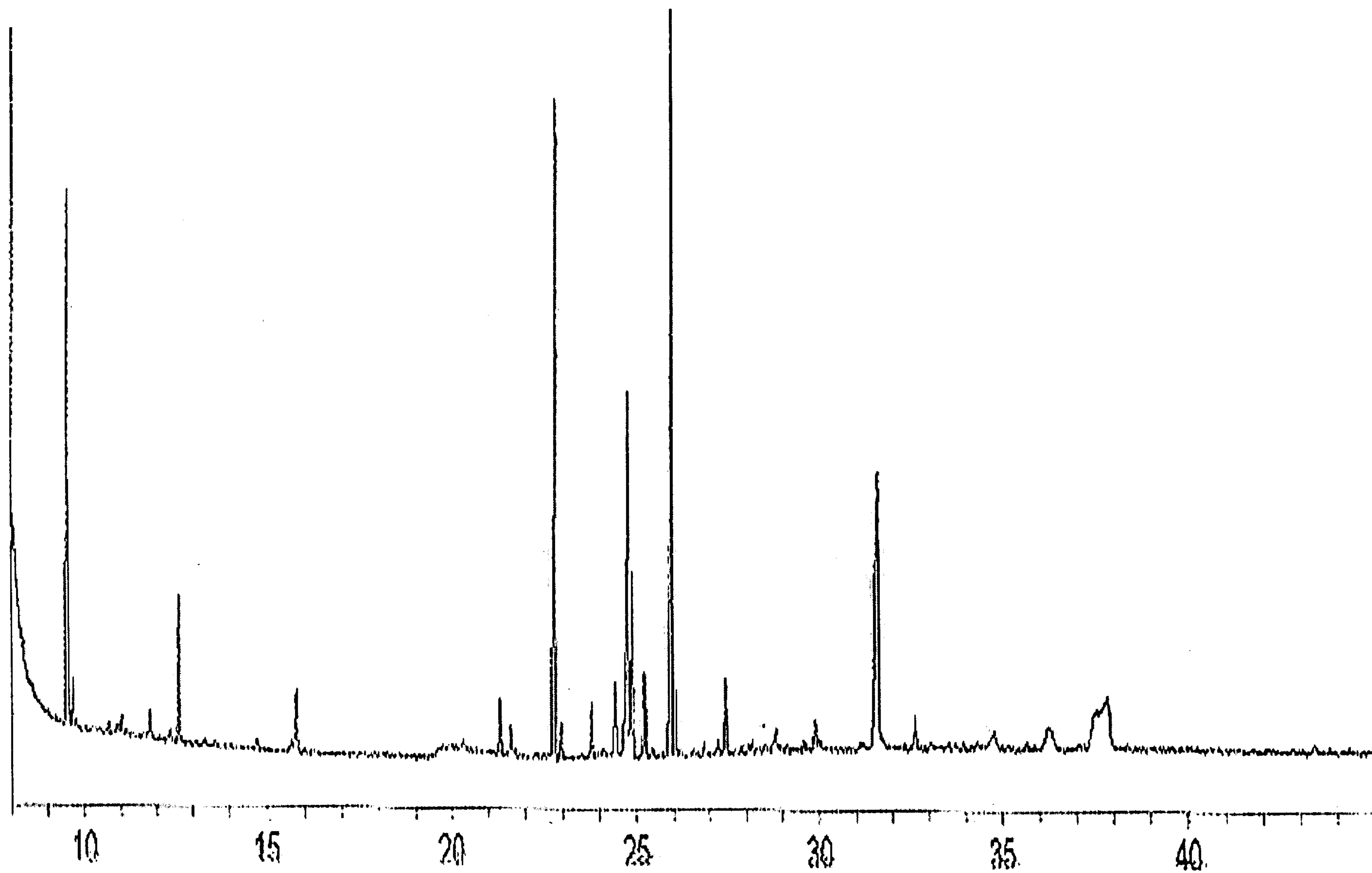


Fig. 15 .1 Gas Chromatogram of the *in vitro* plant

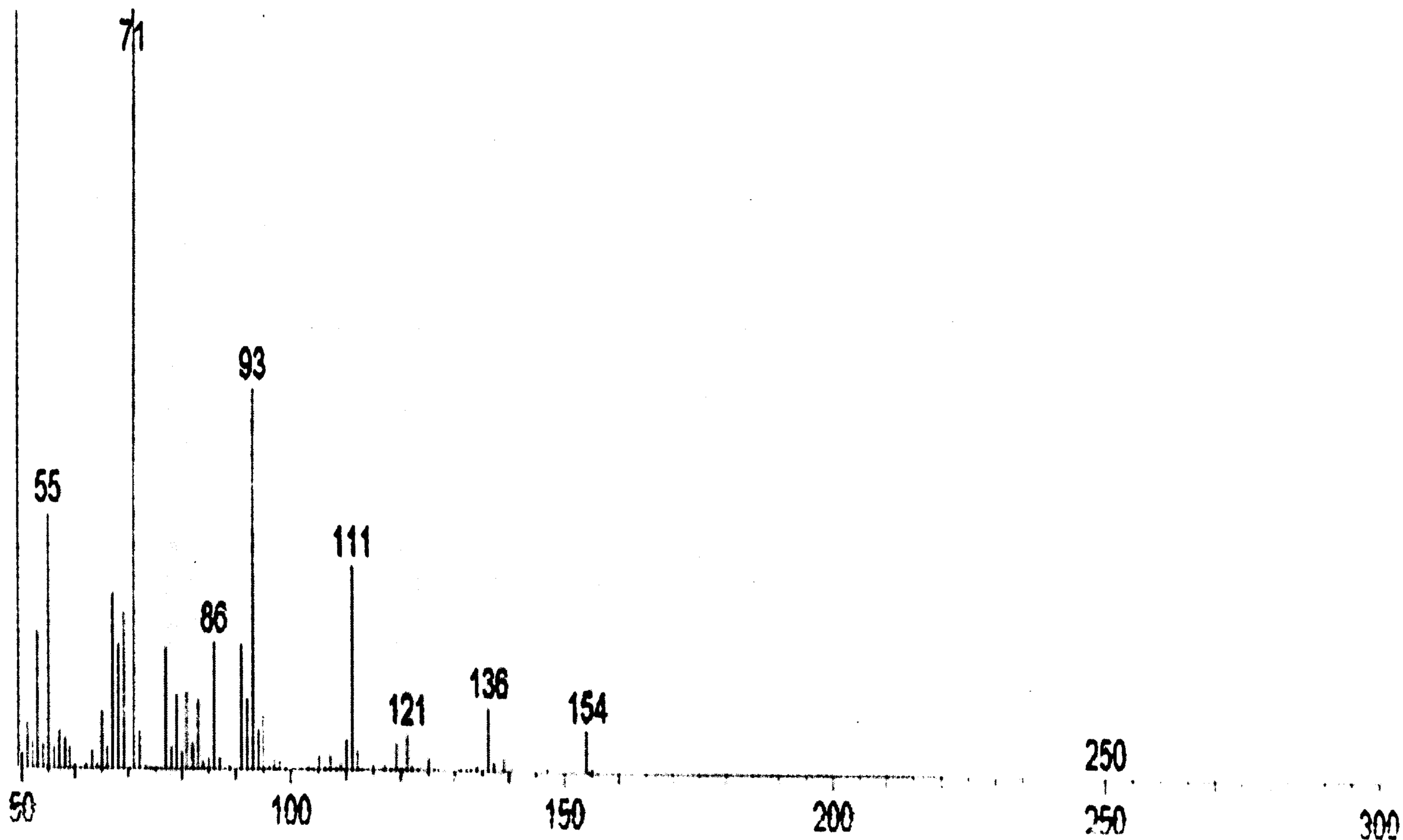


Fig. 16 .1A$_1$ Mass Spectra of β - terpineol

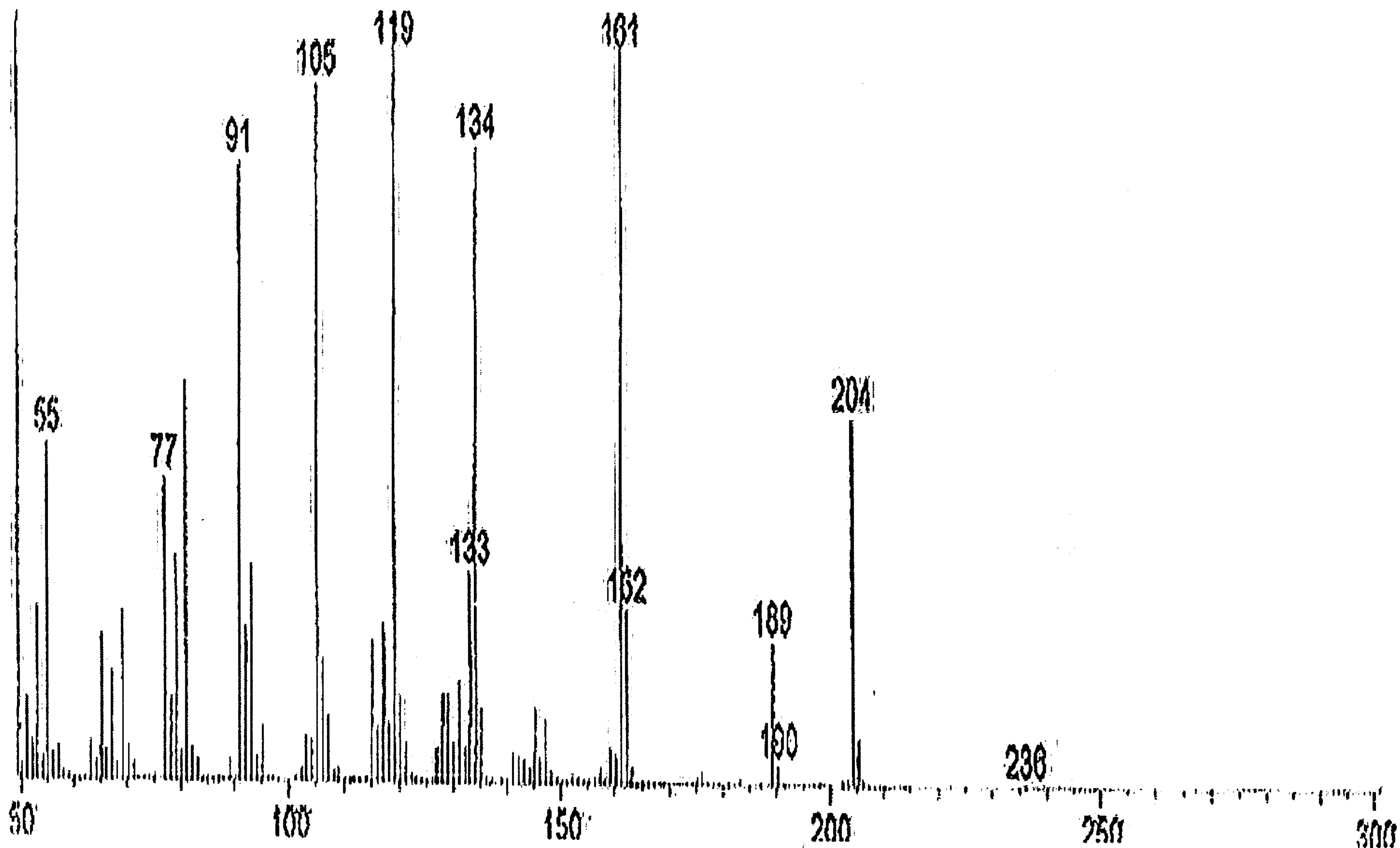



Fig. 16 .1$A_2$ Mass Spectra of δ - cadinene

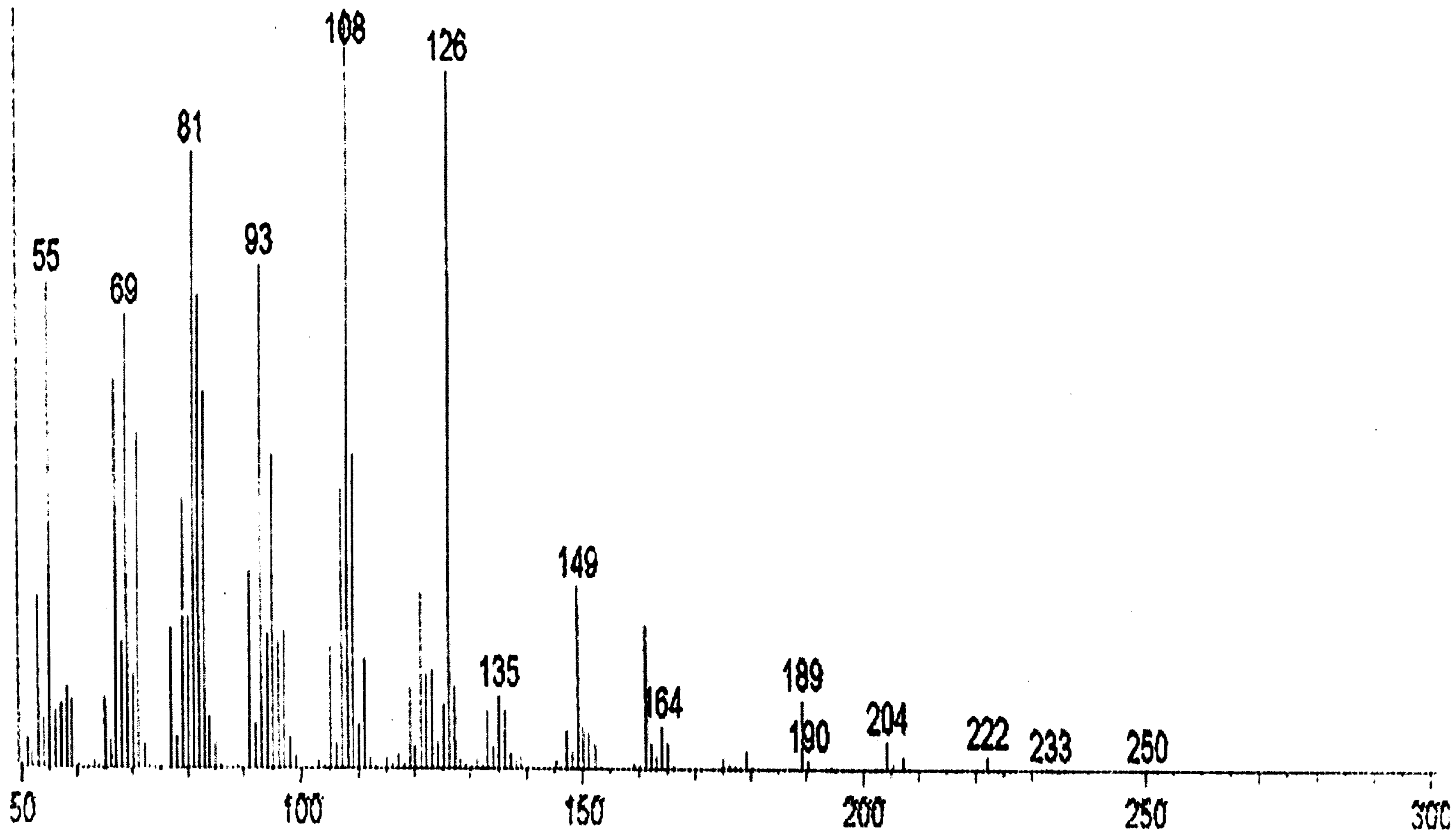



Fig. 16 .1$A_3$ Mass Spectra of epiglobulol

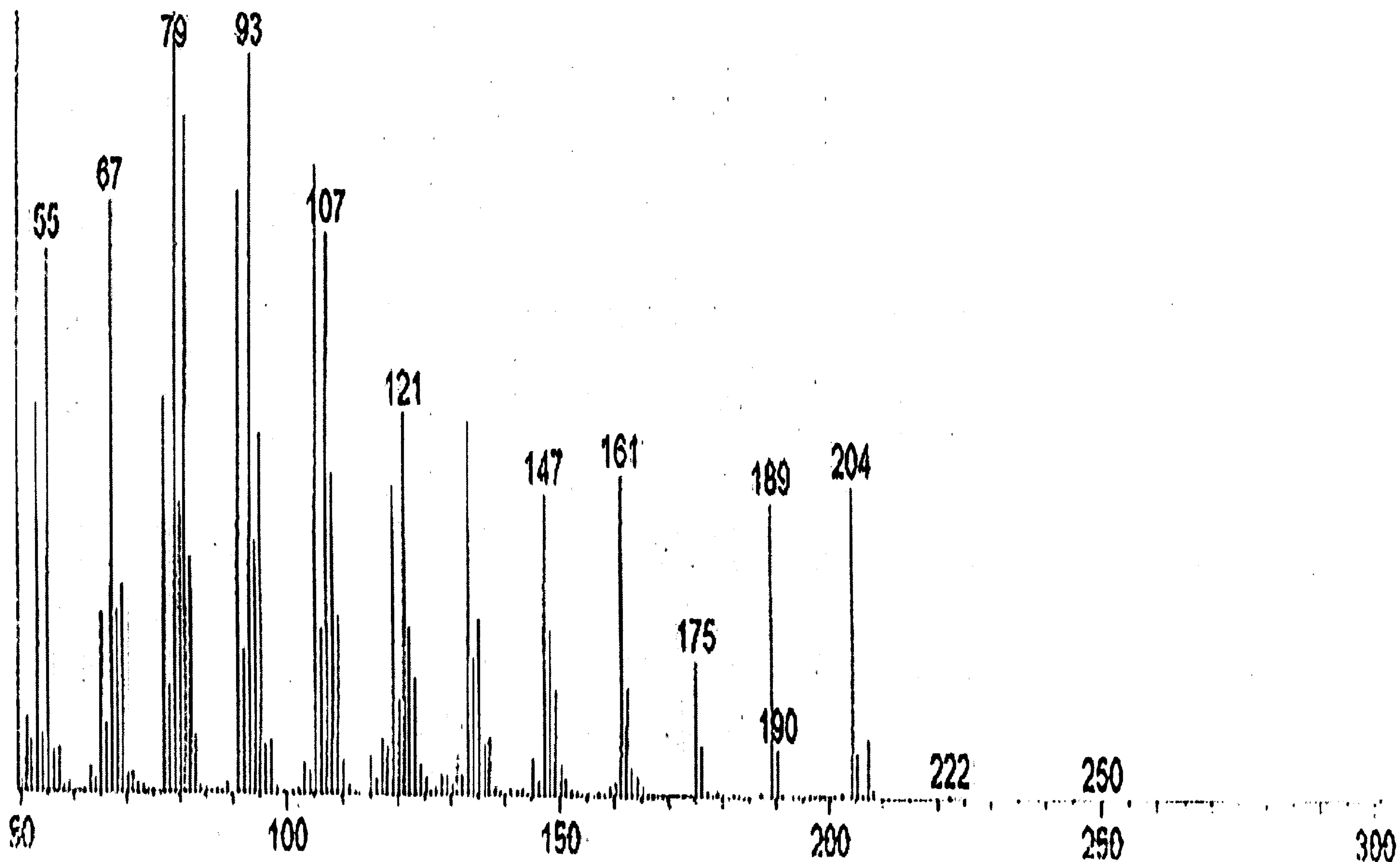



Fig. 16 .1A$_4$ Mass Spectra of eudesma - 4 [14], 11-diene

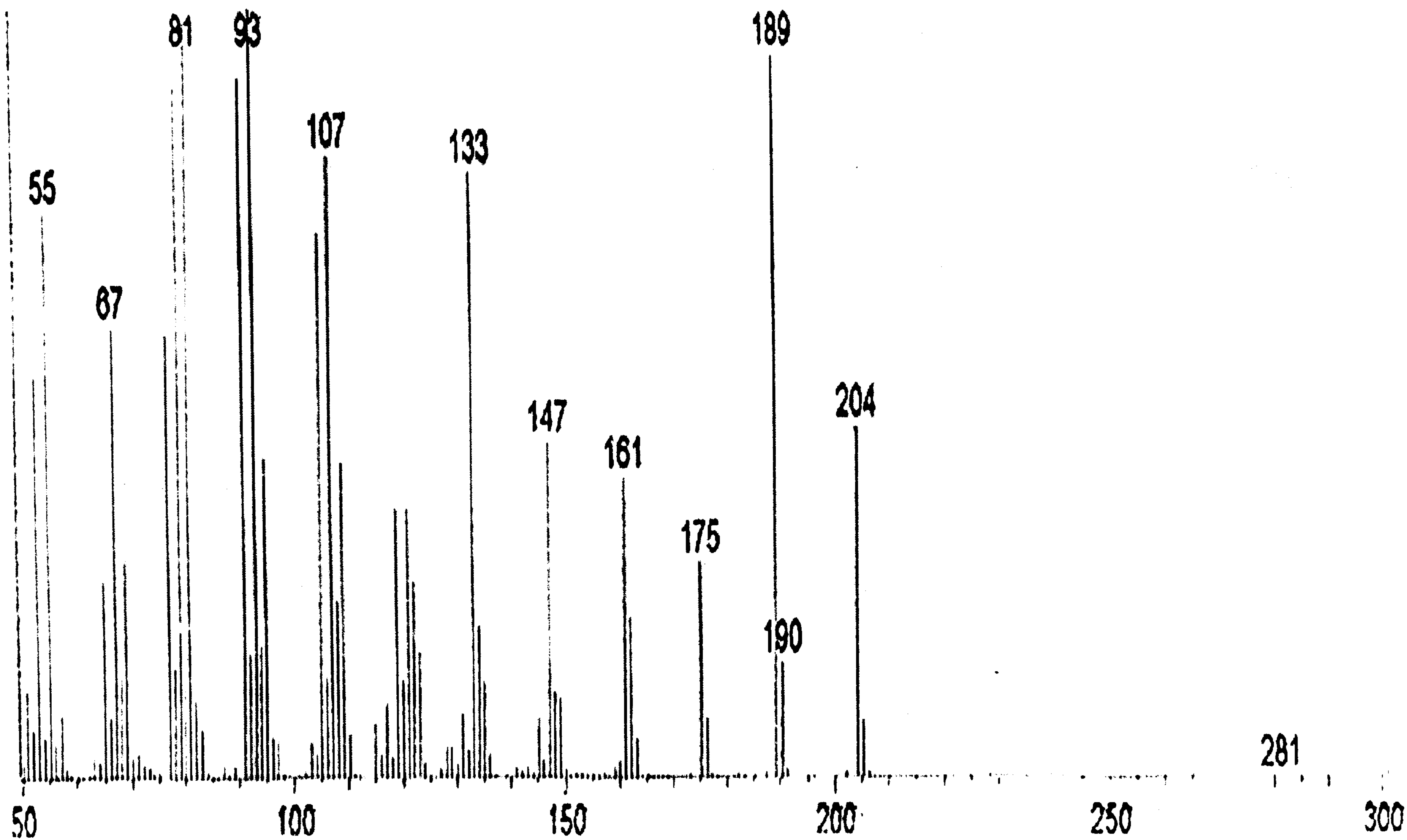



Fig. 16 .1$A_5$ Mass Spectra of aromadendrene

