CHROMOSOME IMAGING, RAPD AND GC-MS ANALYSES ON A SOMACLONAL VARIANT OF *MENTHA ROTUNDIFOLIA* (L.) HUDS.

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By

DEENA MERIA JOSE

Genetics and Plant Breeding Division
Department of Botany
UNIVERSITY OF CALICUT
Kerala, India

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Mentha rotundifolia (L.) Huds.

DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT

Calicut University P. O., Kerala - 673 635, India

Phone : (0494) 400275 PBX 0494 401144, exten. BOTANY Fax : 0494-400269 Grams : UNICAL

Date: 12-08-02

Dr. John E. Thoppil,
Lecturer (Sr. Grade),
Genetics & Plant Breeding Div.

CERTIFICATE

This is to certify that the thesis entitled 'Chromosome Imaging, RAPD and GC-MS Analyses on a somaclonal variant of Mentha rotundifolia (L.) Huds.' is an authentic record of work carried out by Miss Deena Meria Jose in the Department of Botany, University of Calicut during 1999-2002 under my supervision and guidance and that no part thereof has been presented earlier for any other degree or diploma.

12-8-2002
Dr. John E. Thoppil

DR. JOHN E. THOPPIL
Lecturer, Genetics &
Plant Breeding Division
Department of Botany
University of Calicut
673 635, Kerala, India

DECLARATION

I hereby declare that the thesis entitled 'Chromosome Imaging, RAPD and GC-MS Analyses on a somaclonal variant of Mentha rotundifolia (L.) Huds.' submitted for the Ph.D. Degree of the University of Calicut has not been submitted earlier for the award of any other degree or diploma and that it represents the original work carried out by me.

Date:12- 08-02

Deena Meria Jose

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CONTENTS

Abbreviations Used

2,4-D	2,4-Dichlorophenoxy acetic acid
ACE	Acetylcholineesterase
AP PCR	Arbitrarily Primed Polymerase Chain Reaction
BA	Benzyl Adenine
BAP	6- Benzyl Amino Purine
CNS	Central Nervous System
CS	Coefficient of Similitude
CTAB method	Cetyl Trimethyl Ammonium Bromide method
CW	Coconut Water
DI	Disparity Index
dNTP	deoxy Nucleotide Tri Phosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FPP	Farnesyl Pyrophosphate
GA_3	Gibberellic Acid
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GGPP	Geranyl Geranyl Pyrophosphate
GPP	Geranyl Pyrophosphate
HPLC	High Performance Liquid Chromatography
IAA	Indole 3-Acetic Acid
IPP	Isopentenyl Pyrophosphate
KIN	Kinetin (6 furfurylaminopurine)
MS medium	Murashige & Skoog medium
NAA	α-Naphthyl Acetic Acid
NIR	Near Infra Red Spectroscopy
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TAE buffer	Tris Acetic Acid EDTA buffer
TE buffer	Tris HCl EDTA buffer
UI	Unidentified Component
VC	Variation Coefficient

INTRODUCTION

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

INTRODUCTION

Aromatic plants have been of great interest to mankind from the beginning of human civilization. Aromatic plants and their derivatives, particularly the essential oils are now becoming one of the most important export items from many developing countries in Asia. The main reason for such an upswing is the technological advances in the production and processing of the essential oils and their increased use in various medicines. There is great potential to improve the yield and quality of these plants, either by mere selection of existing species or varieties through plant breeding or by other novel methods of plant improvement.

In the dawn of human cultural evolution the art of curing was essentially magical (Naranjo, 1984). Herbal medicines can be viewed as the precursor of modern pharmacology. References to miracle herbs or wonder drugs are often found in old literature. In India, references to the curative properties of some herbs in *Rigveda* seem to be the earliest record of use of plants in medicine. Few plants, that were usually psychoactive, known as magic or psychedelic plants, were used by ancients. Later on empirical medicine arose, using many plants for the treatment of various afflictions. This tendency culminated in the Old World, in the famous work *Materia Medica* by Dioscorides, published in the first century AD. The ancients possessed an extensive vegetal pharmacopoeia. Some of the oldest pre-literature archaeological records in both the Old and New Worlds deal with the medicinal use of plants.

Medicinal plants are the source of various alkaloids, terpenoids and other chemical substances quite essential to mankind. Medicinal plants normally growing in wild habitat show an increasing depletion of natural resources (Tandon, 1994). Keeping this in view, there is an urgent need for the systematic cultivation of medicinal plants through novel techniques for their germplasm conservation, selection of desired genotypes and mass propagation of superior genotypes (Ahuja, 1994; Filippini *et al.*, 1994). Plants are the traditional source of many chemicals used as pharmaceuticals, biochemicals, fragrances, food colours and flavours (Leung, 1980). Most valuable phytochemicals are the products of plant secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult. Procurement, cultivation and regeneration of important medicinal and aromatic plants used in

indigenous systems of medicine are the other aspects of current scientific importance as pharmaceutical industry depends on it for raw material.

Edible materials, which are, used more for their aromatic flavour than for their food value are known as spices. Spices are fragrant or aromatic plant parts used as a whole, ground, paste or in liquid form for flavouring and seasoning foods. They have played a vital role in the world trade due to their varied properties and applications. The flavour and aroma are due to essential oils. Spices serve mainly three purposes: they do not let the food to be monotonous, they disguise the stale flavour of food materials and increase the pleasure of eating and they help to cool the body by increasing the rate of perspiration. Spices by themselves are not high in nutrient value. But spices and herbs can play an extremely valuable role in good nutrition by helping to increase the appeal and appreciation of foods that are nutritionally important to us. Aroma and flavour characteristics are important quality attributes of spices, which are based on the nature of essential oil in them. Spices were sometimes considered so important that they were used in beverages, medicine and even in lieu of money.

Mentha (mint), one of the most important taxa of the family Lamiaceae occurs in all the five continents, although its native occurrence in the New World is restricted to a single species in the North. It is frequent in the tropics (Tyagi & Ahmad, 1989; Tyagi *et al.*, 1992). The genus comprises of about 25 species (Willis, 1973) distributed in the temperate regions of the Old World. According to Bhattacharjee (2000), six species of this genus are distributed in India, majority of them being found in the Western and Eastern Himalayas. Several species are cultivated widely in different parts of the world for their content of menthol and its other important derivatives (Heywood, 1978). *Mentha* species were originally cultivated in Eastern Asia, mainly in Japan and China (Bersaghi, 1945). Regular cultivation of mints started around 1870 in Japan (Mehra, 1982).

The genus has been subdivided into a number of sections, of which by far the largest and taxonomically most complex is Section *Mentha* (=subgenus *Menthastrum* Cossom & Germain). This includes all the better known species of commerce, such as Japanese mint or corn mint or field mint (*M. arvensis*), peppermint (*M. piperita*), common or native spearmint (*M. spicata*), Scotch spearmint (*M. cardiaca*), Bergamot mint or

orange mint (*M. citrata*), apple mint (*M. rotundifolia*) etc. (Tyagi & Ahmad, 1989; Tyagi *et al.*, 1992). In modern taxonomic treatment of the genus, there are five sections (*Mentha* Sect. *Audibertia*, Sect. *Eriodontes*, Sect. *Mentha*, Sect. *Preslia* and Sect. *Pulegium*) containing 19 species and 13 named hybrids involving *M.* Sect. *Mentha.* Formation of hybrid species of the genus *Mentha*, and in particular with those of the subgenus *Menthastrum* Cossom & Germain is a very common phenomenon in wild populations, where more than one species occur (Harley & Brighton, 1977). Identification of mints is difficult due to great phenotypic and genetic variability caused by interspecific hybridization. *Mentha* species are extremely variable and specific limits are hard to define, with consequent unstable nomenclature (Santapau & Henry, 1973). Assumptions of type specimens and the consequential publication of illegitimate names have rendered the nomenclature of this polymorphic genus more difficult. Almost 2300 names have been published for essentially 20 species of this genus (Tucker *et al.*, 1980).

Mints are subdivided into three groups – culinary, medicinal and fragrant. The first group includes the species *M. viridis* (lamb or pea mint), *M. sylvestris* (wild mint, crisped or curled mint) and *M. rotundifolia*. The second group contains *M. piperita*, *M. gentilis* (gingermint) etc. and the third group includes *M. citrata*, *M. aquatica* (water mint) etc. (Genders, 1972).

Theophrastus in the fourth century was the first to use the name 'Minthe' in referring to the mint, *M. viridis*. According to Greek mythology, a nymph called 'Menthe', who was greatly loved by Pluto, was transformed into mint herb by Pluto's jealous wife (Macleod, 1968). The poets celebrate Minthe, a daughter of Cocytus, as being turned into mint by Proserpine in a fit of jealousy (Hereman, 1980). Mints have been used and valued for aromatic purposes for thousands of years. The ancient Greeks and Romans used them. Mints were the favourite herbs of the Roman scholar Pliny. He enjoyed them for their aromatic effect: "the very smell of mints reanimates the spirit" (Doyle, 1998). In the Middle Ages, mints were used for strewing purposes – they were scattered about castles to mask the dreadful odours and to repel insects. As the writer Gerald stated, "the smell rejoiceth the heart of man", for which they used to strew it in chambers and places of recreation, pleasure and repose where feasts and banquets were made (Macleod, 1968).

There are Biblical references also regarding this herb. In the New Testament, in chapter 23, verse 23, Mathew notes their value by quoting Jesus' condemnation of the Pharisees for requiring a tithe of mint, dill and cumin. The philosopher Seneca, though a stoic, died in a bath strewn with mint. The herb's culinary virtues were also known from classical times (Conway, 1973). Mint's ability to prevent milk from curdling was well known (Culpeper, 1999). In Italy, mint was spread on the floor of churches and hence called 'Erba Santa Maria' (Nayar, 1985). Shakespeare mentioned mint along with lavendar and marjoram in 'The Winter's Tale'. In France, mint was called 'Mente de Notre Dame'. Mints were employed much by Victorian ladies as they used bottles of smelling salts to revive anyone in danger of swooning. In Greece, ancients used to scent each part of their body with different perfumes and they reserved mint for their arms. Put into bath with balm and other herbs, mints help to comfort and strengthen the nerves and sinews (Macleod, 1968). They have been found in Egyptian graves and are described in old literature of China. In ancient Greece, the juice of mints was applied to arms as an after bath rubdown (Clarkson, 1972).

The economic importance of mints is due to the production of mint oil as raw material for confectionary, pharmaceutical and cosmetic industries as well as for flavouring food, beverages, tobacco (Rech & Pires, 1986), candies, medicines, tooth pastes, mouthwash, and chewing gums (Chambers & Hummer, 1994; Banthrope, 1996). *Mentha* species are known from time immemorial as kitchen herbs (Mehra, 1982). It is also used for flavouring meat, fish, sauces, soups, stews, vinegar, teas, tobacco and cordials. The fresh leaf tops of all the mints are used in beverages, apple sauce, ice creams, jellies, salads, sauces for fish and meat, chutneys etc. (Pruthi, 1976).

The herbs of mint are much esteemed in India as diaphoretic (Keys, 1976), diuretic (Watt & Breyer-Brandwijk, 1962), carminative, antiseptic, deodorant and stimulant (Dey & Bahadur, 1973; Nadkarni, 1976). In herbalism, mint tea is used to help digestion, to revive appetite and to alleviate rheumatism. The Arabs have always believed that mints increased virility and some modern herbalists still prescribe it in cases of impotence and decreased libido (Conway, 1973). According to Dioscorides, it has a healing, binding and drying quality and therefore the juice taken in vinegar stops bleeding. It stirs up venery or bodily lust, stops vomiting and allays cholera. Applied with salt, it helps to heal the wound

caused by the biting of mad dogs and pain in the ears. It is also used against the poison of venomous creatures, used to wash the heads of young children to cure sores or scabs and to cure the obstructions of liver. It is good against the gravel and stone in kidneys and the stranguary. The smell is comfortable for head and memory (Culpeper, 1999). The leaves are used as a contraceptive and to cure indigestion (Vedavathy *et al.*, 1997). Mints having pungent camphoraceous odour and taste are used to cure headache, rheumatism, neuralgia, cholera, colic, diarrhoea, flatulence, dysmenorrhoea, hiccup, palpitation of the heart, pruritus, diphtheria and toothache. It is also used in pharmaceutical preparations to disguise the taste of evil smelling and unpleasant drugs, and in antiseptic inhalations (Nadkarni, 1976).

Mints are extremely bad for wounds. If too much is taken, it makes the blood thin and wheyish (Culpeper, 1999). Mints are also reported to have antimicrobial (Mimica *et al.*, 1993; Lis Balchin, 1997; Thoppil *et al.,* 2001a), antibacterial (Singh *et al.*, 1992; Patnaik *et al.,* 1995; Sivropoulou *et al.*, 1995; Alippi *et al.,* 1996; Ela *et al.*, 1996; Patnaik *et al.*, 1996; Hassanein & Eldokseh, 1997; Shapiro, 1994; Carvalho *et al.*, 1999), antifungal (Mizutani *et al.*, 1989; Singh *et al.,* 1992; Patnaik *et al.*, 1996; Carvalho *et al.,* 1999), antiviral (Ismail, 1994), antiparasitic (Santana *et al.*, 1992), antinociceptive (Atta & Alkofahi, 1998), acaricidal (Mc Donald & Tovery, 1993; Perucci *et al.*, 1996), insecticidal (Franzios *et al.*, 1997), mosquito repellant (Thorsell *et al.,* 1998), larvicidal (Nadkarni, 1976), nematicidal (Oka *et al.*, 2000), herbicidal (Seidlova & Sarapalka, 1997) and genotoxic (Franzios *et al.*, 1997) activities.

M. rotundifolia (L.) Huds., a perennial pubescent herb otherwise called apple mint (CSIR, 1962) or wooly mint (Usher, 1984) or round leaved mint (CSIR, 1992) is grown for culinary purposes (Genders, 1972). It is a native of Europe and cultivated in Indian gardens (Chopra *et al.*, 1969). It is a diploid natural hybrid (2n=24) between the species *M. longifolia* (L.)(2n=24) and *M. suaveolens* Ehrh. (2n=24). *M. rotundifolia* auct. non (L.) Huds. and *M. suaveolens* Ehrh. are synonyms (Wiersema & Leon, 1999). *M. rotundifolia* is a delicious flavoured mint. It has digestive properties (Genders, 1972) and good for mint sauce (Bhattacharjee, 2000). Mint is used widely in Indian cuisine. The best-known examples are mint coriander chutney, green curries of meat, fish and poultry, mint raita, mint sweet chutney etc. Being a carminative, it is used for cooking peas, dals and other

dishes that are difficult to digest. It is also used for mint tea, mint sherbets etc. In Western cuisine it is used to sprinkle over fruit cups, melon balls, fruit aspic salads and as a garnish in hamburgers, sauces like cranberry, mint jelly etc. It is also mixed with cream cheese, wine punches, liqueurs, fruit juices etc. (Thangam, 1989).

In Vietnam, this plant is used for the treatment of headache, dyspepsia and fever. Pharmaceutical enterprises around the world have produced different balms such as Truong Son (Long Mountain), Sao Vang (Gold star) and Cao con ho (Tiger Balm). It is also used in food and perfume production industry and for making boudon, toothpastes and soaps (Dung & Thin, 1992). Leaves are used for flavouring (Usher, 1984). It helps to digest meat and used in confectionary trade for flavouring cakes (Genders, 1972). A mixture of *M. rotundifolia* (apple mint) and *M. citrata* (orange mint) is used in jellies to make it delicately fragrant (Clarkson, 1972). A hot infusion of apple mint can help at the start of a cold (Anonymous, 1996). *M. suaveolens* is used to cure hepatic complaints, as a tranquillizer and anticatarrhal agent (Gonzales Tejero *et al.*, 1992). *M. rotundifolia* is also reported to have nematicidal (Oka *et al.*, 2000) and cytotoxic (Minija *et al.*, 1999) properties.

Mints are considered today as the most important commercial essential oil bearing plants from the stand point of world wide production (Lawrence, 1985). Of the known flavouring materials in the world, mint is one of the most popular flavour and ranks probably third after *Vanilla* and *Citrus* (Singh *et al.*, 1998). Minty fragrances differ greatly in their organoleptic properties, going from fresh and cool long lasting tastes as those of spearmint to sweeter flavours and fragrances such as peppermint and cornmint (Sacco *et al.*, 1999). To the perfumer, anything that smells cool and fresh, would be classified as minty, and anything minty would fall into the mint category to be used to provide a cool, fresh, natural and clean top note to perfumes. For a perfumer, all mint notes are top note materials because they have high volatility and therefore they can be employed in the initial stage of a perfume's progression (Doyle, 1998). Such a history and economics make this plant a thrust area of growing research.

It has been found that our mint products are rated poor in quality and fetch lesser price in the world market as compared to Brazil, China and USA (Singh *et al.*, 1998).

Owing to their diversified uses, the demand of mint oils is ever increasing. Thus there is an urgent need to increase the production per unit area by developing effective package of practices for developing high oil yielding varieties as well as for developing high quality oil producing varieties. Selection of new clones is needed with improved terpene accumulation and desirable agronomic traits.

Decline of potential for the production of active principle or essential oil content is a common feature with cultivation of medicinal plants and a continuous selection is required to maintain the high yield of active constituents for commercially viable programmes. Therefore selection through conventional breeding as well as through tissue culture are exploited nowadays for obtaining high yielding cultivars. Development in the technology of plant tissue culture since its pioneering experiments by Laibach (1925; 1929), White (1934; 1937), Loo (1941), Skoog (1944) and Murashige & Skoog (1962) have contributed in establishing a strong foundation for the applications of this versatile technology.

M. rotundifolia does not flower due to genetic reasons and thus sexual reproduction and seed setting are lacking (Thoppil, 1993). It is vegetatively propagated through vigorous rhizome system. These vegetative clones fail to exhibit variability. In contrast to the conventional methods of vegetative propagation through rhizomes, the use of *in vitro* propagation enables the production of large number of variants. This is an alternative method for improving mint, which involves the use of tissue culture regeneration and the production of somaclonal variants (Larkin & Scowcroft, 1981; Constabel, 1990). Somaclonal variation is a term coined by Larkin & Scowcroft (1981) to cover all types of variations, which occur in plants regenerated from cultured tissues. Plant tissue culture has the potential to induce genetic variability in mint genotypes through somaclonal variants, somatic hybrids or transgenic plants. However a prerequisite to applied plant biotechnology is the development of a suitable and reproducible plant regeneration system (Jullien *et al.*, 1998). Potential use of cell culture (Drupeau *et al.*, 1987), multiple shoots (Constabel *et al.*, 1982; Endo *et al.*, 1987; Hirata *et al.*, 1987) and improvement of various cultivation conditions (Facchini & Dicosmo, 1991) have been attempted to scale up production of secondary metabolites. The exploitation of tissue culture technique in medicinal plants for the extraction of important

chemical compounds is indeed more advantageous (Tabata, 1977). A wide variety of compounds have been shown to be produced in shoot, callus or cell suspension cultures at levels equal to or higher than the levels in the intact plant sources (Brodelins, 1988; Dodds & Roberts, 1995).

Plant tissue culture has been extensively used to exploit the secondary metabolite it can produce. Growth of a cell in a totally controlled environment of physical and chemical factors provides an excellent system for studying changes in the production of secondary metabolites, which are always present in small quantities. The basic information has provided significant clues about genes and their functioning, leading to genetic manipulation of biosynthetic pathways so as to obtain desired products by either blocking a pathway or enhancing the metabolic reaction (Merillon & Ramawat, 1999).

Exploitation of possible somaclonal variation, which has been observed in *in vitro* cultures, could be used to widen the genetic pool from which to select desirable traits. Application of modern biotechnology can complement conventional breeding techniques and it helps in the development of improved varieties (Chomchalow & Sahavacharin, 1981; Vasil, 1988; Bajaj, 1991). *In vitro* propagation can yield a large number of clonal plants for continuous plant establishment. It is also important for germplasm conservation (Whithers, 1980; Kathiravan & Ignacimuthu, 1999; Kukreja & Dhawan, 2000). Variation is a ubiquitous phenomenon associated with tissue culture (Carlson & Polacco, 1975; Green, 1977). Induced variation is an alternative source to naturally occurring variability for crop improvement (Ansari & Siddiqui, 1995). Tissue culture induced variation is defined as the variation that arises *de novo* during the period of dedifferentiated cell proliferation that take place between culture of an explant and production of regenerants (Munthali *et al.*, 1996). Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin & Scowcroft, 1981), which is often heritable (Breiman *et al.*, 1987). Other reports claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al.*, 1989).

The genus *Mentha*, in view of its medicinal and commercial importance, has been the subject of cytological, genetic and phytochemical studies (Sobti, 1971; Murray *et al.*, 1972; Gill *et al.*, 1973; Sobti, 1975; Tucker & Fairbrothers, 1981; Harley & Brighton,

1992). It exhibits a variety of genetic forms with different sets of chromosomes. Chromosome number proved to be of important systematic value in many groups of Labiatae as in *Mentha* (Harley & Heywood, 1992). A major contribution to the cytology of the genus was the work of Harley & Brighton (1977) which tested chromosome counts of many accessions of almost all the taxa recognized today, with major emphasis on *M.* sect. *Mentha*. It is well known that the DNA content undergoes a multiplication with increase in genome sets (Kundu & Sharma, 1985). However there are certain confirmed reports (Dworick & El Bayoumi, 1969; Grant, 1969) indicating a relative decrease of the amount of DNA at higher ploidy levels. Based on a base number of x=12 (Ikeda & Udo, 1963; Harley & Brighton, 1977) somatic chromosome numbers in sect. *Mentha* range from 2n=24 to 120 (Morton, 1956; Harley & Brighton, 1977). Several workers (Nagao, 1941; Sharma & Bhattacharyya, 1959; Ouweneel, 1968; Tyagi & Naqvi, 1987) have also recorded aneuploid individuals. It is well known that an increase in chromosome number is reflective of an increase in cell size (Stebbins, 1971).

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). Chromosomal variation is a common feature of plant tissue culture. Changes can take place at the ploidy level like the production of aneuploids (Taliaferro *et al.*, 1989), polyploids (Mariotti *et al.*, 1984) and mixoploids (Mariotti *et al.*, 1984; Taliaferro *et al.*, 1989). Karyological studies can bring to light the variations in chromosome number and their size and suggest the direction of chromosomal evolution in specific taxa (Jones, 1978). Chromosomal differences may also cause changes in quality and composition of the essential oils (Guenther, 1949).

Computer aided chromosome image analysis system is a modern technique for karyomorphological analysis. Ordinary karyotype analysis has provided a limited success in chromosome identification. Possibility of making errors is much greater in the conventional method of measuring and characterizing by visual evaluation. These difficulties can be overcome by computer aided chromosome image analysis system. It allows an accurate chromosome pairing mainly in those cases where the chromosome size is very small (Fukui & Kakkeda, 1994). This technique gives a better knowledge of

the cytogenetic constitution of the material under study (Fukui & Kakkeda, 1990; 1994; Fukui & Iijima, 1992; Fukui & Kamisugi, 1995).

Genomic analysis is a prerequisite for establishing the genetic stability and uniformity of a desired clone (Ikeda & Ono, 1967). Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones but most of them have limitations. Karyological analysis can not reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al.*, 1993). The Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) has been the basis of a growing range of newer techniques. PCR allows the specific amplification of DNA sequences, making it ideal for the identification of plant genotypes. Amplification of a genotype specific sequence can take advantage of some of the many features of PCR like speed, simplicity, specificity, sensitivity and cost (Henry, 1997). Molecular markers such as Restriction Fragment Length Polymorphism (RFLP) (Tanksley *et al.*, 1989) and Randomly Amplified Polymorphic DNA (RAPD) (Welsh & Mc Clelland, 1990; Williams *et al.*, 1990) appears to be good, but when compared to RFLP, RAPD appears to provide a better basis for genetic characterization because of simplicity of the necessary procedures (Baird *et al.*, 1992). The approach of using molecular markers including RAPD profiles is a powerful tool not only for the identification of genotypes but also to quantify the extent of genetic variation in any given population. While on one hand the approach of RAPD profiling has been useful in tissue culture methods for detection and selection of somaclonal variants (Munthali *et al.*, 1996), this molecular technique, at the same time with the same logic, is directly utilizable for assessing the population of micropropagated clones from any given explant for genetic uniformity.

Using PCR with short primers of arbitrary sequences, RAPD markers were recently shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991; Roy *et al.*, 1992). This is an alternative approach for finding new DNA based polymorphic markers among closely related genotypes (Welsh & Mc Clelland, 1990; Nymbom *et al.*, 1990; Lindhout *et al.,* 1999). RAPD analysis using PCR with arbitrary oligonucleotide primers (Williams *et al.*, 1990; 1992) has the advantage of being non radioactive, rapid and convenient assay of polymorphism and requires only a small amount of crude DNA. Today, RAPD technique has been adopted

most widely. The main issues associated with the use of these techniques are the problem of ensuring reproducibility of amplification profiles. The nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by any variation in the method used to prepare the DNA template and the exact reaction composition and conditions used in the PCR (Muralidharan & Wakeland, 1993). Obtaining reliable results depends upon standardizing these conditions or identifying combinations of conditions that give consistent results, even when variations in the key variables are encountered. A key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation. Both the quantity and quality of the template DNA preparation have the potential to substantially influence the result.

Polymorphism result from either base changes at the primer binding site (point mutation) or chromosomal changes in the amplified regions (insertions, deletions or inversions) which alter the size or prevent the successful amplification of a target DNA. Southern hybridizations are not required and polymorphisms can also be detected in fragment containing highly repeated sequences, which are recalcitrant to RFLP analysis. The extent of polymorphism detected by RAPDs is therefore greater than that observed by RFLPs (Williams *et al.*, 1992).

Plants represent an unlimited source of phytochemicals such as the metabolites of primary and secondary metabolism. The secondary compounds are of major interest because of their different functions and a wide range of biological activities. Secondary metabolites are compounds that are biosynthetically derived from the primary metabolites and their distribution in the plant kingdom is restricted. These compounds are generally detected in lower volume compared to the primary metabolites and also possess significant biological activities. So they are also termed as the 'higher value – lower volume products' or 'speciality chemicals' (Roja & Rao, 1998).

Essential oils belong to the most vital constituents of many spices and medicinal plants. It is well known that plant derived natural products are extensively used as biologically active compounds. Among them, essential oils were the first preservatives used by man originally in its natural state within plant tissues and then as oils obtained by

distillation. Essential oils are distinguished from fatty oils in that they evaporate or volatilize in contact with air and possess a pleasant taste and strong aromatic odour. They can be removed from plant tissues without any change in composition (Hill, 1972). Most essential oils are very complex mixtures consisting of hundreds of compounds (Bicchi *et al.*, 1999). Essential oils are complex mixtures of odorous and steam volatile compounds which are deposited by plants in the subcuticular space of glandular hairs and in cell organelles like oil bodies, idioblasts, excretory cavities and canals or exceptionally in heartwoods.