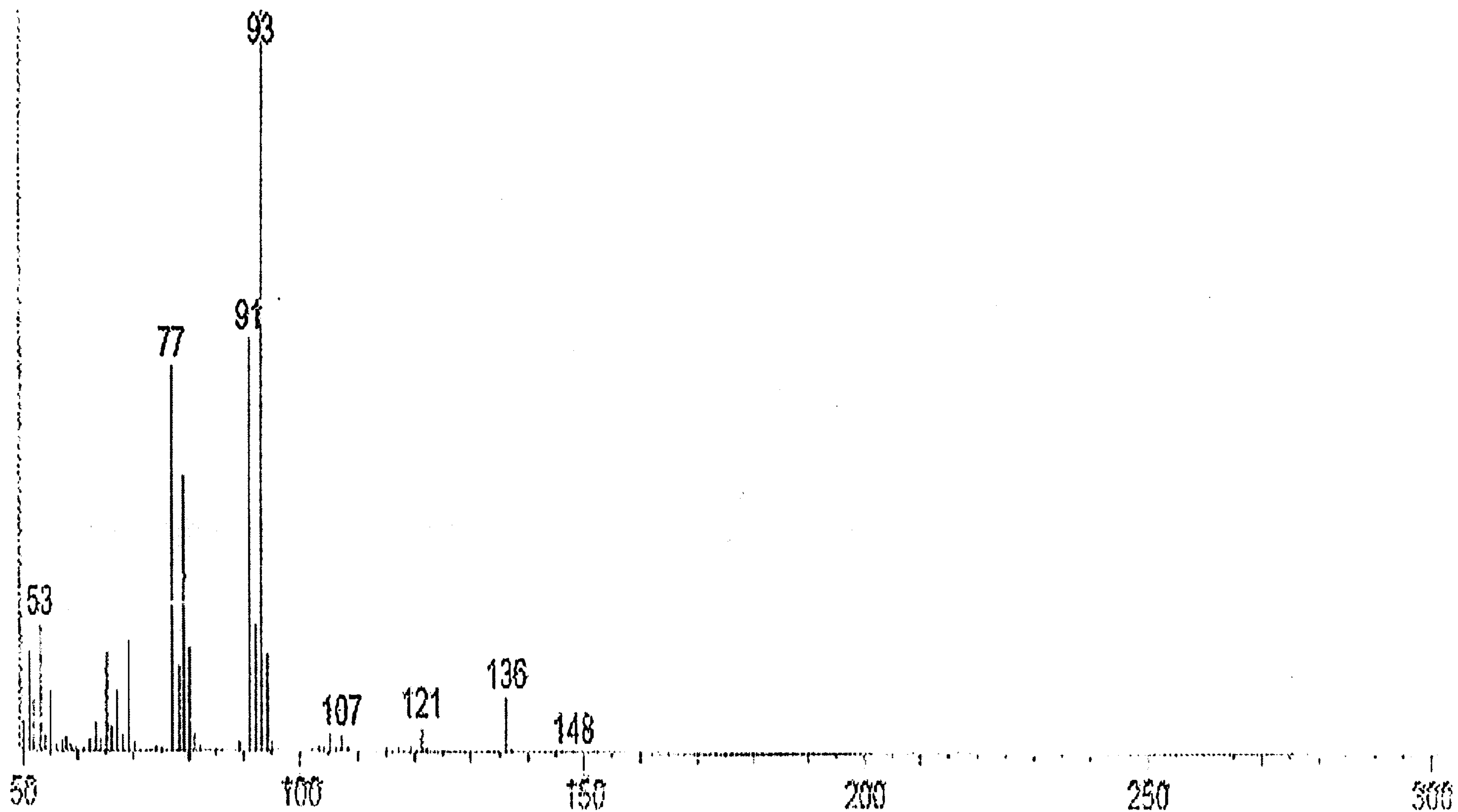



Fig. 16 .1A$_6$ Mass Spectra of thujene

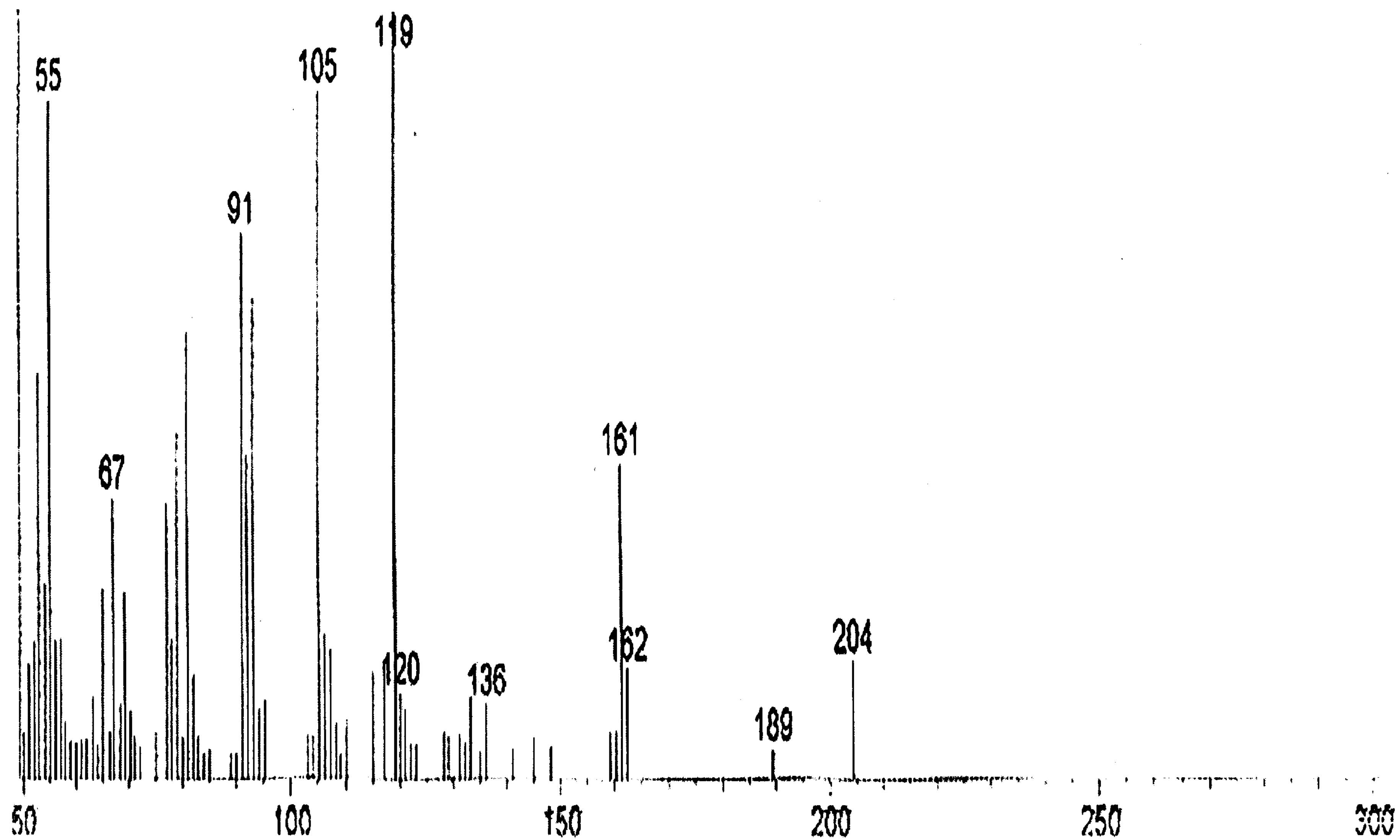



Fig. 16 .1$A_7$ Mass Spectra of germacrene - D

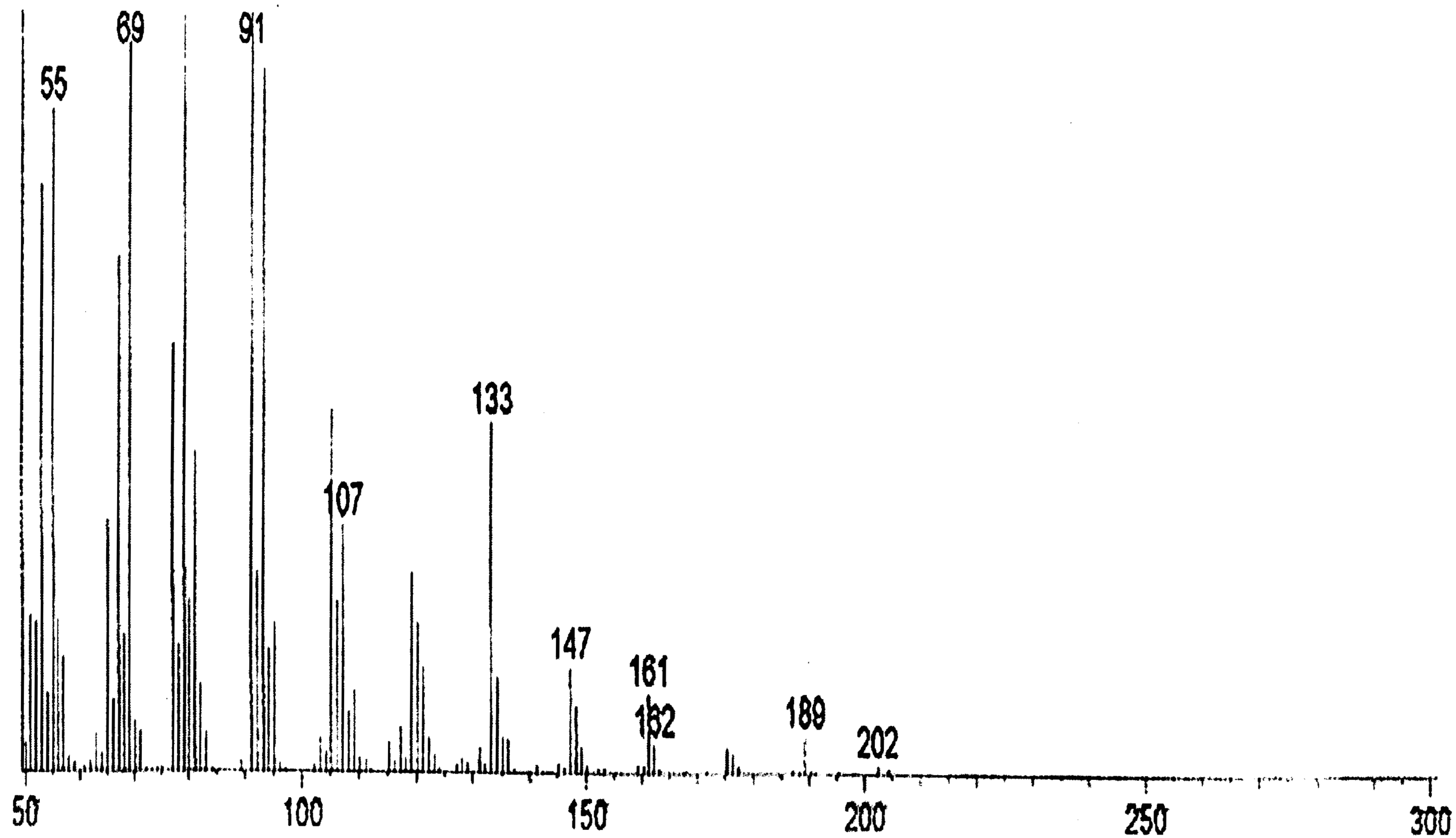



Fig. 16 .1A$_8$ Mass Spectra of β - caryophyllene

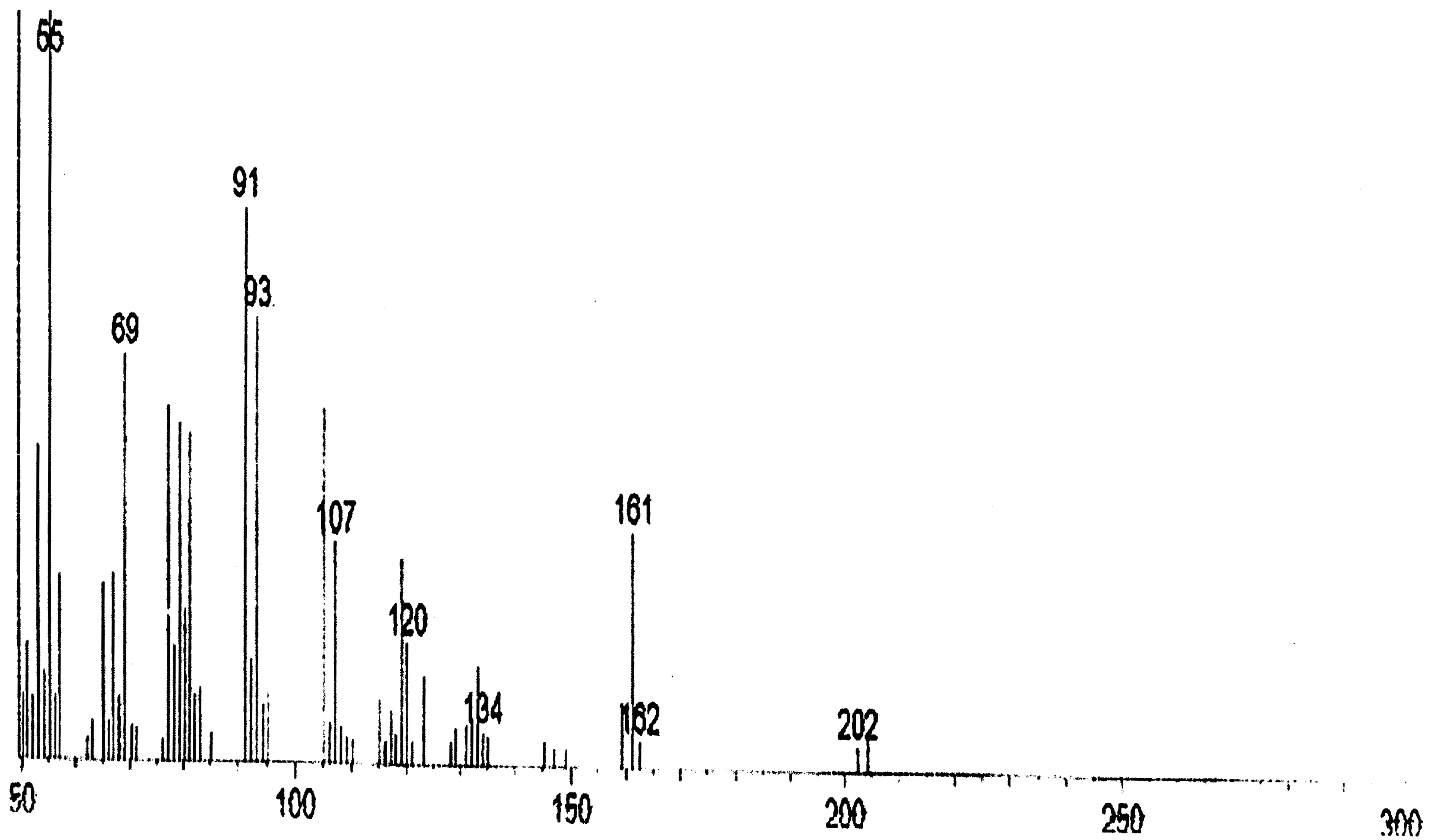



Fig. 16 .1$A_9$ Mass Spectra of α - farnasene

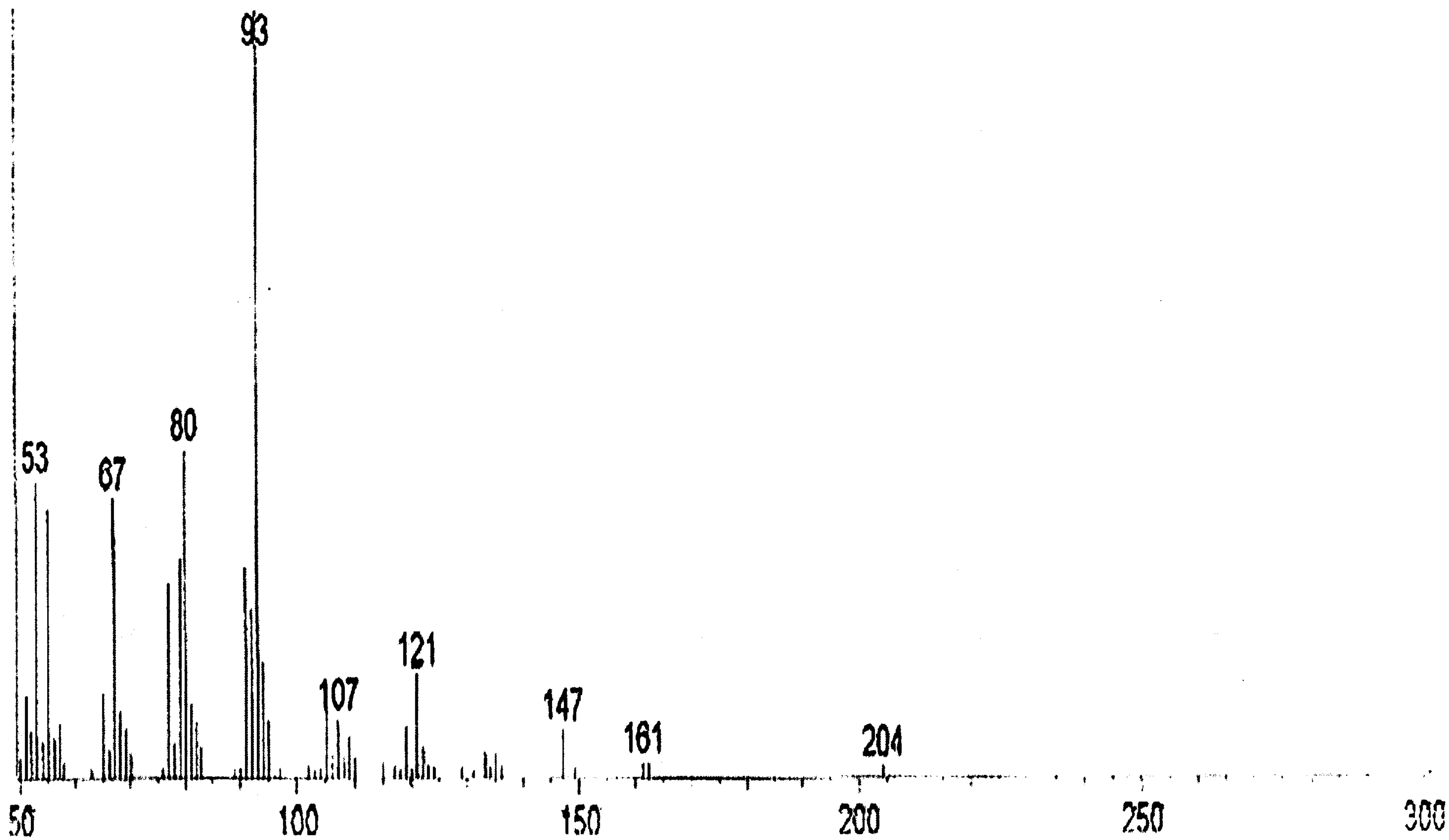



Fig. 16 .1$A_{10}$ Mass Spectra of ocimene

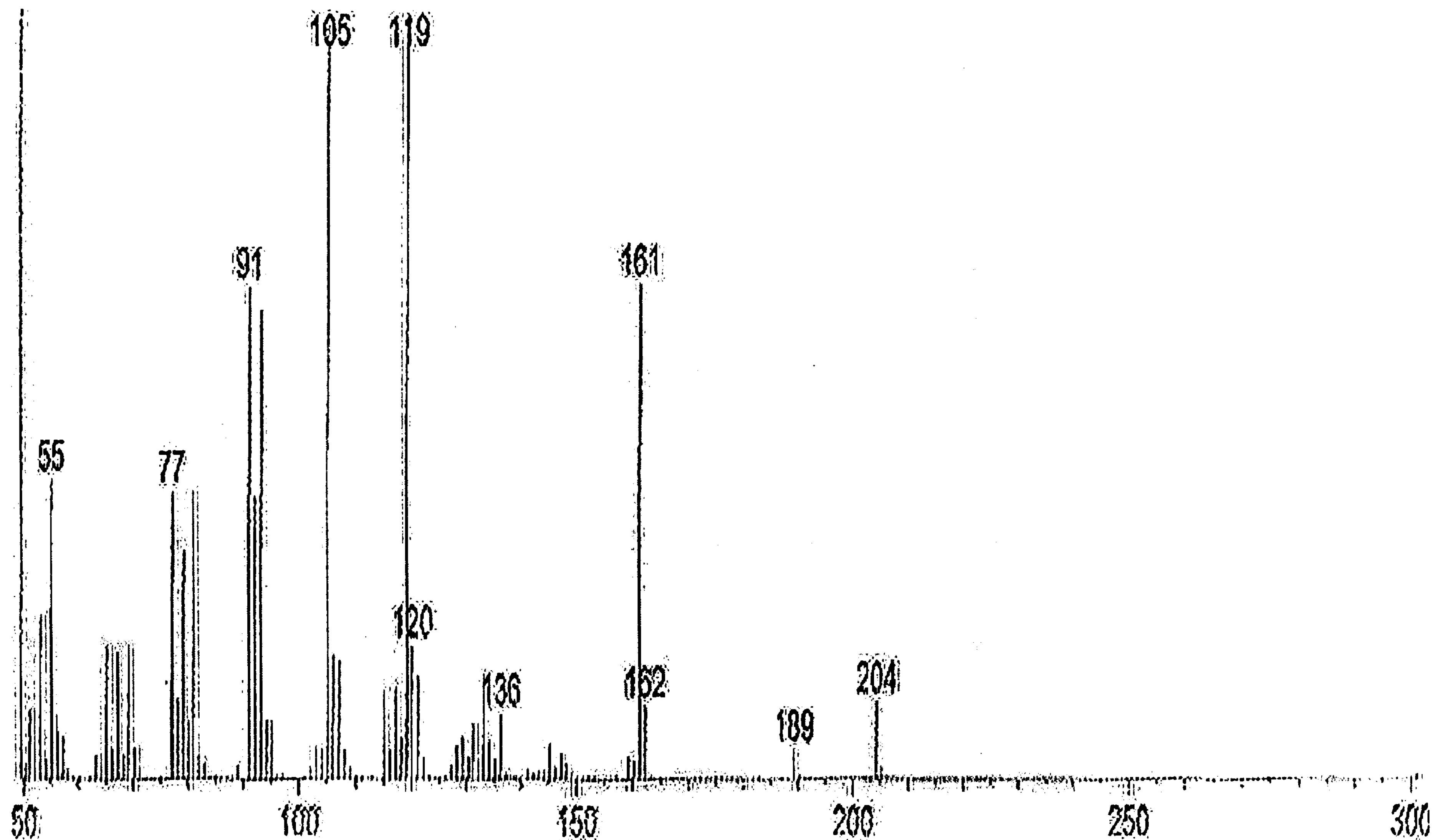



Fig. 16 .1$A_{11}$ Mass Spectra of α - cubebene

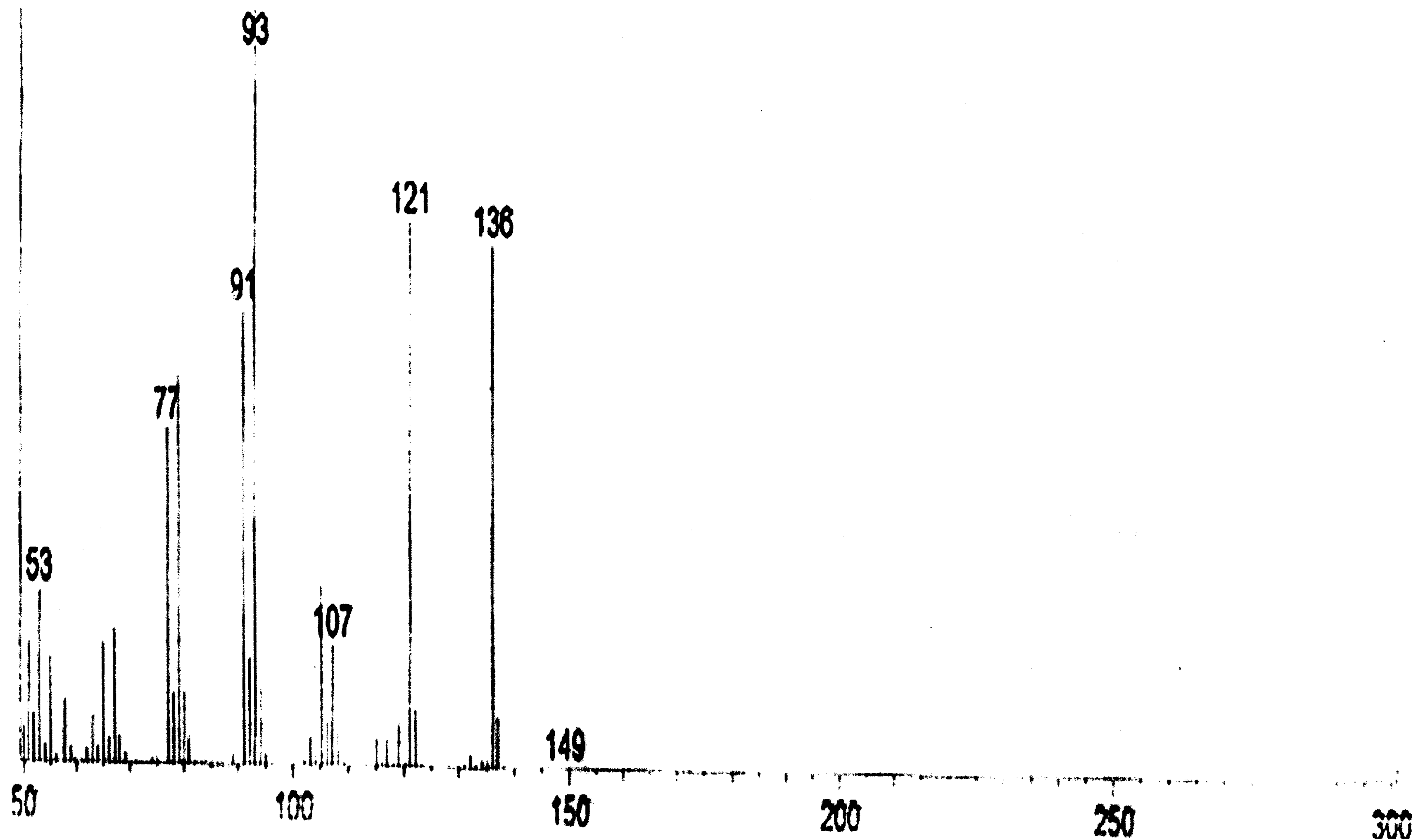



Fig. 16 .1$A_{12}$ Mass Spectra of δ - 3 - carene

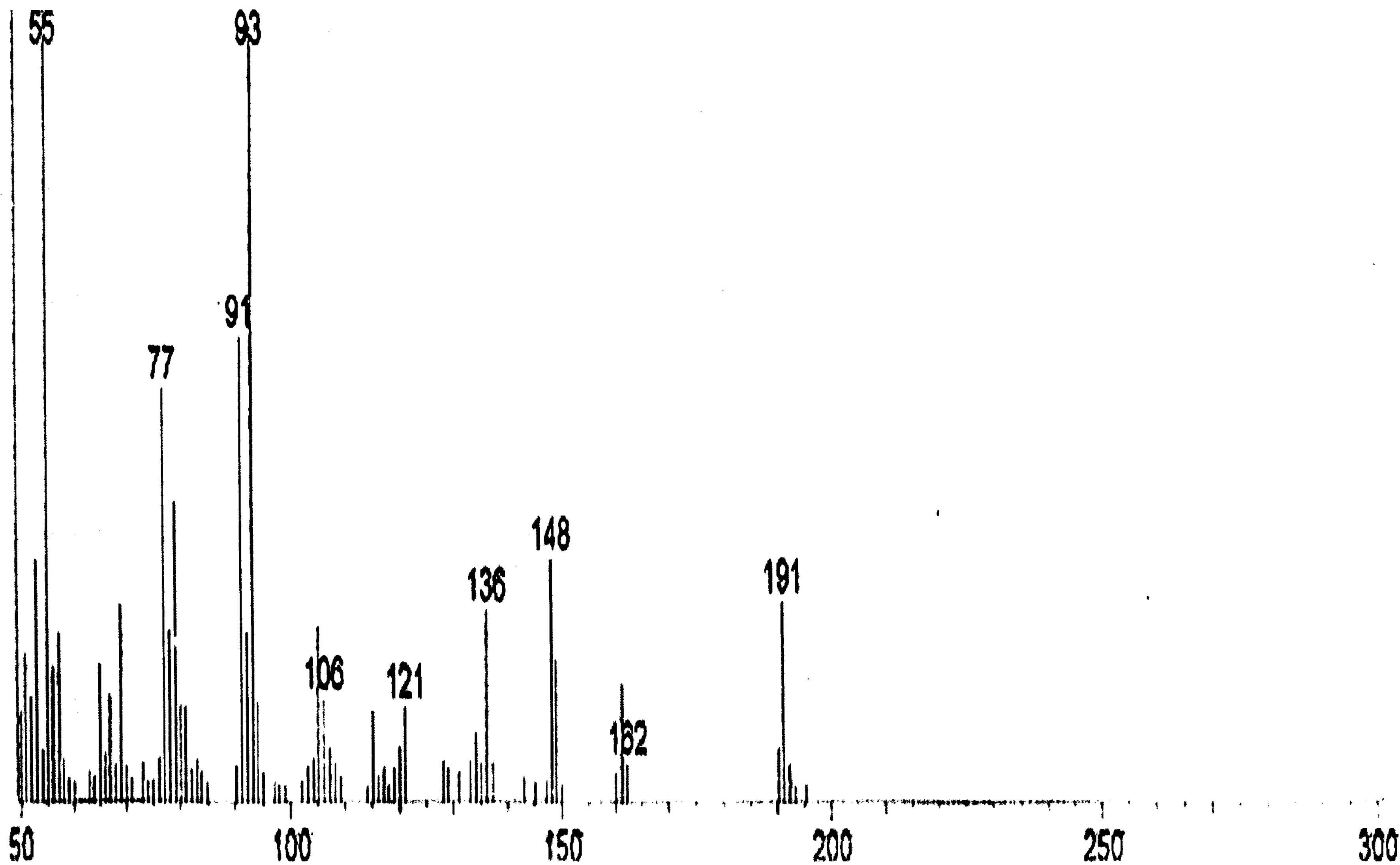



Fig. 16 .1$A_{13}$ Mass Spectra of the unidentified component

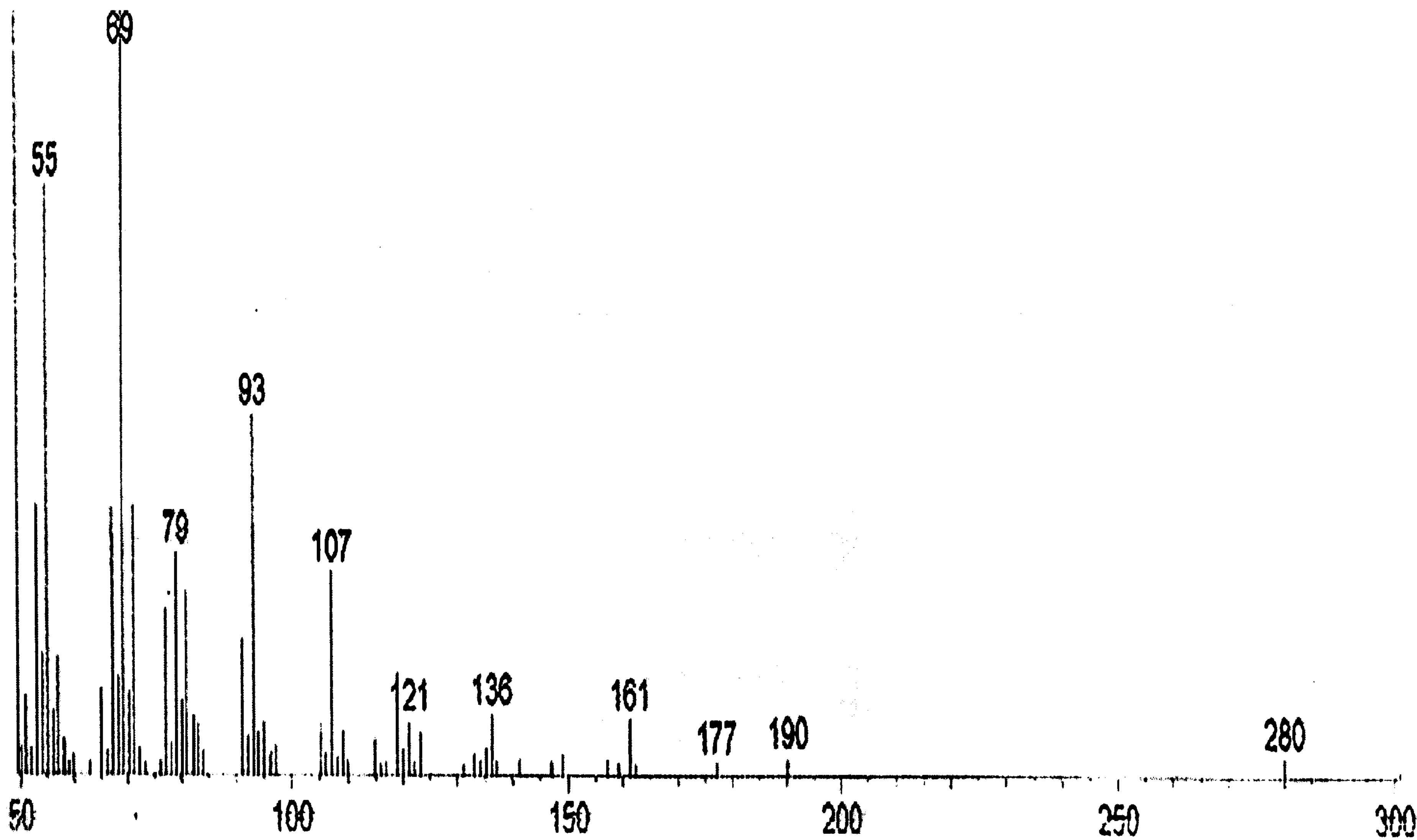



Fig. 16 .1$A_{14}$ Mass Spectra of β - farnasene

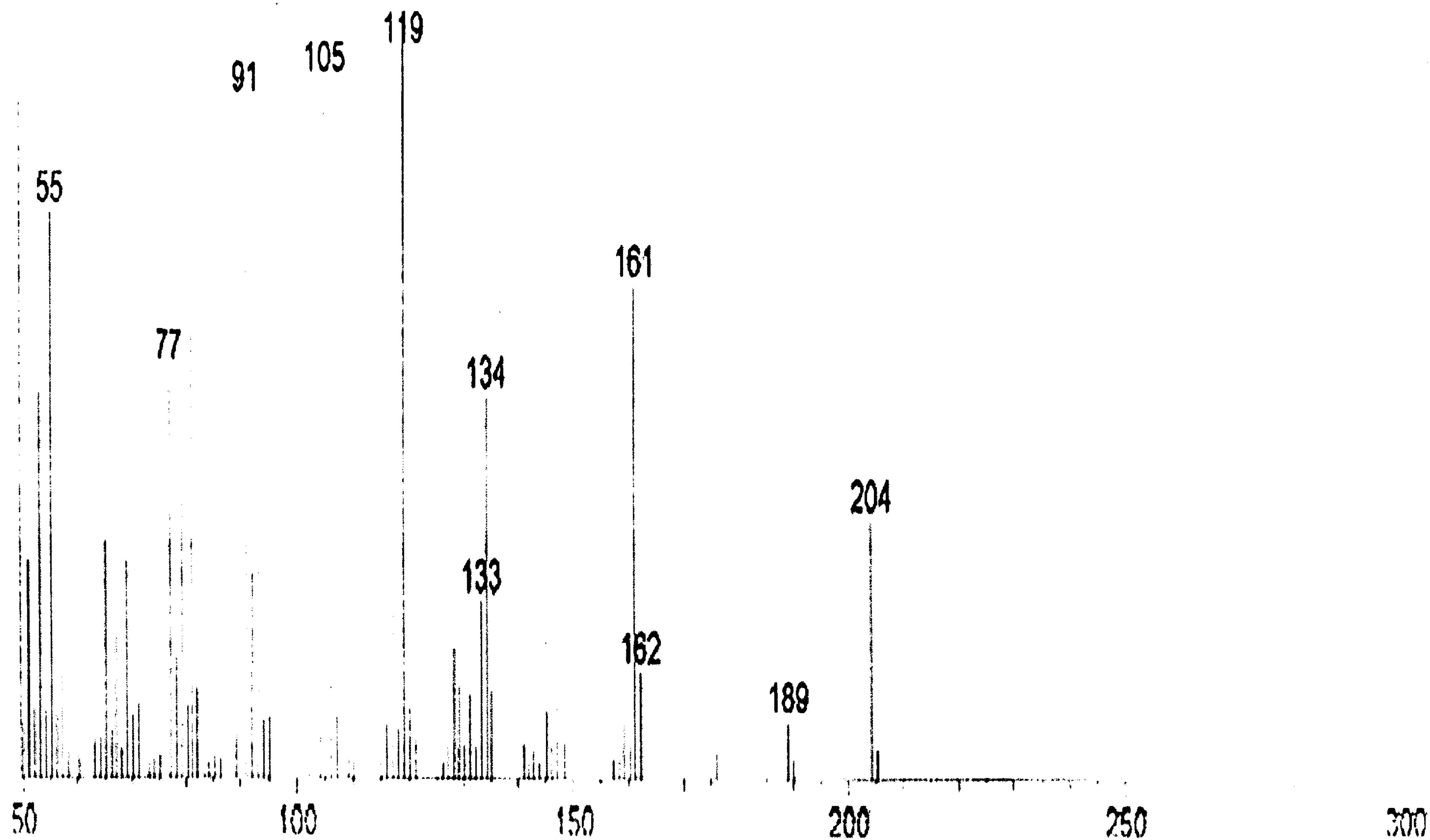



Fig. 16 .1$A_{15}$ Mass Spectra of α - neoclovene

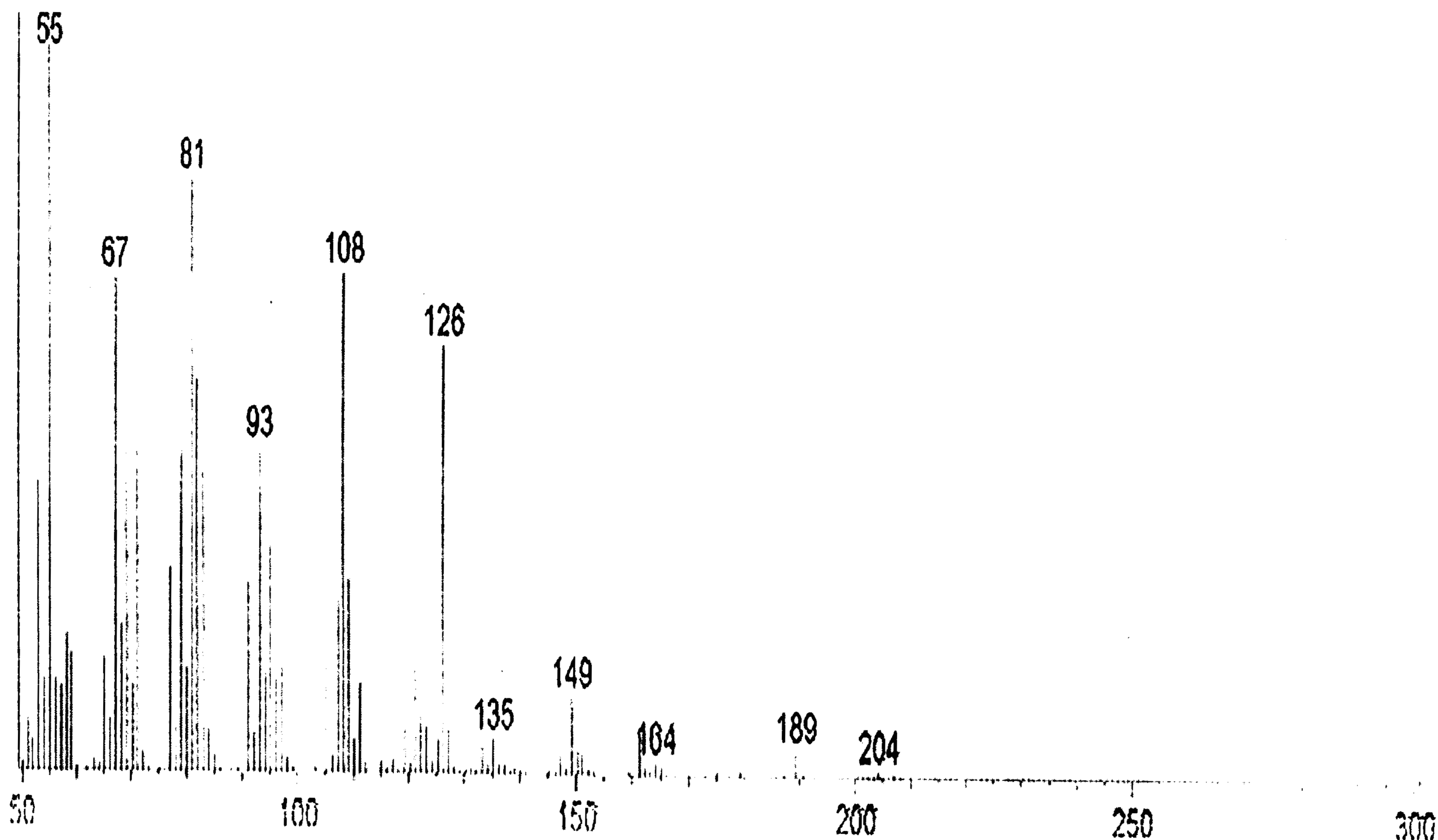



Fig. 16 .1A$_{16}$ Mass Spectra of isothujol

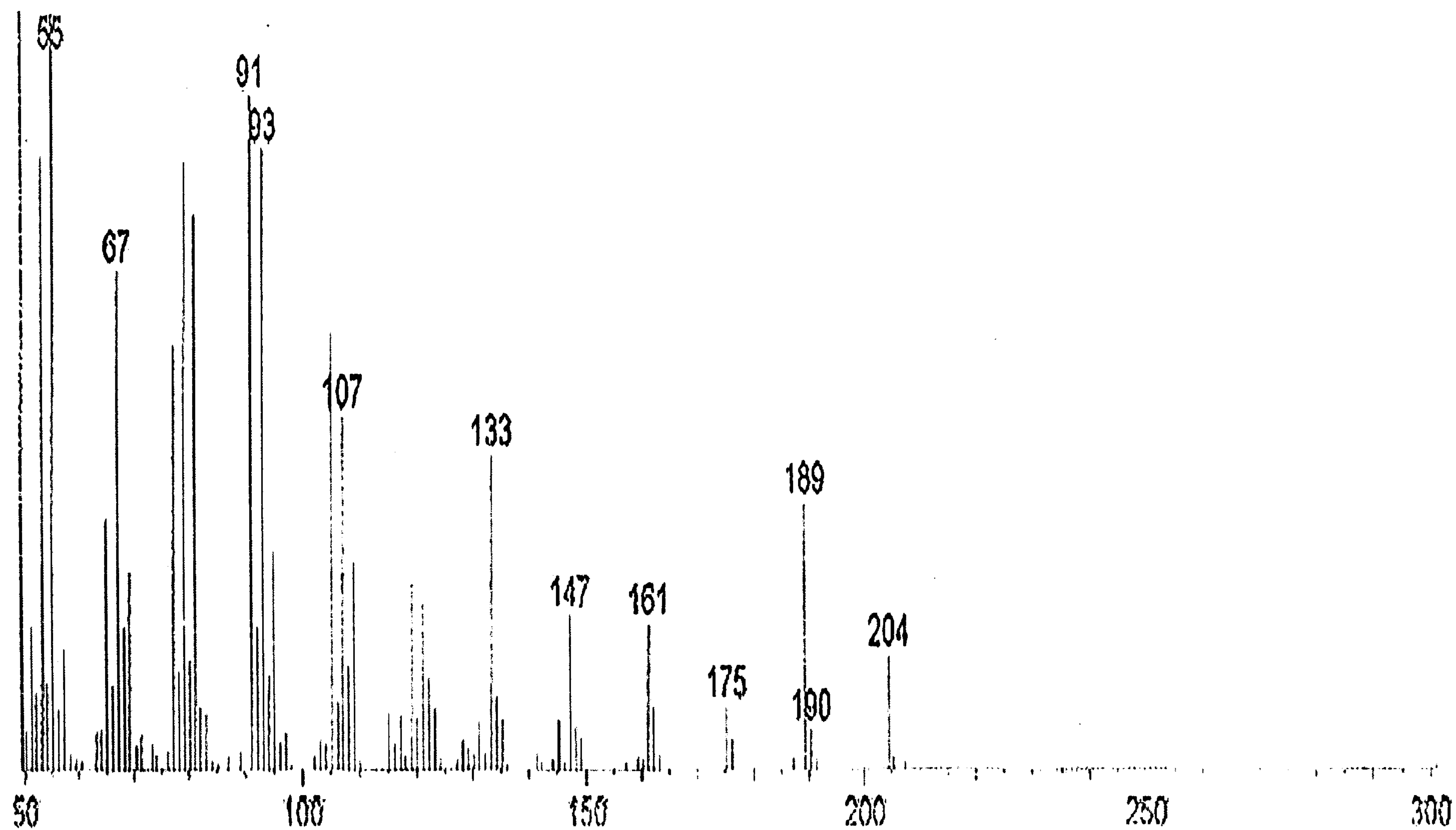



Fig. 16 .1A$_{17}$ Mass Spectra of 1 R, 3 Z - 9 S - 4 -11, 11 - trimethyl 8 - methylene bicyclo [7.2.0] undec-3-ene