Biochemical studies with isolated peltate glandular trichomes have revealed that the secretory cells are not only responsible for the secretion of monoterpenes into the oil storage space, but also serve as the actual site of monoterpene biosynthesis (Gershenson *et al.*, 1992; Mc Caskill *et al.*, 1992). The oil composition especially terpene components are important in chemotaxonomy. Hundreds of different compounds with open chain, cyclic and bicyclic skeletons are known. Most of them have a typical odour, so they are responsible for the aroma of many herbs and spices. Monoterpenes are characteristic plant ingredients. They are also important as raw material for flavouring agents (Faber *et al.*, 1997). The chemical constituents associated with the typical olfactory characteristics of mint oils are monoterpenes and to a lesser extent sesquiterpenes both of which belong to a structurally diverse group of natural products known as isoprenoids (Lange & Croteau, 1999).

The biogenetic origin of most of the *Mentha* oil components has been deciphered in the last 20 years (Murray *et al.*, 1980; Maffei, 1988). It is known that the chemical composition of the oil is greatly affected by extrinsic factors like light, water, temperature, soil and nutrients (Grahle & Hoeltzel, 1963; Burbott & Loomis, 1967; Clark & Menary, 1979a; 1979b; 1981; Murray *et al.,* 1988; Lawrence, 1989) and intrinsic factors like genotype and stage of development of leaves and glands (Lawrence, 1989; Clark & Menary, 1981; Murray *et al.,* 1988; Kokkini, 1991). Oil yield and quality are genotype dependent characteristics (Murray, 1960a; b; Hefendehl & Murray, 1976).

Chemically and biogenetically, the natural product essential oil is heterogeneous. The best known constituents of essential oils are terpenoids, the mono, hemi, sesqui and

diterpenes (C_5, C_{10}, C_{15} and C_{20} respectively), but they may also contain aliphatic and aromatic esters, phenolic compounds and substituted benzene hydrocarbons (Ramawat, 1999). Phenyl propanoids, alkane derivatives, alkanals, alkanoic acids etc. are by far the other ubiquitous essential oil components (Hegnauer, 1982). The volatile fraction of the essential oil, commonly called terpenes, contain a large number of hydrocarbons, all of the formula $C_{10}H_{16}$ (De Mayo, 1959). Some essential oils are attractive to certain animals and insects (Nicholas, 1973) and some are repellant. Sesquiterpenes regulate bud dormancy, abscission and are used as antifungals. Plants having considerable amount of oil are prevented from becoming too warm. Some oils help in wound healing (Guenther, 1949; Hegnauer, 1982).

A multiplicity of ecological roles has been ascribed to essential oils and their constituents. There is a general consensus that these compounds contribute a basic armament in the defensive potential of the plants against excessive water loss (Rhoades, 1977; Stephanou & Manetas, 1995) or biotic attack and act as antiherbivore, antibacterial and antifungal agents. Certain allelopathic functions have also been reported in many cases (Harbone, 1997; Fischer, 1991; Cole, 1992). In recent years essential oils have received much attention as resources of potentially useful bioactive components. They are plant secondary metabolites mainly composed of terpenoid compounds and play an important role in the interactions between plant and insects. The effect of essential oils on insects range from an attraction or repellence to that of toxicity or even lethality (Karpouhtsis *et al.*, 1998). Many of these crude mixtures have been found to have *in vitro* antifungal, antibacterial, cytostatic and insecticidal activities (Janssen *et al.*, 1987; Thompson, 1989; Konstantopoulou *et al.*, 1992; Sivropoulou *et al.*, 1995; Mehmood *et al.*, 1997; Deena & Thoppil, 2000; Thoppil *et al.*, 2001a, b). It is also used as a termiticide (Beckstrom-Sternberg & Duke, 1996). Today there is an increasing interest in the use of 'microbicidal' plants because of the necessity of finding safer insecticides and microbicides in combination with the need of preventing environmental degradation and pollution (Franzios *et al.*, 1997). The mint essential oils and their fractions rich in specific terpenes and semisynthetic products of individual terpenes have been found to possess a variety of antimicrobial, pesticidal and anticancer properties, on which new uses of mint oils are expected to be based (Patra *et al.*, 2001a).

Essential oils are effectively used in aromatherapy. They are believed to stimulate the olfactory nerves and exert influence on the brain centre that controls emotion (Mabey, 1988). Essential oils are used as natural rejuvenating and antiwrinkle agent in aromatherapy (Varshney, 1991). It is suitable in the treatment of pain, psychological disturbances, allergies, skin diseases, gastrointestinal disorders, cardiovascular problems, urinary disorders, gynaecological disturbances, cancer etc. (Jamil, 1997).

Menthol, the major component in mint oils is a crystalline stearopten obtained by cooling the distilled oil and freezing the distillate by ice and salt. It occurs in permanent prismatic or colourless acicular crystals resembling sulfate of magnesium and very slightly soluble in water (Boericke, 1991; Khory & Katrack, 1999). It is an antineuralgic and applied externally in alcoholic solution or in the form of the popular 'menthol care' (Dey & Bahadur, 1973). In India it is taken with betels providing a cooling sensation when chewed (Tyagi & Naqvi, 1987). It is also used against malaria, tape worm infection, giardiasis (Anonymous, 1973), acute nasal catarrh, pharyngitis, laryngitis and neuralgia (Boericke, 1991). Menthol is a stimulant of Central Nervous System (CNS) but it can be toxic when ingested with serious effects to the CNS (Benezra *et al.*, 1985; Der-Marderosian & Liberti, 1988). Because of its refreshing aroma and cooling effects, menthol is used in tooth pastes, mouthwashes, pan masala, hair oils, chewing gums, candies, pain relieving balms (Singh *et al.*, 1998), after shave lotions and medicinal and cosmetic preparations (Tyagi & Naqvi, 1987). It is also used widely in lotions, antiseptics, dentrifuges, cigarettes and certain foods (Schery, 1972).

In addition, the versatile use of several aromatic plants in food and cosmetic industries demands an extensive screening of essential oils and their components. Individual chemicals isolated from essential oils are more often used than the oils (Brud & Gora, 1989). So identification of trace components is very helpful to reveal the quality of the oil. Analysis of essential oils can be easily done using the technique of Gas Chromatography-Mass Spectrometry (GC-MS). GC is a tool for separating the volatile components while analysis depends upon retention characteristics under standard conditions. The mass spectrometer can be used as a detector for a gas chromatograph in which 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 the

GC-MS technique and the routine nature of much of the data obtained make the computer a very useful accessory to the GC-MS unit. With the help of GC-MS technique, it has now been possible to analyze directly the fragrances of natural or artificial materials without the use of heat or solvents and directly by the use of head space analysis (Thappa *et al.*, 1982). MS differs from other types of spectral analysis in that the sample does not absorb radiation from the electromagnetic spectrum. It is highly sensitive and only a small quantity of the sample is required. When coupled with separation techniques like GC or HPLC (High Performance Liquid Chromatography), it is a highly specific way to identify organic compounds (Smith & Busch, 1999).

With regard to mint oil chemical composition, discovery and selection of new landraces or chemotypes of *Mentha* species bring forth novel and unique flavour profiles. These are unique model systems for the study of essential oil biosynthetic pathway regulation and can be applied commercially, fulfilling some special market requirements. In view of great public concern about the safety of synthetic aroma chemicals and also formation of mixture of isomers by synthetic chemicals, essential oil bearing plants shall remain an ideal commercial source for aroma chemicals (Hussain, 1991). The quality and quantity of the aromatic compounds that can be extracted from a sample is a sensitive aspect that depends upon the species, variety, locality, time and method of collection, method of extraction, storage etc., making quality control a very intricate issue. The present study is an attempt to generate somaclonal variants of *M. rotundifolia* by *in vitro* techniques, that differ from the parent plant in quality and quantity of the essential oil and to reveal the genetic basis of variation in them by using the chromosome image analysis system and RAPD technique.

REVIEW OF LITERATURE

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

REVIEW OF LITERATURE

1. Micropropagation

Genetic variability, which forms the basis of the selection for crop improvement, can be generated through hybridisation, polyploidy and induced mutagenesis. Nowadays attention has been focussed on inducing genetic variability through tissue cultures and the plants regenerated from undifferentiated callus cultures have now become a new and useful source of genetic variation.

Tissue culture of peppermint using White's medium supplemented with 2,4-D and coconut water (CW) was attempted as early as in 1951 and by 1960, slow growing cultures of *M. piperita* had been established and maintained on semisolid agar medium containing 15% coconut water and 1 mg/l 2,4-D (Hove, 1951). Callus cultures of peppermint and spearmint were established on a high salt medium containing an extraordinary amount of myo inositol (5 g/l). In addition to this, a complex mixture of vitamins was also used to establish callus cultures of these mints (Lin & Staba, 1961). Tulecke *et al.* (1961) reported the presence of regeneration inducing components in tender coconut water.

Lamba & Staba (1963) investigated the effects of various growth regulators added in solid media on *M. spicata* cell suspension. George & Sherrington (1984) reported the presence of regeneration inhibitors in mature coconut water. Bayliss (1980) also generated genetic variability through cell cultures. Since then the application of plant tissue culture for the induction of stable and heritable variations had been demonstrated in a range of economic plant species (Evans & Sharp, 1985; Bajaj, 1986). Experimental conditions for rapid multiplication of various *Mentha* species (*M. arvensis, M. spicata, M. piperita, M. viridis* and *M. pulegium*) using single node stem segments had also been described (Rech & Pires, 1986). Axillary bud proliferation was observed on MS media supplemented with BAP and kinetin (KIN). Micropropagation of *M. piperita* was also demonstrated by Repcakova *et al.* (1986).

Codaccioni & Vescovi (1987) obtained *in vitro* cultures of *M. viridis* in presence of mannitol. Rodov & Davidova (1987) tried meristem culture in mint. They found that amending the proliferation medium with BA and rooting the microcuttings could increase

the propagation rates. Mariska *et al.* (1987) obtained bud multiplication through *in vitro* culture in *Mentha* spp. Stable and heritable variation was reported by Mathur *et al.* (1988) in plants derived through tissue cultures. Bhaumic & Datta (1988) standardised a new media composition to obtain menthol by tissue culture of Japanese mint. Axillary buds of *M. piperita* had been used as a test system for micropropagation (Ravisankar & Venkataraman, 1988). The role of hormones in tissue culture and micropropagation of mints was reported by Krikorian *et al.* (1988). The propagated plants were true to parent and produced 0.85% oil on fresh weight basis.

Geslot *et al.* (1989) proposed an *in vitro* multiplication method for mints, combining the advantages of apex culture and microcutting techniques. The study concerned six taxa of *Mentha* and the results were comparable in various taxa except for some quantitative differences. Shoot elongation of mints was found to be greatly affected by temperature and photosynthetic photon flux (Karlsson *et al.*, 1989). It was reported that the carbon source in the culture medium had a regulatory role in the *in vitro* shoot multiplication in many plants (Welander *et al.*, 1989).

Plant regeneration from callus cultures of mint depended on explant source, genotype and culture medium components. An exclusive study was made to determine the influence of explant source and medium components on shoot regeneration. Leaf discs from peppermint, lavender mint, orange mint, spearmint and Scotch spearmint were cultured on various MS based media (Van Eck & Kitto, 1992). BAP and CW supported significantly large number of shoots (Van Eck & Kitto, 1990). Banthorpe & Brown (1990) reported the growth and secondary metabolism in cell cultures of *Mentha* in buffered medium. Malek *et al.* (1992) and Moe *et al.* (1990) reported the effect of day and night temperatures on stem elongation of mints. With this background, efforts were initiated to develop high yielding somaclones possessing desired oil composition and resistance to various diseases (Kukreja *et al.*, 1991a; b; 1992; 1998).

Appelgren (1991) reported the correlation between light quality and stem elongation in micropropagation of mints. The effect of photosynthetic photon flux, photoperiod and photoperiod/dark period temperature on *in vitro* stem elongation and shoot length of mints was reported by Kozai *et al.* (1992; 1995; Tutty *et al.*, 1994).

According to Kozai *et al.* (1993), stem elongation of *in vitro* mints was also greatly affected by humidity in the culture vessel.

Micropropagation protocol of *Mentha* species using terminal and axillary buds with modified MS medium had been reported (Cellarova, 1992; Romallo & Gonzalez, 1994). Van Eck & Kitto (1992) obtained efficient regeneration of peppermint and orange mint from leaf disks. Reed *et al.* (1995) detected the presence of endophytic bacteria in mint plants. They have also reported eradication methods to make cultures bacteria free. Buckley *et al.* (1995) characterised and identified these bacteria using biochemical and morphological tests.

According to another report, size of culture chamber and medium volume had influenced the growth rate of shoot tips of spearmint (Tisserat, 1996). A regeneration protocol allowing 51% shooting frequency from microcuttings of peppermint was proposed by Caissard *et al.* (1996). According to Jeong (1996), photosynthetic photon flux and difference between day and night temperatures had greatly affected the stem elongation and growth of *M. rotundifolia.*

Kukreja (1996) had reported a two step procedure for micropropagation and *in vitro* shoot regeneration from leaf and nodal explants of a commercial cultivar of peppermint. Nodal explants cultured on MS medium containing KIN or BAP and IAA produced multiple axillary and adventitious shoots which easily rooted on IAA fortified MS medium.

Bandziuliene & Indrisiunaite (1996) also reported micropropagation of mint. Regenerated buds from *in vitro* cultured leaves of peppermint had been obtained on a ½ MS medium supplemented with saccharose and various concentrations of BAP. Medou *et al.* (1997) used young leaves from *in vitro* micropropagated plants cultivated in the dark for this purpose. Micropropagation was reported in spearmint and peppermint by Sajina *et al.* 1997. Berry *et al.* (1997) obtained regeneration from leaf disks and petioles of *M. citrata, M. piperita, M. spicata* and *M. gracilis.* Somaclones exceeding the control plants in oil and menthol content was produced by micropropagation (Xue *et al.*, 1998). Li *et al.* (1999) obtained efficient plant regeneration from native spearmint.

Tisserat & Silman (2000a) studied the interactions of culture vessels, media volume, culture density and CO_2 level on spearmint shoot growth *in vitro*. High positive correlation occurred between the culture vessel capacity and spearmint fresh weight, leaf number and root and shoot number. Tisserat & Silman (2000b) also reported the enhancement effect of ultra high CO_2 levels on *in vitro* shoot growth and morphogenesis in Labiatae members. Kukreja & Dhawan (2000) tried somaclonal breeding for improvement of herb and essential oil yield in mints.

Entry and establishment of pathogenic bacteria in plant tissues was reported by Billing (1982). Leifert *et al.* (1989; 1991a) found bacterial contamination in micropropagated plant tissues. Presence of endophytic bacteria in mint species is reported by Reed *et al.* (1995). Increasing attention has been directed to the problem of microbial contamination of plant tissue culture by workers seeking to ascertain sources of contamination and to develop procedures for eliminating them by avoidance, rigorous manipulation of environmental and nutritional factors or treatment with antibiotics (De Fossard & De Fossard, 1988; Cassells, 1991; Debergh & Zimmerman, 1991; Viss *et al.*, 1991; Kneifel & Leonhardt, 1992). The popular solution was to use antibacterial substances like antibiotics. This approach had met with varying degrees of success (Cornu & Mitchel, 1987; Falkiner, 1990; Leifert *et al.*, 1991b). In some cases, antibiotic treatment appeared bacteriostatic, resulting in reduced rather than eliminated contamination (Phillips *et al.*, 1981; Bastiaens *et al.*, 1983; Young *et al.*, 1984). In other cases, phytotoxicity of the antibiotic had precluded their use at a level high enough to destroy all contaminants (Falkiner, 1990; Leifert *et al.*, 1991a; 1992). Falkiner (1988) reviewed the desirable features of antimicrobial substances for use in plant tissue culture and outlined the establishment of antibiotic combinations and effective concentrations for treatment of infected tissues. Combinations of antibiotics to control microbial contamination in micropropagated plants were tried by Leifert *et al.* (1992).

2. Cytological Analysis

Regenerability of a callus was often ascribed to numerical and structural changes in the chromosomes (Muir, 1965; Murashige & Nakano, 1965; Torey, 1967).

Chromosomal instability of callus and cell suspension cultures had often been reported (D'Amato, 1977). Chromosomal instability in cells grown *in vitro* not only included numerical variations but also comprised changes in chromosome structure and basic karyotype (Sunderland, 1977).

Variation in callus culture had been observed to be expressed within the regenerants of several plants (Sacristan, 1971; Orton, 1980). Numerical changes had been examined most often among flowering plants (Evans & Reed, 1981; Mohanty, 1990) in tissue culture. Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and regeneration had been reported by Edallo *et al.* (1981). It had been observed that plant regeneration in callus culture was obtained mainly from cells with diploid chromosome number (Larkin & Scowcroft, 1981; Vasil, 1983). Variation within regenerated plants was often reported (Mc Coy *et al.*, 1982; Scowcroft, 1984). The possibility of minor genetic reshuffles in the form of minor structural changes in chromosomes without affecting their original chromosome number in regenerants could not be ruled out. Structural alterations of chromosomes in cultured cells were reported by Mohanty *et al.* (1991).

A detailed analysis of the mitotic and meiotic behaviour of chromosomes of different species and populations of mints (*M. spicata, M. arvensis* var. *javanica, M. arvensis, M. piperita*) was conducted by Kundu & Sharma (1985). The chromosomes were very small in size (0.89 to 2.5μ) with median to nearly median primary constrictions. Chromosomal interchanges had been described to be the basis for obtaining somaclonal variation (Karp & Bright, 1985; Pijnaker & Ferweda, 1987). No direct relationship with chromosome number variation to yield and quality of essential oil was found in *M. arvensis* (Tyagi & Naqvi, 1987). The chromosome number in an F_1 hybrid progeny between *M. spicata* and *M. piperita* ranged from 36-115 (Tyagi & Ahmad, 1989). The normally expected chromosome number (2n=96) was found to be the most frequent.

3. Random Amplified Polymorphic DNA (RAPD) Analysis

Molecular markers had already been used in 1989 to detect the genetic basis and breeding applications of somaclonal variation (Evans, 1989). Bousquet *et al.* (1990)

obtained DNA amplification from vegetative and sexual tissues of plants by PCR. It was reported that molecular markers could be used for differentiation of nearly identical germplasm accessions (Carlson *et al.,* 1991; Potter & Jones, 1991; Weycott & Fort, 1991). RAPDs were used for identification of germplasm (Fukuorka, 1992; Vidal *et al.*, 1999) and in other gene introgression breeding programmes (Frederic *et al.*, 1992).

Shenoy & Vasil (1992) reported that micropropagation through meristem culture was generally associated with low risk of genetic instability because the organised meristems were generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions. Bouman *et al.* (1992), Bouman & Kuijpers (1994) and Al-Zahim *et al.* (1999) also found intraclonal RAPD polymorphism among micropropagated plants.

Many authors using RAPD or RFLP, have failed to observe intraclonal variations in various plant species (Merkle *et al.*, 1988; Shenoy & Vasil, 1992; Isabel *et al.*, 1993; Valles *et al.*, 1993; Choudhury & Vasil, 1993; Rout *et al.*, 1998). Recently DNA markers were utilised effectively in plant improvement programmes (Paterson *et al.*, 1991; Waugh & Rowell, 1992; Rafalsky *et al.*, 1994).

Molecular analysis of plants regenerated from embryogenic cultures was conducted by Choudhary *et al.* (1994). Virk *et al.* (1995) used RAPD for the study of diversity within plant germplasm. Rani *et al.* (1995) found RAPD variations among 23 micropropagated plants originating from the same clone which were morphologically similar. The variation of monomorphic bands in micropropagated plants by using different primers had been reported earlier (Potter & Jones, 1991; Angel *et al.*, 1996).

Sato *et al.* (1996) carried out somatic hybridisation between peppermint and gingermint by protoplast fusion. Chromosome counts and RAPD analysis indicated that it was an interspecific somatic hybrid. Among the reports on the use of RAPD analysis for the molecular analysis in the micropropagated clones, emphasis has been laid on the confirmation of genetic homogeneity of the raised plantlets. Wallner *et al.* (1996) had applied this technique to compare the field grown and *in vitro* micropropagated plants.

RAPD markers were generated and used in plant improvement programmes (Rafalsky *et al.*, 1996) and in assessment of botanical diversity (Karp *et al.*, 1996). Identification of plants using sequence characterised amplified region DNA markers were also reported (Xong & Bakalinsky 1996; Zheu *et al.* 1996; Shoyama *et al.* 1997). Similarly from the conservation point of view, Parani *et al.* (1997) used RAPD approach to compare the mother plant and micropropagated progenies to maintain the fidelity of elite genotypes that were to be conserved.

Kumar *et al.* (1997) developed a high menthol yielding hybrid clone of *M. arvensis*. The uniqueness of its genotype was evident at molecular level from its RAPD profile generated by a set of 12 primers. Recently a procedure that permits rapid isolation of somaclones of cultured genotypes in *M. arvensis* had been developed (Khanuja *et al.*, 1998). RAPD analysis of the phenotypically differing somaclones confirmed alteration at genetic levels also.

PCR amplification technique was used to detect the transgenic peppermint plants regenerated after cocultivation with *Agrobacterium* (Lin *et al.*, 1998). Shasany *et al.* (1998) have defined media and incubation conditions for highly efficient regeneration of shoots from internodal explants of slow and fast growing cultivars of *M. arvensis*. The genetic homogeneity of regenerated plants was ascertained with the use of RAPD technique.

Krasnyanski *et al.* (1998) used RAPD technique to identify the somatic hybrids between *M. piperita* and *M. spicata* produced by protoplast fusion. Diemer *et al.* (1999) produced *Agrobacterium tumefaciens* mediated transgenic *M. spicata* and *M. arvensis*. The presence and structure of transgenes were studied by the use of PCR analysis and Southern blot hybridisation. Direct sequencing of RAPD products was used as a practical tool for marker assisted selection of wheat (Hernandez *et al.*, 1999).

Krasnyanski *et al.* (1999) tried *Agrobacterium tumefaciens* mediated and direct gene transfer into protoplasts to produce transformed peppermint plants with limonene synthase gene. Both type of transformation resulted in transgenic plants, which were detected using PCR, and confirmed by Southern blot hybridisation. RAPD profiles were

unambiguously used to establish the distinct identity as different from the parent plant in many new varieties of *M. arvensis* (Khanuja *et al.*, 2001a; Khanuja *et al.*, 2001b), *M. spicata* var. *viridis* (Khanuja *et al.*, 2001c), *M. piperita* (Dwivedi *et al.*, 2001a) and *M. gracilis* var. *cardiaca* (Dwivedi *et al.*, 2001b). RAPD analysis had also been used for the confirmation of hybridity (Patra *et al.*, 2001c). Molecular characterization of several other plants have been done successfully by RAPD technique (Ayana *et al.*, 2000; Pradeepkumar *et al.*, 2001; Dwivedi *et al.*, 2001c; Patra *et al.*, 2001b).

4. Essential Oil Analysis

Accumulation of monoterpenes has been found to vary in *Mentha* cultures both between the taxa and between the different cell lines from the same species. A number of reports described that *in vitro* cultures of *M. spicata, M. piperita,* and *M. pulegium* failed to produce volatiles (Lin & Staba, 1961; Wang & Staba, 1963; Staba *et al.*, 1965; Becker, 1970) Monoterpene interconversions in a cell free system from *M. piperita* was reported by Bhattaile *et al.* (1968).

Bricout & Poupardin (1975) and Bricout *et al.* (1978) studied the biosynthesis of monoterpenes in six *Mentha* species. Monoterpene formation was lower in plant cultured *in vitro* than in control plants. In *M. rotundifolia* and *M. piperita* the products synthesized *in vitro* were more oxidised than the natural forms. *M. rotundifolia* produced epoxy piperitone in place of menthone and *M. piperita* elaborated menthofuran and pulegone in cultures instead of menthol and menthone. Terpenes did not accumulate in suspension cultures of six *Mentha* chemotypes (Aviv & Galun, 1978). None of the monoterpenes characteristic of essential oil of intact plant of *M. piperita* was found in the callus culture derived from this species.

Callus cultures established from the leaves of the allopolyploid form of *M. piperita* had the capacity to synthesize oil of intact plants (Kireeva *et al.*, 1978). The callus released the oil into the culture medium. They observed that under the normal conditions of cultivation, biosynthesis of terminal products (menthone and menthol) was inhibited and their precursors (piperitone and pulegone) were accumulated. Malnikov *et al.* (1979) also investigated formation of terpenes in callus cultures of genetically different lines of *M.*

spicata. Karasawa & Shimizu (1980) described the effect of KIN and NAA in the media on the composition of triterpenes between the callus tissue and intact plants of *M. rotundifolia, M. arvensis* and. *M. spicata*. There were some other cases where no terpenoid accumulation was reported in cultured tissue (Suga *et al.*, 1980).

Charlwood & Charlwood (1983) described the results of an investigation concerning the monoterpene production by tissue cultures of several *Mentha* species. They observed that many callus cultures accumulated only the early precursors of these compounds such as geraniol, linalool etc. But some other cultures synthesized monoterpenes characteristic of intact plants. Rodov & Reznikova (1982) used peppermint cell suspension cultures as model systems for studying the pathways of essential oil synthesis.

Essential oil composition of *in vitro* propagated *M. piperita* changed markedly during the culture period leading to the recognition of 3 phenotypes (Mucciarelli *et al.*, 1995). Chaput *et al.* (1996) developed a procedure to regenerate plants from leaf protoplasts of two micropropagated hybrid species of mint, *M. piperita* and *M. citrata* in order to determine whether the *in vitro* treatment could influence the monoterpene composition. In the field trial, a decrease in the amount of menthone and menthol and an increase of carvone levels were noticed in all protoplast-derived plants.

Jullien *et al.* (1997) applied biotechnological tools for the improvement of mints and essential oils. Karousou *et al.* (1998) tried *in vitro* propagation in two chemotypes of *M. spicata*. Abou-Mandour & Binder (1998) studied the effect of exogenous growth regulators on plant regeneration from tissue cultures of *M. spicata* and production of volatiles.

Essential oil obtained from shoot tip cultures of *M. spicata* by Hirata *et al.* (1990) showed that carvone and limonene were higher in the cultured plant than the parent plants. The production of these monoterpenoids was influenced by NAA and thiamine hydrochloride. New approaches directed to unravelling monoterpene metabolism and secretion and recent progress in transformation protocols had set the stages for the

systematic genetic engineering of essential oil production in mint (Lange & Croteau, 1999).

Volatile oil yield and composition of *M. spicata* obtained by solvent extraction and supercritical CO_2 extraction were compared by Pino *et al.* (1999a). Sensory profiles and GC-MS analysis of both extracts were performed and their results were compared with those obtained by steam distillation. The difference in aroma of the extracts was quite noticeable and was attributed to qualitative and quantitative differences in components.

The relation between oil yield and free menthol content (Ellis & Gaylard, 1944) and time of culturing and free menthol content (Ellis, 1945) were reported very earlier. Murray & Reitsema (1954) revealed genetic basis of the biosynthesis of ketones, carvone and menthone in *M. crispa*. Murray (1960a) reported genetic basis for conversion of menthone to menthol in Japanese mint.

Concerning the biosynthetic pathway leading to the formation of isomenthone, two possible precursors- piperitone (Burbott & Loomis, 1967) and pulegone (Katsuhara, 1966; Lawrence, 1978) were reported. Genetic basis for the reduction of piperitenone to the ketone pulegone, pulegone to menthone and menthone to menthol was revealed by Hendriks *et al.* (1976). Monogenic basis for the reduction of (+)-pulegone to (-)-menthone in *Mentha* oils was revealed by Lincon & Murray (1978).

According to Aviv & Galun (1978), biotransformation of pulegone to isomenthone occurred in *Mentha* cell lines. The relation between mint physiology and essential oil production was reported by Loomis & Croteau in 1979. According to Croteau & Martinkus (1979), (+)-neomenthyl β-D-glucoside was the major metabolite of (-)-menthone in peppermint. Multiple allele control of the biosynthesis of (-)-menthone and (+)-isomenthone stereoisomers in *Mentha* species was reported by Murray *et al.* (1980).

Limonene was the first cyclic intermediate in the biosynthesis of oxygenated p-menthane monoterpenes in *M. piperita* and other *Mentha* species (Kjonaas & Croteau, 1983). Kokkini (1983) reported a carvone chemotype of the diploid hybrid, *M. rotundifolia* found in a mixed population with its parental species *M. longifolia* and *M. suaveolens* growing in Greece.

Research published to date strongly suggested that the qualitative production of mint essential oils was clearly controlled by simple genetic systems (Lincon *et al.*, 1986).

Dmitrev *et al.* (1988) used GC-MS technique to reveal the chemical components of volatile substances released by plants. Bharadwaj (1989) made correlation studies on yield and quality characters of essential oil in peppermint.

Gershenzon *et al.* (1989) could localize the monoterpene biosynthesis in glandular trichomes of spearmint using biochemical and histochemical methods. Variation between the essential oil components in commercially exploited and wild populations of *M. spicata* was further reported by Kokkini & Vokou (1989). They characterised four different chemotypes in this species.

Isopentenyl diphosphate (IPP) was the intermediate essential oil precursor leading to the formation of over 22000 known isoprenoids (Connolly & Hill, 1992). Maat *et al.* (1992) analysed the essential oil composition of *M. mirennae* by GC-MS technique. The basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes were revealed by Gershenzon & Croteau (1993). Genetic control of monoterpene biosynthesis in mints was reported by Croteau & Gershenzon (1994).

Biochemistry and molecular biology of isoprenoid biosynthetic pathway was revealed by Chappel (1995). Biosynthesis of monoterpenes and sesquiterpenes from plastid derived isopentenyl diphosphate was described by Mc Caskill & Croteau (1995). The identification of monoterpenes and sesquiterpene hydrocarbons in *M. piperita* using GC and GC-MS techniques was done by Zenkevich (1996).

Bohlman *et al.* (1998) revealed the molecular biology and phylogenetic analysis of plant terpenoid synthesis. Schulz *et al.* (1999) studied the chemical composition of secondary metabolites in the leaves of different *Mentha* species using Near Infra Red spectroscopy (NIR). Biosynthesis of monoterpenes in mint from Geranyl pyrophosphate (GPP) (Wise & Croteau, 1999), sesquiterpenes from Farnesyl pyrophosphate (FPP) (Cane, 1999) and diterpenes from Geranyl Geranyl pyrophosphate (GGPP) (MacMillan, 1999) were reported recently.

Table: 1

Previous reports on chromosome counts of *M. rotundifolia*

Name of taxa	Chromosome No.	Authority	Year
M. rotundifolia (L.) Huds.	2n=54	Schurhoff	1929
,,	2n=24	Ruttle	1931
,,	,,	Junell	1937
,,	2n=18	Heimans	1938
,,	2n=24	Nagao	1941
,,	,,	Junell	1942
,,	2n=54	Delay	1947
,,	2n=24	Suzuka & Koriba	1949
,,	,,	Morton	1956
,,	,,	Murray	1958
,,	2n=24, 36	Arora	1960
,,	,,	Murray	1960b
,,	2n=24	Love & Love	1961
,,	,,	Gadella & Kliphuis	1963
,,	,,	Sobti	1965
M. rotundifolia (L.) Huds. var. *variegata* Sole.	,,	,,	,,
M. rotundifolia (L.) Huds.	2n=24, 36	Ouweneel	1968
M. rotundifolia (L.) Huds. var. *nullata* Briq.	2n=36, 48	Sacco & Scannerini	,,
M. rotundifolia (L.) Huds.	2n=24, 36	Dahlgren *et al.*	1971
,,	2n=24	Sobti	1971

,,	,,	Harley & Brighton	1977
M. rotundifolia (L.) Huds.	,,	Gill	1981
	,,	Roy *et al.*	1983
	,,	Queiros	1985
	,,	Chambers & Hummer	1994
	,,	Thoppil	1993

Table: 2

Previous reports on chemical components of *M. rotundifolia* essential oil

Name of taxa	Part used	Chemical component	Authority	Year
M. rotundifolia (L.) Huds.	Whole plant	piperitenone oxide pinene limonene	CSIR	1962
M. rotundifolia var. *bullata*	Whole plant	piperitenone oxide	Sacco & Scannerini	1968
M. rotundifolia (L.) Huds.	Whole plant	rotundifolone neoisopulegol	Chopra *et al.*	1969
M. rotundifolia (L.) Huds.	Whole plant	piperitone piperitenone oxide	Hendriks	1971
M. rotundifolia (L.) Huds. Chemotype 1	Aerial parts	α-pinene camphene β-pinene sabinene myrcene limonene 1,8-cineole β-terpinene p-cymene terpinolene 3-octyl acetate 3-octanol nonanal hexylisovalerate menthone octyl acetate isomenthone β-bourbonene 1-terpinene-4-ol caryophyllene menthol germacrene D piperitone oxide δ-cadinene piperitenone piperitenone oxide α-pinene	Kokkini & Papageorgiou	1988
Chemotype 2	,,	camphene	,,	,,

M. rotundifolia (L.) Huds. Chemotype 2		β-pinene sabinene myrcene limonene 1,8-cineole cis-ocimene trans-ocimene γ-terpinene p-cymene terpinolene 3-octyl acetate 3-octanol nonanal hexylisovalerate menthone isomenthone linalool menthyl acetate neoiso menthyl acetate caryophyllene menthol pulegone α-terpineol germacrene D piperitone piperitenone	Kokkini & Papageorgiou	1988
M. rotundifolia (L.) Huds.	Whole plant	isopiperitenone carvone	Rastogi & Mehrotra	1990
,,	,,	piperitone oxide 1,2-epoxy menthyl acetate piperitenone piperitenone oxide limonene carvone pulegone menthone isomenthone cineole limonene o-cresol p-cresol	,,	1991
,,	,,	piperitenone oxide 1,2-epoxy neomenthol 1,2-epoxy neomenthol acetate 4-hydroxypiperitone		

M. rotundifolia (L.) Huds.		luteolin luteolinidin pelargonidin cyanidin delphinidin petunidin	Rastogi & Mehrotra "	1993 "
M. rotundifolia (L.) Huds.	Aerial parts	menthone isomenthone	Thoppil	1993
M. suaveolens Ehrh.	Fresh leaves	α-thujene α-pinene camphene sabinene β–pinene myrcene 3-octanol α-phellandrene α-terpinene p-cymene limonene 1,8-cineole γ-terpinene cis-linalool oxide trans-linalool oxide linalool camphor menthone isomenthone isopulegone menthofuran neomenthol menthol terpinen-4-ol isomenthol α-terpineole pulegone piperitone isopulegyl acetate neomenthyl acetate menthyl acetate piperitenone δ-elemene α-cububene α-copaene β-cububene β-bourbonene β-elemene β-caryophyllene	Velasco-Negueruela & Perez-Alonso	1996

M. suaveolens Ehrh.		aromadendrene β-humulene allo aromadendrene germacrene D viridiflorol α-cadineol	Velasco-Negueruela & Perez-Alonso	1996
M. rotundifolia (L.) Huds.	Aerial parts	piperitenone oxide pulegone carvone limonene 1,2-epoxy menthol acetate 1,2-epoxy neomenthol acetate allo-cymene α-phellandrene α-pinene apigenin β –pinene borneol acetate caffeic acid camphene cineole citral cyanidin delphinidinin dihydrocarvone diosphenol diosphenolene isoneopulegol isoneopulegol acetate isopiperitenone menthofuran menthol menthone neoisopulegol octan-3-ol octan-3-ol acetate p-cymene pelargonidin petunidin piperitenone piperitenone oxide piperitone piperitone oxide pulegone rotundifolone thujone	Beckstrom-Sternberg & Duke	1996

M. rotundifolia (L.) Huds	Air dried leaves	1-octen-3-ol myrcene p-cymene limonene (Z)-β-ocimene cis-sabinene hydrate trans-sabinene hydrate linalool isoamyl isovalerate 1-octen-3-yl-acetate veratrol menthone isomenthone borneol neomenthol menthol terpinen-4-ol α-terpineol 1-menthoxy-4-propyl benzene methyl chavicol carvone piperitone piperitine oxide bornyl acetate thymol piperitone oxide α-copaene 2,4(8),6-p-menthatrien-2,3-diol β–bourbonene cyperene β–caryophyllene β–gurgunene geranyl acetone α-humulene γ-muurolene germacrene D cis-β-gualene bicyclogermacrene α-muurolene γ-cadinene δ-cadinene germacren D-4-ol viridiflorol cubenol α-cadinol	Beckstrom-Sternberg & Duke	1996

M. rotundifolia (L.) Huds.		1,4-hydroxy-α-muurolene khusinol acetate hexadecanol hexadecanoic acid phytol	Pino *et al.*	1999b
M. rotundifolia (L.) Huds.	Fresh herb	pinene limonene	Bhattacharjee	2000

Table: 3

Previous reports on the biological activities of phytochemicals of *M. rotundifolia* essential oil detected in the present study.

Component	Biological activity	Authority	Year
α-pinene	allelochemic allergenic	Mitchell & Rook	1923
	irritant	Harbone & Baxter	1983
	cancer preventive	Stitt	1990
	insectifuge	Jacobson	1990
	herbicide	Keeler & Tu	1991
	coleoptiphile expectorant antiflu	Castleman	1991
	anti-inflammatory antiviral bactericide flavour insectiphile perfumery sedative tranquillizer	Beckstrom-Sternberg & Duke	1996
	bactericide	Srivastava *et al.*	2000
	insecticidal fumigant ovicidal	Singh *et al.*	2000
β-bisabolene	abortifacient	Pei-Gen & Nai-Gong	1991
	antiulcer	Yamahara *et al.*	1992
	antirhinoviral antiviral	Denyer *et al.*	1994
	perfumery	Beckstrom-Sternberg & Duke	1996

β-elemene	anticancer (cervix)	Leeuwenberg	1987
β-terpineol	insectifuge	Jacobson	1990
	perfumery	Beckstrom-Sternberg & Duke	1996
carvone	allergenic	Mitchell & Rook	1923
	antiseptic	Wagner & Wolf	1977
	perfumery	Harbone & Baxter	1983
	insectifuge insecticide	Jacobson	1990
	CNS stimulant cancer preventive	Stitt	1990
	carminative	Yamamoto *et al.*	1993
	flavour motor depressant nematicide sedative vermicide	Beckstrom-Sternberg & Duke	1996
	antibacterial antifungal	Hassanein & Eldokseh Oka *et al.* Aggarwal *et al.*	1997 2000 2002
limonene	nematicide	Beckstrom-Sternberg & Duke	1996
	larvicidal insecticidal	Singh *et al.*	2000
	allergenic antialzheimeran	Mitchell & Rook	1923
	sedative	Wagner & Wolf	1977
	expectorant	Harbone & Baxter	1983
	ACE inhibitor Insecticide	Grundy & Still	1985

	cancer preventive	Stitt	1990
	insectifuge	Jacobson	1990
	herbicide	Keeler & Tu	1991
	candidistat fungistat	Kang *et al.*	1992
	nematicide	Nigg & Seigler	1992
	antitumour	Yu *et al.*	1995
	viricide spasmolytic irritant fungiphilic enterocontractant bactericide antiviral anticancer antiflu antilithic antimutagenic	Beckstrom-Sternberg & Duke	1996
	ovicidal insecticidal	Singh *et al.*	2000
	antibacterial antifungal	Aggarwal *et al.*	2002
methyl chavicol	hepato carcinogenic	Bisset	1994
	insecticide	Beckstrom-Sternberg & Duke Singh *et al.*	1996 2000
myrcene	allergenic	Mitchell & Rook	1923
	insectifuge perfumery	Harbone & Baxter	1983
	antinociceptive	Rao *et al.*	1990
	fungicide	Keeler & Tu	1991
	analgesic antimutagenic	Kauderer *et al.*	1991

citronellol	antioxidant bactericide spasmolytic insecticide repellant	Beckstrom-Sternberg & Duke Singh *et al.*	1996 2000

MATERIALS AND METHODS

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

MATERIALS AND METHODS

1. Micropropagation

M. rotundifolia plants cultivated in the Nilgiris were collected and grown in the net house of Botany Department, University of Calicut. The plant was authenticated at the Herbarium of Botany Department, University of Calicut where a voucher specimen was deposited (CALI 86003). Nodal cuttings (1-1.5 cm) from three months old potted plants were used as explant to initiate the cultures. Nodal cuttings were collected in water. Expanded leaves were removed and the stem cuttings were washed with labolene detergent for 15 min. The tissue was again thoroughly washed in running water followed by a quick rinse (30 seconds) in 70% alcohol. This material was again washed in double distilled water. Surface sterilization was done using 0.1% mercuric chloride for 6 min. The stem cuttings were then washed 5-8 times in double distilled water to remove the traces of $HgCl_2$ The tissue was trimmed again and dipped in 15% streptomycin for 5 min. to eliminate the endophytic bacteria found commonly in *Mentha* species (Reed *et al.*, 1995) and implanted on to the culture medium. 1-2 week old leaves collected from the potted plants were used as explants for callus induction. The leaves were surface sterilized in 0.1% mercuric chloride for 4 min. 1 cm^2 pieces cut from the basal portion of the leaves were dipped in 15% streptomycin for 5 min. and inoculated on to the surface of the medium.

Murashige & Skoog (1962) basal medium (Table 4) with 3% sucrose, 100mg/l myoinositol and 0.8% agar was used. MS basal medium was supplemented with different concentrations of auxins, cytokinins, different combinations of both these and also 15%-50% coconut water alone or in combination with BAP. The pH of the medium was adjusted to 5.8. The media were sterilised at 120^0C for 20 min. 10-15 replicates of each hormonal combination were tried. The cultures were grown at 25 ± 3^0C with 55%-60% humidity under fluorescent day light tubes emitting 2000 lux for 16/8 h light/dark period and were subcultured every 5-7 weeks.

Table: 4

Murashige & Skoog Basal Medium

Stock Chemical	mg/l	Stock Concentration	Stock g/l
I NH_4NO_3	1650.00	50 X	82.50
KNO_3	1900.00		95.00
KH_2PO_4	170.00		8.50
$MgSO_4.7H_2O$	370.00		18.50
II $CaCl_2.2H_2O$	440.00	50 X	22.00
III Na_2EDTA	37.30	100 X	3.70
$FeSO_4.7H_2O$	27.80		2.80
IV $MnSO4.4H_2O$	22.30	100 X	2.23
$ZnSO4.7H_2O$	8.60		0.860
H_3BO_3	6.20		0.620
KI	0.83		0.083
$Na_2MoO_4.2H_2O$	0.25		0.025
$CoCl_2.6H_2O$	0.025		0.0025
$CuSO_4.5H_2O$	0.025		0.0025
V VITAMINS		100 X	
Glycine	2.00		0.200
Nicotinic acid	0.50		0.050
Pyridoxine-HCl	0.50		0.050
Thiamine-HCl	0.10		0.010

Establishment of Plants in Soil

Four to six weeks old micropropagated plants were subcultured in ½ MS medium for rooting. Root system with 5-10 roots (2-6 cm long) was developed after 3 week's growth in ½ MS medium for 7-8 days. The rooted plants were taken out from the culture tubes and planted in a sterilised mixture of sand and soil (1:1) in plastic cups and initially covered with polythene bag to control humidity (Das, 1993). These plants were initially irrigated with ½ strength liquid MS medium for 7-8 days. Well-established plants

were transplanted to earthen pots (Figs. 1.26-1.29) kept in the net house and watered regularly (Figs. 1.26-1.29).

2. Cytological Analysis

(i) Squash Preparation

Cytological preparations were made using improved techniques (Sharma & Sharma, 1990). Young healthy root tips were collected at the time of peak mitotic activity (10-11 AM) from both the parent plant and the field transplanted micropropagated plant. Two weeks old white friable callus was also used for squash preparation. The root tips and callus were washed thoroughly with distilled water and pre treated in cytostatic chemicals. Chilled saturated solution of para dichlorobenzene with a trace of aesculin was used as pre-treatment chemical. Small quantity of saponin was added to remove the oil content from the cells. The pre-treatment solution was initially chilled at 0-5^{0}C for 5 min. and root tips and callus were dipped in it. This was kept at 12-15^{0}C for 3 h. After this, the root tips and callus were washed thoroughly with distilled water and fixed in Carnoy's modified fluid (1 acetic acid : 2 ethanol) for 2 h.

The fixed root tips and callus were washed in distilled water and hydrolysed with 1N HCl for 20 min. and 30 min. respectively at room temperature. Traces of acid were removed by thorough washing in distilled water. The hydrolysed root tips were stained in 2% aceto orcein (Sharma & Sharma, 1990) for 3-4 h. The stained root tips and callus were washed in 45% acetic acid to remove excess stain and squashed in 45% acetic acid. Slides were scanned under LEICA GALLEN III microscope and photographs were taken with a Pentax camera system attached to it.

(ii) Karyomorphological Analysis

Karyograms were prepared from the microphotographs using computer based programs such as Adobe Photoshop, Auto CAD and data based analysing system (Microsoft Excel). Photographs were scanned and stored as digital images. These digital images were converted to grayscale images using Photoshop program. Identification

numbers were allotted to each chromosome and then loaded to Auto CAD for karyomorphometrical analyses. After determining the centromeric position, arm lengths of each chromosome were measured and centromeric indices were calculated. On the basis of arm ratio and centromeric indices, homologous chromosomes were identified and were classified (Table 5) according to Abraham & Prasad (1983). The images were loaded to Photoshop and karyograms were generated.

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

Disparity Index (DI) of the chromosomes were calculated with the method of Mohanty *et al.* (1991) by using the formula

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

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

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

Table: 5

Details of 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 to 0.61	1.01 to 1.63	49.99 to 38.01	50.01 to 61. 99
Nearly Submedian	Nsm(-)	0.60 to 0.34	1.64 to 2.99	38.00 to 25.01	62.00 to 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly Submedian	Nsm(+)	0.32 to 0. 23	3.01 to 4.26	24.99 to 18.20	75.01 to 81.80
Nearly Subterminal	Nst(-)	0.22 to 0.15	4.27 to 6.99	18.19 to 12.51	81.81 to 87.49
Sub Terminal	ST	0.14	7.00	12.50	87.50
Nearly Subterminal	Nst(+)	0.13 to 0.01	7.01 to 14.38	12.49 to 5.01	87.51 to 94.99
Nearly Terminal	Nt	0.06 to 0.01	14.39 to 19.99	5.00 to 0.01	95.00 to 99.99
Terminal	T	0	α	0	100.00

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

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

3. Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD is an improved technique used to reveal sequence polymorphism between template DNAs based on selective amplification of DNA sequences. The template DNA (genomic DNA) can be prepared with any purification protocol appropriate for the biological sample under study.

(i) DNA Extraction

Total DNA was extracted from the parent plant and the cultured plants using CTAB method of Doyle & Doyle (1987) with minor modifications (Ausubel *et al.*, 1995). The steps involved are described below.

- Fresh young leaves of the parent and cultured plants of *M. rotundifolia* were collected in ice boxes and ground immediately in liquid nitrogen with ice cold mortar and pestle. The powder obtained was mixed with 16 ml of extraction buffer (extraction buffer was prepared by mixing 120 ml of 2% CTAB buffer [100 mM Tris HCl pH 8, 1.4 M NaCl, 20 mM EDTA pH 8, 2% CTAB] with 240 µl of 0.2% β mercapto ethanol) taken in oakridge tubes.

- The mixture was incubated at 65^{0}C for 1 h. in a water bath. After this an equal volume (16 ml) of chloroform: isoamyl alcohol (24:1) was added to it and mixed thoroughly. This mixture was centrifuged at 1000 rpm for 10 min. at 4^{0}C in a refrigerated centrifuge (Hitachi, Himac CR 21, Japan). The two phases seemed to separate.

- The clear upper aqueous phase was transferred into a fresh oakridge tube. 2/3rd volume of ice cold isopropanol was added into it and mixed gently. The DNA threads were visible.

- This mixture was kept at –20^{0}C for 15 min. for better precipitation and centrifuged at 1000 rpm for 10 min. at 4^{0}C.

- The supernatant was discarded and the tubes were inverted on paper towels for 5 min. Then the pellet was washed with 70% alcohol twice by centrifugation.

- The pellet was kept overnight for air-drying. The dried pellet was rehydrated again in TE buffer (10 mM Tris HCl pH 8, 0.1 mM EDTA pH 8).

(ii) Purification of Isolated DNA (Removal of RNA & Protein)

- RNase (0.8 µl) was added to 200 µl of dissolved unpurified DNA and the mixture was incubated overnight (~ 15 h) at 37^0C.

- An equal volume (200 µl) of a mixture of phenol, $CHCl_3$ & IAA (25:24:1) was added to the DNA & RNase mixture and centrifuged at 1000 rpm for 10 min. at 4^0C.

- The supernatant was transferred to a fresh tube and added equal volume of $CHCl_3$: IAA (24:1) mixture and centrifuged at the same conditions above.

- The upper aqueous phase was taken and $1/10^{th}$ volume (20 µl) of 3M CHCOONa (pH 5.2) was added into it. The DNA was precipitated with $2/3^{rd}$ volume of cold isopropanol. All the tubes showed transparent gel like precipitate. This DNA was pelleted by centrifugation under the same conditions above.

- The pellets were washed twice with 70% ethanol and kept for air-drying.

- The dried pure DNA was dissolved in 250-µl TE buffer at room temperature. This pure DNA was quantified by running in agarose gel. The quantity was assumed by comparing bandwidth of the sample with that of the weight marker (Fig. 3.1).

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

Arbitrarily Primed Polymerase Chain Reaction (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. Six different decamer oligonucleotide primers (OPA 02, OPA 08, OPB 07, OPD 19, OPE 14 & OPF 05) were used for the present investigation. The sequences of these primers are given in Table: 13.

PCR was performed using 25 µl reaction mixture. The reaction mixture contained 2.5 µl assay buffer (10 nM 3-Tris (hydroxymethyl) methyl aminopropane

sulphonic acid pH 8, 50 mM KCl), 0.3 µl dNTPs, 0.8 µl Taq polymerase, 1.25 µl $MgCl_2$, 2 µl primer and 1 µl genomic DNA. This mixture was made upto 25 µl using 17.15 µl autoclaved double distilled water. Amplification was carried out in a BIO RAD Gene Cycler (Japan) programmed for 35 cycles as follows: I^{st} cycle of 2 min. at 94°C, 1 min. at 40°C, 2 min. at 72°C; followed by 34 cycles each of 1 min. at 94°C, 1 min. at 40°C, 1 min. at 72°C and one final extension cycle of 15 min. at 72°C. The amplification products were size separated by electrophoresis (75 V for 4 h) in 2% agarose gels with 1 X TAE (Tris Acetate EDTA) buffer stained with 3 µl of bromophenol blue. The gels were scanned under UV light in an UV transilluminator. In all cases λ phage DNA digested with EcoRI & Hind III was used as size marker.

(iv) Amplified DNA Marker Scoring

Amplified DNA markers were scored as present or absent in each micropropagated plant. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

4. Essential Oil Analysis

(i) Essential Oil Extraction

Shade dried aerial plant parts of both the parent and the somaclonal variant were hydrodistilled separately in a Clevanger (Clevanger, 1928) apparatus at 100°C for 4 h as prolonged extraction normally increases the yield (Gildemeister & Hoffman, 1961). 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°C. The percentage of essential oil was calculated on a dry weight basis to avoid faulty estimation that may arise due to different water content of the tissues analysed each time (Von Rudloff, 1972).

(ii) Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS was carried out on a Shimadzu QP-2000 instrument at 70 eV and 250^{0}C. GC Column: ULBON HR-1 equivalent to OV-1, fused silica capillary – 0.25 mm x 50 m with film thickness 0.25 μ. The other conditions were: carrier gas – Helium, flow rate 2 ml/min., temperature programme: initial temperature – 100^{0}C for 6 min. and then heated at the rate of 10^{0}C per min. to 250^{0}C. Mass spectral identification was based on published spectra (de Brauw *et al.*, 1979-1988).

(iii) 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

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RESULTS

1. Micropropagation

Murashige & Skoog (MS) basal medium (Table: 4) with different hormonal combinations was used for micropropagation. The two types of explants – leaves and nodal cuttings – responded positively, but the response was extremely different in both cases. For callus induction and multiple shoot regeneration, medium with auxins (2,4-D, IAA and NAA) alone, cytokinins (BAP and KIN) alone and different combinations of both these were used. Besides these, MS medium with 15-50% coconut water alone or in combination with BAP was also tried (Tables: 6-9).