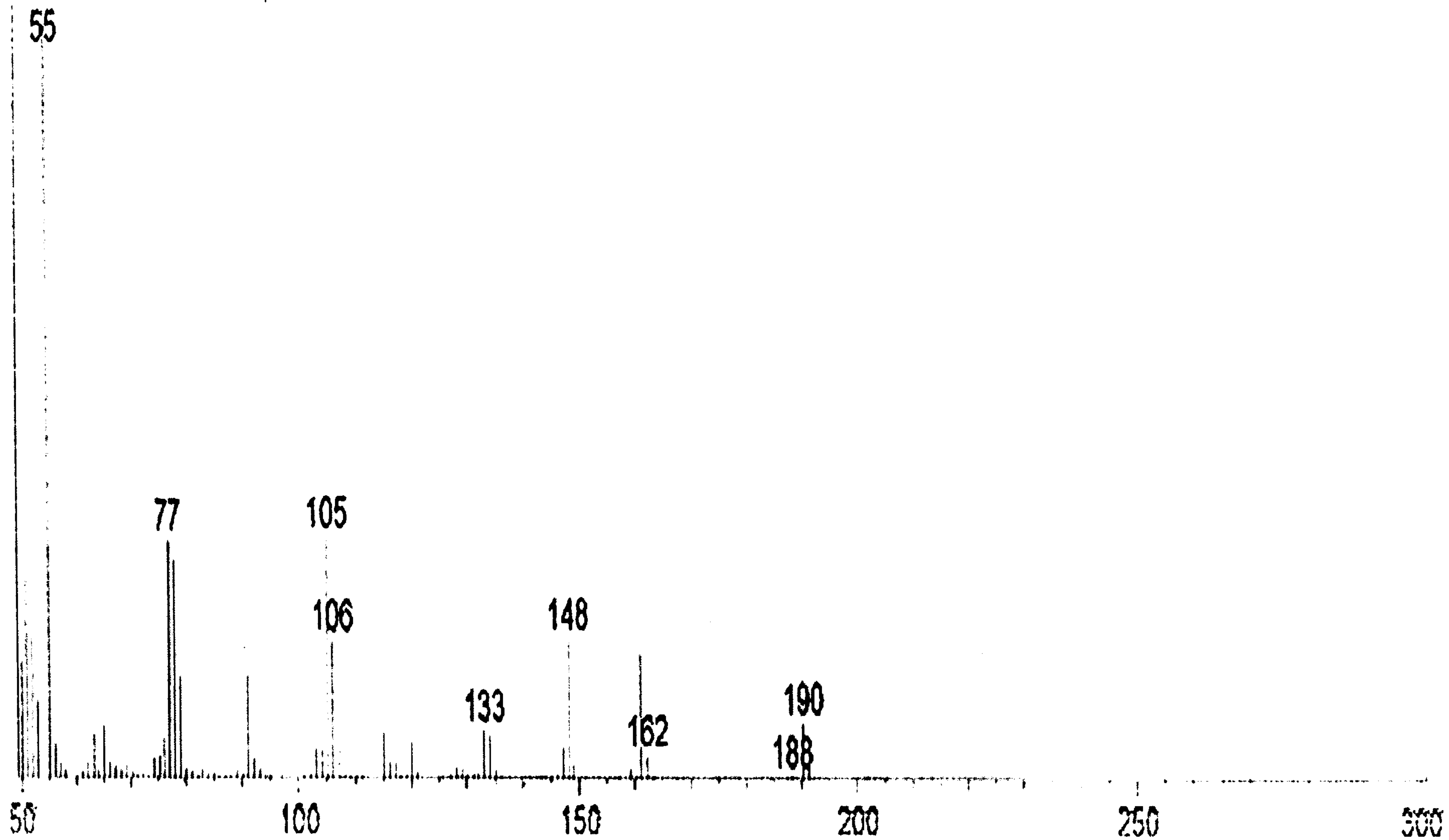


Fig. 16 .1$A_{18}$ Mass Spectra of α - β - epoxycumene

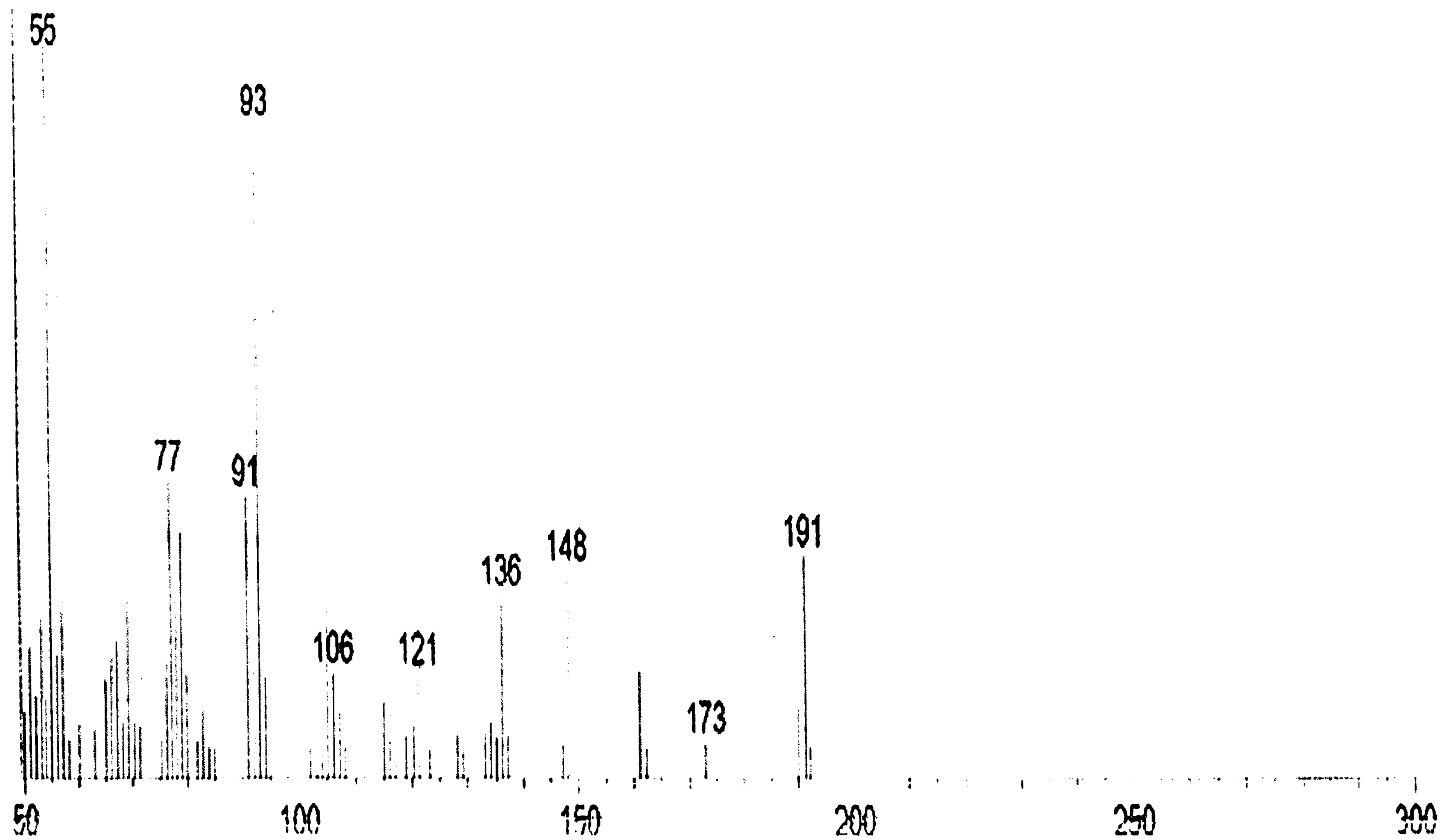



Fig. 16 .1A$_{19}$ Mass Spectra of α - bergamotene

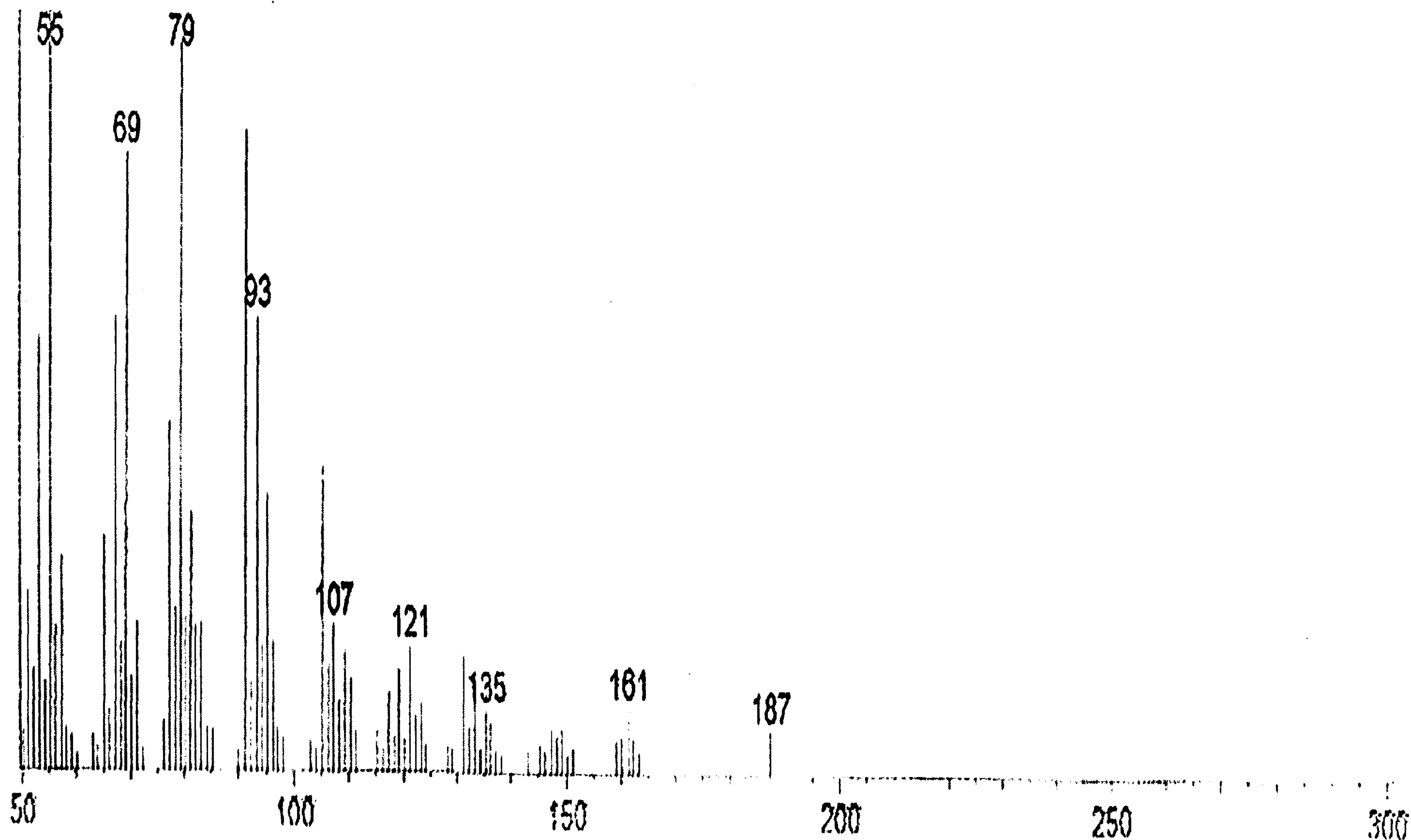



Fig. 16 .1A$_{20}$ Mass Spectra of α - bisabolene oxide

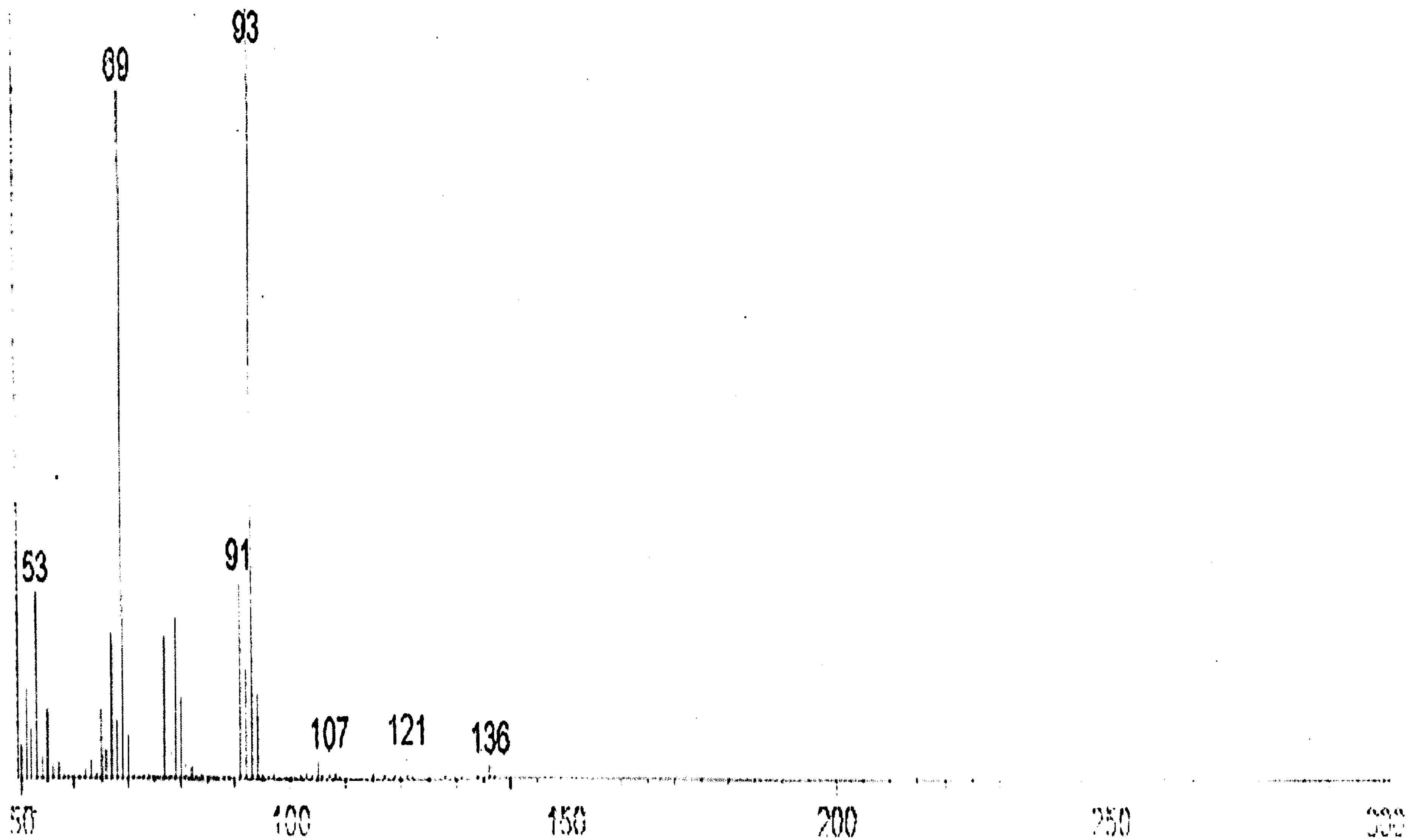



Fig. 16 .1$A_{21}$ Mass Spectra of β - myrcene

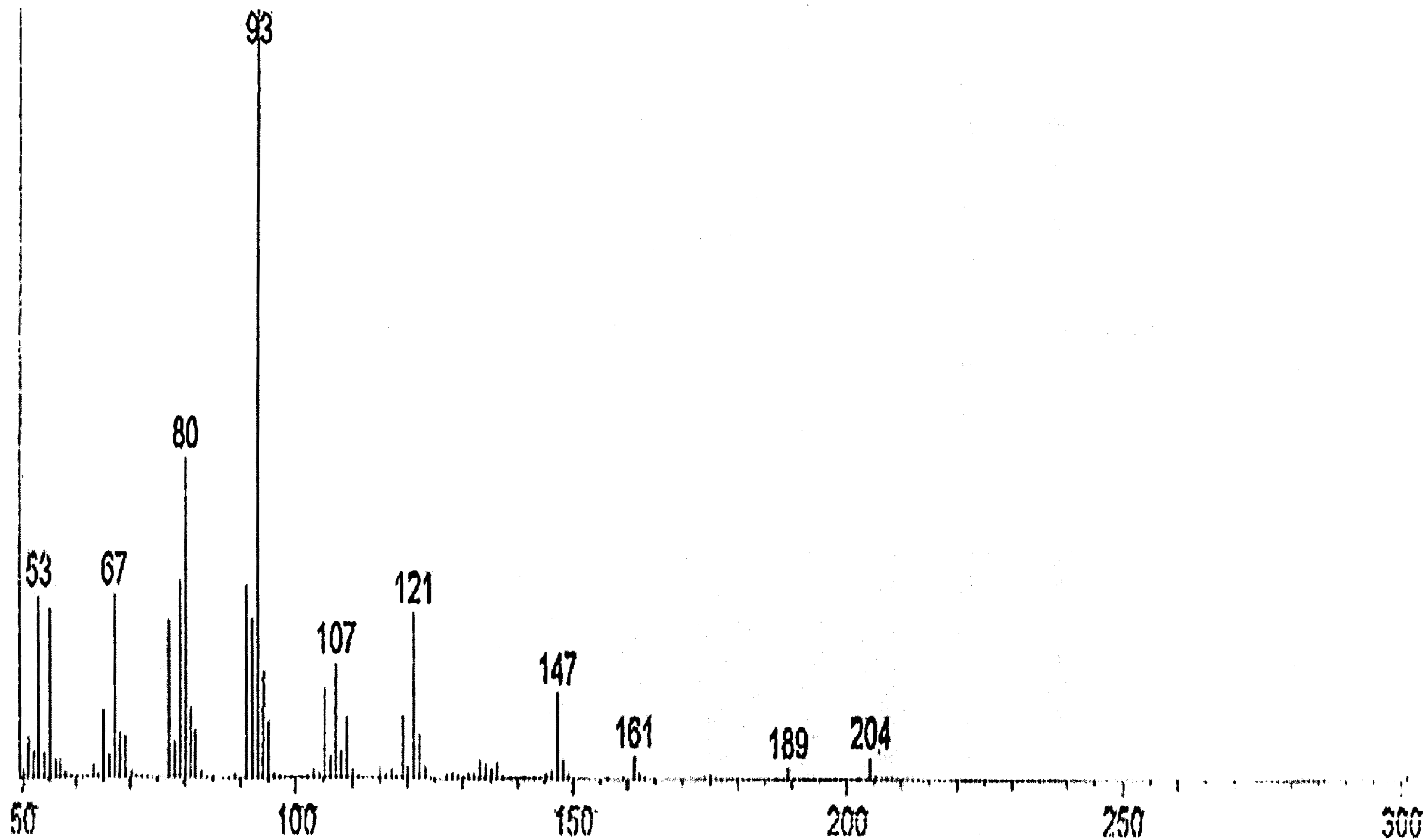



Fig. 16 .1$A_{22}$ Mass Spectra of α - caryophyllene

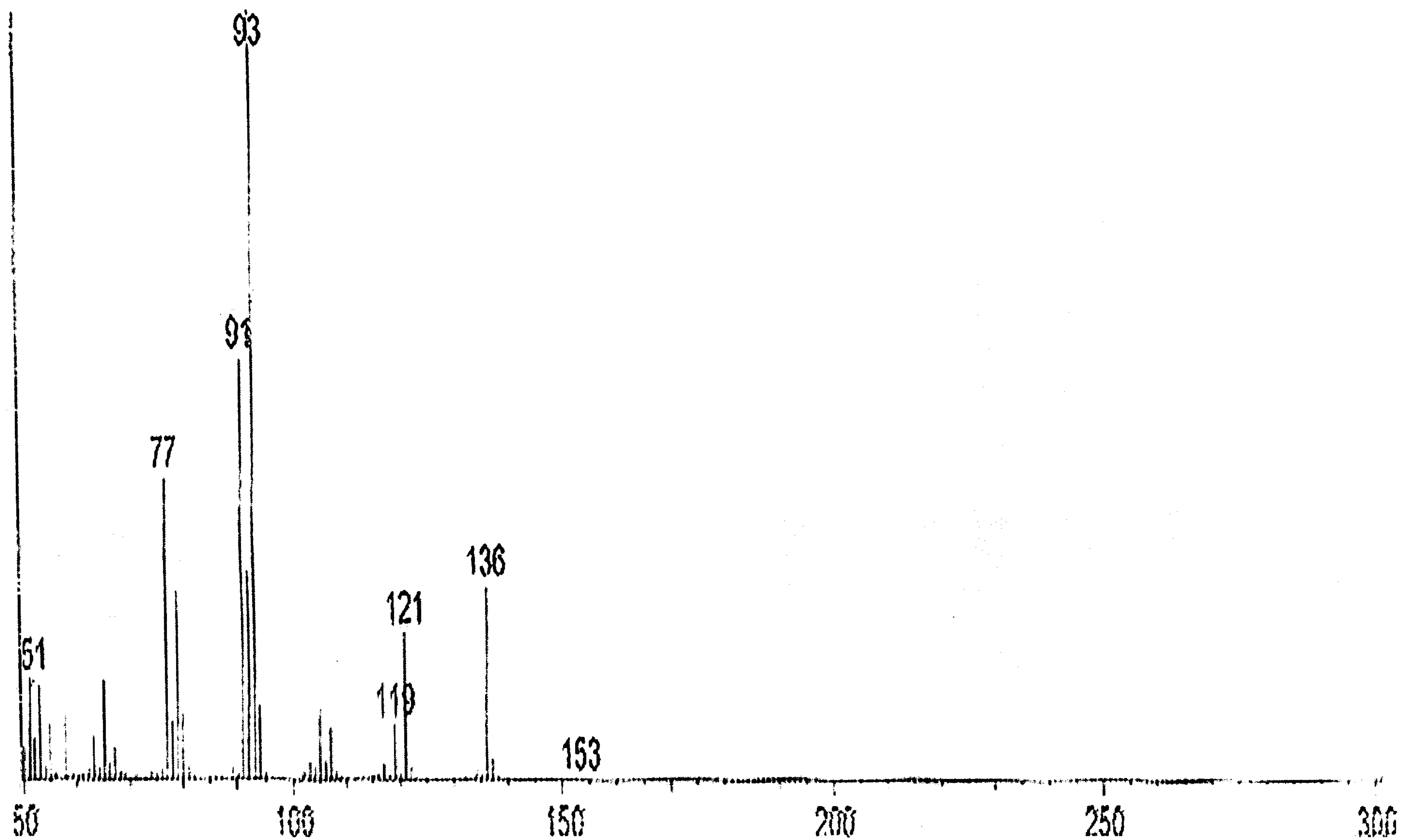



Fig. 16 .1$A_{23}$ Mass Spectra of γ - terpinene

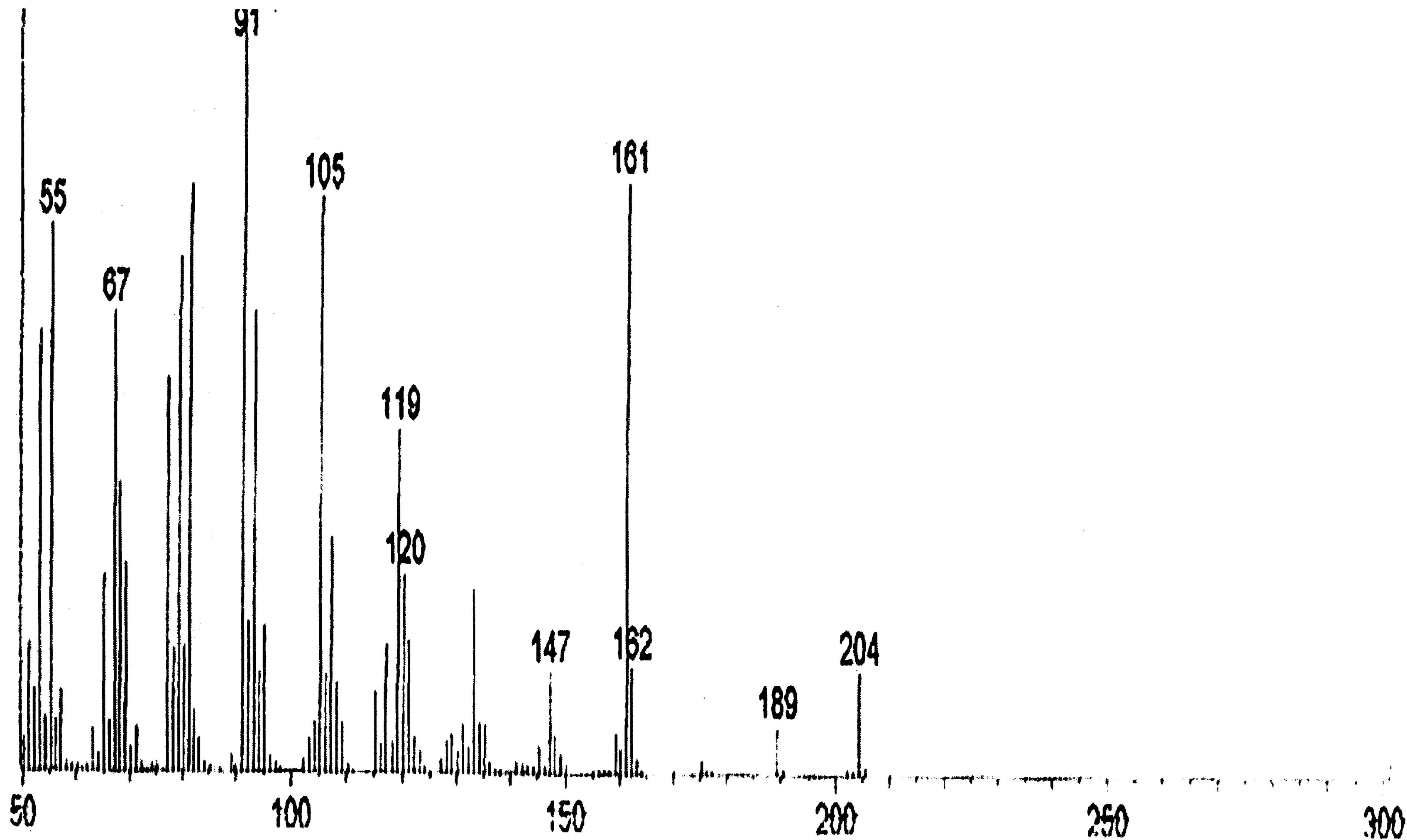



Fig. 16 .1$A_{24}$ Mass Spectra of cedrene

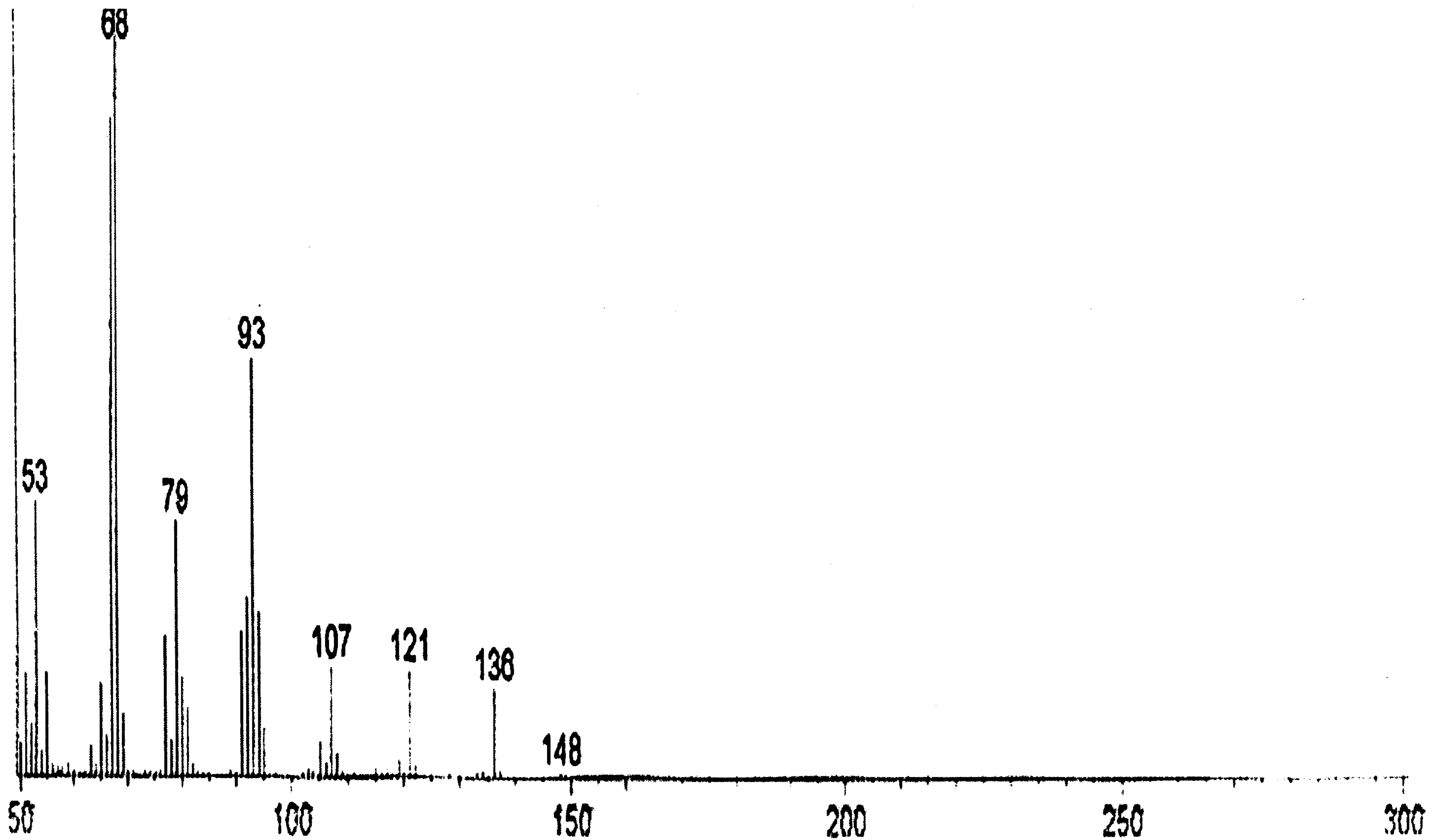



Fig. 16 .1$A_{25}$ Mass Spectra of limonene

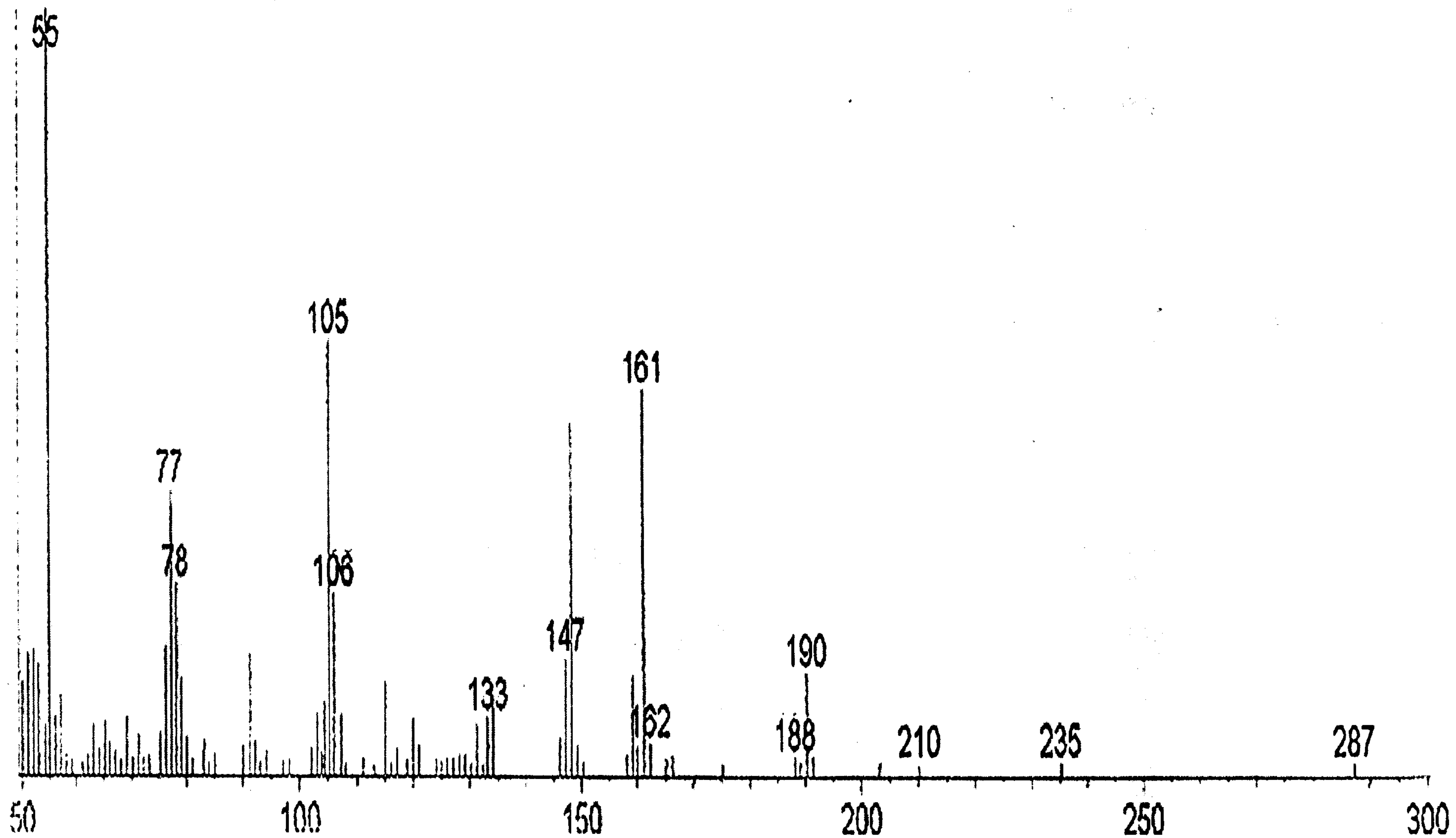



Fig. 16 .1$A_{26}$ Mass Spectra of aromadendrene oxide

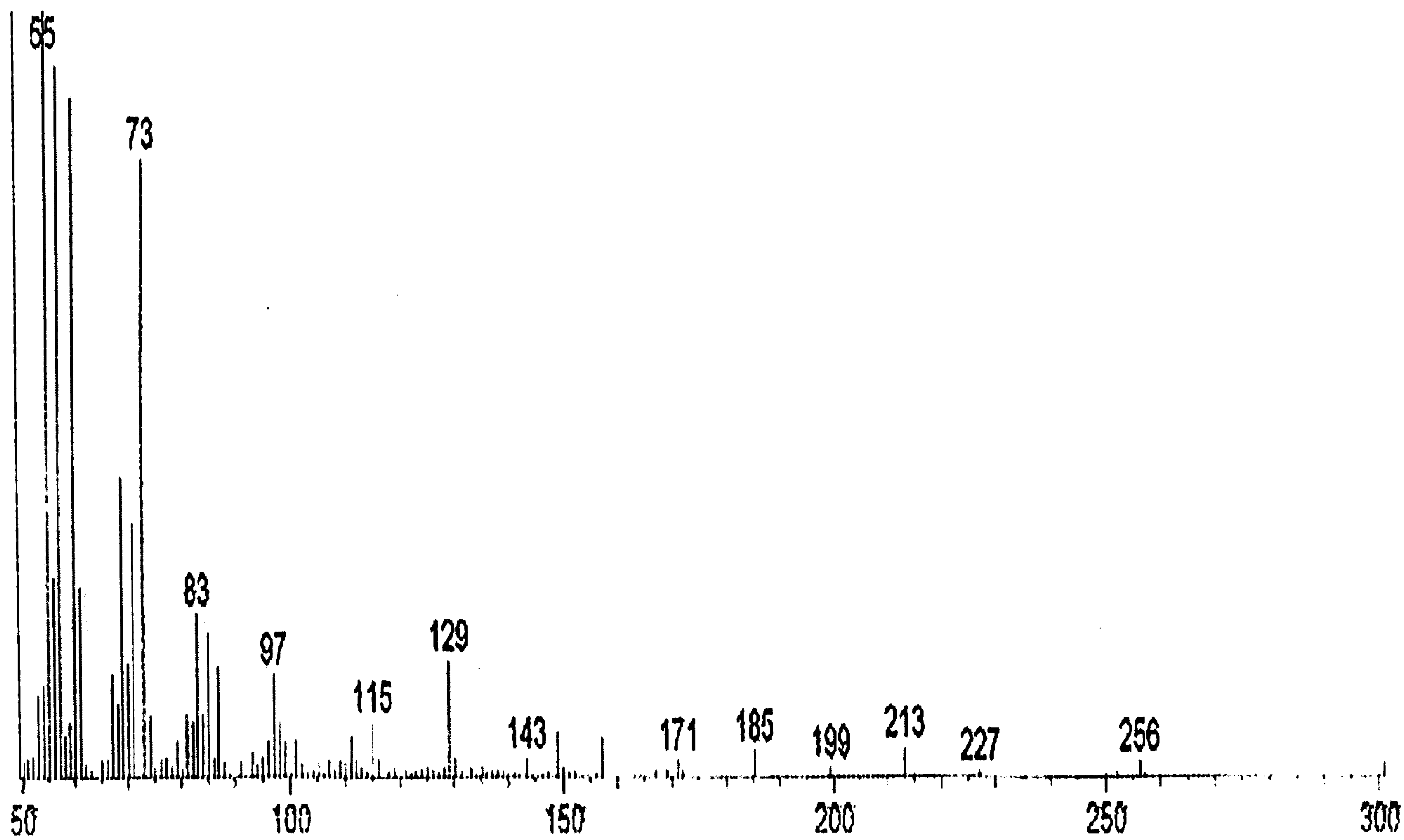


Fig. 16 .1$A_{27}$ Mass Spectra of n - hexadecanoic acid

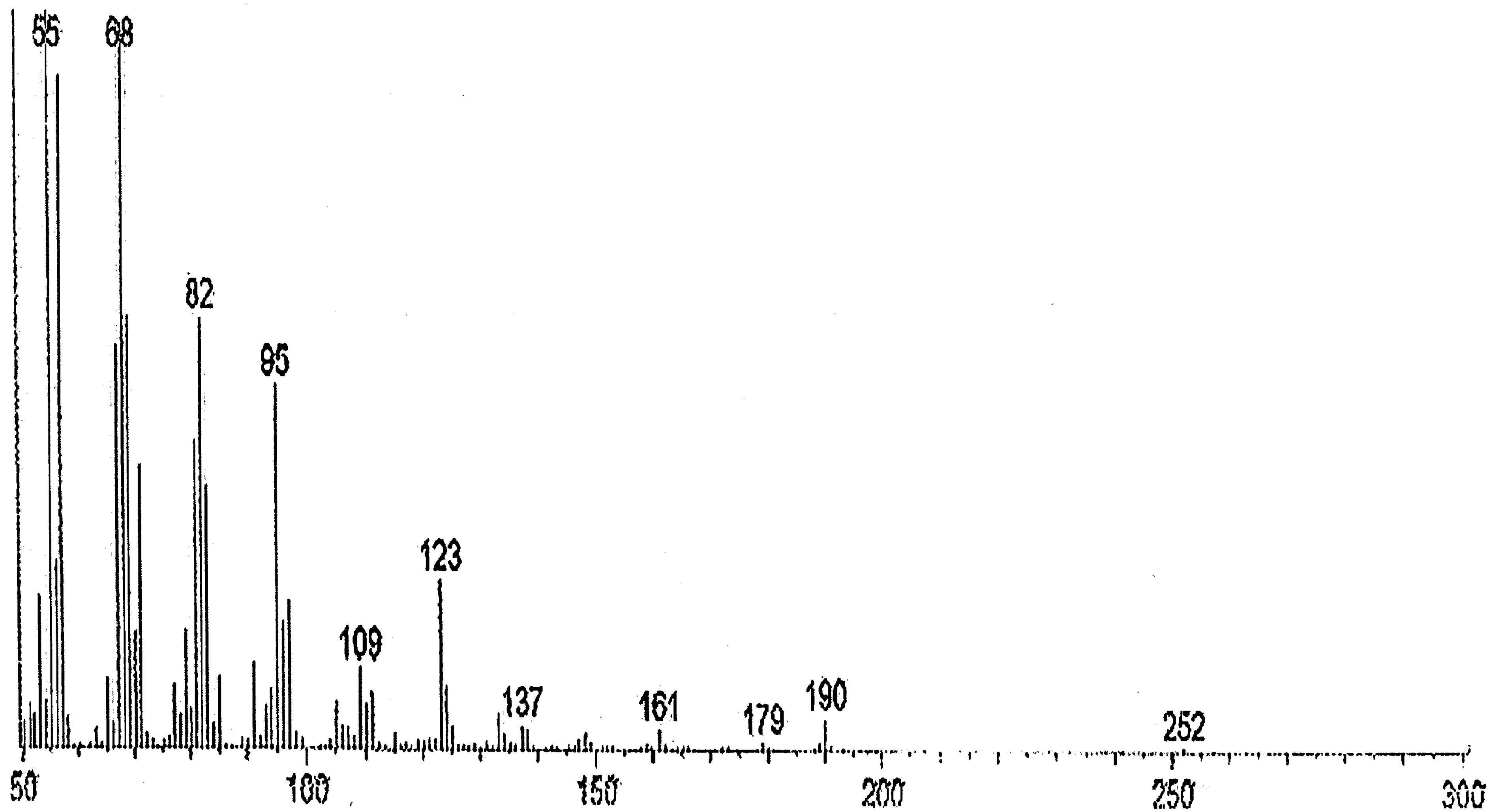



Fig. 16 .1$A_{28}$ Mass Spectra of tetradecanol

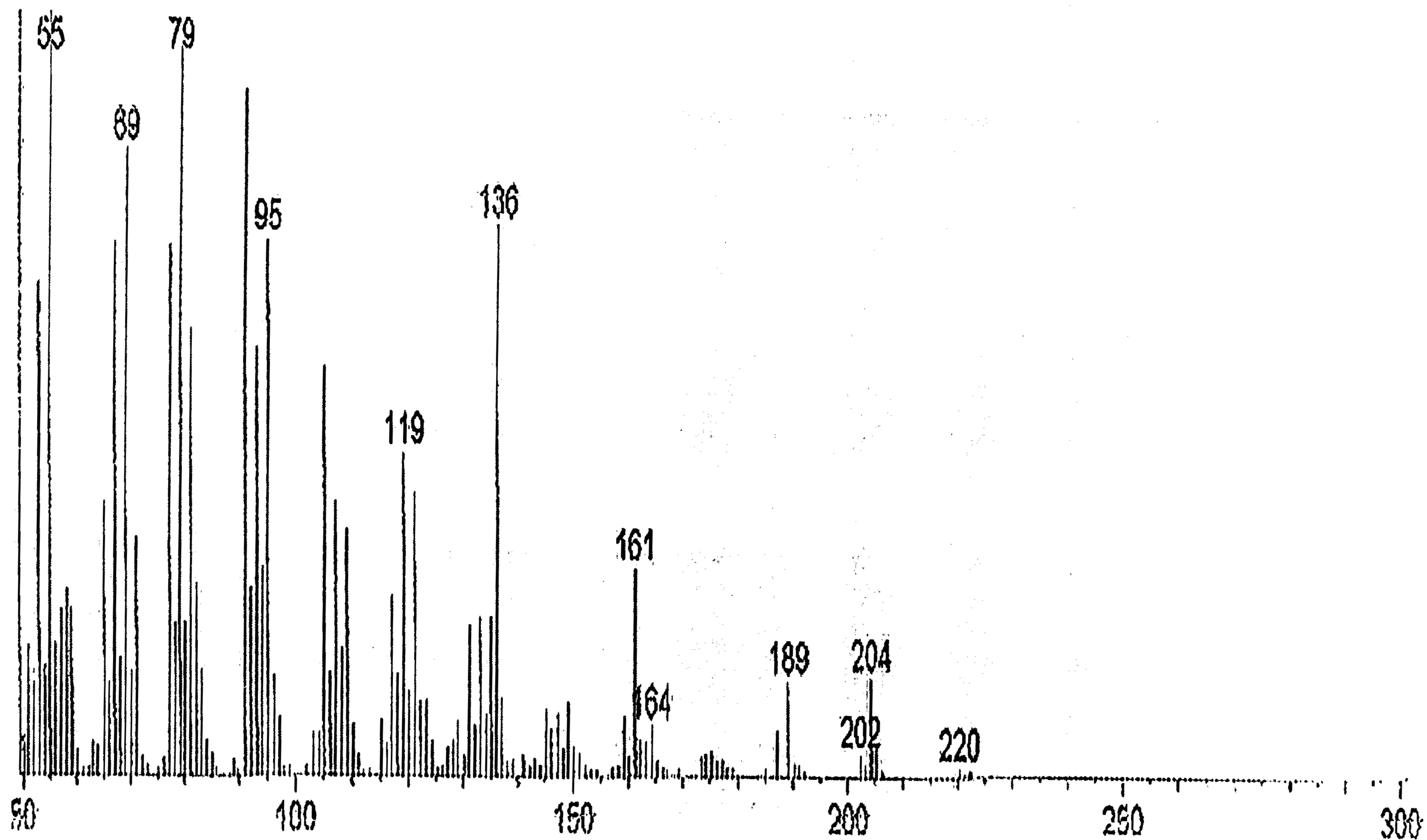



Fig. 16 .1A$_{29}$ Mass Spectra of patchoulane

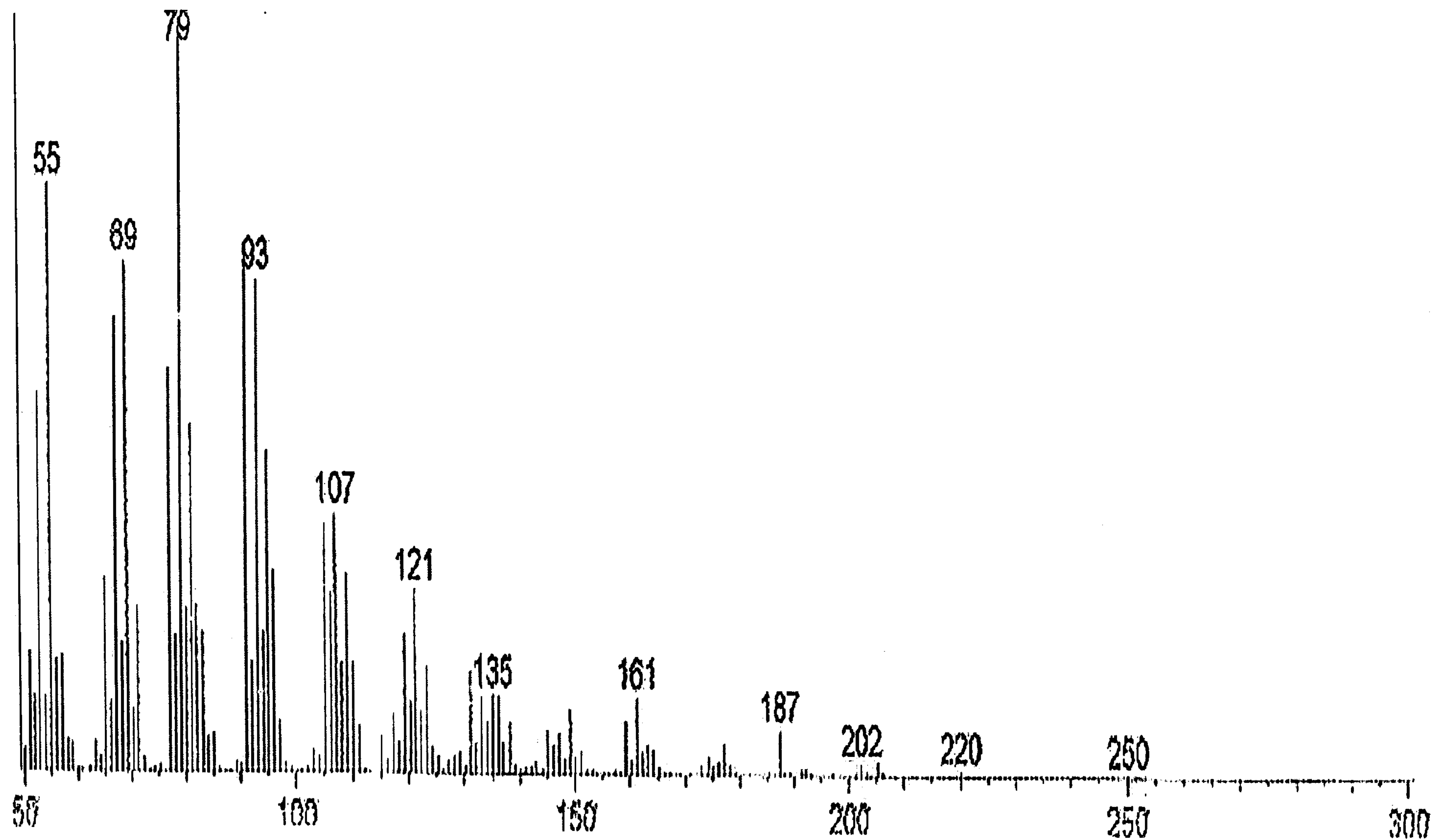



Fig. 16 .1$A_{30}$ Mass Spectra of caryophyllene oxide

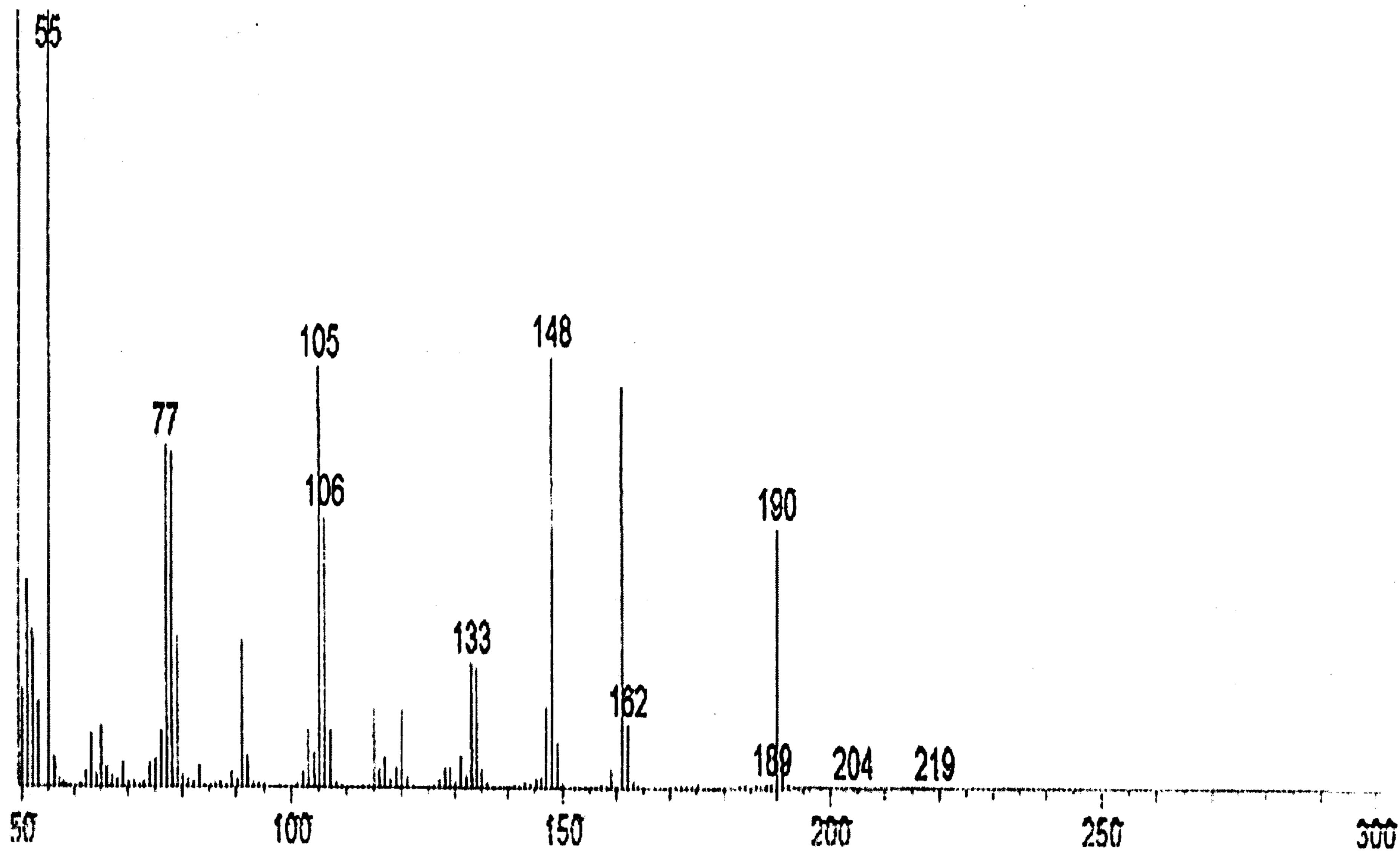



Fig. 16 .1A$_{31}$ Mass Spectra of β - methyl benzene propanal

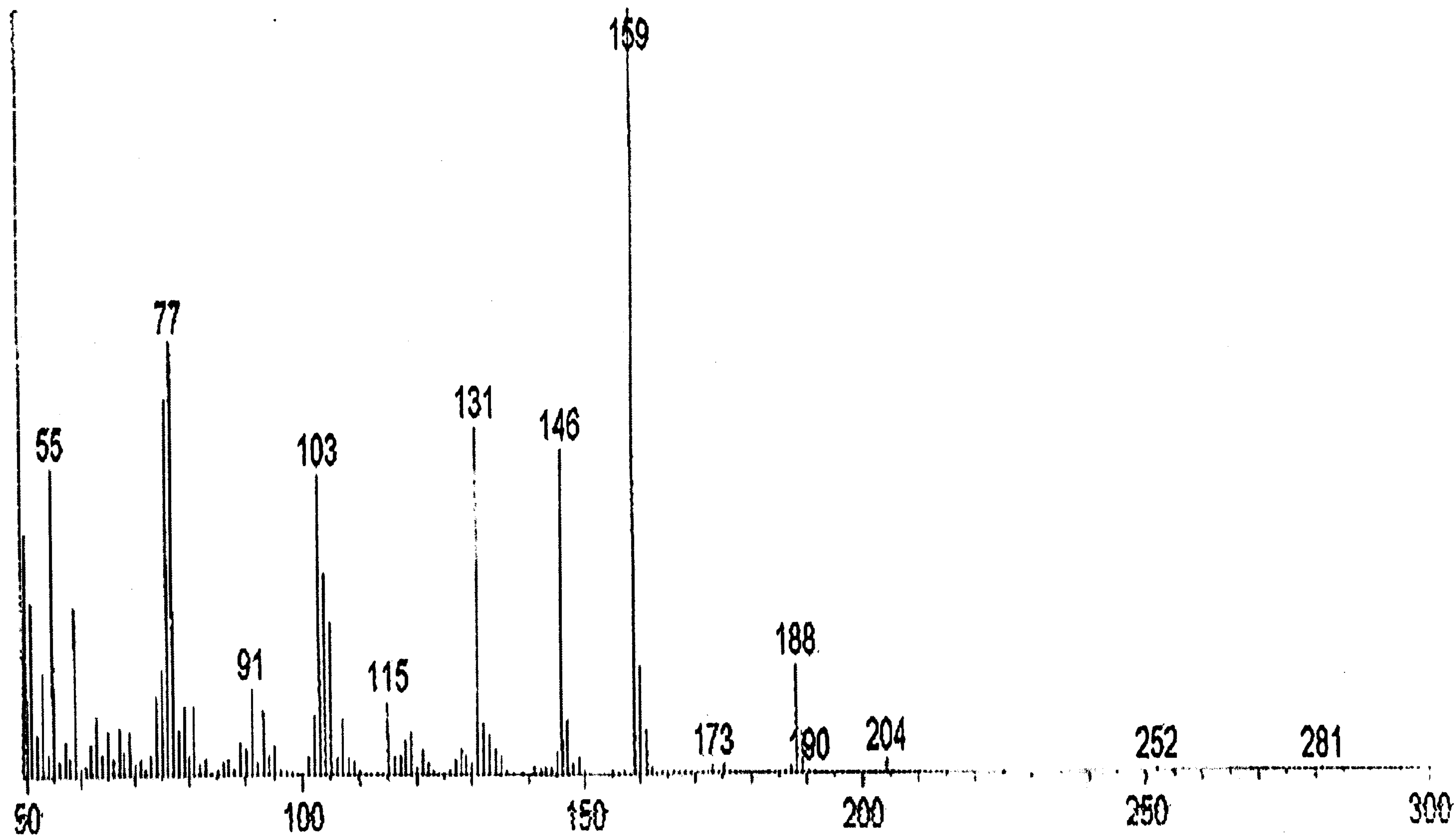



Fig. 16 .1$A_{32}$ Mass Spectra of isobenzofuranone

# DISCUSSION

Sreeranjini K. “Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)” Thesis. Department of Botany, University of Calicut, 2004

# DISCUSSION

## Micropropagation

Plant tissue culture techniques have become a powerful tool for studying and understanding the basic and applied aspects in plant biotechnology. Tissue culture techniques are gaining increasing importance as a valuable supplement to the conventional methods for genetic improvement besides clonal propagation (Bajaj, 1990 b). The regeneration of plants from cell and tissue culture is an important and essential component of biotechnology that is required for the genetic manipulation and improvement of plants. Plants regenerated from tissue culture undergo some form of variation usually from original cultivars (Bayliss, 1980; Larkin & Scowcroft, 1981; Brown, 1989). Data recorded on the somaclonal variants (Seeta *et al.*, 2000) for both qualitative and quantitative characters including the seed oil content and fatty acid composition clearly suggests that somaclonal variation serve as a potential, novel and an alternative source in generating variability for subsequent utilization in conventional breeding programmes.