Of the two types of explants, the leaf explants produced white friable callus in medium supplemented with 2,4-D 1mg/l + KIN 0.2mg/l (Fig. 1.6)and greenish hard callus (Fig. 1.4) with 2,4-D 1.5mg/l + KIN 0.2 mg/l after a period of 4-5 weeks in culture. Both these leaf-derived calli did not respond to the shoot regeneration trials with different hormonal combinations. Leaf explants inoculated on media with 2,4-D 0.5mg/l produced only swelling and crumbling of the tissue and with 1.5 mg/ml produced a little white callus at periphery. Medium with BAP 0.2 mg/l + NAA 1 mg/l could help only in the rooting of the explant and no further development occurred in this leaf tissue. BAP 0.2 mg/l + IAA 0.5 mg/l combination resulted in swelling and crumbling of leaf tissue. A combination of BAP 1 mg/l + NAA 1.5 mg/l also gave the same result. Medium with BAP 1 mg/l + 2,4-D 1.5 mg/l produced very little callus at the cut ends of the leaf tissue. Combinations like BAP 1 mg/l + IAA 1.5 mg/l; BAP 2 mg/l + IAA 2 mg/l; KIN 0.5 mg/l + IAA 1 mg/l and KIN 1 mg/l + NAA 1.5 mg/l gave only swelling and crumbling of the leaf tissue (Fig. 1.8). 30% of the explants produced single shoots when a medium with BAP 2 mg/l + IAA 2.5 mg/l was used (Table: 6).

Small whole leaves collected from the *in vitro* elongated axillary buds were also used as explants. They induced direct multiple shoots in a medium with 25% coconut water, but the frequency of shoot induction was very low (3-5 shoots/leaf)(Fig 1.9). Combinations like CW 15% + BAP 0.5 mg/l; CW 35% + BAP 1 mg/l; CW 40% + BAP 1.5 mg/l; CW 45% + BAP 1.5 mg/l and CW 30% alone resulted only in swelling of the explant. Medium with CW 50% + BAP 1.5 mg/l produced callusing at periphery of the leaf tissue.

CW 20% + BAP 1 mg/l combination could produce single shoot from 40% of the leaf explants (Fig. 1.10; Table: 7).

The nodal cuttings also produced white friable callus in a medium with 2,4-D 1mg/l + KIN 0.2 mg/l (Fig. 1.1). The stem-derived callus showed a low frequency of shoot regeneration when subcultured into medium with BAP 0.5 mg/l (Fig. 1.2). The regeneration frequency in this case was very low (2-3 shoots). Nodal explants taken from this callus regenerated plants produced a large number of multiple shoots when inoculated in a medium with BAP 2mg/l + IAA 2.5mg/l (Fig. 1.24-1.25). A few calli produced large number of small hairy roots but no shoots in medium with NAA 0.5mg/l (Fig. 1.3). Direct multiple shoot induction was obtained from the nodal cuttings on medium having a hormonal combination of BAP 2mg/l + IAA 2.5mg/l after 3-4 weeks culture period (Fig. 1.11-1.15). Frequency of shoot induction was very high. About 20-40 shoots arose from each node. 95% of the explants responded positively in this medium. Medium having a hormonal combination of BAP 0.2 mg/l + NAA 1 mg/l produced greenish swelling of the explant. A combination of BAP 0.2 mg/l + IAA 0.5 mg/l produced yellow hard callus (Fig. 1.15). Media with BAP 0.5 mg/l + IAA 1 mg/l and BAP 1 mg/l + IAA 1.5 mg/l produced single shoots from the explants. A combination of BAP 2 mg/l + 2,4-D 2 mg/l resulted in rooting of 80% of the explants. White hard callus was produced on combinations like KIN 0.2 mg/l + 2,4-D 1.5 mg/l and KIN 0.2 mg/l + 2,4-D 2 mg/l (Fig. 1.7). The *in vitro* developed white friable calli produced small roots when subcultured on to a medium with KIN 0.5 mg/l + 2,4-D 2.5 mg/l. A combination of KIN 0.5 mg/l + NAA 1 mg/l resulted in bulging of the explant. Combinations like KIN 1 mg/l + NAA 1.5 mg/l and KIN 1 mg/l + IAA 1 mg/l produced calli at cut ends of the nodal explant. Low concentrations of BAP alone could only bring forth the elongation of axillary buds, but higher concentrations produced some abnormal tissue growth (Table: 8) .

A comparatively low frequency of shoot induction (5-10 shoots from each node) was obtained after 2-3 weeks time when 25% coconut water was used instead of hormones. Medium with CW 35% produced only 1-2 shoots from 50% of the explants. Combinations like CW 20% + BAP 0.5 mg/l; CW 25% + BAP 1mg/l; CW 30% + BAP 1 mg/l; CW 40% + BAP 1 mg/l; CW 45% + BAP 1 mg/l and CW 50% + BAP 1 mg/l resulted

in axillary bud elongation from the nodal explants. A combination of CW 15% + BAP 0.5 mg/l resulted in swelling of the nodal region and callusing at the cut ends (Table: 9).

Nodal cultures in both the above mentioned multiple shoot induction media (BAP 2mg/l + IAA 2.5mg/l and 25% CW) produced morphologically variant plants with varied number of leaves at the nodes, ranging from 1-4 leaves at each node (Fig. 1.16-1.23). The frequency of production of morphological variants was 10-20%.

The cluster of multiple shoots was separated and subcultured for rooting on ½ MS medium (Fig. 1.26). A cluster of 3-7 roots was developed after 2-3 weeks. Rooted plantlets were transferred to a sterilized mixture of sand and soil (1:1) (Figs. 1.27-1.28). The field survival frequency was 80%. Only those plants, which were morphologically similar to the parent plant, could establish successfully in the soil. The field-transplanted plants produced a little larger sized leaves compared to the parent plant. The plantlets obtained full maturity after a growth period of 2 months in the pots (Fig. 1.29). The cultured plants also did not flower.

Vigorous vegetative propagation by rhizome cutting was noticed in the cultured plant also. Since no remarkable morphological variation was noticed among the field established plants, further analyses at cytological, molecular and phytochemical levels were conducted to search for the possible somaclonal variation.

Table: 6

Effect of hormones on shoot multiplication and callus induction from leaf explants

Cytokinins		Auxins			% frequency of response		Nature of response	% of morpho-logical variants
BAP mg/l	KIN mg/l	NAA mg/l	IAA mg/l	2,4-D mg/l	shoot	callus		
0.2	-	1	-	-	-	40	Rooting of explant	-
0.2	-	-	0.5	-	-	80	Swelling & crumbling	-
1.0	-	1.5	-	-	-	50	Rooting of explant	-
1.0	-	-	-	1.5	-	10	Very little callus at cut ends	-
1.0	-	-	1.5	-	-	70	Swelling	-
2.0	-	-	2.0	-	-	70	Swelling	-
2.0	-	-	2.5	-	30	-	Single shoot	-
-	0.2	-	-	1	-	90	White friable callus	
-	0.2	-	-	1.5	-	80	Greenish hard callus	-
-	0.5	-	1	-	-	70	Swelling	-
-	1.0	1.5	-	-	-	60	Swelling	-
-	-	-	-	0.5	-	90	Swelling & crumbling	-
-	-	-	-	1.5	-	80	Little white callus at periphery	-

Table: 7

Effect of coconut water alone and in combination with BAP on shoot multiplication from leaf explants

CW (%)	BAP mg/l	Nature of response	% frequency of response		% of morpho-logical variants
			shoot	callus	
15	0.5	Swelling	-	50	-
20	1.0	Single shoot	40	-	-
25	-	3-5 multiple shoots	60	-	10
30	-	Swelling	-	70	-
35	1.0	Swelling	-	60	-
40	1.5	Swelling	-	40	-
45	1.5	Swelling	-	10	-
50	1.5	Swelling & callusing at periphery	-	10	-

Table: 8

Effect of hormones on shoot multiplication and callus induction from nodal explants

Cytokinins		Auxins			% frequency of response		Nature of response	% of morpho-logical variants
BAP mg/l	KIN mg/l	NAA mg/l	IAA mg/l	2,4-D mg/l	shoot	callus		
0.2	-	0.5	-	-	-	65	Callus with numerous small hairy roots	-
0.2	-	1	-	-	-	55	Greenish swelling	-
0.2	-	-	0.5	-	-	50	Yellow hard callus	-
0.5	-	-	-	-	10	-	2-3 shoots from callus on subculture	-
0.5	-	-	-	-	20	-	Axillary bud elongation	-
0.5	-	-	1	-	40	-	Single shoot	-
1.0	-	-	-	-	45	-	Axillary bud elongation	-
1.0	-	-	1.5	-	60	-	Single shoot	-
1.5	-	-	-	-	30	-	Axillary bud elongation	-
2	-	-	2.5	-	95	-	20-40 shoots	30
2	-	-	-	2	-	80	Rooting of explant	-
2.5	-	-	-	-	-	60	Abnormal tissue growth	-
3	-	-	-	-	-	20	Abnormal tissue growth	-
-	0.2	-	-	1	-	90	White friable callus	-
-	0.2	-	-	1.5	-	70	White hard callus	-
-	0.2	-	-	2	-	30	White hard callus	-
-	0.5	1	-	-	-	50	Bulging	-
-	0.5	-	-	2	-	80	Rooting of callus	-
-	1	1.5	-	-	-	60	Callus at cut ends	-
-	1	-	1	-	-	40	Very little callus at cut ends	-

Table: 9

Effect of coconut water alone and in combination with BAP on shoot multiplication from nodal explants

CW (%)	BAP mg/l	Nature of response	% frequency of response		% of morpho-logical variants
			shoot	callus	
15	0.5	Swelling and callusing at cut ends	-	50	-
20	0.5	Axillary bud elongation	20	-	-
25	1	Rapid axillary bud elongation	30	-	-
25	-	5-10 multiple shoots	40	-	10
30	1	Axillary bud elongation	40	-	-
35	-	1-2 shoots	50	-	-
40	1	Axillary bud elongation	10	-	-
45	1	Axillary bud elongation	20	-	-
50	1	Axillary bud elongation	10	-	-

2. Cytological Analysis

The cultured plant was analysed for any karyomorphological changes and to detect the cytological basis of any variation, if present. The ploidy level of the parent plant, callus and the cultured plant was invariably the same. They were found to be diploid (2n=2x=24). Neither chimeral nor aneuploid variations were obtained. Average chromosome length of the parent plant (1.0017 μm) was slightly smaller than that of the somaclonal variant (1.1561 μm). Average chromosome length of the callus was still smaller (0.9379 μm). The total chromosome length of the somaclonal variant was 27.74 μm and that of the parent plant was 24.04 μm. The total chromosome length of the callus was 22.52 μm. A single pair of chromosomes with secondary constriction was detected in all the samples analyzed (Figs. 2.1.A1-2.3.A3). The disparity index of the parent plant

1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
1.10

Fig. 1.11-1.15: Different stages of direct multiple shoot development from nodal Explant (BAP 2mg/l + IAA 2.5 mg/l)

Fig. 1.16: Morphological variant with single leaf at each node

Fig. 1.17: Morphological variant with 4 leaves at basal nodes and 2 leaves at upper nodes

Fig. 1.18-1.20: Morphological variants with 3 leaves at each node

1.11
1.12
1.13
1.14
1.15
1.16
1.17
1.18
1.19
1.20

Fig. 1.21-1.23: Morphological variants with 3 leaves at each node

Fig. 1.24- 1.25: Multiple shoots developed from nodal explants of callus regenerated plants

Fig. 1.26: Isolated plantlet in ½ MS medium for rooting

Fig. 1.27: Transplantation of the rooted plant to sand : soil (1:1) mixture

Fig. 1.28: Development of the plant in plastic cup

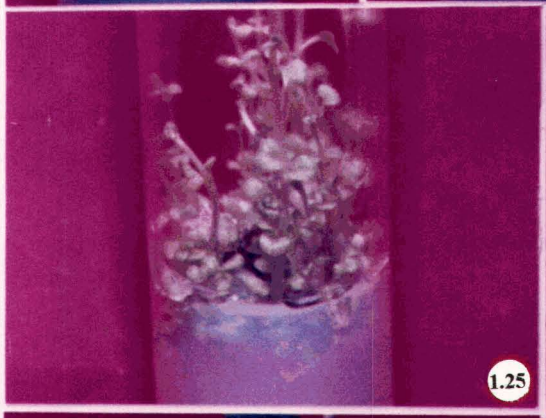
Fig. 1.29: Field established somaclonal variant


1.21


1.22


1.23


1.24


1.25


1.26


1.27


1.28


1.29

was 35.48, somaclonal variant was 17.76, and that of the callus tissue was 31.37. The variation coefficient was higher in the case of callus (20.20), when compared to the parent plant (17.36) and the somaclonal variant (11.60). The Total Forma percentage of the parent plant was 37.60, the somaclonal variant was 36.50 and the callus was 44.31. A general description of the common chromosome types found in the parent plant, somaclonal variant and callus is given below.

Type A : Chromosomes with secondary constriction ranging from 1.4019 μm to1.2582 μm with nearly submedian/ nearly median primary construction

Type B : Chromosome ranging from 1.3783 μm to 0.5991 μm with nearly median primary constriction.

Type C : Chromosome ranging from 1.2236 μm to 0.9525 μm with nearly submedian (-) primary constriction.

Type D : Chromosome having a length of 0.8388 μm with nearly submedian (+) primary constriction.

The karyotype formula of the parent plant was $A_2B_{12}C_8D_2$, of the callus was $A_2B_{20}C_2$ and of the somaclonal variant was $A_2B_{10}C_{12}$. Diagrammatic representation of the different chromosome types observed in the present investigation is shown in Fig. 2.4). Detailed karyotype description (Tables. 10-12), microphotographs of mitotic metaphase stages (Fig. 2.1-2.3), computer scanned images of karyotypes (Figs. 2.1.A1-2.3.A3), karyograms (Fig. 2.1.B-2.3.B) and idiograms (Figs. 2.1.C-2.3.C) of the parent plant, somaclonal variant and the callus are given below.

3. Random Amplified Polymorphic DNA Analysis

DNA was isolated from the parent plant and three cultured plants, of which TC 1 is the somaclonal variant mentioned hitherto. 20-30 ng of template DNA is required to amplify a specific DNA segment by PCR. The total DNA extracted was quantified by comparing the bandwidth of the isolated genomic DNA with that of the weight marker after running in agarose gels. The amount of DNA was found to be greater (~75ng) in the

Figs 2.1-2.3: Mitotic metaphases

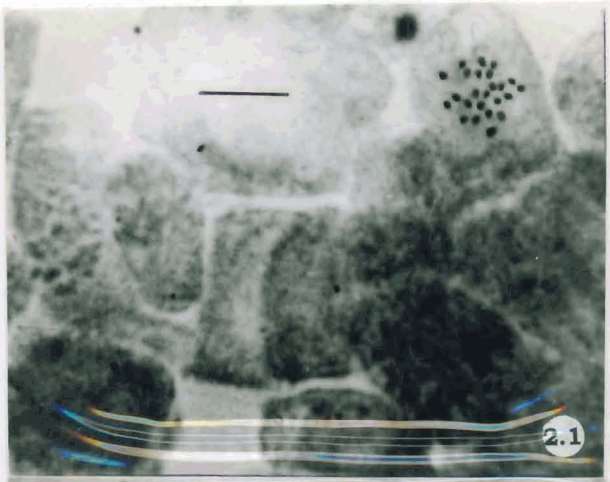
Fig. 2.1: *Mentha rotundifolia* (L.) Huds. Parent plant (2n=24)

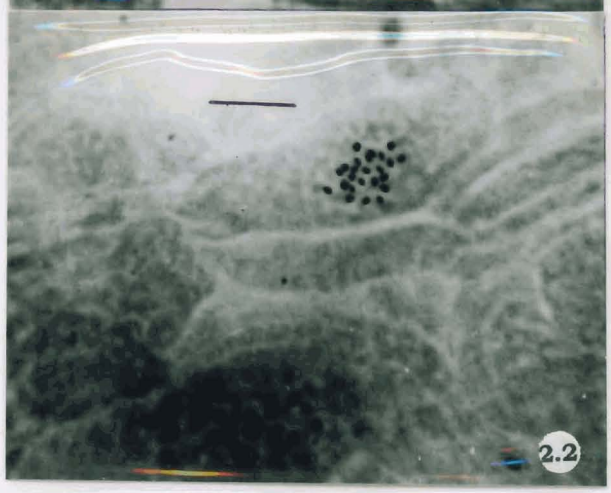
Fig. 2.2: M*entha rotundifolia* (L.) Huds. Callus (2n=24)

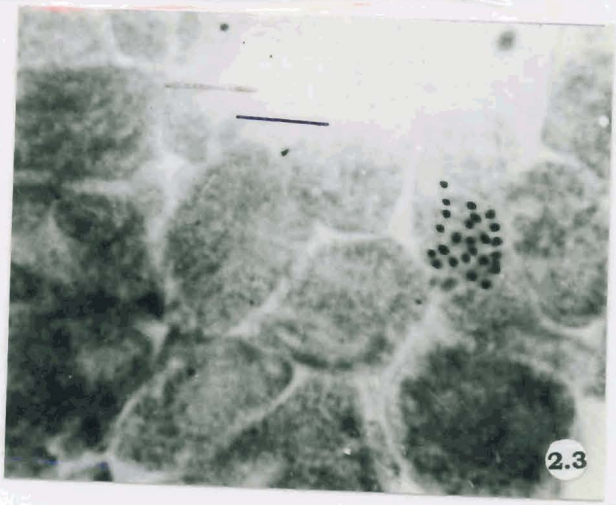
Fig. 2.3: *Mentha rotundifolia* (L.) Huds. Somaclonal variant (2n=24)

Bar = 10 μm

55B

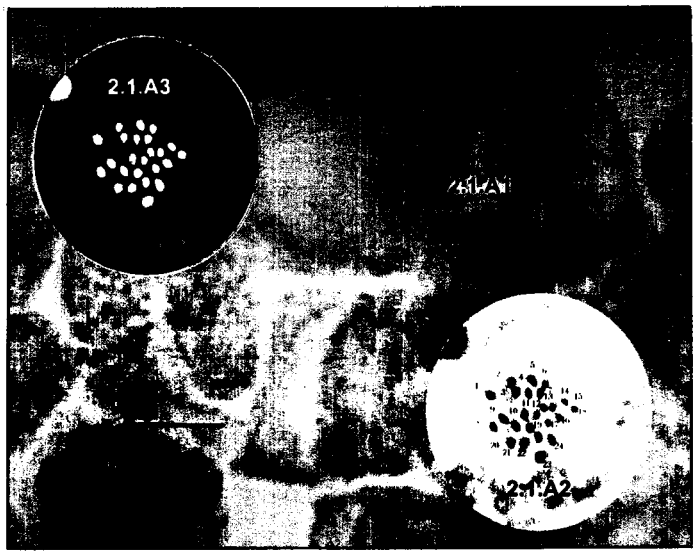




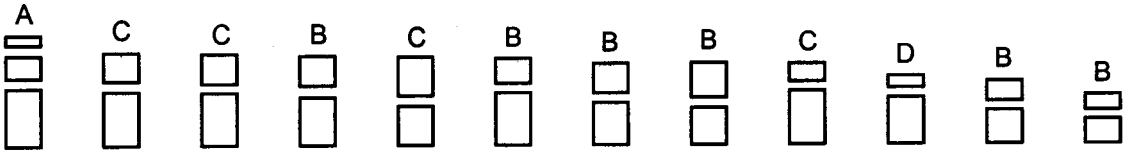


19

Mentha rotundifolia (L.) Huds.
Parent Plant



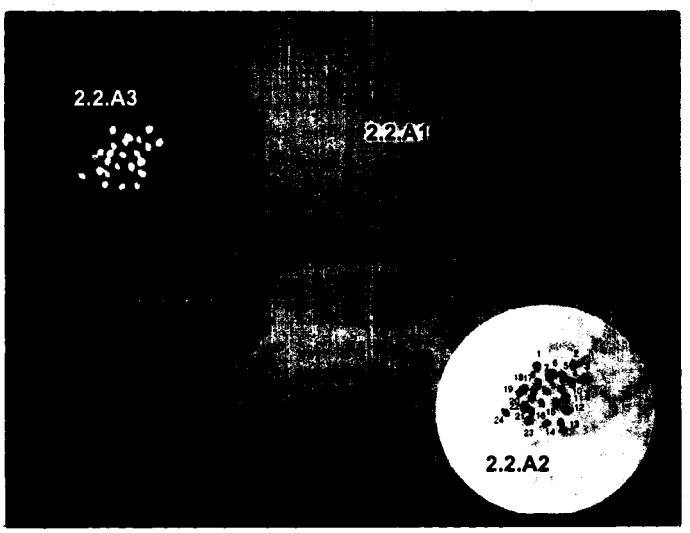
2.1.B Karyogram



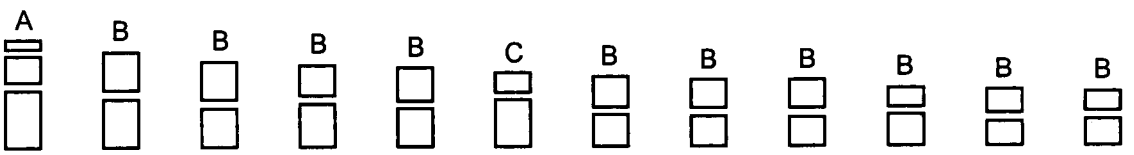
2.1.C Idiogram

Fig. **2.1.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.1.A2**: Resolved image
Fig. **2.1.A3**: Inverted image

Mentha rotundifolia (L.) Huds.
Callus



2.2.B Karyogram



2.2.C Idiogram

Fig. **2.2.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.2.A2**: Resolved image
Fig. **2.2.A3**: Inverted image

55F

Mentha rotundifolia (L.) Huds. ($2n = 2x = 24 = A_2B_{20}C_2$)

Callus

Normal somatic chromosome number	: 24
Chromosome pair with secondary constriction	: 1
Total chromosome length	: 22.52 μm
Range of chromosome length	: 1.3098 μm-0.6842 μm
Average chromosome length	: 0.9379 μm
Disparity Index	: 31.37
Variation Coefficient	: 20.20
TF value	: 44.31

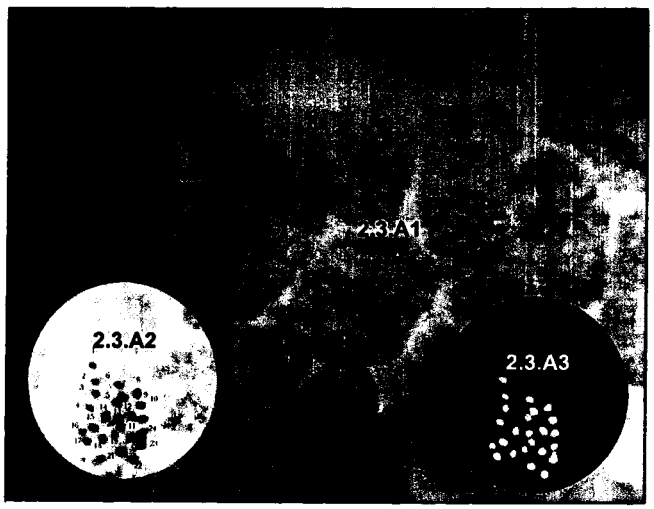
Table: 11

Detailed karyomorphometrical data

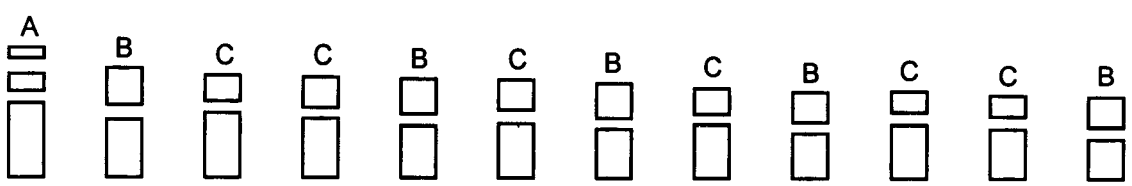
Chromosome type	No. of pairs	Total length (μm)	s (μm)	l (μm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of primary constriction
A	1	1.3098	0.5358	0.7740	0.6922	1.4445	40.90	59.09	Nm
B	1	1.1906	0.5332	0.6574	0.8110	1.2329	44.78	55.21	Nm
B	1	1.0716	0.5313	0.5403	0.9833	1.0169	49.58	50.41	Nm
B	1	1.0248	0.4295	0.5953	0.7214	1.3860	41.91	58.08	Nm
B	1	1.0121	0.4763	0.5358	0.8906	1.1249	57.06	52.94	Nm
C	1	0.9525	0.2976	0.6549	0.4544	2.2006	31.24	68.75	Nsm(-)
B	1	0.8929	0.4378	0.4551	0.9619	1.0395	49.03	50.96	Nm
B	1	0.8355	0.4062	0.4293	0.9462	1.0567	48.61	51.38	Nm
B	1	0.8335	0.4067	0.4268	0.9529	1.0494	48.79	51.20	Nm
B	1	0.7438	0.2893	0.4545	0.6365	1.5710	38.89	61.11	Nm
B	1	0.7054	0.3482	0.3572	0.9748	1.0258	49.36	50.63	Nm
B	1	0.6842	0.2976	0.3866	0.7697	1.2990	43.49	56.50	Nm

S : short arm
L : long arm
R_1 & R_2 : arm ratios
I_1 & I_2 : centromeric indices

Mentha rotundifolia (L.) Huds.
Somaclonal variant



2.3.B Karyogram



2.3.C Idiogram

Fig. **2.3.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.3.A2**: Resolved image
Fig. **2.3.A3**: Inverted image

Mentha rotundifolia (L.) Huds. ($2n = 2x = 24 = A_2B_{10}C_{12}$)

Somaclonal variant

Normal somatic chromosome number	: 24
Chromosome pair with secondary constriction	: 1
Total chromosome length	: 27.74 µm
Range of chromosome length	: 1.4019 µm - 0.9790 µm
Average chromosome length	: 1.1561 µm
Disparity Index	: 17.76
Variation Coefficient	: 11.60
TF value	: 36.50

Table: 12

Detailed karyomorphometrical data

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	1.4019	0.4268	0.9751	0.4376	2.2846	30.44	69.55	Nsm(-)
B	1	1.3783	0.6098	0.7685	0.7934	1.2602	44.24	55.75	Nm
C	1	1.2236	0.3659	0.8577	0.4266	2.3440	29.90	70.09	Nsm(-)
C	1	1.2196	0.4268	0.7928	0.5383	1.8575	34.99	65.00	Nsm(-)
B	1	1.1823	0.4878	0.6945	0.7023	1.4237	41.25	58.74	Nm
C	1	1.1586	0.4269	0.7317	0.5834	1.7139	36.84	63.15	Nsm(-)
B	1	1.1560	0.4852	0.6708	0.7233	1.3825	41.97	58.02	Nm
C	1	1.0977	0.3659	0.7318	0.5000	2.000	33.33	66.66	Nsm(-)
B	1	1.0505	0.4295	0.6210	0.6916	1.4458	40.88	59.11	Nm
C	1	1.0367	0.3049	0.7318	0.4166	2.4001	29.41	70.58	Nsm(-)
C	1	0.9895	0.3055	0.6840	0.4466	2.2389	30.87	69.12	Nsm(-)
B	1	0.9790	0.4302	0.5488	0.7838	1.2756	43.94	56.06	Nm

s : short arm
l : long arm
R_1 & R_2 : arm ratios
I_1 & I_2 : centromeric indices

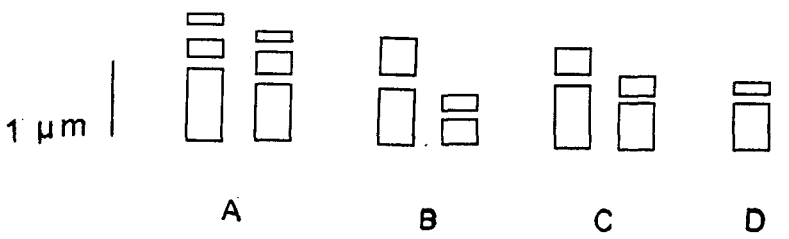


Fig. 2.4 Diagrammatic representation of the chromosome types observed in *Mentha rotundifolia* (L.) Huds. (Parent plant, callus and somaclonal variant).

somaclonal variant than in the parent plant (~50ng) (Fig. 3.1). 1µl of the template DNA was used for the preparation of reaction mixture. To detect the somaclonal variation at the molecular level, RAPD analysis was carried out using six primers of arbitrary sequences (Figs: 3.2-3.7). Of the six primers used, all successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from a minimum of 3 to a maximum of 11. The size of the amplification products also differed and ranged from approximately below 0.5 Kb to 3 Kb. The primers and the characterization of consistent bands are listed in Table: 13.