Plants regenerated from relatively undifferentiated callus cultures possess a vast array of genetic changes. Such variations can result in useful agricultural and horticultural products. The presence or absence of variations depends on the source of explants and method of regeneration (Larkin *et al.*, 1985). According to Mc Clintock (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 micropropagation protocol was attempted on *Trachyspermum roxburghianum* and generated a variant *in vitro* plant (Figs. 1-47). For the micropropagation studies, nodal segments (Fig. 1) of *T. roxburghianum* (DC.) Craib were mainly used, which was subjected to callus development and shoot initiation. Cultivation of *in vitro* plants is often practiced for the biosynthesis of secondary metabolites (Ikuta *et al.*, 1974).

Clonal multiplication of *Trachyspermum roxburghianum* acquires significance because of the added medicinal virtues of the plant. The cultured explants regenerate shoots either via callus or directly. Emergence of shoots directly from cultured explants and from their callus will be useful in the propagation of true-to-type plants and also in the induction of variations, respectively.

The morphogenic response of explant was dependent on the type and concentration of growth hormones. Tissue culture studies on a number of medicinal plants (Irawati & Nyman, 1986; Kumar, 1992; Nirmal 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, $p^H$ of the cultures and addition of organic acids to buffer media have been found to affect the concentration of the propagules in plant tissue culture (Dougall, 1981). There was maximum multiplication in the medium with 1 mg/l BA and 7 mg/l IAA (Figs. 5 & 6). The present result indicate that a combination of BA and IAA was practically effective in inducing multiple shoots in *T. roxburghianum* (DC.) Craib. With KIN the result was unsatisfactory with regard to multiplication though occasional shooting was noticed. BA shows more activity than KIN for shoot proliferation (Lundergan & Janic, 1980; Rahman & Blake, 1988; Sen & Sharma, 1991). Two different auxins viz., NAA, IAA was used with KIN and BA for shoot multiplication. The auxin, 2, 4-D was also used first singly and later medium was changed to IAA and BA. There was no significant synergistic effect of any auxin treatment except in the set of IAA with BA. Of the two cytokinins tested, BA was found to be comparatively more effective than KIN in inducing multiple shoots from nodal segments. Regeneration of plants from callus is a necessary prerequisite for biotechnological approaches in crop improvement (Shrivastava & Chawla,

2001). The success of regeneration in any crop depends upon the type of medium used in each phase of culture from callus initiation to maintenance and during regeneration. The shoot forming ability has been improved by using auxin and benzyl aminopurine (Chawla & Wenzel, 1987). The same result is observed in the present study. The expression of regeneration potential of primary callus depends on the type of explant and ploidy level. The media composition, especially an optimum combination of auxin and cytokinin may affect an additional increase in the expression of the morphogenetic potential (Heszky *et al.*, 1991). It is observed that various plant species respond to concentration dependent auxin / cytokinin ratios to show morphogenesis (Mohan *et al.*, 1995). In the present study also, simultaneous inclusion of auxin and cytokinin was essential for shoot generation (Table 3).

Yeung *et al.* (1981) showed that shoots might be induced from meristematic tissues since actively dividing cells are highly susceptible to hormonal treatments, which alter their normal developmental pathways. Auxin-cytokinin balance was known to regulate the growth of callus tissues and also the process of organogenesis as reported earlier for some tree species as *Dalbergia sisso* (Datta & Datta, 1983) and *D. latifolia* (Laksmisita *et al.*, 1986). Hu & Wang (1983) proved superiority of 2,4-D over other auxins for induction of callus and strongly antagonize any organized development. In the present investigation, 2,4-D (2 mg/l) proved better than NAA (2 mg/l) for compact callus formation. Guimaraes *et al.* (1989) pointed out that the lower concentration of 2,4-D stimulated only callus proliferation from hypocotyls of *Cyphomandra betcea.* This is in agreement with the present study since callus proliferation was induced in the medium containing 2,4-D (2 mg/l). Similar findings were reported in *Rauwolfia serpentina* (Perveen & Elahi, 1987) and *Plumbago rosea* (Harikrishnan & Hariharan, 1996). Regeneration did not occur in 2,4-D (1 mg/l) and BA (2 mg/l) supplemented medium, due to some

intrinsic factors. Skoog & Miller (1957) reported that shoot bud regeneration depends on quantitative interaction between various growth regulators, viz., auxins and cytokines. In the present investigation, BA (1 mg/l) and IAA (7 mg/l) proved the effective growth regulator combination for indirect organogensis from callus (Table 3). Reynolds (1987) have reported that high levels of auxins promote callus formation. This is in conformity with the callus formation in MS medium with IAA (7 mg/l) and BA (1mg/l). According to Von & Woodward (1988), Hakman & Fowke (1987) presence of auxin together with cytokinin is indispensable for the induction and formation of organogenic callus, which is in conformity with the results of the present study. Studies have shown that optimum concentration of BA required for shoot multiplication varies in different cultivars (Hussey, 1977; Dantu & Bhojwani, 1987; Grewal *et al.*, 1990). This could be attributed to variation in endogenous hormone levels in the plant. The younger tissues are known to contain high auxin levels (Sheldrake, 1973) that could promote callus formation. Using various hormonal levels can therefore, bring about high degree of regeneration. The superiority of BA over other cytokinins for multiple shoot formation has been reported in many fruit plants (Lundergan & Janic, 1980). Generally, a cytokinin or a combination of cytokinins and auxin is required for *in vitro* shoot proliferation (Thorpe & Patel, 1984).

Axillary shoot initiation and development was observed in medium containing BA (1 mg/l) and IAA (7 mg/l). The role of cytokines in overcoming the apical dominance of the terminal shoot bud and enhancing the branching of the lateral buds from axils was observed. It is known that BA is the most effective synthetic cytokinin for stimulating axillary shoot proliferation for different plant systems (Bhojwani, 1980; Hasegawa, 1980; Kitto & Young, 1981; Welander *et al.*, 1989; Nadel *et al.*, 1991; Devi *et al.*, 1994; Gangopadyay *et al.*, 1998). The stimulatory effect of BA on multiple shoot formation was reported in *T. ammi* by Ajita *et al.* (2001). The

stimulatory effect of BA in the present investigations in multiple shoot formation was in consonance with reports on *Trachyspermum*. Present investigation revealed BA in combination with auxin as effective for axillary bud formation, which agree with findings of Mujib *et al.* (1995) in *Catharanthus roseus*. Reynolds (1987) assessed that adenine based cytokinins resulted in adventitious shoots or expression of axillary shoots. This result agrees with that of the present study in the formation of axillary shoot in nodal explants in the medium with BA (1mg/l) and IAA (7mg/l). Hussey (1976) reported that precocious out growth could be stimulated by the bud present in the leaf axil by adding an appropriate concentration of BA in the culture medium. In the present study, shoot bud proliferation has been achieved in the leaf axil, by the addition of BA (1mg/l) in combination with IAA (7 mg/l).

The variability observed in the regenerants usually included morphological changes in plant and organ size, leaves and flowers, pubescence and anthocyanin pigmentation and composition of essential oil components. The variability observed might be due to segregation of chimera tissue, euploid changes and heritable changes involving individual chromosomal aberrations or gene mutations. Variability observed in the present study included very minute morphological changes but clear-cut cytological and essential oil variation. A particular concentration of growth regulators was required for multiple shoot generation in the present study. 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 & Jagadishchandra, 1999).

Immature inflorescence has been recognized as an important source of totipotent cultures in many dicots and monocots (Vasil, 1982; Eapen & George, 1997). Peduncle was used as an explant in the present study and

positive response was obtained (Fig. 35). The inflorescence explants and the isolated flower buds of turmeric differentiated to plants under *in vitro* conditions (Salvi *et al.*, 2000). Single flowers were also used as explants in MS medium with IAA (7 mg/l) and BA (1 mg/l). Isolated flower buds showed swelling at the basal portion of the bud, which included ovary in the buds cultured in BA. This calli later developed to plantlet. Since floral buds are modified vegetative buds, it is quite likely that immature floral buds have the capacity to revert back to vegetative buds under appropriate *in vitro* conditions (George & Sherrington, 1984). Suryanarayanan & Pai (1998) observed green callus formation from flower explants, which gave rise to shoots when grown in combination of NAA and KIN. A similar tendency of very little callus growth was observed in the production of shoots from flowers of African violet on MS medium with 5.4 μ M NAA and 4.4 μ M BA (Grout, 1990). Organogenesis from callus is a useful tool for induction of somaclonal variations such as increase in secondary metabolite production. Though axillary shooting and indirect regeneration from callus of nodal explants was successful, the use of flowers for micropropagation obviates excision of stem and leaf explants.

*In vitro* flowering is observed in the present study from nodal explants, peduncle *via* callogenesis resulting in plantlets as well as directly from axillary bud developed in the MS medium supplemented with IAA (7 mg/l) and BA (1 mg/l) (Table 5). Phytohormones influence many diverse developmental processes ranging from seed germination to root, shoot and flower formation (Mc Court, 1999). The effect of BA and KIN on *in vitro* flowering of shoot tips observed by Wang *et al.* (2001) are consistent with those described by Prakash (1977) in *in vitro* culture of floral buds in bittermelon. The observations that different kinds of cytokinins have different effect on *in vitro* flowering has also been made by Wagner *et al.* (1989), who showed in tobacco explants KIN promoted flower formation, whereas zeatin

promoted leafy shoot formation. The influence of cytokinins on *in vitro* flowering was considerable but specific. Plant flowering transition is a complex process, which is controlled by various factors (Bernier, 1988; Weigel, 1995; Weller *et al.*, 1997). Since growth regulators have been implicated in flowering, *in vitro* techniques are being employed to understand the process in a more precise way. Purohit *et al.* (1995 a, b) reported that low auxin (IAA) and a high cytokinin (KIN) concentration induced flowering in *Ammi majus*. Nutritional, hormonal as well as environmental factors have been envisaged important in the past for initiation and subsequent development of floral organs (Kennet *et al.*, 1985; Chailakhyan & Krikorian, 1987).

Flower bud initiation frequently observed in the present study, from the vegetative tissue cultured *in vitro* is consistent with many of the earlier reports (Narasimhulu & Reddy, 1987; Shobha *et al.*, 1987; Reddy & Narasimhulu, 1988). In several earlier cases, shoot buds differentiated on cytokinin (BA) alone or auxin (IBA) and cytokinin (BA) rich nutrient medium directly produced flowers *in vitro* (Mc Hughen, 1980; Causson & Van, 1981; Narasimhulu & Reddy, 1984). According to Chambers *et al.* (1991), Nadgauda *et al.* (1997) BA was found to be necessary for induction of flowering in bamboos. Media without BA or with other cytokines could not induce flowering *in vitro*. This is in conformity with the result of the present study, where *in vitro* flowering was observed in medium containing BA and IAA (Figs. 8 & 9). A drop in the $p^H$ of the culture is also attributed to be a cause of development of *in vitro* flowers (Nadgauda *et al.*, 1997). The tissue culture process could cause the apex to undergo a morphogenetic change, which results in the formation of pseudo-inflorescence as an evocation of phenotypic plasticity rather than as a clear-cut transition from juvenile to mature stage, characterized by flowering (Gielis, 1999).

Various explants showed differential response to media for multiple shooting. The difference was not only in the frequency of the responding explant, but also in the number of shoot produced per explant (Thanh & Trinh, 1990). This is in conformity with the present study. Organogenesis depends on factors like explant, physiological state of donor plant/organ and endogenous levels of plant hormones. Moreover, every tissue has its own requirements, and sometimes less defined adjuvants plays a part in this (Narayanaswamy, 1994). Auxins promote the growth of axillary bud at lower concentrations (Hopkins, 1999). Cytokinins are known to affect orientation of cell division and induction of organs or organoid development (Szweykowska, 1974).

The incorporation of an auxin in the medium generally promotes rooting (Gautheret, 1945). Roots have been reported to originate from elaborate callus tissue (Hubakova, 1986; Hartman *et al.*, 1990). The relative levels of auxins have been known to greatly influence morphogenetic responses like rooting (Sitborn *et al.*, 1993). The observation that IBA generally performed better as an auxin for rhizogenesis than IAA emphasized the fact that auxin types differ in their morphogenic ability and organogenetic effect on plant tissues in culture (Nagasawa & Finer, 1988). Rooting was observed in IAA and IBA by Purohit *et al.* (1995 a, b). This is in conformity with Zhang *et al.* (1987) that for rooting, a root-inducing medium is to be inoculated. In a number of species, IAA is known for its promotory role on rhizogenesis (Thorpe, 1978). In the present study, IAA induced rooting at the base of regenerated plantlets as well as from callus. IBA, generally employed in rhizogenesis (Chalupa, 1974; Cosio *et al.*, 1981; Amin & Rahman, 1994; Pijut Pula *et al.*, 1994) was effective in the present study in inducing rhizogenesis (Table 4).

Biological phenomena in plants are closely related to the chemical and physical characteristics of macromolecules occurring in them. Their dynamic equilibrium may be attributed to the alterations, which continuously occur in plants – the bearers of genetic information in processes of plant metabolism, biosynthesis, accumulation, decomposition of alkaloids, flavonoids, terpenes etc. 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.*, 1995).

According to Bajaj (1990 b) any genetic variability brought about by *in vitro* culture may be included in the term somaclonal variation. Somaclonal variation has been extensively documented in many species of cereals to trees (Bajaj, 1990 a; Phillips *et al.*, 1994). Simultaneously, little effort has been made so far among the essential oil yielding plants to obtain somaclonal variants (Sreenath & Jagadishchandra, 1991; Sahoo & Dabata, 1995; Patnaik *et al.*, 1999). Various types of changes in cultures namely phenotypic, genetic/epigenetic, karyotypic, physiological, biochemical or at molecular level may be derived from changes already existing in the explant from which the culture is initiated or induced *in vitro* by culture media. The genetic variation that is created in the tissue culture plant according to Berlyn *et al.* (1990) may be 'benign i.e., a sequence of dedifferentiation, rejuvenation or redifferentiation may be transient or may be persistent'. They are not readily reversible, are stable and can be enhanced by mutagens or tissue culture environment. Factors promoting instability are prolonged culture time, high level of growth regulators and environment (Bajaj, 1990 a). The frequency of genetic changes in somaclones is much higher than the spontaneous genetic changes brought about in the entire plant (Prat, 1983).

Phenotypic variability between cell and tissue culture derived regenerants may be attributed to genetic, epigenetic and chromosomal

changes induced by the culture conditions (Evans & Reed, 1981; Sibi, 1984; Oono, 1991). Epigenetic variations are acquired traits, due to the results of culture stress and these variations are not transmitted from generation to generation. Thus these changes are not genetically controlled. The genetic variations are due to single nuclear gene mutations, induced during culture. The mutants exhibit Mendelian inheritance. The epigenetic and genetic changes form the central themes of somatic cell inheritance. According to Larkin (1987), somaclonal variation can also be due to either the variation induced by mutagenic action of culture media or variation induced by stress (leading to the activation of mobile elements). Variation shown by plants regenerated by tissue culture from donor plant may be due to changes in the chromosome number and structure, dominant and recessive mutations, or changes in chloroplast and mitochondrial genomes (Bingham & Mc Coy, 1986). Stress induced by tissue culture process (Hormone effect, nucleotide pool imbalance etc.) cause alterations in the DNA. These alterations could affect the expression of specific genes (Kaeppler & Philips, 1993 a). Conditions in the artificial environment of cell culture may enhance the spontaneous mutation rate. The mutation rate may also be enhanced by leakage of toxic byproducts and exudates from the calli into the surrounding medium (Olhoft & Philips, 1999). It has already been recognized that the nature of growth regulators used in the medium may result in the occurrence of somaclonal variation (Patel & Berlyn, 1982). One of the possible mechanism suggested to explain somaclonal variation is the activation of different classes of mobile genetic elements such as those reported during tissue culture of maize (Peschke & Philips, 1991), tobacco (Hirochika, 1993) and rice (Hirochika *et al.*, 1996). Transposable element activation has been shown to be induced by genomic shock (Mc Clintock, 1984). Epigenetic variation is another important cause of somaclonal variation in plants. Epigenetic aspects of somaclonal variations involve mechanism of gene

silencing or gene activation that was not due to chromosomal aberrations or sequence change (Kaeppler *et al.*, 2000).

The stress during *in vitro* culture activates transposable elements, which would be responsible for high frequency of chromosome breakage, recessive mutations and expression of silent genes (Lee & Philips, 1987; D'Amato, 1991). Only some callus cells were able to regenerate, so the lack of regeneration ability in long-term callus cultures may be due to some genetic alterations induced by somaclonal variation (Molina & Garcia, 1998). Methylation is one of the mechanism causing mutations in plants regenerated from tissue culture as reported and reviewed by Kaeppler & Philips (1993 b) and Rasmusson & Philips (1997). Methylation was also observed in original cultivars but with less intensity (Brown, 1989). The age of the explant is an important factor in morphogenesis (Abhyankar & Chinchanikar, 1996).

According to Larkin & Scowcroft (1985) and Larkin (1987) the origin of somaclonal variation may be due to the following; -

1. Genetic variation already present in the mother plant tissue
2. Variation induced by mutagenic action of the culture media.
3. Epigenetic variation
4. Variation induced by stress (eventually leading to activation of mobile elements)

High concentration of growth regulators in the medium and long-term culture are thought to be the main causes of variation in plants cultured *in vitro* (George & Sherrington, 1984). It is accepted that *in vitro* manipulation do cause genetic aberrations (Vajrabhaya, 1977). The possibility of genetic changes occurring in plants raised from callus cultures can be used as a potential source of somatic variation (Pillai & Hildebrandt, 1969; Bush *et al.*,

1976). The frequency of variation is also influenced by culture, duration, concentration of cytokinins and number of plants produced from each explant (Reuveni *et al.*, 1986; Vuylsteke *et al.*, 1988). Somaclonal variation has been extensively documented in many species of cereals to trees (Bajaj, 1990 b; Phillips *et al.*, 1994). Skirvin (1978) studied natural and induced variation and 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. Much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and other chromosomal abnormalities (Conger, 1987).

Revealing the mechanisms of mutation will lead to a better understanding of genomic change in response to stress, factors contributing to genomic stability and methods to control variation among tissue culture regenerants. Any step made towards understanding the basis of tissue culture induced genetic variation should be helpful in developing a more stable and manipulable somatic cell systems.

## Cytological Analysis

### Mitosis

The chromosomes seen during mitosis in cells of eukaryotes offer visible evidence of the genetic architecture of the organism as the number of chromosomes and their linear morphology can be directly related to the number of linkage groups. Knowledge of the karyotype of a species i.e., the number of chromosomes, their size and morphology is necessary for a full understanding in plant genetic studies and plant improvement. Karytoype is useful to understand the origin and nature of chromosome variations (Gu *et al.*, 1984). It has been clear that since early in the development of plant tissue

culture methodology, a consequence of *in vitro* growth was the appearance of dividing cells with chromosome numbers and karyotypes not usually found within intact plant (Melchers, 1965; Kunakh, 1974; D'Amato, 1975, 1977; Sunderland, 1977). There have been claim that random genetic changes evidenced by chromosomal differences can have some use in plant improvement (Skirvin, 1978). 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 & Collins, 1976) through mitotic evidence of chromosomal rearrangements (Cummings *et al.*, 1976) to complete polyploid or aneuploid plants (Sacristan & Melchers, 1969). The genetic alterations in the plants grown in artificial environment are manifested as increased frequencies of single gene mutations, chromosome breakages, transposable element activation, quantitative trait variation and modification of normal DNA methylation patterns (Kaeppler & Philips, 1993 b). Genomic changes usually appear as the basis for phenotypic alterations (Phillips *et al.*, 1994). Plants regenerated via organogenesis are usually associated with genetic and chromosomal variability. Chromosome rearrangements are frequently found in plants regenerated from tissue cultures. The alterations, which are the result of chromosome breakage events, have been well characterized in maize and oats (Benzion *et al.*, 1986). A variety of mutational types have been explained which most likely are responsible for the observed variations. These changes include cytological abberations which are primarily the result of chromosome breakage or single base changes, changes in copy number of repeated sequences and alterations in DNA methylation patterns. Chromosome breakage followed by the reunion of the broken ends leads to translocations, inversions, duplications and deletions (Benzion & Philips, 1988; Lapitan *et al.*, 1988; Phillips *et al.*, 1994). Among these abnormalities, translocations were the most frequently observed (Kaeppler *et al.*, 2000). 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 & Scowcroft, 1983; Orton, 1983 a, 1984; Evans *et al.*, 1984). There is also accumulating evidence that specific DNA sequences can be altered in copy number (Culis, 1990). 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 rearrangements, somatic crossing over with sister chromatid exchange, activation of transposable sites along certain regions of nuclear DNA and rearrangements of nucleotides in both nuclear and organellar DNA (Mantell, 1986).

The chromosome base numbers of Apiaceae is x = 9, 10 or 11 (Liu *et al.*, 2003). The haploid chromosome number of *T. roxburghianum* is x = 22. It seems probable that the plant is of polyploid origin, with the original base number x = 11. The tetraploid chromosome complement of *T. roxburghianum* reveals the existence of autopolyploidy from the secondary basic chromosome number of $x_2$ = 11. An increase in the number of chromosomes through autopolyploidy provides increased possibilities for new gene combinations which are of considerable importance in evolution (Reese, 1961). Since the family Apiaceae, seems to be actively engaged in karyotype evolution, the karyological studies seem quite relevant.

In the present study the chromosome number of parent (Fig. 1 .1 & 1 .1C), and the variant (Fig. 5 .1 & 5 .1C) was invariably found to be 2n = 44, which is in consonance with the report of Hore (1971). Some of the callus cells exhibited a chromosome number of 2n = 8x = 88, 2n = 2x = 22 amidst cells with chromosome number of 2n = 4x = 44 (Figs. 2 .1 – 4 .1 &

2 .1C - 4 .1C). Karyotypic variations are exhibited by the diploid, tetraploid, octaploid calli and the *in vitro* plant (Figs. 2 .1D – 5 .1D), on comparison with the parent plant (Fig. 1 .1D). The idiograms of the diploid, tetraploid, octaploid calli and the *in vitro* plant (Figs. 2 .1E – 5 .1E) clearly reveal their differences from the karyotype of the *in vivo* plant (Fig. 1 .1E). Variation in the karyotype resulted in changes in the centromeric position and total length of chromosomes (Table 6 -10). The total chromosome length of the parent plant was noticed as 43.0762 μ m and that of the variant was 28.8582 μ m. The total chromosome lengths of diploid, tetraploid and octaploid calli were 16.6340 μ m, 28.0124 μ m and 54.1692 μ m respectively. Karyotype formulae deduced for all (parent, somaclonal variant, calli with diploid, tetraploid and octaploid condition) showed variations in the type of chromosomes. In the present investigation, four different types of chromosomal categories were deduced, viz., A, B, C & D (Fig. 6 .1E). The differences in the chromosome length and volume may be attributed to differential spiralization and condensation of chromosome along with the content of protein and DNA. Changes in length of the chromosome may also arise by translocations and deletions. The change in the chromosome length may be the aftermath of cryptic changes, probably duplications, which may arise due to *in vitro* stress produced in the altered culture environment. Moreover, retrotransposon activation and inversions may significantly contribute to the change in the physical size of the genome (Olhoft & Philips, 1999). Similar reports are available in *Allium cepa* (Sekerka, 1977) and *Papaver somniferum* (Bajwa & Wakhlu, 1986). Reports on karyotypic variations are also available in *Haplopappus gracilis* (Singh, 1981) and *Triticum durum* (Gupta & Ghosh, 1983). Chemnaveeraiah & Habib (1966) reported the structural rearrangements of chromosomes in cultures of *Capsicum annuum*. The difference in the length of chromosomes in the *in vitro* plant and also in the different calli exhibiting different ploidy levels (diploid, tetraploid and

octaploid), which has been observed when compared with the karyotype of the *in vivo* plant may be due to any of these above-mentioned reasons.