The RAPD fingerprint of the somaclonal variant (TC 1) differed from the parent plant with 2 primers (OPE 14 and OPF 05) used (Figs. 3.6-3.7). A few bands were found to be missing in the somaclonal variant when these two primers were used. No additional bands could be detected in the variant by this marker screening. Amplification products with OPE 14 generated no bands above 0.75 Kb length in the somaclonal variant but bands were clearly visible in the parent plant's amplification products in this region. When the primer OPF 05 was used for amplification, a fragment with nearly 1 Kb length was found missing in the somaclonal variant which was clearly present as a prominent band in the parent plant's finger print. In both these cases, no additional bands were present in the variant. The other primers used could not generate any polymorphism, but certain intensity differences in the bands were noticed in the amplification products of all the six primers tested.

In the case of TC 2 and TC 3, certain band differences were noticed when the primer OPE 14 was used. TC 2 showed minor band differences when the primer OPF 05 was used for amplification. The reproducibility of these genomic DNA bands were consistent in the case od TC 1 alone. Though TC 2 and TC 3 showed band differences, they were inconsistent in successive repetitions.

Fig. 3.1

P: Parent plant

TC 1: Tissue Cultured plant 1 (Somaclonal variant)

TC 2: Tissue Cultured plant 2

TC 3: Tissue Cultured plant 3

Fig. 3.1

Agarose gel electrophoresis of pure genomic DNA for quantification

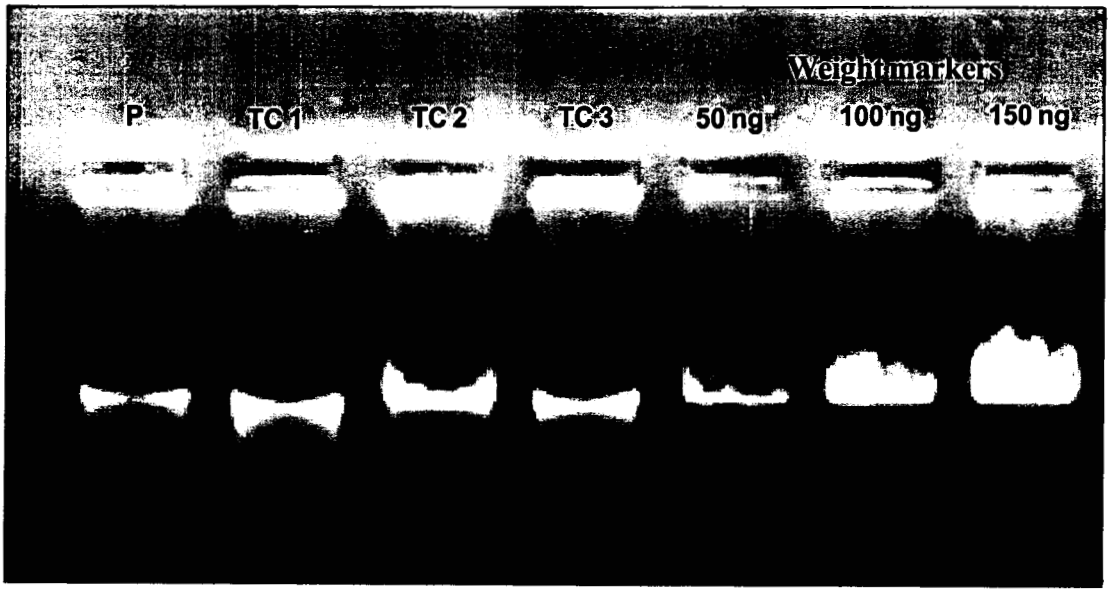


Fig. 3.2

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.2

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPA 02 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

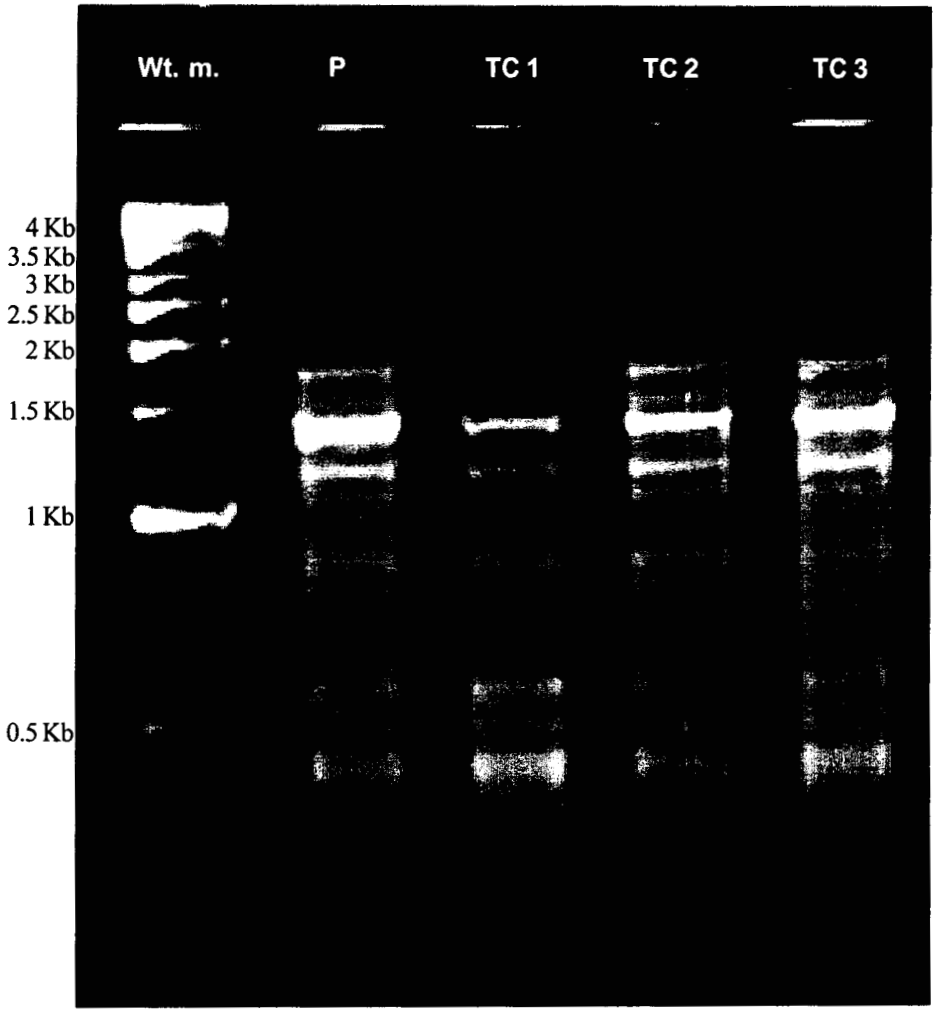


Fig. 3.3

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.3

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPA 08 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

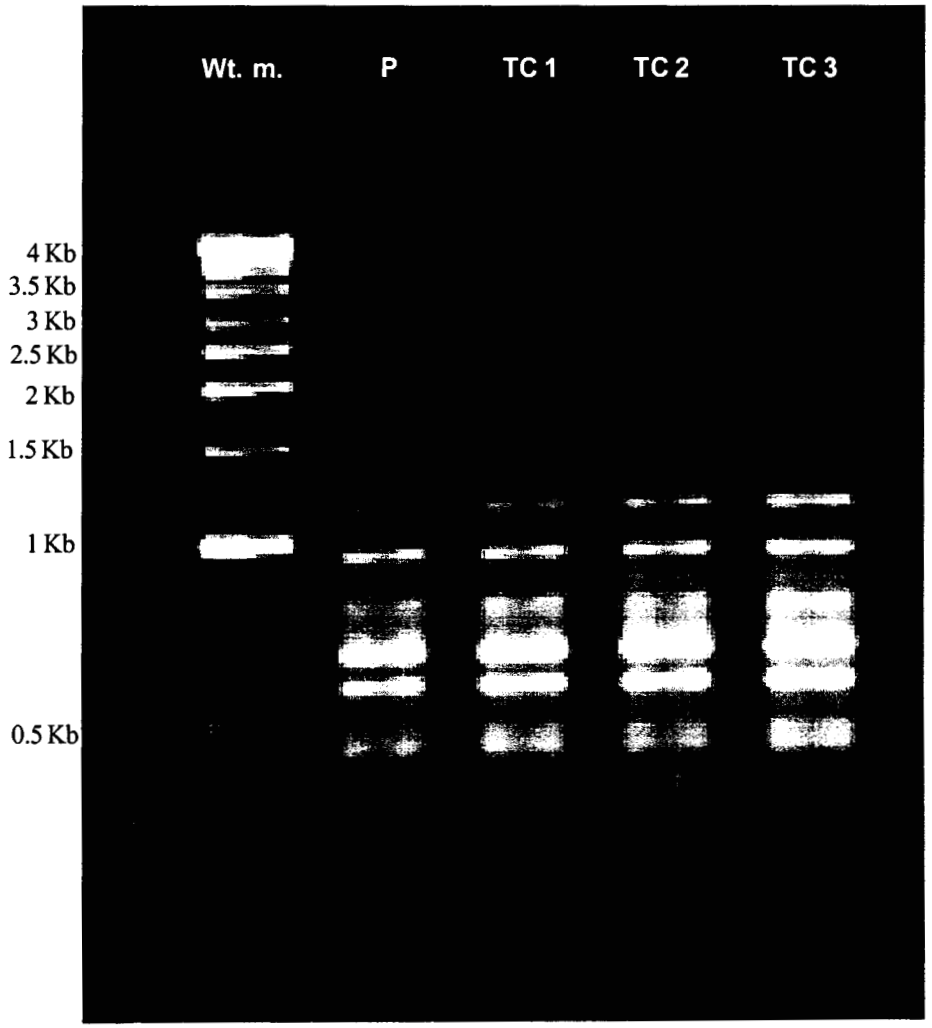


Fig. 3.4

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.4

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPB 07 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

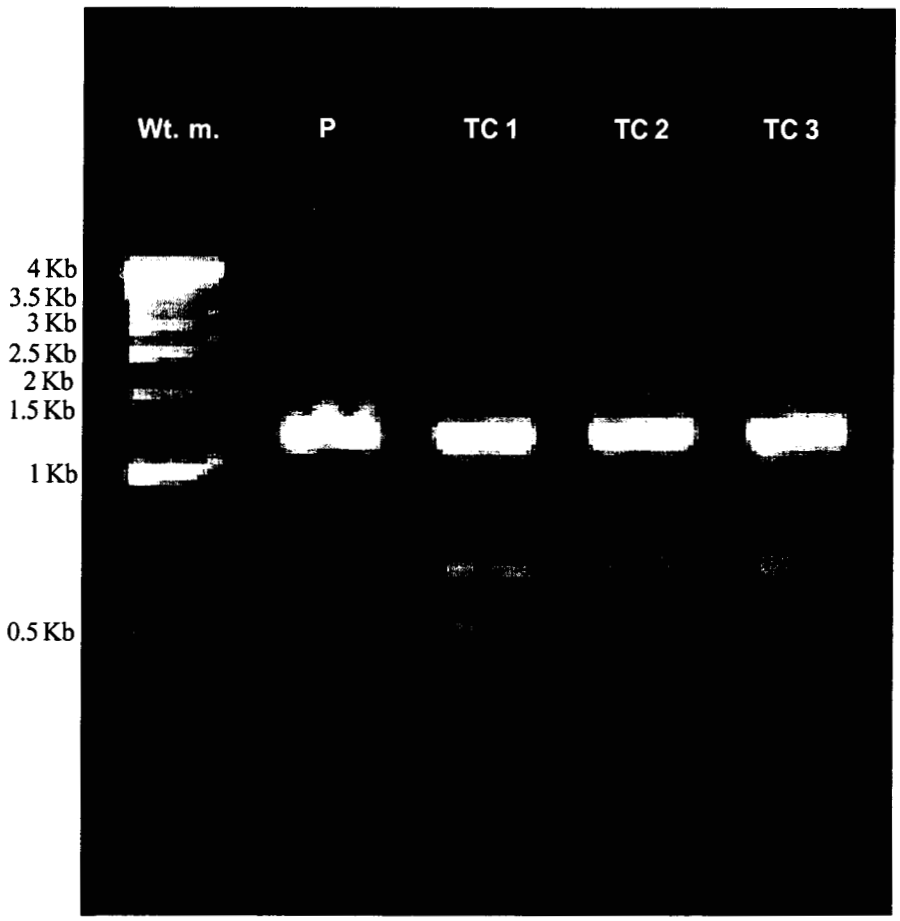


Fig. 3.5

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.5

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPD 19 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

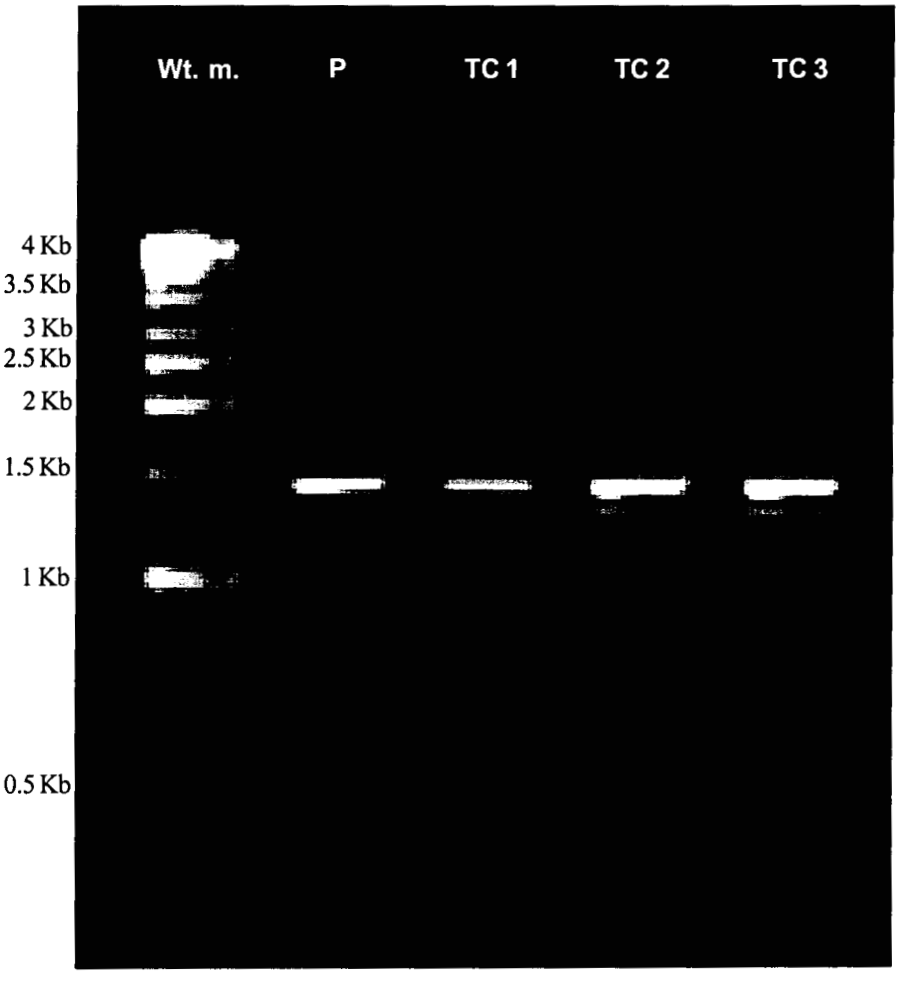


Fig. 3.6

Wt. m.: Weight marker

P: Parent plant

TC 1: Tissue Cultured plant 1 (Somaclonal variant)

TC 2: Tissue Cultured plant 2

TC 3: Tissue Cultured plant 3

Arrow represents absence of a prominent band in the somaclonal variant (TC 1) when compared to the parent plant, TC 2 and TC 3.

Fig. 3.6

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPE 14 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

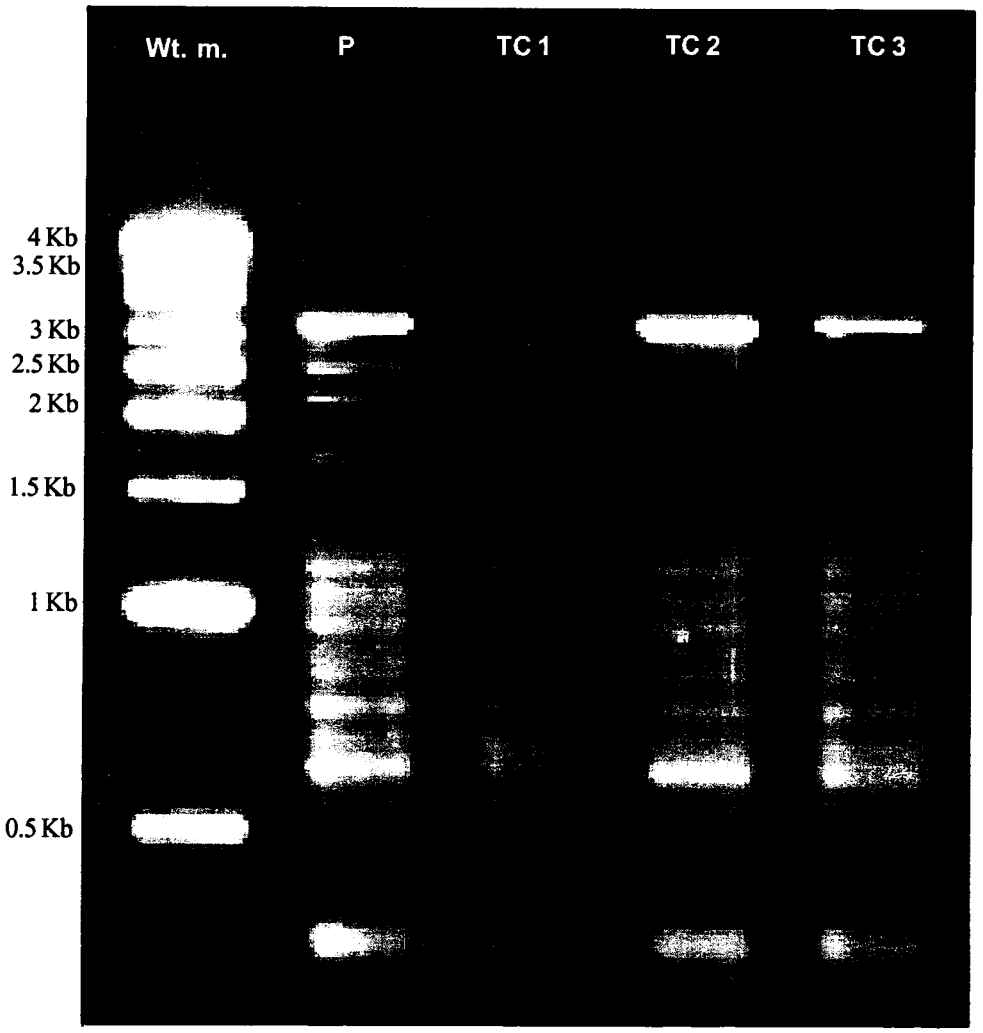


Fig. 3.7

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Arrow represents absence of a band in the somaclonal variant (TC 1)

Fig. 3.7

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPF 05 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

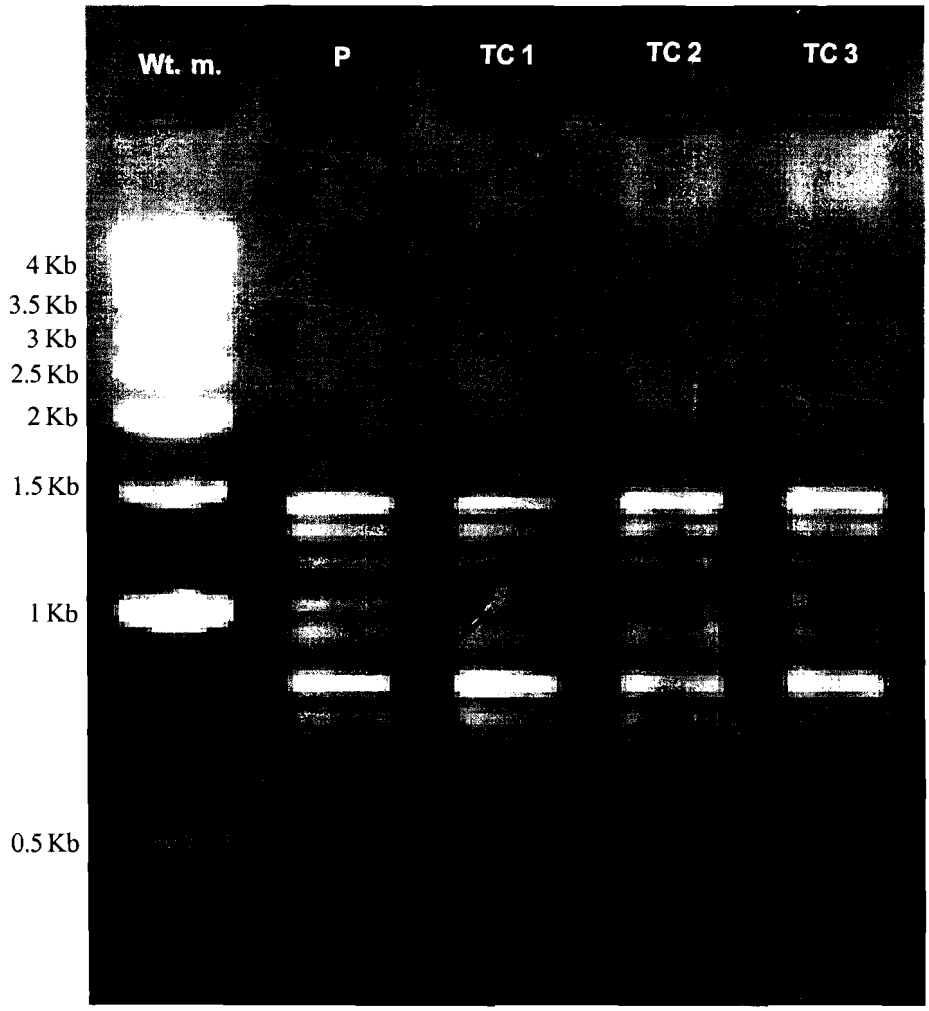


Table: 13

Primers and characterization of consistent bands in parent and three tissue cultured plants of *M. rotundifolia* (L.) Huds..

Primer	Sequence	Number of markers				Size Range in Kb (kilo base)
		Parent	TC 1*	TC 2	TC 3	
OPA 02	5′ TGCCGAGCTC 3′	7	7	7	7	~0.5-1.75
OPA 08	5′ GTGACGTAGG 3′	6	6	6	6	~0.5-1.25
OPB 07	5′ GGTGACGCAG 3′	3	3	3	3	~0.5-1.25
OPD 19	5′ CTGGGGACTT 3′	8	8	8	8	~0.5-1.5
OPE 14	5′ TGCGCCTGAG 3′	8	3	6	6	~0.25-3.25
OPF 05	5′ CCGAAT TCCC 3′	7	5	5	7	~0.75-1.5

* Somaclonal variant hitherto mentioned.

4. Essential oil Analysis

RAPD analysis revealed a notable deviation in the genetic make up of the somaclonal variant, TC 1 (Figs. 3.6-3.7) than TC 2 and TC 3. Moreover, repeatability was consistent only in the case of TC 1. So the essential oils of the parent plant and the somaclonal variant TC 1 were analyzed qualitatively and quantitatively to search for any biochemical variations in the secondary metabolism.

The oil yield of the parent plant was 1%. The somaclonal variant (TC 1) contained a higher quantity of oil (1.3%). The oil yield of TC 2 and TC 3 were almost equal to that of the parent. The oil of the parent plant was light yellow in colour and that of the somaclonal variant, TC 1was almost colourless. The essential oils of the parent as well as the somaclonal variant possess a warm herbaceous odour with a minty topnote.

(i) Gas Chromatography-Mass Spectrometry Analysis

The results of Gas Chromatography-Mass Spectrometry analyses of the parent plant and the somaclonal variant are listed in Table: 14. GC-MS analyses revealed 21 components each in the parent plant (Figs. 4.1.1 - 4.1.22) and in the somaclonal variant (Figs. 4.2.1 - 4.2.22). There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant).

α-pinene (tr.), sabinene hydrate (0.081%), ethyl 9,12 octa decadienoate (tr.), trans-2-octenal (tr.), isopulegol (tr.), citronellol (tr.), alloaromadendrene (tr.), β-caryophyllene (1.1), and β-gurgunene (0.7%) were the unique components detected from the parent plant. The somaclonal variant was characterized by a set of new components like methyl chavicol (tr.), isoborneol (tr.), cis 6 nonenal (0.176%), 2,3 diethyl 6 methyl pyrazine (0.031 %), p-mentha trans 2,8 dien 1 ol (tr.), γ-caryophyllene (0.4 %), β-bisabolene (1.0 %), 1 ethynyl-2-trimethylsilyl benzene (tr.), α-amorphene (0.091%), juniper camphor (0.081%) and di isobutyl phtalate (tr.). Components like myrcene, limonene, carvone, α-terpinyl acetate, β-elemene, α-cububene, aromadendrene, trans-2-cis-6-nonadien-1-ol, and β-terpineol were found in both *in vivo* and *in vitro* developed plants. In addition to these, three unidentified components in the parent plant and one unidentified component in the somaclonal variant were also noticed.