A high TF% represents a symmetrical karyotype, which is a primitive condition (Stebbins, 1959). Comparatively longer chromosomes in the karyotype seem to be a primitive characteristic feature (Dasgupta & Datta, 1976). The higher individual chromosome length in the *in vivo* plant seems to be in consonance with this, which may suggest considering it as primitive when compared to the other samples. A high TF% is shown by the *in vivo* plant, which in turn suggests its primitive nature. The number of submetacentric chromosomes were found to be higher in the *in vitro* plant (Table 10) when compared with that of *in vivo* plant (Table 6). An increase of submetacentrics at the expense of metacentrics leads to asymmetry of the karyotype, which is an advanced condition (Stebbins, 1958). Other karyomorphometrical parameters used show slight fluctuations in the cytotypes studied.

The culture conditions imposed upon the explant tissues are likely to exert considerable trauma on the surviving cells. The chemical composition of the culture medium has shown to affect the cytogenetic behaviour of plant cells *in vitro* (Bennici *et al.*, 1970; Singh, 1972; Singh & Harvey, 1975; Bajwa & Wakhlu, 1986; Karp, 1992). Mineral deficiencies, chelating agents, some heavy metal ions and anaerobic conditions have been implicated in inducing chromosomal breakage and rearrangements in plants (Steffensen, 1961). Some studies suggest that auxin and / or cytokines induced chromosomal abnormalities probably leading to chromosome number variation in cultured tissues (Bayliss, 1973; Ogura, 1982). The concentration and type of hormones in the culture medium influence the pattern of methylation in cultured *Daucas carota* L. genomes (Lo Schiavo *et al.*, 1989; Arnholdt-Schmitt *et al.*, 1991). Singh (1976) reported chromosomal changes

induced by KIN in *Haplopappus gracilis*. The effects of cytokinins and auxins in inducing chromosome changes were studied in tissue culture of *Nicotiana* (Ronchi *et al*., 1976). Singh (1986) reported that the chromosome variation in callus cultures of barley was possibly due to media components. The significance of nutrient medium in chromosomal behaviour of *Allium* and *Capsicum* cultures was studied by Nair *et al*. (1993) and Nair & Kumar (1998). The growth regulator 2,4-D is known to induce somatic crossing over (Turkula & Jalal, 1985). This increase in the frequency of somatic crossing over could amount for many of the spontaneously occurring variations in chromosome structure seen in tissue-cultured cells. The changes in the number or structure of chromosomes in tissue cultured plants could be due to culture environment or due to naturally occurring events (Griesbach, 1987). Among media components, auxins and cytokines have been found to contribute to ploidy changes (Torrey, 1961). The stress during *in vitro* culture activates transposable elements, which would be responsible for high frequency of chromosome breakage, recessive mutations and expression of silent genes (Lee & Philips, 1987; D'Amato, 1991). Chromosomal aberrations induced in culture are the result of direct influence of chemical substances present in the medium. Majority of reports agree that chromosome aberrations are generated during culture by growth hormones such as 2,4-D (Venkateswaran & Spiess, 1963; Shimada & Tabata, 1967; Ziauddin & Kasha, 1990), IAA (Nishiyama & Taira, 1966), NAA (Zhang *et al*., 1987) and KIN (Ronchi *et al*., 1976; Singh, 1993). Auxins or cytokinins, which are known to promote cell division, also caused chromosomal abnormalities.

A genetic change commonly observed in tissue culture is the alteration of the chromosome number, which has also been included among the types of genetic events responsible for some of the commercially important plant chimeras (Marcotrigiano & Gouin, 1984). In this study, some of the callus cells exhibited change in chromosome number (Fig. 4 .1C & 2 .1C)

(2n = 8x = 88; 2n = 2x = 22). However, the variant plant showed the normal chromosome number (Fig. 5 .1C) (2n = 4x = 44), but showed structural rearrangements. A previous report confirm this chromosome number in *T. roxburghianum* (Table A). Even then, these results require molecular evidences for genomic variation. The examination of solid-stained chromosomes can only reveal changes in number and gross morphology. Molecular evidences for genomic variation can be deduced to some extent with the aid of RAPD fingerprinting (Isabel *et al.*, 1996; Piccioni *et al.*, 1997; Yang *et al.*, 1999).

In tissue culture, repeated sub culturing induces genetic changes resulting in somaclonal variation of varied nature and origin i.e., polyploidy, aneuploidy, gene amplification, chromosome elimination, translocation etc. (Semal, 1986). These changes in cell cultures may either be derived from changes already existing in the explants from which the culture is initiated or induced *in vitro* by the culture media and environment (Bajaj, 1990 b).

There have been many studies on chromosomal aberrations in the cultured plant cells and their regenerants using various plant materials (Singh, 1993). Joachimiak *et al.* (1993 a, b, 1995) recently reported chromosome alterations in callus cells of *Allium fistulosum*. It has been reported frequently 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 & D'Amato, 1978; Bennici, 1979). Several literature reviews dealing with ploidy, instability and related phenomena in *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 diploid and octaploid cells are observed among tetraploid callus cells. The time interval between transfers, media composition, atmospheric condition and light are known to influence chromosome constitution in callus

cultures (Constantin, 1981). The changes in the number or structure of the chromosomes may be due to the stress induced in the culture environment or due to naturally occurring events (Griesbach, 1987). Chromosome instability induced in the culture and plant regenerability is often influenced by the genotype of the explant (Ohkoshi *et al.*, 1991; Ruiz *et al.*, 1992).

The chromosome irregularities may result in the alteration of basic chromosome numbers (Jauhar & Joshi, 1969) and repatterning of karyotypes (Jauhar, 1974). The variation in somatic chromosome number among calli may be due to the irregularities in spindle mechanism (Haque & Ghoshal, 1981). Moreover, somatic cells are known to show more variation in chromosome number than meiotic cells as also reported in the genus *Commelina* (Patwary *et al.*, 1987). Structurally changed karyotypes were due to deletions and / translocations (Lee & Ono, 1999). In general, numerical and structural aberrations in chromosomes are attributed to spindle failure that causes endoreduplication, c-mitosis, nuclear fragmentation etc. These changes are induced by media composition, age of callus (morphogenic vs. nonmorphogenic), genetic background of explants and kinds of media (solid vs. liquid) (Bayliss, 1973, 1980; Evans & Reed, 1981; D'Amato, 1985; Ogura, 1990; Geier, 1991). Chromosome aberrations induced in cultures are the result of the direct influence of chemical substances present in the medium. The reasons cited above may be the reason for the chromosomal variations observed in the present study.

The chromosomal instability in *in vitro* may be influenced by the type of explants, which give rise to the callus, as suggested by Partanen (1965). Somatic polyploidy is a widespread phenomenon in a number of plant species (Nagl, 1974; D'Amato, 1975) and it is very likely that pre existing polyploid cell from the primary explant start its division under *in vitro* conditions (Novak, 1974; Novak & Vyskot, 1975). The genotype and partly also culture

conditions, especially growth regulators in the medium may influence the karyologic constitution of proliferating cell population and selection processes taking place *in vitro* (Novak, 1981).

Li *et al.* (1986) and Heszky *et al.* (1990) postulated that the phenotypic manifestations of molecular and chromosomal changes (somaclonal variation) depends on the origin and ploidy level of initial explant and primary callus. The genetic variability or instability of callus cells is well characterized by the variation in chromosome number. Several factors are said to play an important role in the chromosome constitution of cultured tissues. They are nuclear condition of original explants, composition of medium especially kinds and concentrations of plant growth regulators, age of culture, variation due to plant species, karyotypic changes, etc.

Reese (1961, 1966) suggested that an increase in the number of chromosomes provides increased possibilities for new gene combinations, likewise, he concluded that polyploidy results in increase in the genes controlling characters favourable for natural selection, when these characters are already present in the plant. According to Gottschalk (1978) and Stebbins (1980), ploidy is a mechanism, which involves multiplication of whole chromosome complement. An increase in gene number produces an entirely different well-adapted genome. Polyploidy is associated with a marked dimunition in chromosome length. The size of chromosomes is highly reduced in all the tetraploid species while in diploids the chromosomes are comparatively longer (Menacherry & Jose, 2001). The polyploid nature may be attributed either to the selective growth of normally non dividing polyploid cells that pre-existed in the original explant or to the induction of polyploidization by the types of phytohormones used in culture medium (Nagl,1990). Polyploid cells may arise by nuclear fusion or depend on the ploidy nature of explant or endomitosis (Geitler, 1939). Any of the above-

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cited reasons may be responsible for the formation of polyploid cell in the present investigation.

The formation of a diploid callus from the tetraploid explant can be accounted for by several mechanisms, as multipolar spindle formation, nondisjunction or lagging of chromosomes. Marchetti *et al.* (1976) proposed that shift in ploidy level observed *in vitro* may be due to selection of normally nondividing polyploid and aneuploid cells occurring in the *in vivo* plant. This may be the probable reason responsible for the development of diploid callus in *T. roxburghianum* in the present study.

Chromosomal modifications are common, involving not only the change of chromosome number, but structural changes as well (Larkin *et al.*, 1985). This somaclonal variability could lead to production of beneficial plants with novel genotypes or chromosomal complements for basic study and plant breeding.

Studies on chromosome variability among regenerated plants showed that regenerated plants exhibited less chromosome variability than the original callus cells (Sacristan & Melchers, 1969; Orton, 1980). It is, therefore, evident that selection in favour of specific chromosome constitution(s) is operating during the course of plant regeneration from calli. The intensity of the selection seems to vary depending on the materials used; very strong selection is evident for euploids in maize, common wheat and carrot (Shimada *et al.*, 1969; Mok *et al.*, 1976; Gengenbach *et al.*, 1977) and moderate selection in tobacco (Novak & Vyskot, 1975). The present study reveals that in the *in vitro* plant of *T. roxburghianum,* selection for euploids (tetraploids) was rather strong. The morphogenetic ability of a callus depends on the chromosome constitution of the cells. Balanced chromosome numbers in a callus is a prerequisite for generating plants. This is in agreement with the results reported in *Daucas carota* (Smith & Street, 1974), *Hordeum vulgare*

(Singh, 1986; Scheunert *et al.*, 1987) and *Zea mays* (Balzan, 1978). Generally, a high frequency of regenerants from diploid species carries normal chromosome complements. During shoot and root morphogenesis in diploid species, cells with unbalanced chromosome numbers cannot compete with balanced cells during cell division. This enhances the regeneration of a large number of plants with normal chromosome complements. It has been observed that plant regeneration in callus culture was obtained mainly from cells with balanced chromosome number (Larkin & Scowcroft, 1981; Vasil, 1983). 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 & 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). These reasons may be responsible for the development of tetraploid plant in *T. roxburghianum*, since no plants with variation in chromosome number were observed in the study. The same results were observed by Mitra *et al.*, (1960) in *Daucas carota* and Karp *et al.* (1987) in *Hordeum vulgare.*

During successive passages *in vitro*, chromosomal aberrations such as polyploidy, aneuploidy and structural changes commonly occur in cultured plant cells and morphogenetic capacity of cells are often lost (Bayliss, 1973; D'Amato, 1977, 1985). Murashige & Nakano (1967) proposed that chromosome aberrations appeared to be one of the factors causing the loss of morphogenetic capacity in cultured cells (Mitra *et al.*, 1960). In the present study, it was observed that callus cells exhibit varying degrees of ploidy and when sub cultured for a long period could not regenerate plants, which is in agreement with the above report.

In plants derived from cell and tissue cultures, major changes in chromosome complement often do not appear to be accompanied by corresponding changes in the phenotype of the plant. The gain or loss in chromosomes sometime may not be sufficient to cause a large change in morphological character and it is also possible that changes can also occur that are not visibly expressed (Liu & Chen, 1976). This may be the reason for the absence of considerable morphological variation in the regenerated plant of *T. roxburghianum* in the present report.

According to Darlington (1937,1939) when the karyological aberrations affect the genic system the structural changes in the chromosomes act mainly as a means of holding together certain favourable gene combinations and therefore of promoting immediate fitness at the expense of flexibility. Chromosomal interchanges have been described to be the basis for obtaining somaclonal variation (Karp & Bright, 1985; Pijnaker & Ferweda, 1987). The epigenetic (Meins & Binns, 1977) and genetic (Ryan & Scowcroft, 1987) alterations of plant species occurring during tissue or cell culture cycle are transmitted by some regenerants into offsprings through gametes (Maliga, 1984). This indicates that cultured tissues are able to produce novel chromosomal, genic and physiological variations caused by mitosis, which are different from those occurring through meiotic cell cycle. Pardue (1991) has hypothesized that genomic stability is not the default state but is the result of a rather finely tuned system of checks and balances. The tissue culture environment may cause a general disruption of the cellular controls, leading to the numerous genomic changes present in the tissue culture regenerants. Variations could have been induced by *in vitro* process or by added biochemicals and stresses (Swartz, 1990) and therefore understanding the events, which give rise to such variations need more study.

## Meiosis

The chromosomal behaviour during meiosis is considered to be one of the most reliable indices for estimating the potency of mutagens and response of a genotype to mutation. The fertility of an individual depends on the efficiency of the meiotic process. Studies carried out on different plant species have demonstrated that the decline in seed production is correlated with meiotic irregularities (Paggliarini & Pereira, 1992; Paggliarini *et al.*, 1992, 1993). Countless factors have been shown to lead to the occurrence of meiotic irregularities in higher plants. Among them, are lack of chromosome homology resulting from hybridization, polyploidy and genetic as well as environmental factors. Polyploidy has been reported to be one of the major factors carrying sterility (Lewis, 1980; Evans & Davies, 1982). The meiotic irregularities brought about by multiple chromosome associations lead to pollen sterility and consequently to low seed production. In karyological aberrations, occurrence of structural changes in chromosomes chiefly inversion and translocation induce certain amount of inviable gametes. Such structural difference scattered through chromosome complements in natural populations of plants cause relatively a slight reduction of fertility. But when plants are heterozygous for considerable number of inversions and translocations, the effect of crossing over and chromosomal segregation will result in the production of gametes containing duplication and deficiencies and therefore forms inviable seeds (Darlington, 1937; Sturtevant & Beadle, 1939). Because they are lethal in the gametic stage, cause formation of inviable gametes inducing sterility in plants.

Meiotic observations provide various details of chromosomal associations. Analysis of chromosome pairing and chiasma formation is known to provide valuable informations for evolution of species and potentialities for genetic recombination. It is well established that

chromosome pairing is under genetic control (Riley & Law, 1965; Feldman *et al.*, 1966). The increased frequency of bivalents may be due to the adjustment of genes affecting chromosome pairing (Singh *et al.*, 1977). Sybenga (1975) have indicated that the genetic process of crossing over, which leads to chiasmata observed during diplotene, diakinesis and metaphase of first meiotic division, is also influenced by genes. Chiasma frequency is known to have a profound effect on the distribution of the various chromosome configurations at meiosis. Hazarika & Rees (1967) found that in autotetraploid rye an increase in chiasma frequency was accompanied by an increase in quadrivalent frequency and a decrease in bivalent, trivalent and univalent frequency.

It is established that early meiotic events are most susceptible to change by intrinsic and extrinsic factors. A mutation in the gene responsible for normal pairing of chromosomes may bring about a homozygous recessive condition resulting in desynapsis (Jauhar & Singh, 1969). Frequency of meiotic aberrations increased with increasing concentration of NAA in onion (Kumar, 1970). Pollen sterility was observed by Choudhary & George (1962) and Kumar (1970) with NAA. In the present investigation, multivalent associations especially tetravalents were observed in the meiosis (diakinesis and metaphase I stages) of *in vitro* plant (Figs. 8 .1 & 9 .1) when compared with the *in vivo* plant (Fig. 6 .1 & 7 .1). Aberrant meiotic associations may be due to some spontaneous multiple translocations which might have arisen due to change in many genes or due to the special genetic constitution (Chaudhari, 1968) or changes in the internal or external environment (Meshram *et al.*, 1981) leading to the abnormal associations in the PMCs.

The meiotic failures leading to the formation of unreduced gametes either in one or two division is not uncommon in plants. Harlan & DeWet (1975) have discussed various methods of formation of unreduced gametes.

Wagenaar (1968) has shown clearly the formation of 2n nucleus or restitution nucleus in meiosis due to poor pairing as well as delayed divisions.

In the present study, the donor plant showed no cytological abnormalities in meiotic cells and formed 22 normal bivalents at diakinesis (Fig. 6 .1) and metaphase I (Fig. 7 .1) stage of PMCs. The 22 bivalents shown by the spore mother cells indicate that it is an autotetraploid, since the basic chromosome number of *Trachyspermum* is 11. On the other hand, the regenerated plants revealed a wide variation of chromosome associations. The *in vivo* plant showed 22 bivalents at metaphase I of PMCs. On the other hand the metaphase I of PMCs of the *in vitro* plant appeared to be aberrant when compared with the bivalents observed in the former. The diakinesis stage of prophase I of *in vivo* flower showed normal number of chromosomes, i.e., 22 bivalents, hence the diploid chromosome number is 44. The diakinesis stage (Fig. 8 .1) of *in vitro* flower consisted of 10 bivalents and 6 quadrivalents. The aberrant meiotic metaphase I (9 .1) of the *in vitro* flower showed 18 bivalents and 2 quadrivalents. The diakinesis and metaphase I of the *in vitro* flower was abnormal with quadrivalents. The same result was obtained by Oghihara (1995) in *Haworthia*. The results available for barley, tomatoes and other higher plants and in animals like *Drosophila,* and human beings, shows that a polygenic group of genes, possibly more than a hundred are responsible for pairing of homologous chromosomes. A mutation in one of the above desynaptic/asynaptic polygenes acting independent to each other may lead to desynapsis/ asynapsis leading to irregular associations and thus sterility (Gottschalk, 1978). In addition to genic control of synapsis, desynapsis is known to occur due to many factors such as drastic temperature fluctuations (Wilson, 1959; Utkhede & Jain, 1974), ageing, reduced water content, ionizing radiations and chemicals (Sybenga, 1969). Desynapsis have been reported in large number of plants occurring in nature and it has been also induced by various physical and chemical mutagens in a variety of plant

species (Verma & Raina, 1979). Recent investigations have clearly demonstrated that there is now unmistakable evidence to believe that synapsis between homolgous chromosomes, which is a prerequisite for crossing over, is controlled by genes at different stages of meiosis (Riley & Law, 1965; Rhoades & Dempsey, 1966; Riley, 1966; Gottschalk, 1978). Desynapsis has been attributed to a variety of causes such as gene action, loss of chromosome pair, apomixes, structural and numerical changes of chromosomes in addition to environmental causes like temperature, humidity etc. (Singh & Gupta, 1981).

Pairing of homolgous chromosomes at zygotene is a rule in meiosis. This delicate, but a complicated phenomenon of pairing at zygotene known as synapsis, has been a fascinating subject of study for the cytologists. Contrary to this, exceptions are also known where either pairing does not occur at all or homologous chromosomes synapse at zygotene but dissociate at diplotene, diakinesis, metaphase I and consequently variable number of univalents are present at metaphase I. Complete failure of pairing has been termed as asynapsis and where some pairing is apparent at early stages of prophase I and as the meiotic division progresses, the chromosome may start falling apart, the term desynapsis has been used. Desynapsis has been reported in a number of plants like *Colocasia antiquorum* (Krishnan *et al.*, 1970), *Amorphophallus campanulatus* (Magoon & Sadasivaiah, 1967) and *Sorghum* (Sadasivaiah & Magoon, 1965).

Various types of associations among the meiotic chromosomes of *in vitro* plant like rings and chains of chromosomes were observed in the present study. The result of such type of pairing can be easily explained if the presence of multiple translocations is assumed. Rings are found when the interchanged parts of chromosomes are long and cross over had occurred in each of the arms of chromosomes present (Burnham, 1956). If the chiasmata

are not formed in all the arms of chromosomes, chains instead of rings are produced. Similar types of observations have been made by Sarkar (1955) in grasshopper. When the interchanged segments are very small, this may attribute to the formation of bivalents (Meshram *et al.*, 1981).

One of the most striking aspects in the analysis of chromosome pairing is the variability that occurs from cell to cell especially as to the number of bivalents and quadrivalents. The presence of multivalents in the normal tetraploid species leads to the assumption of structural heterozygosity involving segmental interchanges. The same was reported by Sansome (1929) and Srivastava & Naithani (1964). Because the chromosomes are seen at metaphase I held together by chiasmata, Koul (1964) suggested that variability of a bivalent or quadrivalent association is directly caused by the variability in chiasma formation. Jauhar (1970) suggested that the reduction of multivalent formation might be due to a natural selection of genes, which contain regular meiosis. In the present investigation, variable number of quadrivalents and bivalents appeared at diakinesis and metaphase I stages of the variant, may be attributed to any of the above reasons. From the evolutionary standpoint, meiotic instability may be considered as an evidence of variation.

## RAPD

Recent works provides evidence that when compared to RFLP, RAPD fragments can be used as informative bands if necessary criteria are met (Reeve *et al.*, 1992; Williams & Clair, 1993). Genetic variability indicates the amenability of a given character for its improvement. A DNA based diagnostic assay like RAPD is able to identify the genotypes directly and can therefore, help mitigate complications arising from earlier cytological and morphological studies.

PCR amplification to detect target DNA sequences has many applications in plant genotyping, gene mapping, diagnosis and diversity assessment (Kreader *et al.*, 2001). It is evident from the electrophoretic gels, (Figs. 10 .1 – 13 .1) that somaclonal variation at DNA level is present in the hitherto mentioned variant of *Trachyspermum roxburghianum* (TC1). A few bands were found missing in the somaclonal variant when seven primers (OPA 02, OPA 06, OPA 09, OPA 11, OPC 03, OPC 11, OPD 01) were used. Additional bands were also detected in the variant for six primers OPA 02, OPA 06, OPA 09, OPA 11, OPC 03 and OPC 11. Similar results were obtained by Wang *et al.* (1993) and Hashmi *et al.* (1997) using RAPDs. The efficacy of RAPD markers in detecting genetic changes after *in vitro* culture is well understood (Piccioni *et al.*, 1997; Olhoft & Philips, 1999; Kaeppler *et al.*, 2000). The method is reported to be an efficient tool in detecting somaclonal variation (Al-Zahim *et al.*, 1999).

Variations in the RAPD bands in the *in vitro* plants of *T. roxburghianum* may be caused by genetic or epigenetic phenomena. Of the 27 primers used in the present study, ten primers showed successful amplification. Failure of primers or probes tested in the investigation to detect variation may indicate that either the gene/altered gene responsible for the abnormality has no homology with the primers or probes or the abnormality in the clones may be caused by an epigenetic phenomenon, i.e, a change in the expression of the gene (due to hypo or hypermethylation) (Philips *et al.*, 1990), but not the content of the gene. Amplification of a certain sequence is based on the sequence of a single nucleotide used as a primer as well as on the GC content (Lodhi *et al.*, 1997). Failure of amplification of different lines may be due to a single base change or completely different sequences (Vierling & Nguyen, 1992). RAPD technique has been used to analyze somaclonal variation, while it can also detect single base mutations and deletions at the level of primer target or insertion/ deletion without amplified

fragments (Gallego *et al.*, 1997). The genetic variations can be explained by change in DNA sequence of the clone, because of successive sub culturing processes or even by other manipulated conditions (Rani *et al.*, 1995).

Polymorphisms in amplified bands were observed in the present study. Polymorphism in the amplification products represents changes in the sequence of primer binding site (eg. Point mutations) or change which alter the size or prevent the successful amplification of a target DNA (eg. insertions, deletions and inversions). DNA amplification products, which represent one allele per locus, 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 (Rout *et al.*, 1998). Presence of RAPD markers at a specific locus in both genotypes indicates a high level of homology at that site. The sequence difference between two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint (Williams *et al.*, 1990).

The tissue culture environment may show a general disruption of the normal cellular controls, leading to numerous genomic changes present in the tissue culture regenerants (Phillips *et al.*, 1994). As far as the genetic stability of the proliferated tissue is concerned, RAPD markers are efficient tools for detection of somaclonal variations in tissue culture. Direct analysis of the DNA by use of RAPD markers proved a very sensitive technique for evaluating genetic changes after *in vitro* culture (Piccioni *et al.*, 1997). RAPD analysis using arbitrary 10-mer oligonucleotide primers was employed in order to investigate the genetic variability of somaclonal variant of *T. roxburghianum* (DC.) Craib.