(ii) Chemotaxonomic Evaluation

The total number of chemical components detected by GC-MS in both the *in vivo* and *in vitro* grown plants was found to be 33. However the number of similar components which occur both in the parent and the somaclonal variant (TC 1) was found to be 9. Coefficient of similitude between the parent plant and the somaclonal variant was found to be 27.27.

Table:14

List of essential oil components detected in the present investigation

Sl.No	Components	Class	Composition %	
			A	B
1	α-pinene	Monoterpene	tr.	-
2	myrcene	,,	0.4	tr.
3	limonene	,,	6.2	8.4
4	sabinene hydrate	,,	0.081	-
5	ethyl 9,12 octa deca dienoate	Ester	tr.	-
6	trans-2-octenal	Aldehyde	tr.	-
7	isopulegol	Monoterpene	tr.	-
8	citronellol	,,	tr.	-
9	carvone	,,	82	87.4
10	UI		1.282	-
11	α-terpinyl acetate	,,	0.3	0.2
12	UI		tr.	-
13	alloaromadendrene	Sesquiterpene	tr.	-
14	β-elemene	,,	2.6	1.0
15	β-caryophyllene	,,	1.1	-
16	trans-2-cis-6-nonadien–1-ol	Unsaturated alcohol	1.0	0.3
17	α-cububene	Sesquiterpene	0.9	0.4
18	UI		2.9	-
19	β-gurgunene	Sesquiterpene	0.7	-
20	β-terpineol	Monoterpene	tr	tr.
21	methyl chavicol	Phenol	-	tr.
22	isoborneol	Monoterpene	-	tr.
23	cis-6-nonenal	Unsaturated aldehyde	-	0.176
24	UI		-	tr.
25	2,3 diethyl-6-methyl pyrazine	Heterocyclic compound	-	0.031
26	p-mentha trans 2,8 dien-1-ol	Monoterpene	-	tr.
27	γ-caryophyllene	Sesquiterpene	-	0.4
28	aromadendrene	,,	tr	0.056
29	β-bisabolene	,,	-	1.0
30	1 ethynyl-2-trimethylsilyl benzene	Benzene derivative	-	tr.
31	α-amorphene	Sesquiterpene	-	0.091
32	juniper camphor	Monoterpene	-	0.081
33	di isobutyl phtalate	Ester	-	tr.

A Parent plant of *M. rotundifolia* (L.) Huds.
B Somaclonal variant of *M. rotundifolia* (L.) Huds.
UI Unidentified component
tr. Trace component

Fig. 4.1.1 Gas chromatogram of the essential oil of *Mentha rotundifolia* (L.) Huds. Parent plant

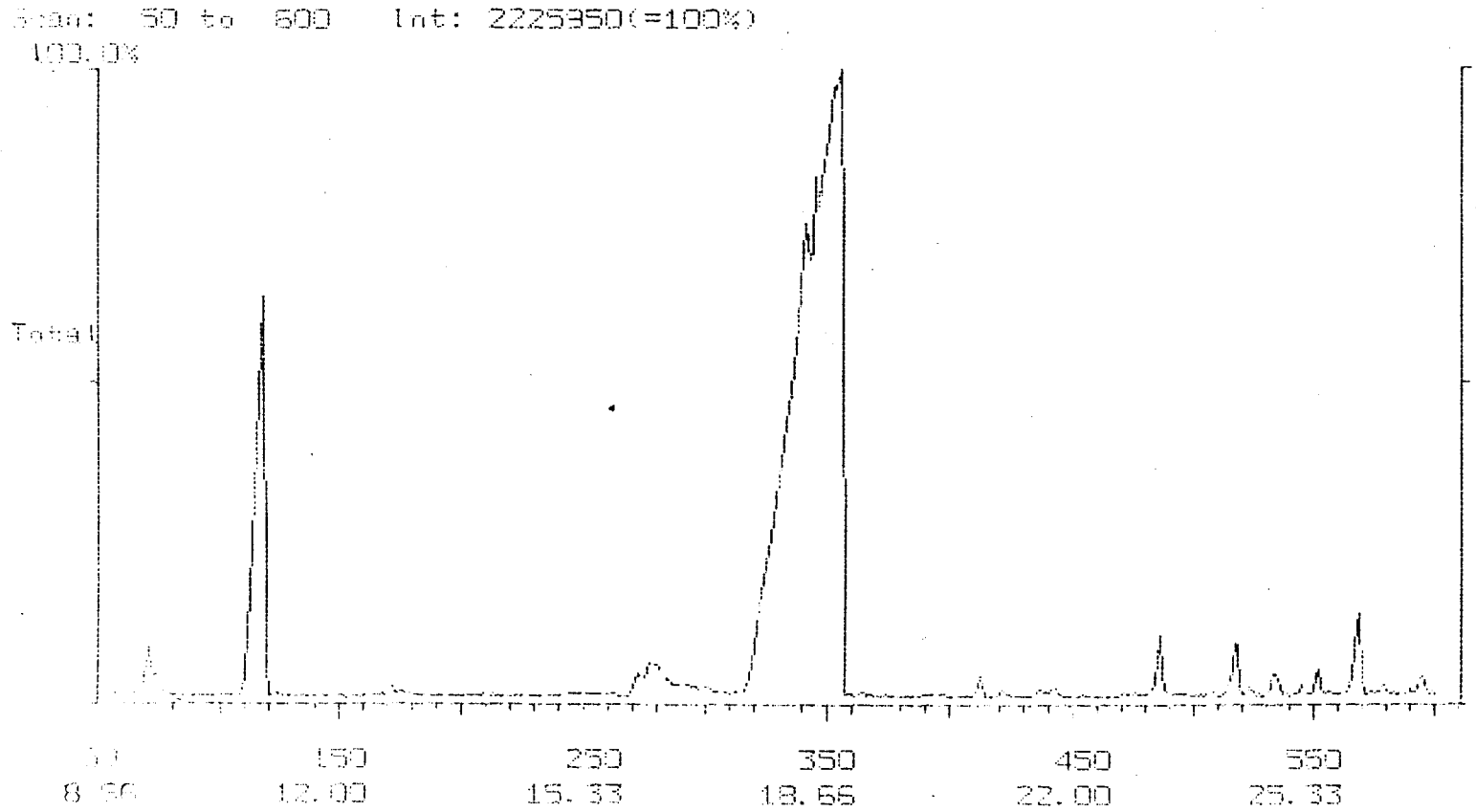


Fig. 4.1.2 Mass spectrum of α-pinene

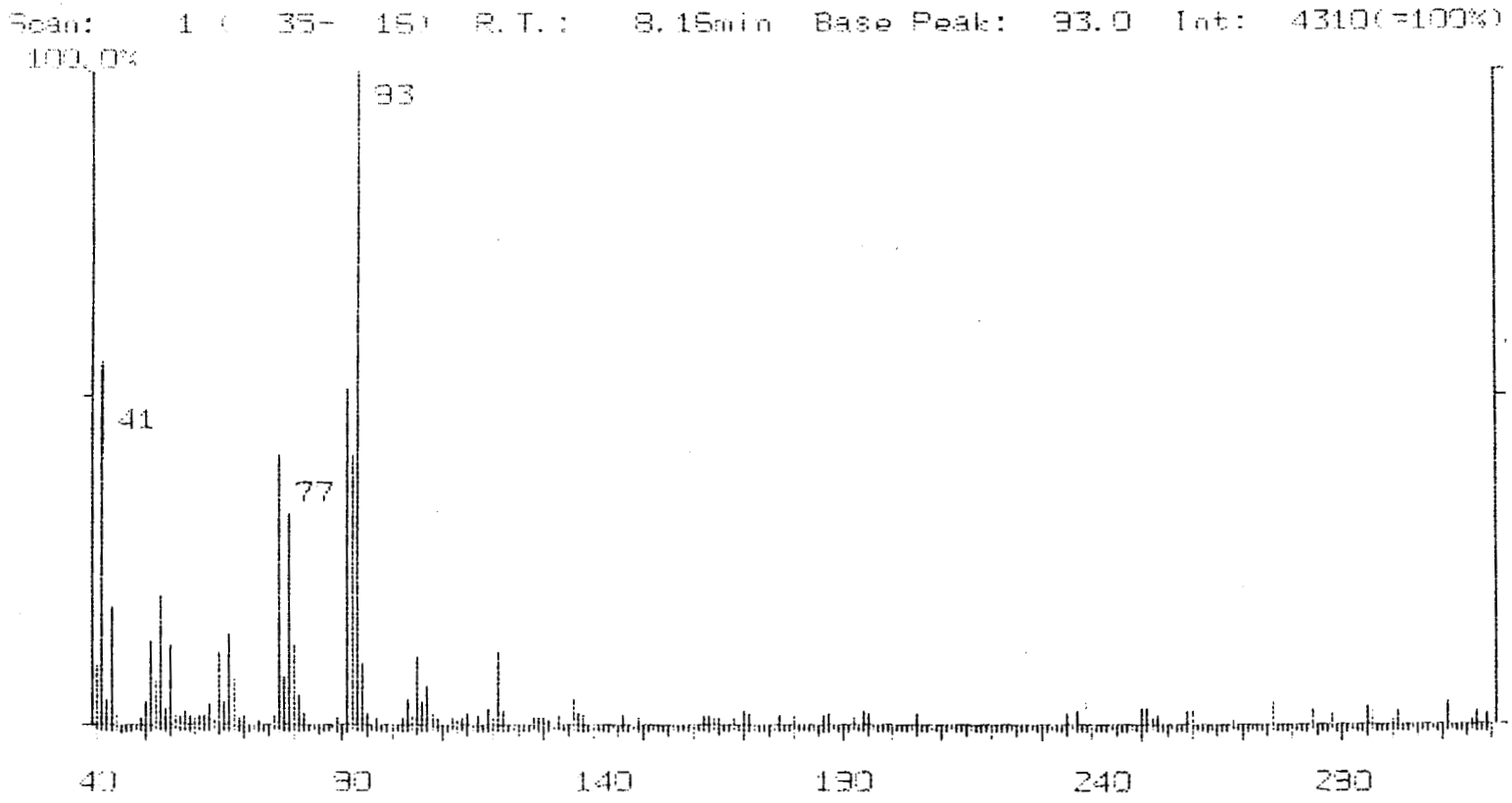


Fig. 4.1.3 Mass spectrum of myrcene

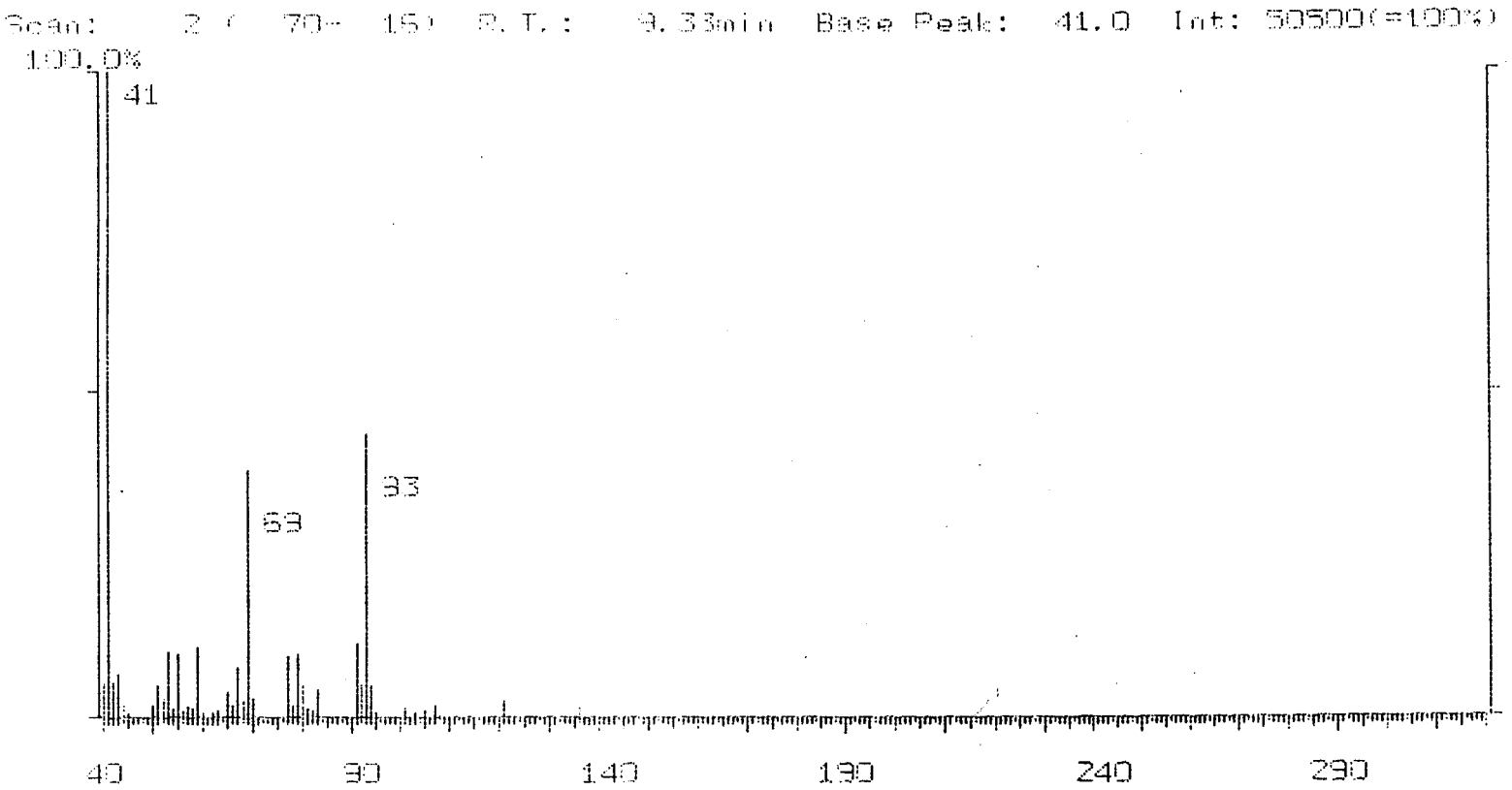


Fig. 4.1.4 Mass spectrum of limonene

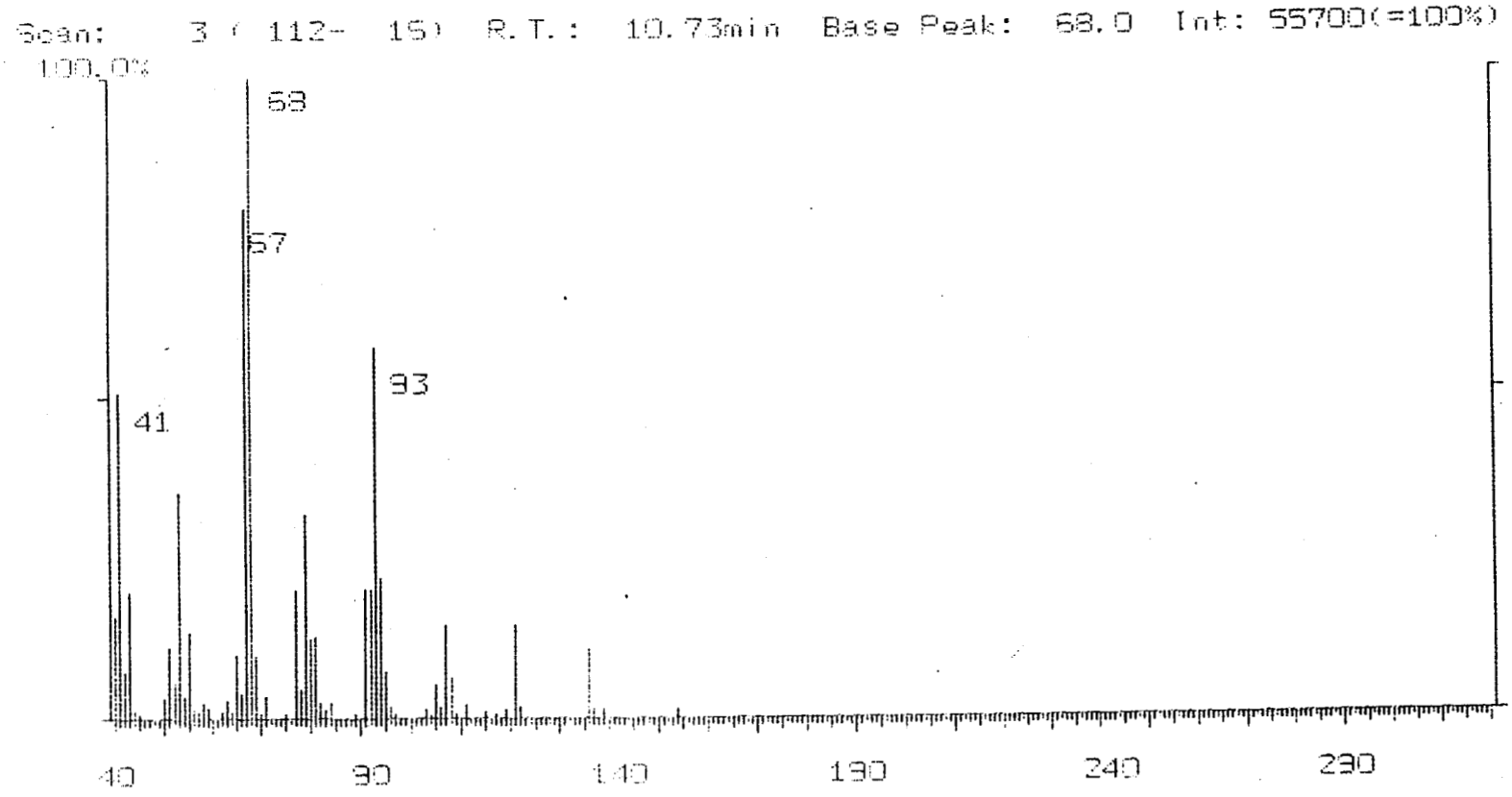


Fig. 4.1.5 Mass spectrum of sabinene hydrate

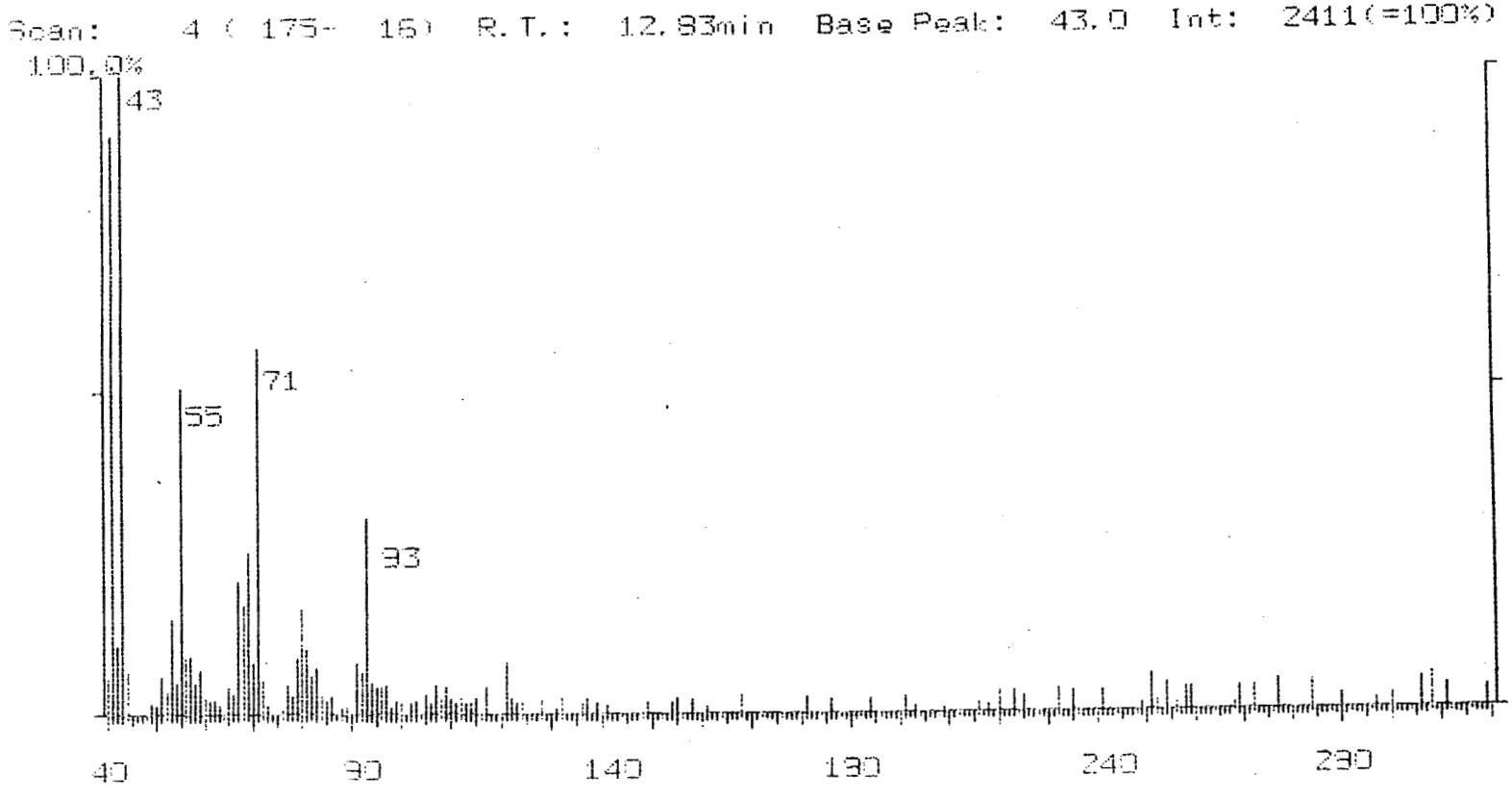


Fig. 4.1.6 Mass spectrum of ethyl 9,12 octa deca dienoate

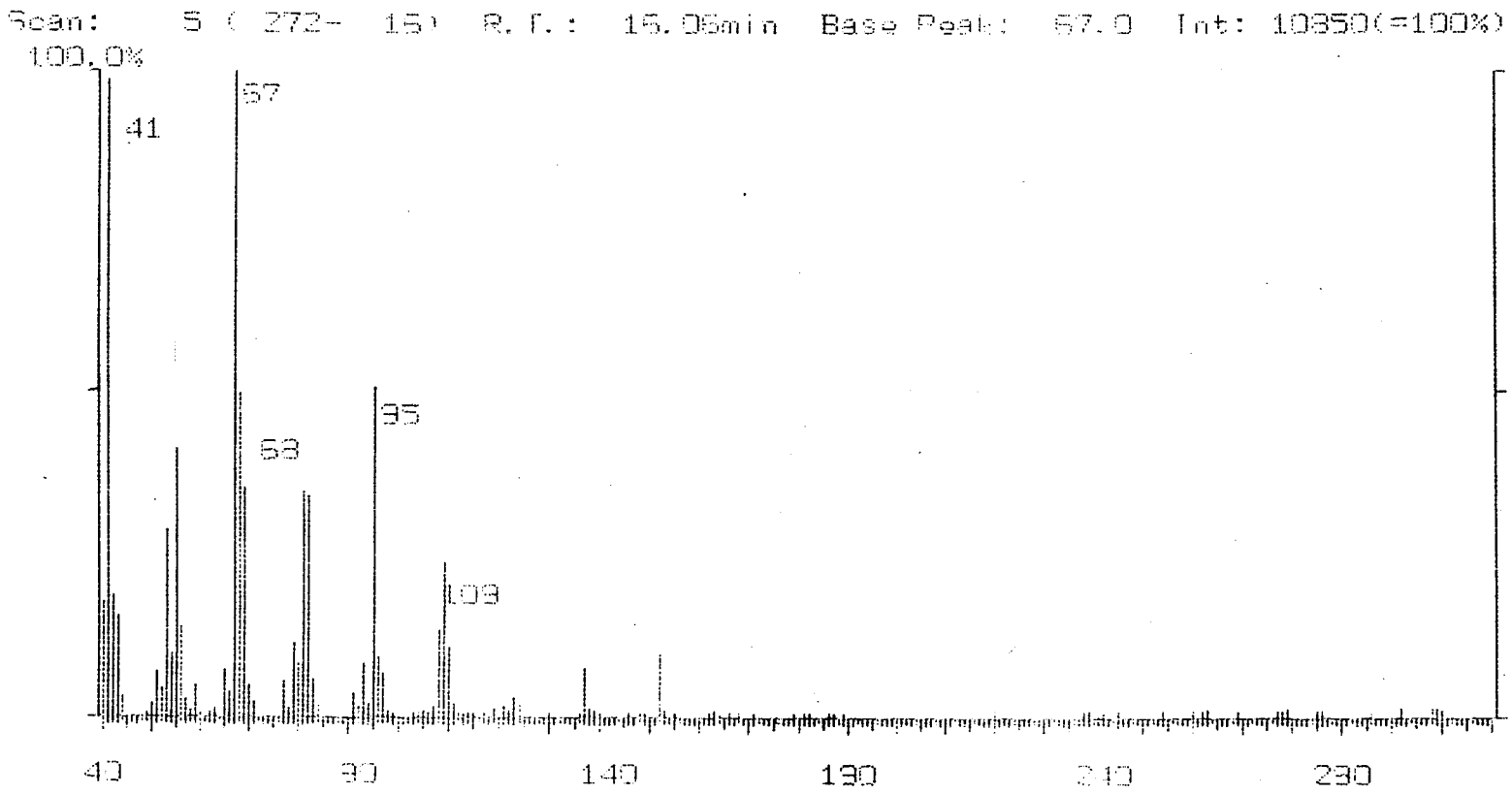


Fig. 4.1.7 Mass spectrum of trans-2-octenal

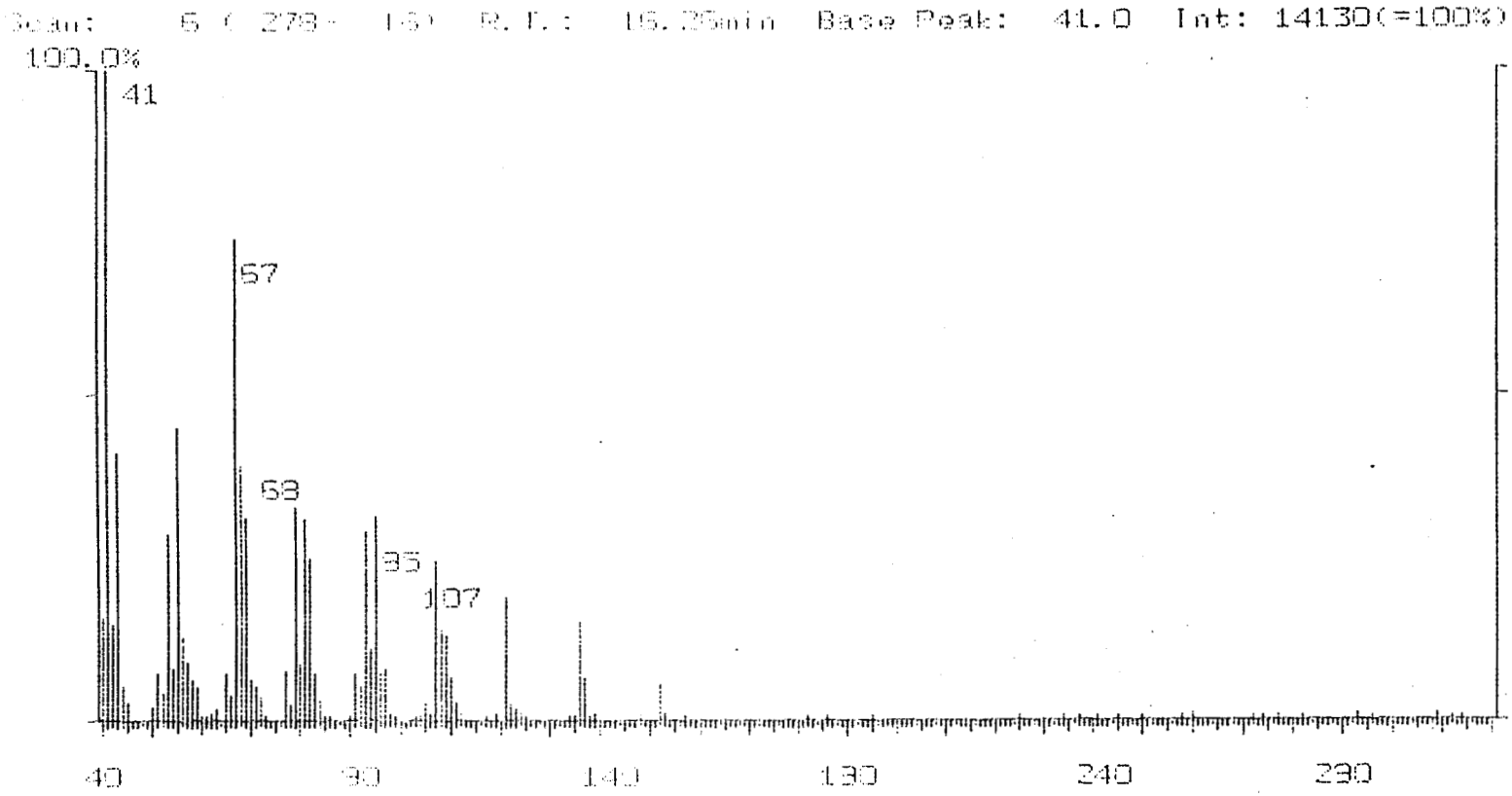


Fig. 4.1.8 Mass spectrum of isopulegol

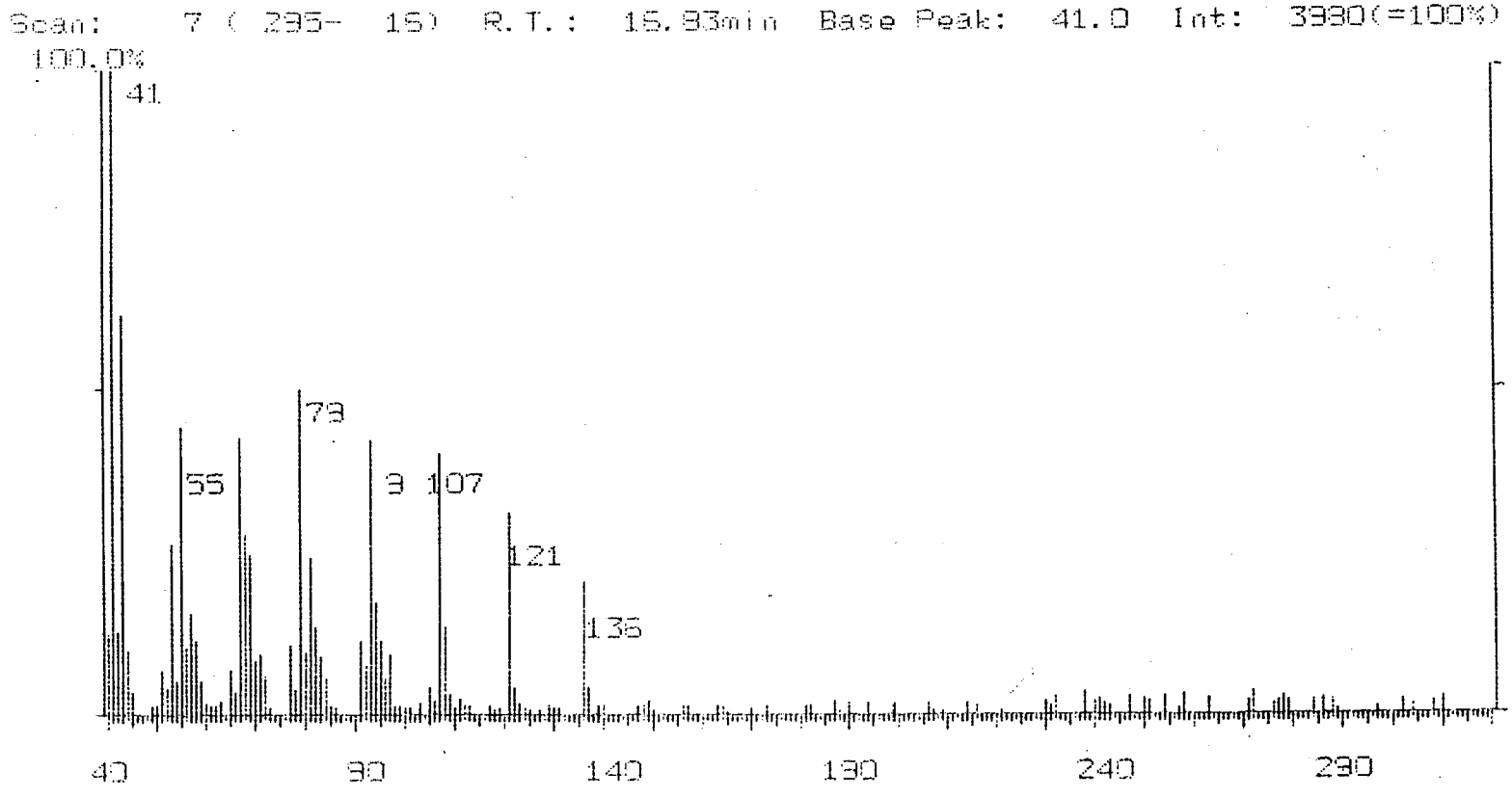


Fig. 4.1.9 Mass spectrum of citronellol

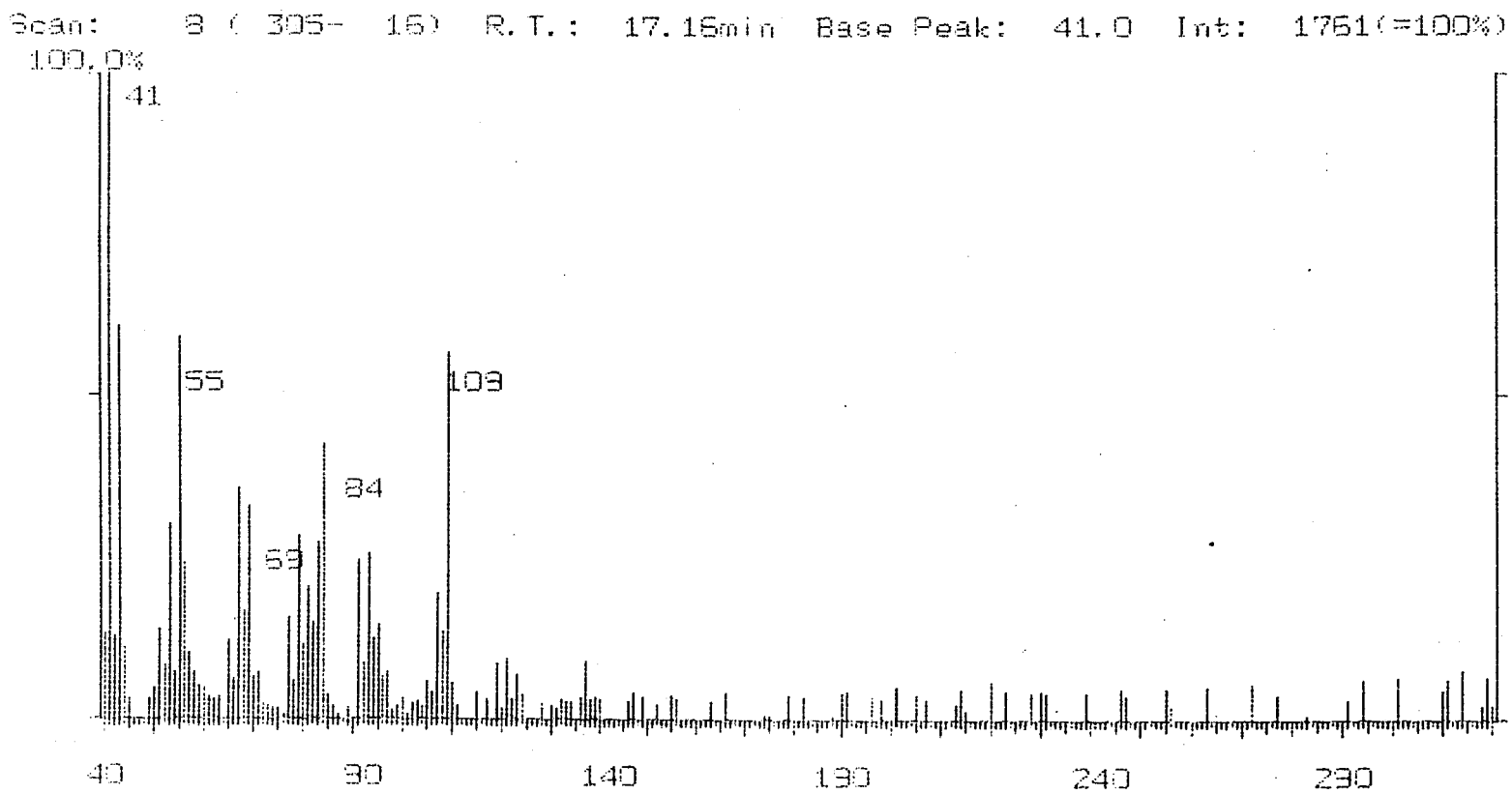


Fig. 4.1.10 Mass spectrum of carvone

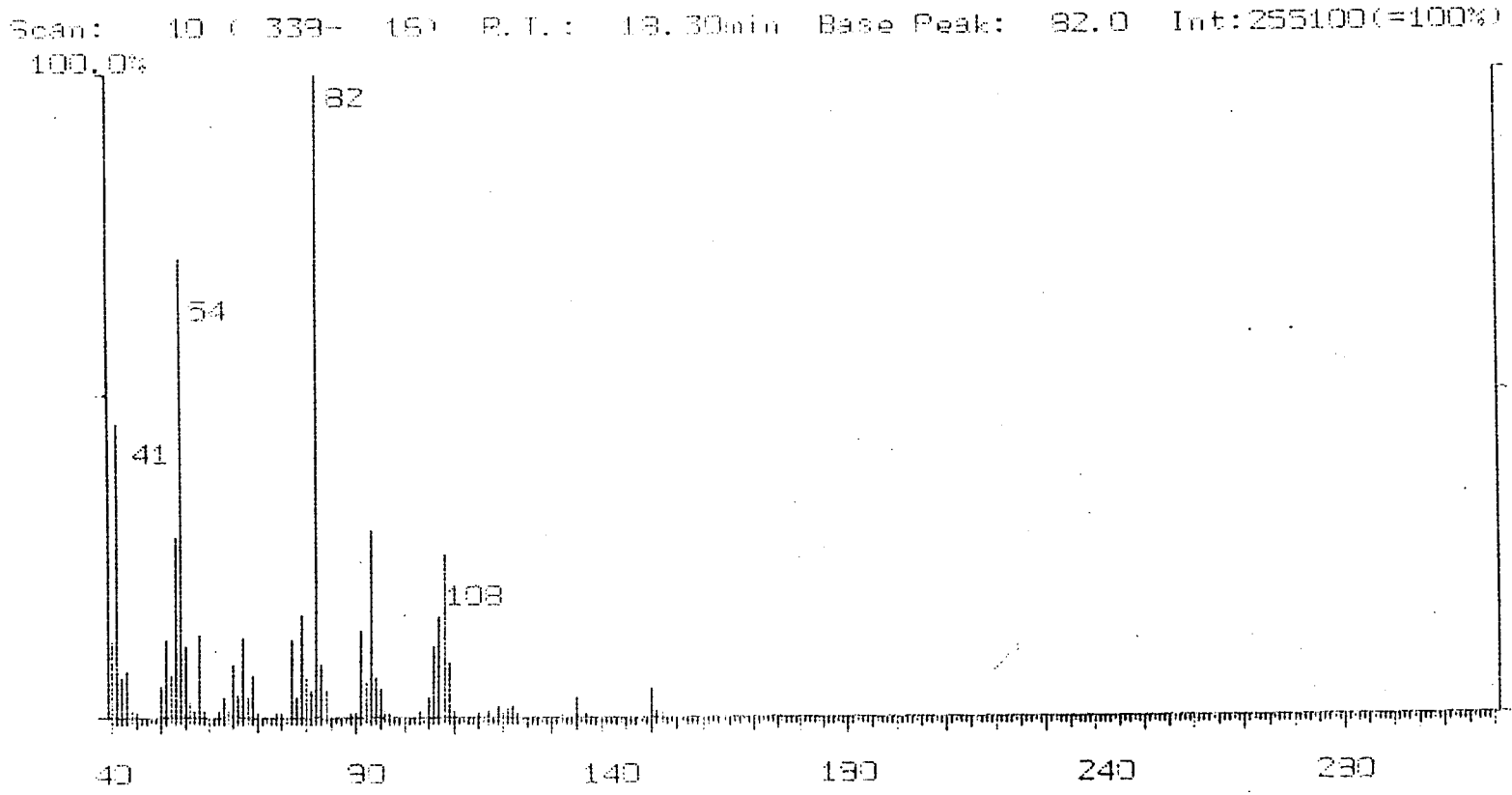


Fig. 4.1.11 Mass spectrum of Unidentified component