Three types of polymorphism were observed in the study, such as presence of additional bands, absence of existing bands as well as band intensity differences (Figs. 10. 1 – 13 .1 & Table 11). The variant *in vitro*

plant (TC1) of *T. roxburghianum* (DC.) Craib showed one additional band for OPA 09 and five for OPA 11 when compared to the parent plant. Using OPC 11, one additional band was observed in the variant and TC2, which again was not detected in the parent. With OPC 03, one additional band was detected in variant, which was absent in the parent as well as TC2. Four bands for OPA 02 and OPA 11, three bands for OPA 09 were absent in the variant when compared to the parent. The parent showed one additional band for OPC 03, two for OPC 11 and two for OPD 01 when compared to the variant and TC2. Sequence variation arising through the culture process has been detected in several plants using different ways including genome scanning with RAPDs (Kaeppler *et al.*, 1998).

This is the first report on the molecular basis of variation detected by RAPD in the micropropagated plants of *Trachyspermum roxburghianum*. The results suggest that RAPDs are useful for establishing the genetic basis of somaclonal variation and strengthens the idea of variant development by tissue culture.

## Essential Oil Analysis

### Gas Chromatography-Mass Spectrometry

Culture stress may induce variation in tissue cultured plants which are sometimes associated with useful agronomic characters such as oil yield, oil content etc. (Patnaik *et al.*, 1999). In most cases, study of somaclonal variation has been limited to phenotypic variation and has been associated with changes in chromosome number and structure. Somaclonal variations in yield and quality have utmost importance in crop improvement. The present study shows that there is significant change in the essential oil content and composition of the variant of *T. roxburghianum* (DC.) Craib. (Table 12). Plant tissue culture has the potential to perform biochemical reactions when

organic compounds are added to the medium. It is possible to transform a substance from a lower to a higher, scientific, commercial or economic value and also to produce a new compound (Kukreja *et al.*, 2000). The capacity of plant cells to serve as catalysts for biochemical reactions such as epoxidation, esterification, glycosylation, methylation, isomerization and dehydrogenation of organic compounds was comprehensively reviewed (Reinhard, 1974; Furuya, 1978). Over the years, de novo syntheses of many commercially important chemical compounds have been reported (Nair *et al.*, 1986; Calleboutet *et al.*, 1990).

Volatile oils are chemically complex mixtures often containing 100 or more individual components. Most oils have one to several components, including sesquiterpenoids, monoterpenoids and phenols, out of which the major components impart characteristic odour and taste. But the minor products also play their part in the final product (Waterman, 1993). The economic properties may be due to the essential oil components present in them, which can be effectively exploited to produce fragrance (Tisserand, 1990) and flavouring agents (Heath, 1981). A number of reports on correct decipherence of biosynthetic pathways for the terpenoids have already been appeared (Akhila *et al.*, 1991; Croteau *et al.*, 1991; Lange *et al.*, 2000). Isopentenylpyrophosphate (IPP) is the immediate precursor leading to the formation of over twenty two thousand known terpenoids (Connolly & Hill, 1992). Monoterpenoids may be acyclic, monocyclic, bicyclic or involved in the formation of furanochromes, iridoids, cannabinoids or pyrethroids (Bhagyalakshmi, 1999).

In the present study, GC-MS analysis revealed twenty five components in the parent plant and 17 in the somaclonal variant (Table 12). The gas chromatogram of the parent and the *in vitro* plant revealed remarkable variation in the pattern of peaks (Figs. 14 .1 & 15 .1). Altogether 32 essential

oil components were detected in the *in vivo* and the *in vitro* essential oils of *T. roxburghianum* (Figs. 16 .1 $A_1$ – 16 .1$A_{32}$). The main chemical compounds identified in the present investigation belong to terpenes, aldehydes, alcohols, furan derivative, epoxy compound etc. Previous reports confirm many of these chemicals in *T. roxburghianum* (Table B). Alcohols possess antiseptic and antiviral properties. Aldehydes possess an uplifting quality. Terpenes possess anti-inflammatory and bactericidal properties (Baudoux, 2001). Several biological activities are reported on the essential oil components of *T. roxburghianum* (Table C).

There was clear difference between the essential oils of *in vivo* and *in vitro* plants tested (Figs. 14. 1 & 15 .1). The essential oil of the parent plant showed components like β-myrcene (0.35%), limonene (0.31%), γ-terpinene (0.52%), α-cubebene (1.55%), α-caryophyllene (1.69%), eudesma-4 [14], 11-diene (10.94%), aromadendrene (4.26%), δ-cadinene (1.74%), epiglobulol (19.55%), caryophyllene oxide (1.86%), aromadendrene oxide (0.45%), isobenzofuranone (1.35%), β-methyl benzene propanal (15.74%), tetradecanol (0.73%) and n-hexadecanoic acid (1.70%). The *in vitro* plant carries to its credit components like ocimene (1.10%), α-farnasene (1.96%), 1,R, 3Z-9S-4, 11,11-trimethyl 8-methylene bicyclo [7.2.0] undec-3-ene (3.81%), isothujol (16.42%), β-farnasene (1.35%), α-β -epoxycumene (14.72%) and α-bergamotene (3.37%). The common components found in the essential oil of both plants were thujene, δ-3-carene, β-terpineol, germacrene-D, β-caryophyllene, cedrene, α-neoclovene, α-bisabolene epoxide, patchoulane and an unidentified compound. Among these, the amount of thujene, germacrene-D, cedrene, α-bisabolene epoxide and patchoulene were found to be higher in the variant than the parent plant whereas δ-3-carene, β-terpineol and β-caryophyllene were found to be present in lesser amounts. The percentage of the unidentified component was greater in the variant

(Table 12). These results clearly justifies that the *in vitro* plant of *T. roxburghianum* exhibits somaclonal variation.

The studies conducted on somaclonal variants of essential oil yielding plants such as *Mentha piperita* (Holm *et al.*, 1989; 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 revealed favourable variation in oil content (Tokumasu & Kato, 1979; Jain *et al.*, 1989).

The lesser value of coefficient of similitude (31.2724) obtained on comparing the essential oils of parent and variant, shows the dissimilar nature of essential oil composition of these oils (Table 12). This dissimilarity may be probably due to variation in the biosynthetic pathways of essential oils, which are genetically controlled. In both samples, the major component was different. This indicates that the genetic changes due to culture stresses of hormones used affected the biosynthetic pathway of major components. The absence of some constituents may be due to some hindrance in the biosynthesis of these components. The appearance of certain new compounds in essential oil of variant may be due to triggering of certain diverged biosynthetic pathways. Almost all the secondary metabolites - the monoterpenes, sesquiterpenes etc. arises from one of the three biosynthetic pathways or from a combination of two or more of these pathways. These are the acetate, mevalonate and shikhimate pathways (Waterman, 1993). Changes in these pathways lead to variation in the chemical composition of essential oils. In the present investigation there is marked change in the minor components also which may be due to the above-cited reason. The marked difference in the essential oil composition may be due to the fact that biosynthesis of volatile aromatic chemicals is genetically controlled (Heffendehl & Murray, 1973). In the present investigation, it seems probable

that the remarkable difference in the essential oil composition of *in vivo* and *in vitro* plants of *T. roxburghianum* can be due to their genetic constitution. 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 & Charlwood, 1986). The absence of some bands in the RAPD analysis in the somaclonal variant can be accounted to this reason, which in turn may be responsible for the absence of certain components in the essential oil of *in vitro* plant of *T. roxburghianum*.

The production of each component of the essential oil is affected by the genetical as well as environmental factors. Variation in essential oil components may be attributed to segregation of chimeral tissue, polyploid changes and heritable changes, which may involve individual chromosomal aberrations or single gene mutations.

Production of plant products in cultures largely depends on various factors like physiological, biochemical and environmental conditions of cell cultures. Source of origin of plant tissue, carbon sources, precursor feeding, ethylene changes, etc. influence the production of secondary metabolites in cultures (Narayanaswamy, 1994). Light is also said to influence secondary metabolite production (Hahlbrock & Wellman, 1970; Berlin, 1984; Luckner & Diettrich, 1987). It has been shown that diurnal change in temperature is an important factor of influence regarding the oil composition (Burbott & Loomis, 1967; Hahlbrock & Wellman, 1970; Berlin, 1984). The above factors may have played an important role in the variation of essential oil content and composition of the *in vitro* plants of *T. roxburghianum* studied.

The genetic basis of biosynthesis of mono and sesquiterpenoids has already been reported (Lincoln *et al.*, 1986). The metabolism of monoterpenes is strongly influenced by environmental factors. It is generally accepted that the definition of interspecific chemical races may concern the presence or

absence of a particular component in the secondary metabolism (Tetenyi, 1973; Harborne & Turner, 1984). Plant cell cultures have produced (i) new components previously not known in the intact plant, (ii) new derivatives of known compounds and (iii) new component by biotransformation of molecules incorporated in the medium. It is presumed that production of new component or derivatives might be due to altered gene function in cultured cells when compared to the mother plant (Merillon & Ramawat, 1999). The production of new components in the *in vitro* plant can be accounted to the above-mentioned reason.

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). The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. The nutritional components of various culture media have been known to affect secondary product formation. The forms in which nitrogen is provided and the concentration supplied have profound effect on the production of secondary metabolites (Fujita *et al.*, 1981). Since the growth of plant cells depend on the phytochrome content of the corresponding culture media, numerous studies were carried out about the dependence of secondary metabolism in phytochrome content. High doses of growth promoters can increase the content of secondary metabolites (Staba, 1980; Misawa, 1985). Direct evidence for the involvement of auxin in enzyme activation was reported by Hino *et al.* (1982). The switching of precursors from primary to secondary is operated in *in vitro* via a change in the organization of cells. Alternatively, the growth rate itself may be the determining factor in secondary product formation by allowing diversion of precursors from primary pathway to secondary pathway (Yeoman *et al.*, 1982). The type of growth regulators in culture medium can affect the production of secondary metabolites in cultured

cells quite dramatically (Cline & Coscia, 1988). The gaseous environment, availability of oxygen and carbondioxide play an impotant role in production of secondary metabolites (Narayanaswamy, 1994; Chand *et al.*, 1999). Sucrose in the medium is said to influence the production of secondary metabolites (Fowler, 1983). Chemical composition of the culture media may influence the production of secondary metabolites. Increase or decrease of macro and micronutrients also play an important role (Chand *et al.*, 1999). The stress induced by the culture conditions may be one of the reasons for changes in the essential oil composition of the *in vitro* plant of *T. roxburghianum* in the present study.

It is clearly evident that the biosynthesis of secondary plant products is controlled by genetic factors (Franz, 1989). Genetic basis of biosynthesis of monoterpenoids and sesquiterpenoids in *Ocimum* (Khosla *et al.*, 1985) and *Mentha* (Lincoln *et al.,* 1986) were already proved. In the present investigation the essential oil variation may be the after effect of genetic changes, which was revealed by chromosome imaging studies and RAPD analysis.

Chromosomal differences can cause changes in quality and composition of essential oils (Guenther, 1949). Changes in the constituents of *in vivo* and *in vitro* plants are influenced by various nongenetic factors (Gerhardt, 1972). But there are also reports on tissue culture plants that match the parent plant in their biosynthetic capacities (Kireeva *et al.*, 1978; Charlwood & Charlwood, 1983). In the present investigation, the marked differences observed in the essential oil composition of the parent plant and the variant may be due to their respective biosynthetic pathways. Changes in the essential oil composition of *in vivo* and *in vitro* plants owe to changes in their respective biosynthetic pathways (Tetenyi, 1973).

With the role of spices in pharmaceutical and food industry steadily increasing, conservation of their germplasm is a matter of great significance. Advances in technology, improved basic knowledge, changing technical principles, boosted awareness of potential of cultured cells, and safety of natural products has recently changed the strategies of micropropagation. The advantages of plant cells, tissues and organs cultured *in vitro* for the production of high value natural compounds are many (Bhagyalakshmi & Bopanna, 1998; Bhagyalakshmi *et al.*, 1998).

There have now been a number of reports on somaclonal variation associated with molecular modifications such as gene amplification, activity of transposable element, point mutations and alteration of multigene family expression (Larkin *et al.*, 1985; Brettel *et al.*, 1986; Cooper *et al.*, 1986; Davies *et al.*, 1986; Ryan & Scowcroft, 1987). These provide impulses for plant genetics and contribute to new breeding strategies (Shepard *et al.*, 1980). The various beneficial factors substantiate the use of modern techniques like tissue culture for obtaining quantitatively and qualitatively superior plants. The tools of plant cell culture are increasingly being applied to a wide range of biotechnology ventures and in particular to the clonal propagation and genetic improvement of crop plants. Research on plant tissue cultures, regenerated plants and progenies of regenerated plants has revealed a rich array of culture induced genetic variants. The powerful technique in plant cell and tissue culture, recombinant DNA, bioprocess technologies etc. coupled with most sophisticated techniques such as NMR, HPLC, GC-MS, LC-MS etc. have offered mankind the great potency of exploiting the totipotent biosynthetic and biotransformation capabilities of plant cells under *in vitro* conditions (Stockigt *et al.*, 1985).

The phenomenon of production of a particular compound is fixed in the genome of a plant. Better understanding of genes, factors involved in the

regulation of pathways and isolation of biosynthetic enzymes provides a feasible application of medicinally important plant products isolated from cells and tissues for commercial exploitation. The new century will witness the surge for new plant based active principles and plant biotechnology offers an excellent opportunity to explore this area of research.

It is thought that chromosome structural rearrangements could facilitate both speciation and adaptive evolution at the organismal level by functioning as cytogenetic, reproductive isolating mechanisms, by altering patterns of gene expression (regulation), or by creating adaptive super genes which cannot be easily broken up by recombination (Grant, 1971; White, 1973, 1977, 1978; Wilson, 1975, 1976; Bush *et al.*, 1977).

Today the modification of oil composition in aromatic plants by micropropagation and genetic manipulation is a realistic phenomenon. Biochemical and molecular knowledge could allow finer investigations on the regulation of terpenoid metabolism if an efficient regeneration technique is available. This type of approach to understand control mechanisms regulating the phase of monoterpenic compounds will be of great use to essential oil industry. The market drive for natural product pharmaceuticals and gradually evolving stringent quality standards for herbal medicines may encourage investment in intensive R & D for *in vitro* production of phytochemicals. Phytochemical investigation of plants has been an interesting area of research leading to the isolation of several new compounds. Being end products of plant cell metabolism, essential oil can be used as good markers for preliminary chemotaxonomical classification.

In conclusion, this study revealed a shoot regeneration method for the production of somaclonal variants having better essential oil profiles and it also revealed the cytological and molecular basis for the phytochemical variations in them. Moreover, it can be stated that the *in vitro* plant of

*Trachyspermum roxburghianum* (DC.) Craib shows remarkable differences from the *in vivo* plant in the following aspects.

1. The karyomorphological studies *via* the image analysis of *in vivo* and *in vitro* plants showed variation in total chromosome length and in the types of chromosomes, whereas the ploidy of the calli varied from diploid, tetraploid to octaploid condition, all of which can be attributed to cryptic numerical [polyploidy/ euploidy (hypo & hyperploidy)] and structural [translocations, deletions, inversions, retrotransposon activation or differential spiralization or condensation of chromosomes] aberrations, which in turn may have resulted from the *in vitro* stress produced in the altered culture environment.

2. The meiotic studies in the *in vivo* flower revealed normal chromosome complement of 22 bivalents exhibiting a normal meiotic behaviour. The diakinesis and metaphase I of the *in vitro* plant exhibited multivalent (tetravalent) association along with bivalents. The probable cause of these aberrant conditions may be associated with increase in chiasma frequency resulting in increased number of quadrivalents or structural heterozygosity involving segmental interchanges giving rise to multivalents in normal tetraploid species. The meiotic instability is considered as an evidence of variation from the evolutionary standpoint.

3. The Random amplified polymorphic DNA (RAPD) fingerprints of the *in vivo* and the *in vitro* plants with ten primers show successful amplification, with seven primers showing polymorphisms were detected. The polymorphisms can be assumed to be due to change in the sequence of primer binding

site or change which alters the size, preventing successful amplification of the target DNA, both of which occurred due to tissue culture stress, that may show a general disruption of normal cellular controls, leading to numerous genomic changes in the tissue culture regenerants.

4. The GC-MS analysis of the *in vivo* and the *in vitro* plants showed a coefficient of similitude of 31. 2724, with 10 common components, out of a total of 32 essential oil components detected. 15 essential oil compounds were detected in the essential oil of *in vivo* plant, whereas 7 occur exclusively in the essential oil of *in vitro* plant of *T. roxburghianum.* The low value of coefficient of similitude showing dissimilar nature of essential oil composition may be due to variation in the genetically controlled biosynthetic pathways, which might have occurred due to either loss in genetic ability or repression of relevant genes under culture conditions.

*On the basis of these findings it seems probable that the in vitro plant of T. roxburghianum exhibits somaclonal variation. These findings emerging from this study has a practical utility for creating variability in Trachyspermum roxburghianum as well as to screen out the variants, which can be used for conventional breeding programmes if found stable after studying its genetics.*

Barbara McClintock quoted in her Nobel lecture; " The treatment from isolation of the cell or cells of a plant, to callus must inflict on the cells a succession of traumatic experiences. Resetting of the genome in these instances may not follow the same orderly sequence that occurs under natural conditions. Instead the genome is abnormally reprogrammed, or decidedly restructured. These restructurings can give rise to a wide range of altered

phenotypic expressions. Some altered phenotypes clearly reflect genomic restructuring and various levels of this have been observed. The many levels of genome modification that already are known and expressed as changed genotypes and phenotypes could be potent sources of selection for plant breeder, and incidentally for theoretical ponderings by the biologist."

Higher plants are still "the sleeping giants of drug development" (Farnsworth & 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).

# SUMMARY

Sreeranjini K. "Chromosome imaging, rapd and gc-ms assays on in vitro and in vivo plants of trachyspermum roxburghianum (dc.) craib (Apiaceae)" Thesis. Department of Botany, University of Calicut, 2004

# SUMMARY

The family Apiaceae, constitutes an important part of the plant kingdom because of the abundance of plants with aromatic and medicinal properties. *Trachyspermum,* a member of the family is an annual herb, which is grown in parts of Asia for its seeds, as they are much valued as spices. The genus *Trachyspermum* has about 25 or more species distributed in temperate regions. The essential oil of *T. roxburghianum* (DC.) Craib is used in medicines widely. The herb is also valued as medicine in Ayurvedic and Yunani systems.

In the present study, attempt is done to present the cytogenetical aspects and the related phytochemical effects of somaclonal variation in *T. roxburghianum* (DC.) Craib. Cytogenetical assays include mitotic and meiotic studies and random amplified polymorphic DNA (RAPD) analysis, whereas the phytochemical studies include Gas Chromatography-Mass Spectrometry (GC-MS).

## *In vitro* multiplication

For the induction of multiple shoot regeneration, nodal explants were inoculated in the MS medium with combination of cytokinins and auxins. The multiple shoot induction was noticed in the medium 2 mg/l BA and 8 mg/l IAA. The frequency of shoot induction and percentage of initiation was higher in the medium containing 1 mg/l BA and 7 mg/l IAA. The micropropagated plants were used for further analysis.

## Cytological Analysis

### Mitosis

The ploidy levels of both the donor plant and the somaclonal variant were invariably tetraploid (2n = 4x = 44). Octaploid (2n = 8x = 88) and diploid (2n = 2x = 22) cells were observed among the cells with normal

chromosome number (2n = 4x = 44) in the callus. Chromosome morphology of the regenerated plant showed slight variation. Some of the chromosomes were not the exact replicas of the standard chromosomes of the parent plant and exhibited structural changes, thereby change in basic length and average chromosome length. Changes in DI, VC and TF % were also noticed.

**Meiosis**

Studies were conducted in the flowers of parent plant as well as the *in vitro* plant. Tetravalent chromosome associations were observed along with the bivalents in the variant PMCs of *in vitro* plant whereas parent plant showed normal bivalent association.

**RAPD analysis**

To obtain more information on the genetic diversity between the variant and the parent plant, RAPD analysis was carried out using 27 primers of arbitrary sequence. Of the 27 primers, only 10 successfully amplified the extracted DNA with consistently reproducible banding. The number of bands resolved per primer ranged from a minimum of 2 to a maximum of 12. The size of amplification product also differed and ranged from approximately 100 bp to 900 bp in the 1000 bp ladder and approximately 300 bp to 3000 bp in the 4000 bp ladder.

The RAPD fingerprint of the variant differed from the parent plant with seven primers used. Altogether, four bands for the primer OPA 02 and OPA 11, three bands for OPA 09 were absent in the variant in comparison with the parent. The variant also showed one additional band for OPA 09 and five additional bands for OPA 11 than the parent. With OPA 06 the variant showed five additional bands than the parent. With OPC 03, one additional band was detected in variant, which was absent in the parent as well as TC2. Using OPC 11, one additional band was observed in the variant and TC2,

which again was not detected in the parent. The parent showed one additional band for OPC 03, two for OPC 11 and two for OPD 01 when compared to the variant and TC2. Altogether, these primers generated thirteen new RAPD bands in the variant that were absent in the parent plant fingerprints while sixteen bands that were present in the parent plant fingerprints were not scored in the variant.

## Essential Oil Analysis

### Gas-Chromatography-Mass Spectrometry

The essential oils of *T. roxburghianum* (DC.) Craib and its variant were analysed quantitatively and qualitatively. The oil yield of variant (1.2%) was slightly higher than that of the donor plant (0.9 %). The essential oils were analysed by GC-MS and number of compounds were identified which included monoterpenes and sesquiterpenes. The GC-MS pattern of variant essential oil was distinctly different when compared with the pattern of parent plant oil.

The quantitative and qualitative differences observed in the essential oil directly influenced the colour of the oil distilled. The oil from the *in vivo* grown plant have light yellow colour whereas essential oil of *in vitro* plant showed deep yellow.

There is clear difference between the compositions of two oils analysed. The analysis of the oil samples revealed a wide range of variations in their constituents. The major components identified from both oils are different (epiglobulol in the *in vivo* plant and isothujol in the *in vitro* plant), showing remarkable variation in percentage composition (19.55% & 16.42% respectively).

The *in vivo* plant is characterized by the presence of β-myrcene (0.35%), limonene (0.31%), γ-terpinene (0.52%), α-cubebene (1.55%), α-caryophyllene (1.69%), eudesma-4 [14], 11-diene (10.94%), aromadendrene (4.26%), δ-cadinene (1.74%), epiglobulol (19.55%), caryophyllene oxide (1.86%), aromadendrene oxide (0.45%), isobenzofuranone (1.35%), β-methyl benzene propanal (15.74%), tetradecanol (0.73%) and n-hexadecanoic acid (1.70%) which were found to be absent in the *in vitro* plant. The *in vitro* plant possess ocimene (1.10%), α-farnasene (1.96%), 1,R, 3Z-9S-4, 11,11-Trimethyl 8-methylene bicyclo [7.2.0] undec-3-ene (3.81%), isothujol (16.42%), β-farnasene (1.35%), α-β-epoxycumene (14.72%) and α-bergamotene (3.37%) which were not detected in the *in vivo* plant. In addition, ten common components were detected in both the oils including one unidentified component.

In the current study it is observed that the combination of IAA and BA produced plants showed very little morphological variation from the donor plant. Plants regenerated from tissue or organ culture could diverge from donor plants for one or more phenotypic traits as a result of different mechanisms of somaclonal variations which include changes in chromosome number and structure, dominant and recessive mutations, changes in chloroplast and mitochondria.

Tissue culture induced variation has usually been based on phenotypic differences in regenerated plants and their progenies. However, genomic changes appear to be basis for the phenotypic alterations. The chemical composition of the culture medium has been shown to affect the cytogenetic behaviour of plant cells *in vitro*. Chromosome aberrations especially duplications are found in plants regenerated from tissue cultures which may complement to change in their chromosome size. However, these assumptions require molecular evidences.

Direct analysis of DNA by using RAPD markers proved to be a very sensitive technique for evaluating genetic changes after *in vitro* culture. Three types of polymorphisms were observed in this study such as band intensity differences, additional bands and the absence of fragments. The presence of bands in two genotypes indicates a high level of sequence homology at that site. In the chromosome, where the marker was present in one genotype but not in the other, there is the certainty of sequence difference. Failure of amplification of different lines may be due to a single base change or completely different sequences.

In most cases, study of somaclonal variations has been limited to phenotypic variation and has been associated with changes in the chromosome structure and function. The somaclonal variations in yield and quality characters have utmost importance in crop improvement. The present analysis shows there is a significant change in the essential oil content and composition in the somaclonal variant of *T. roxburghianum* (DC.) Craib. The biosynthesis of secondary plant products is controlled by genetic factors. Because of the close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites. In the present investigation, the essential oil variation may be the effect of genetic changes revealed by chromosome studies and RAPD analysis.

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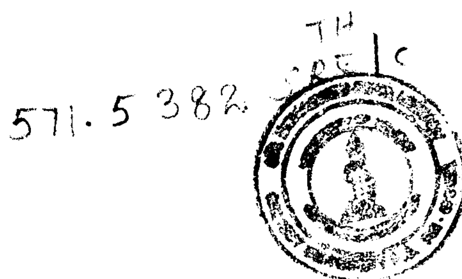
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* Not consulted in original.

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