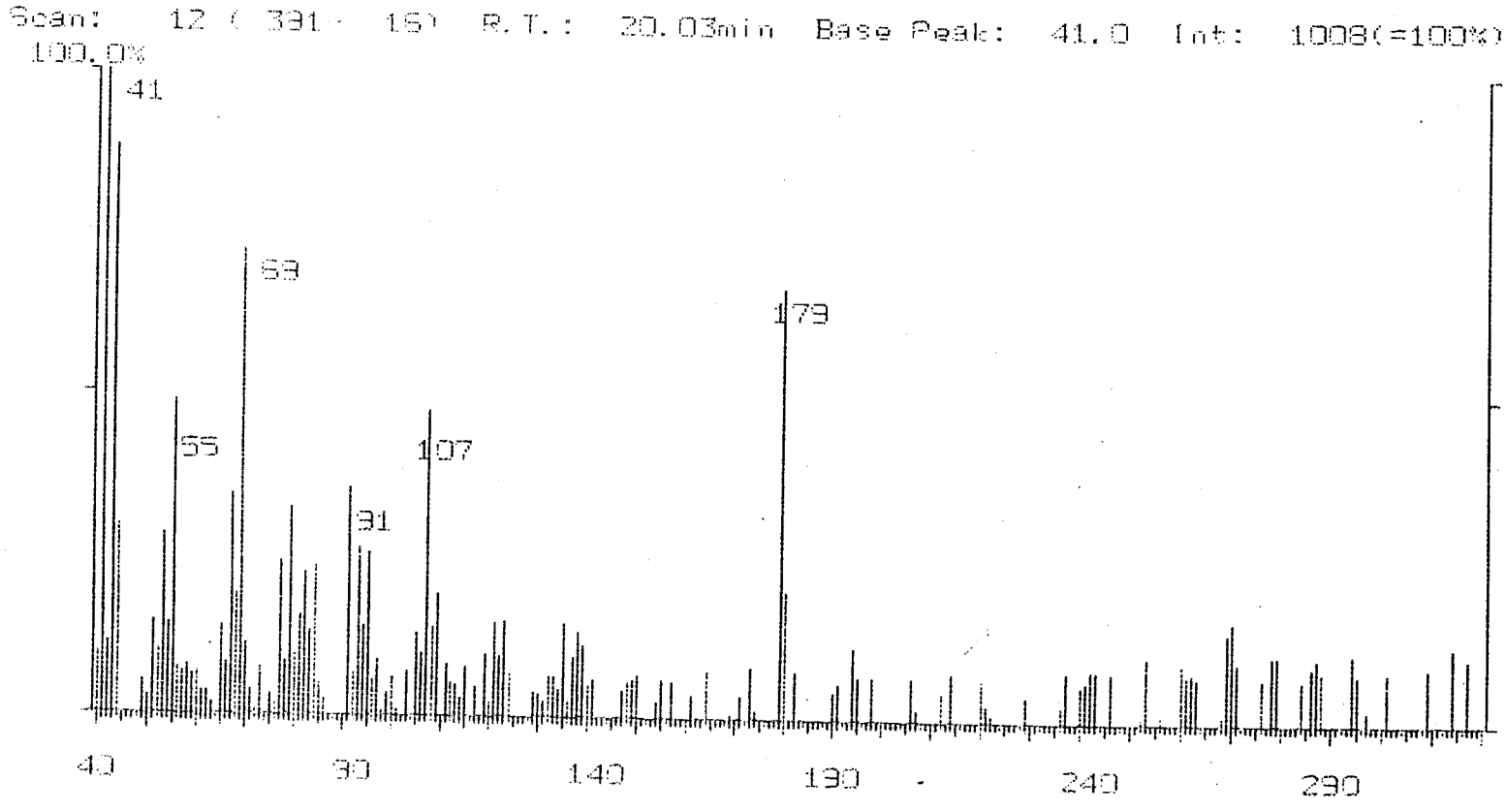


Fig. 4.1.12 Mass spectrum of α-terpinyl acetate

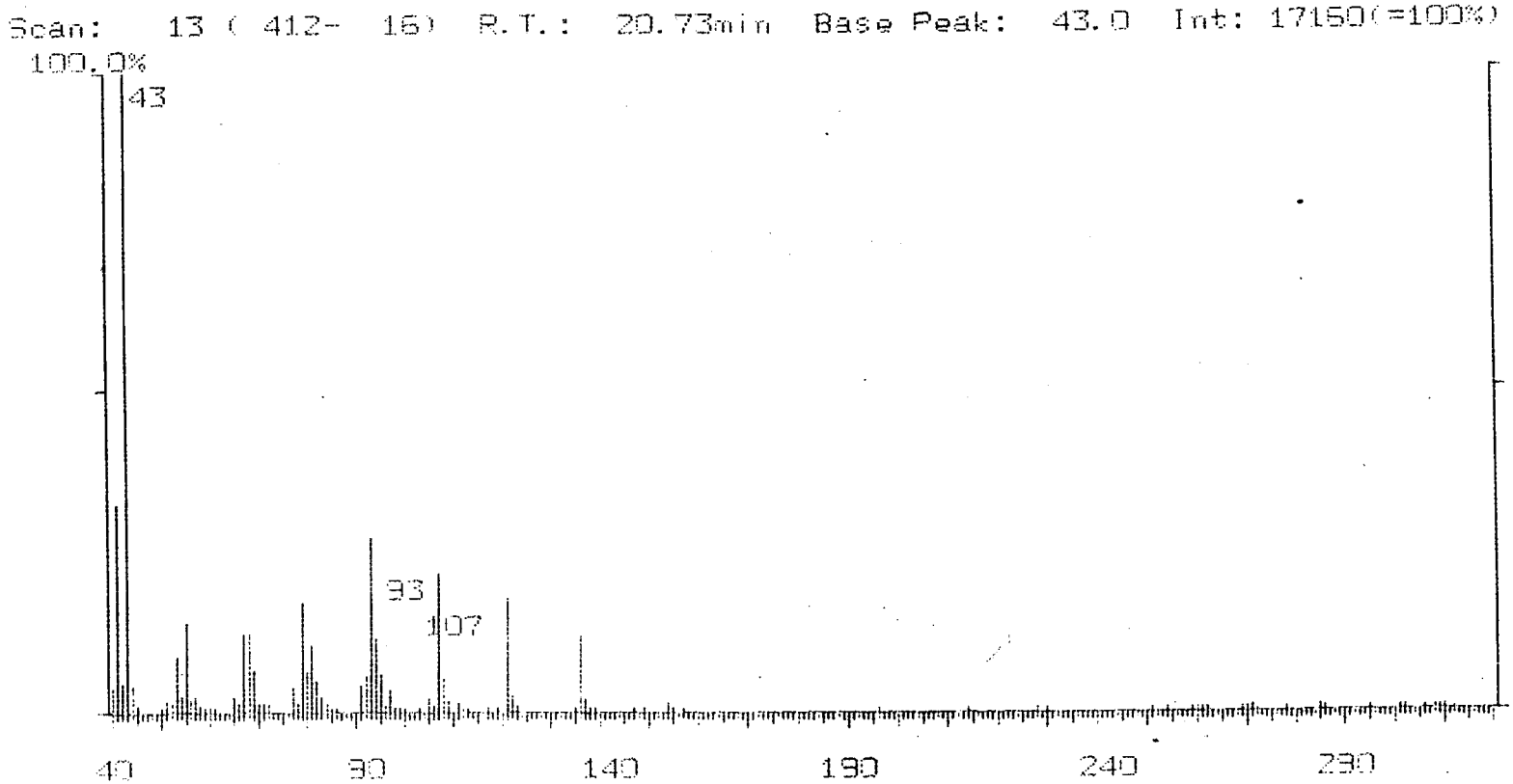


Fig. 4.1.13 Mass spectrum of Unidentified component

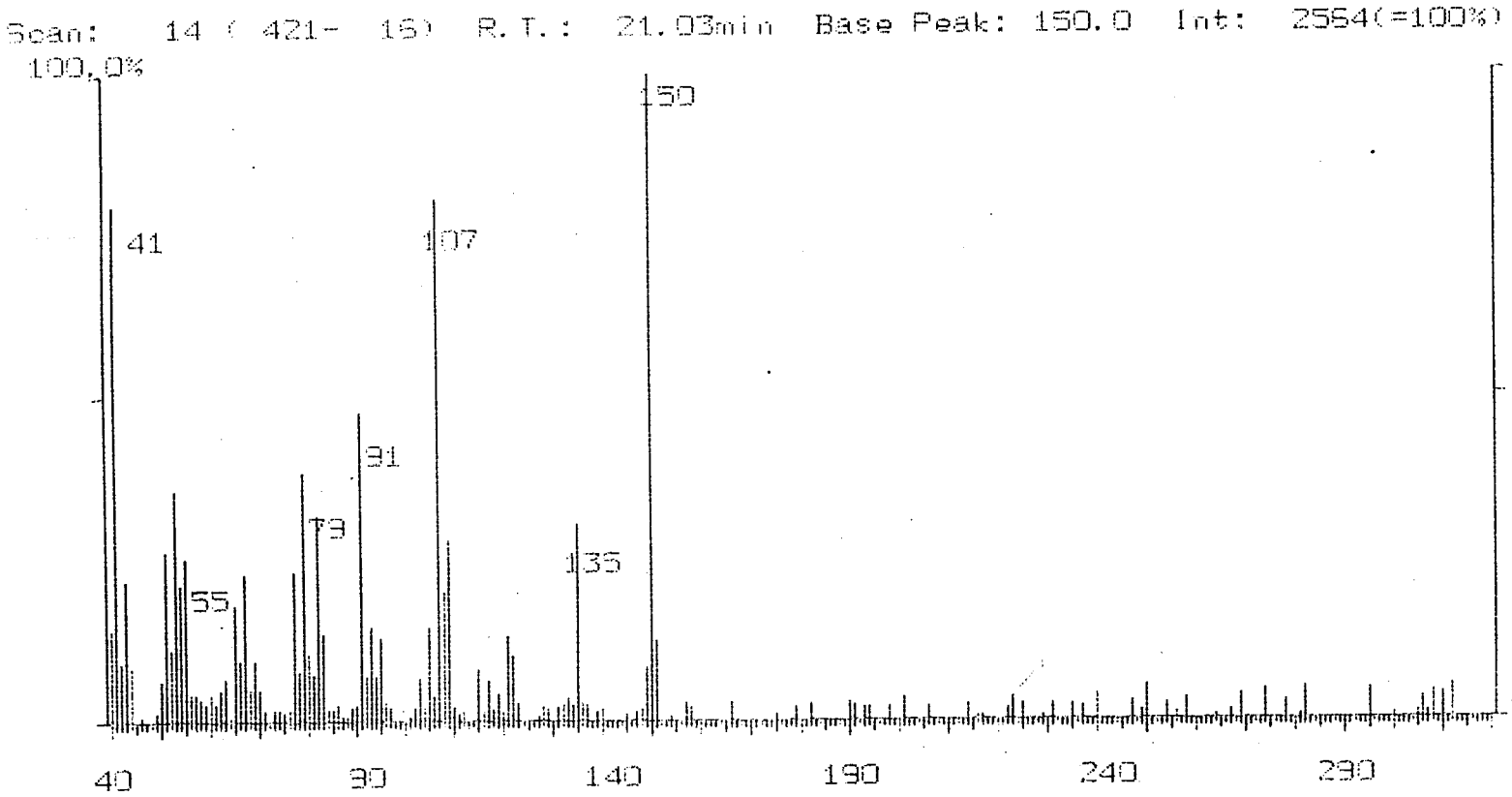


Fig. 4.1.14 Mass spectrum of alloaromadendrene

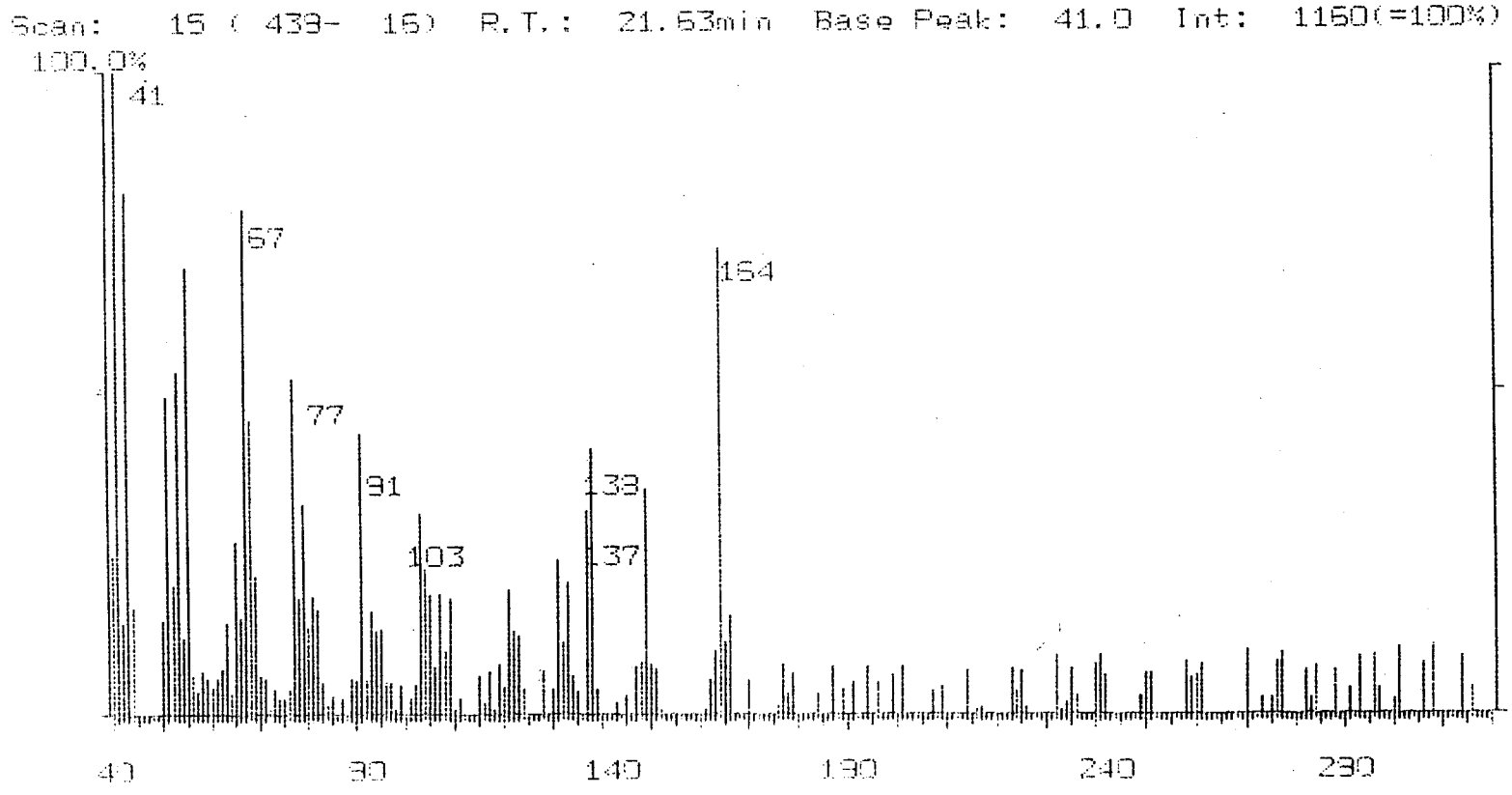


Fig. 4.1.15 Mass spectrum of β-elemene

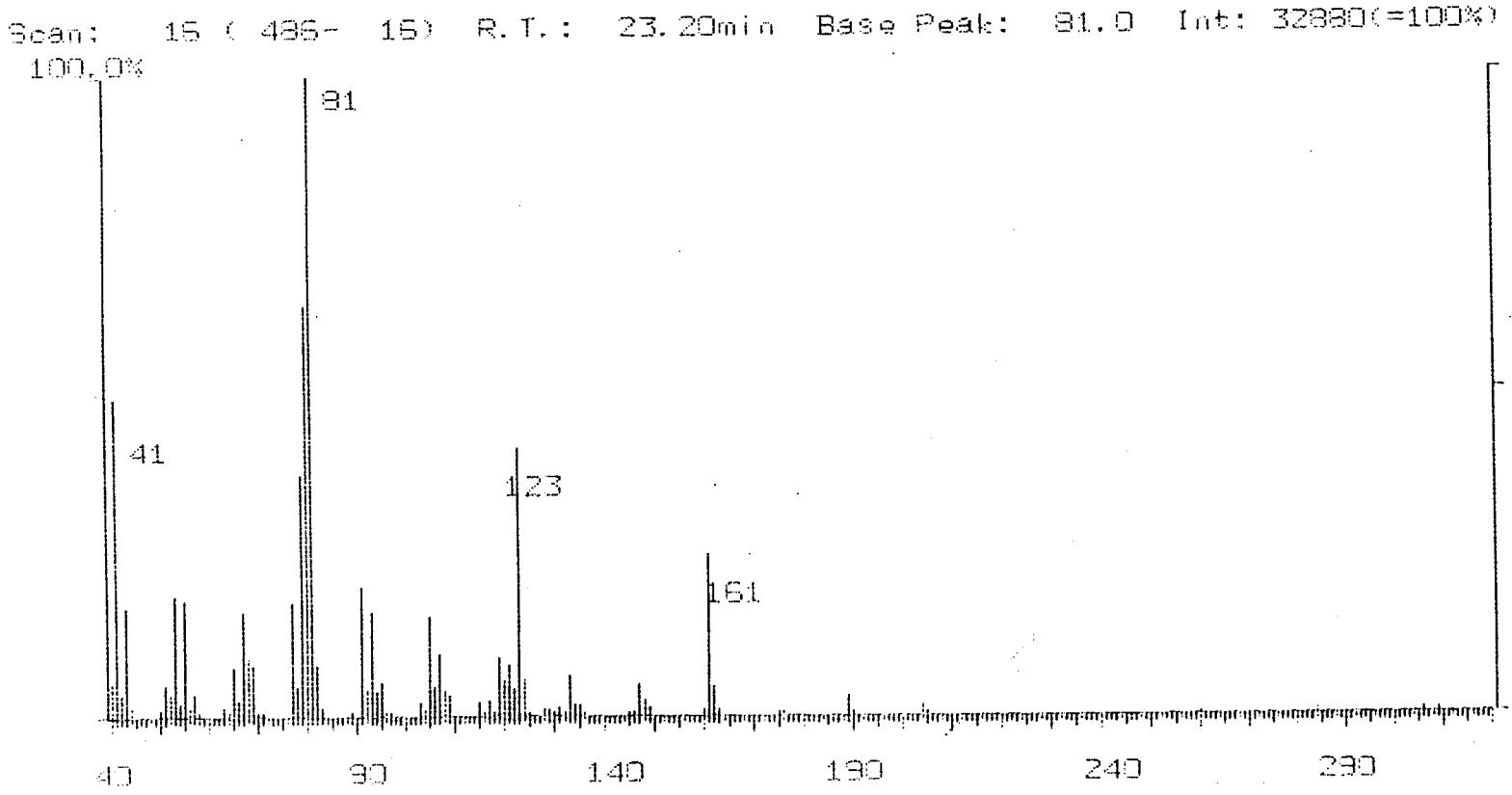


Fig. 4.1.16 Mass spectrum of β-caryophyllene

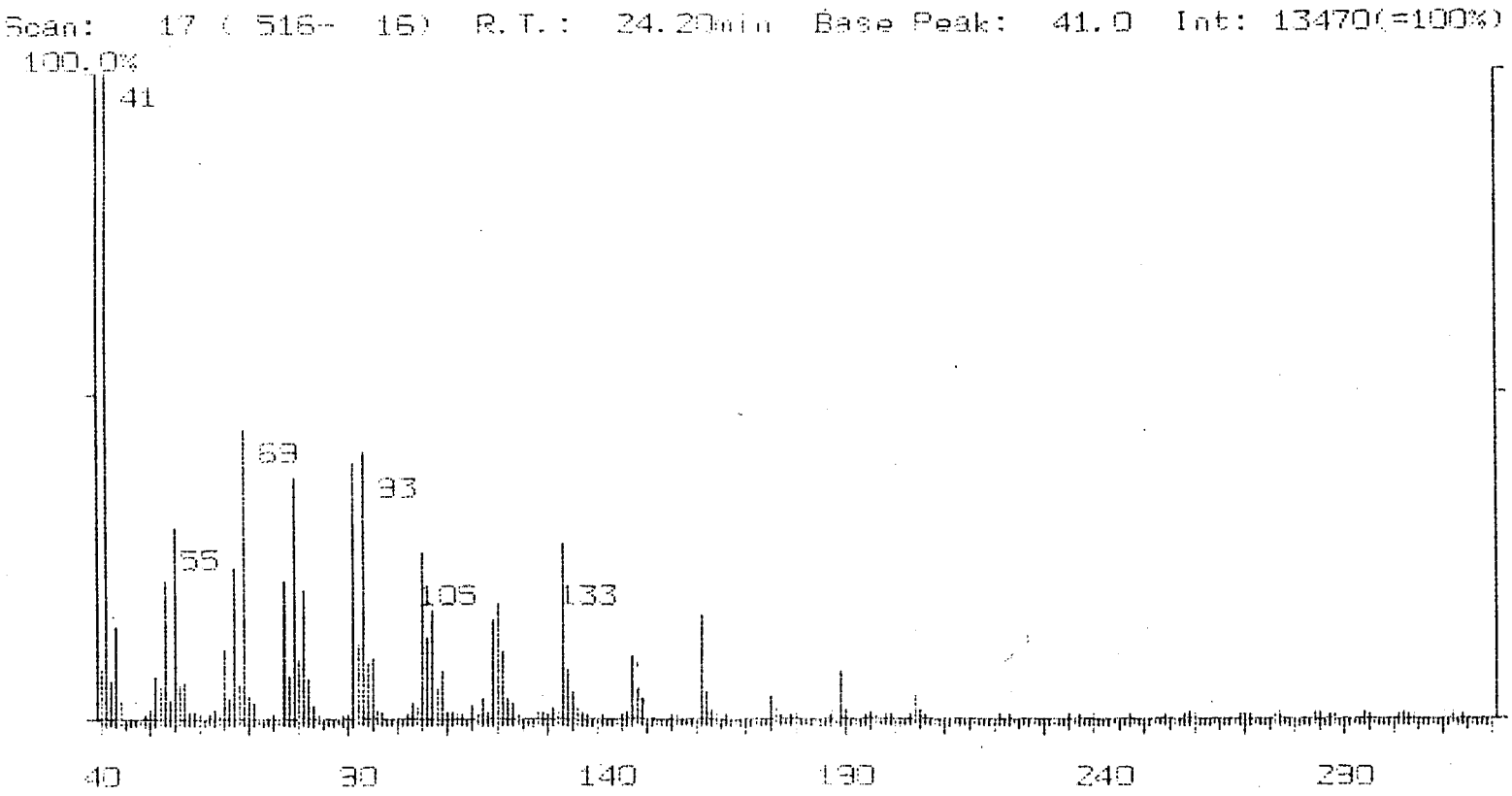


Fig. 4.1.17 Mass spectrum of trans-2-cis-6-nonadien–1-ol

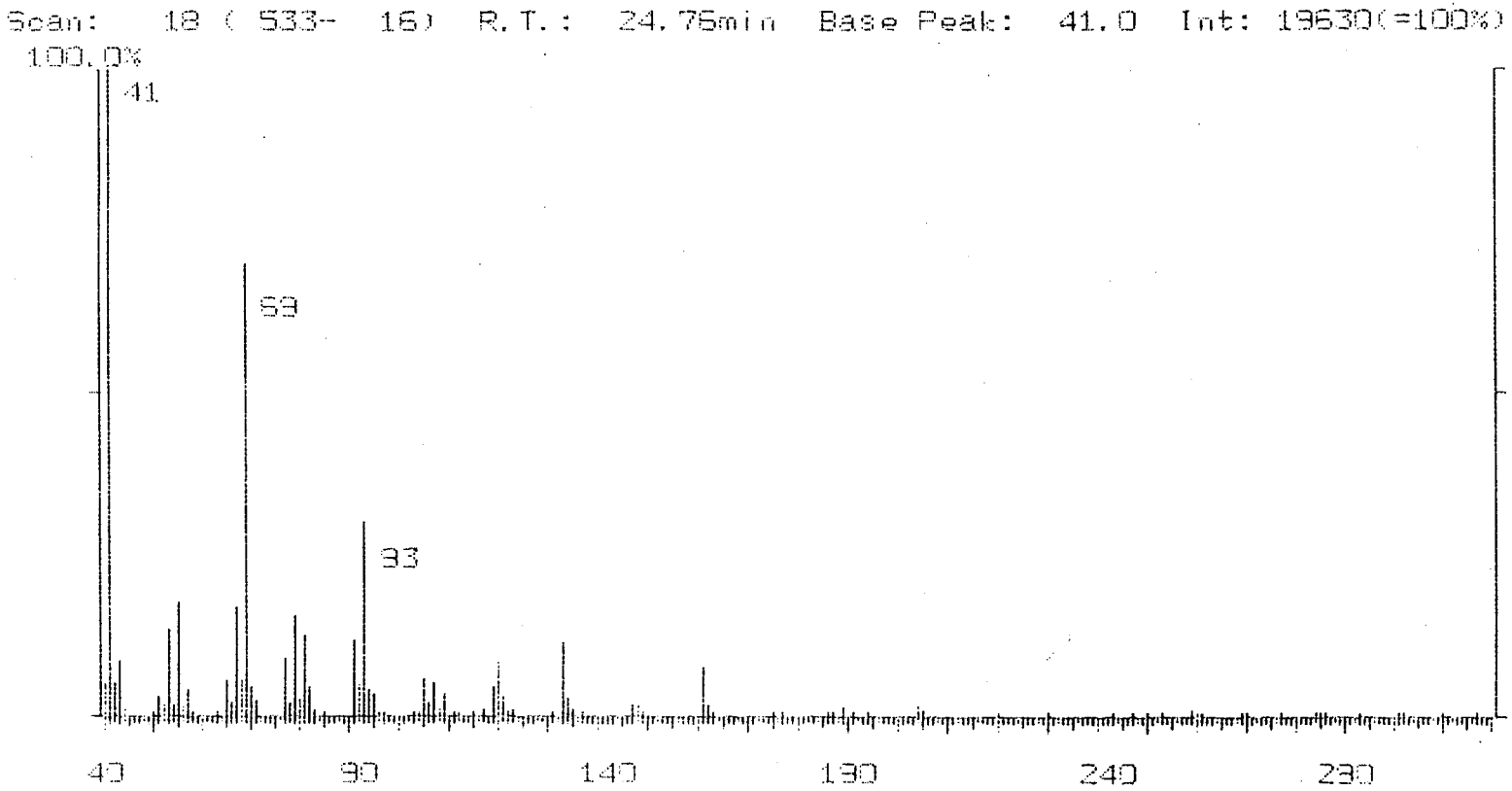


Fig. 4.1.18 Mass spectrum of α-cububene

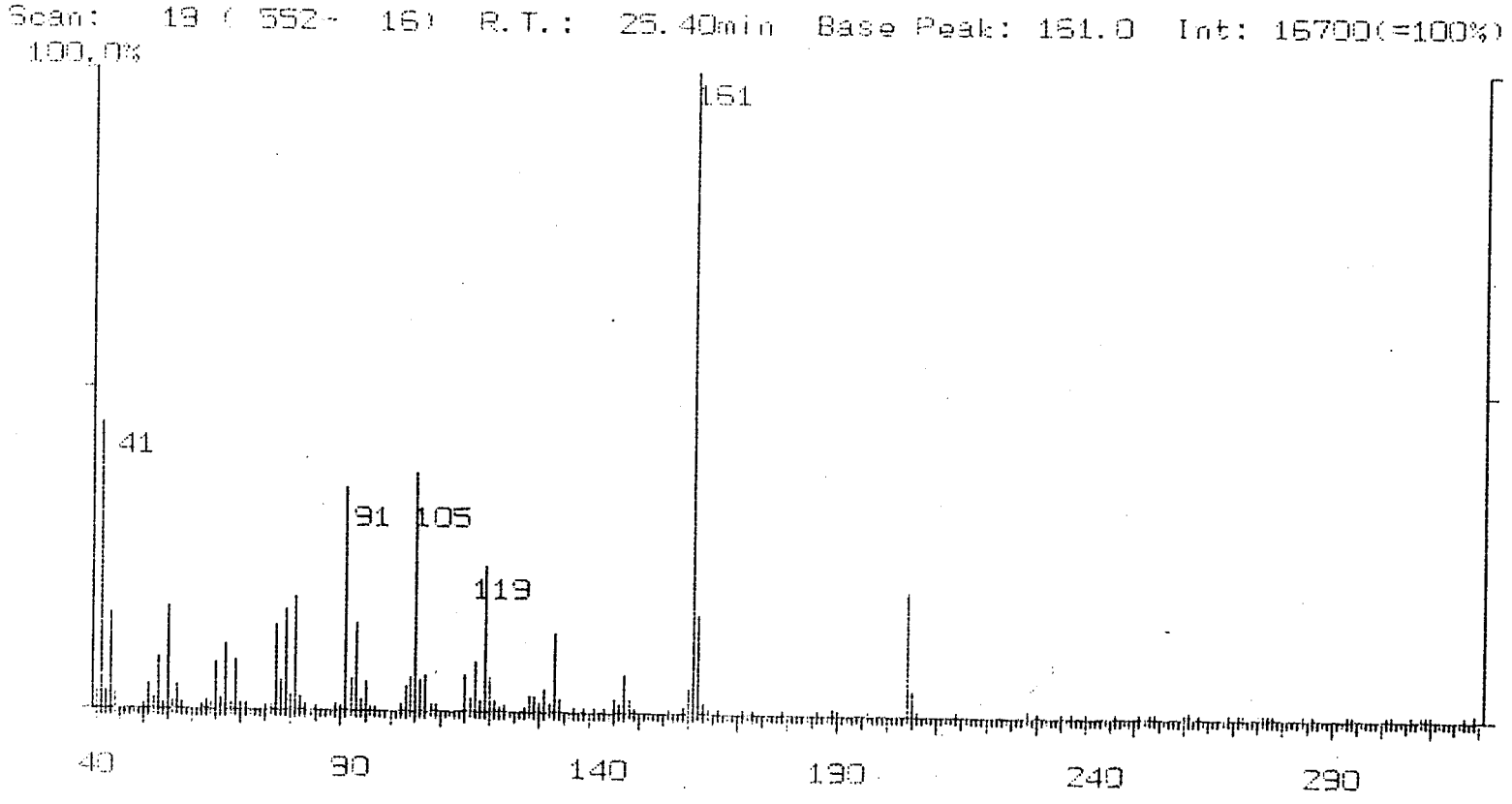


Fig. 4.1.19 Mass spectrum of Unidentified component

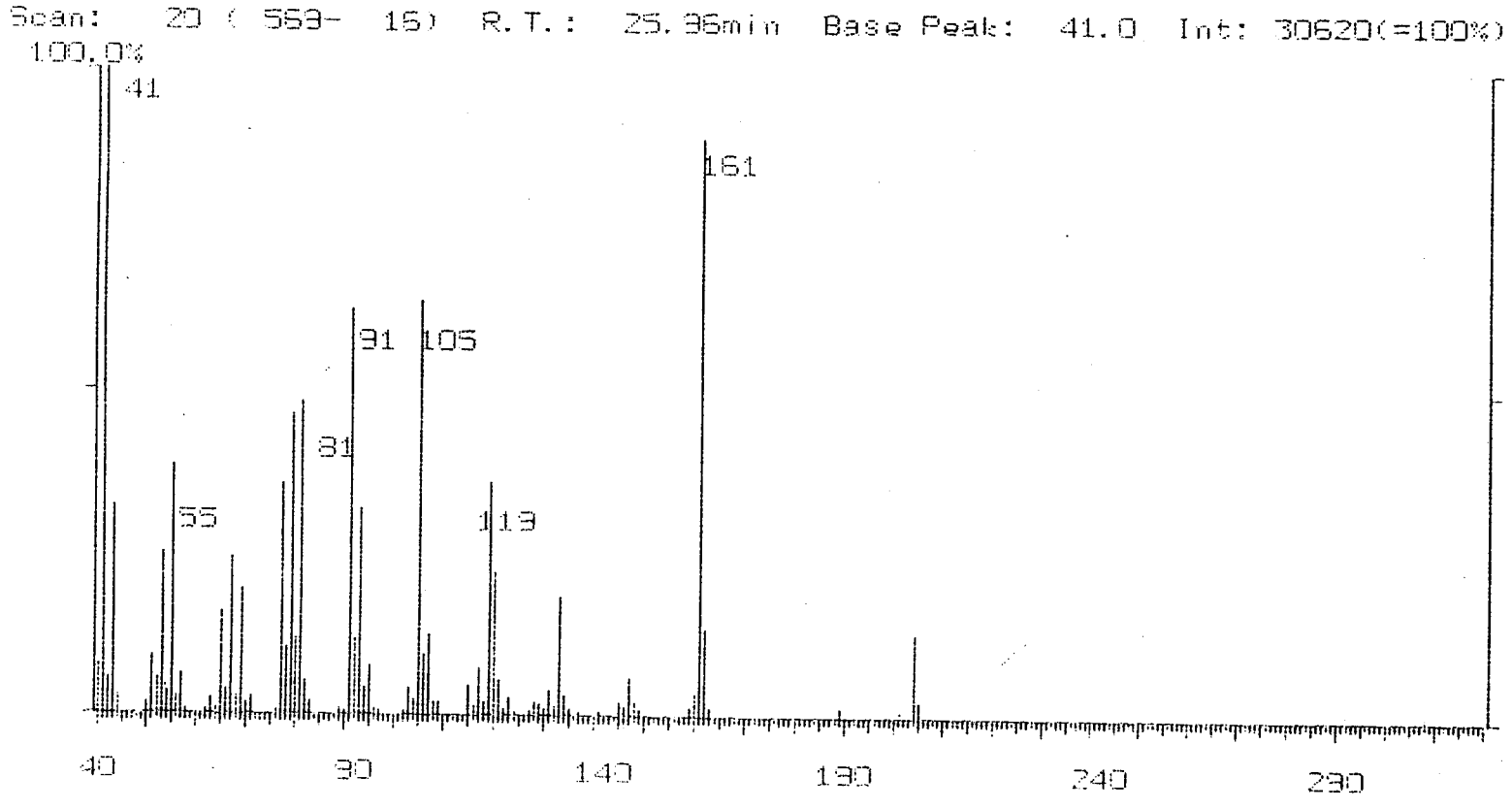


Fig. 4.1.20 Mass spectrum of β-gurgunene

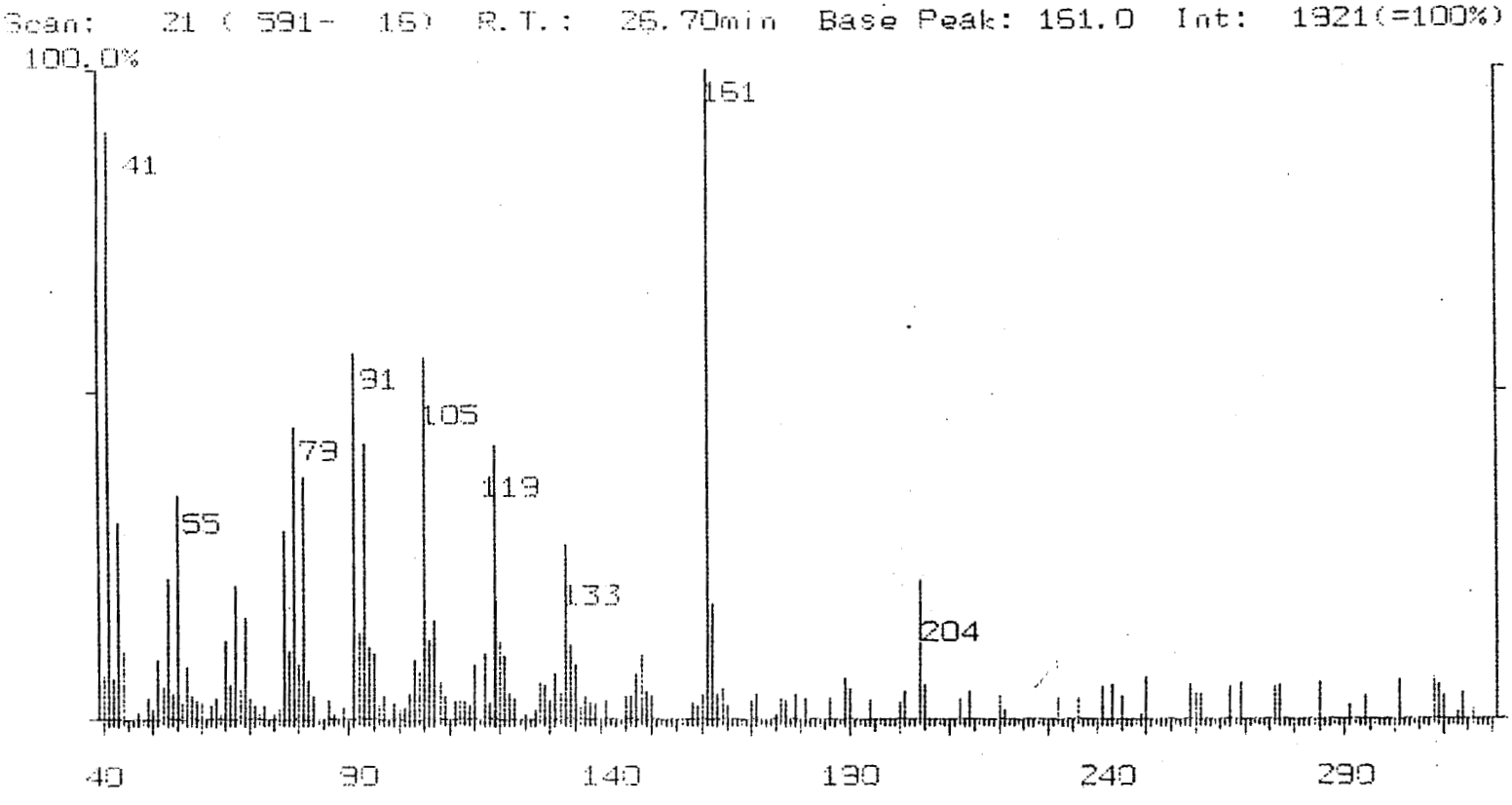


Fig. 4.1.21 Mass spectrum of aromadendrene

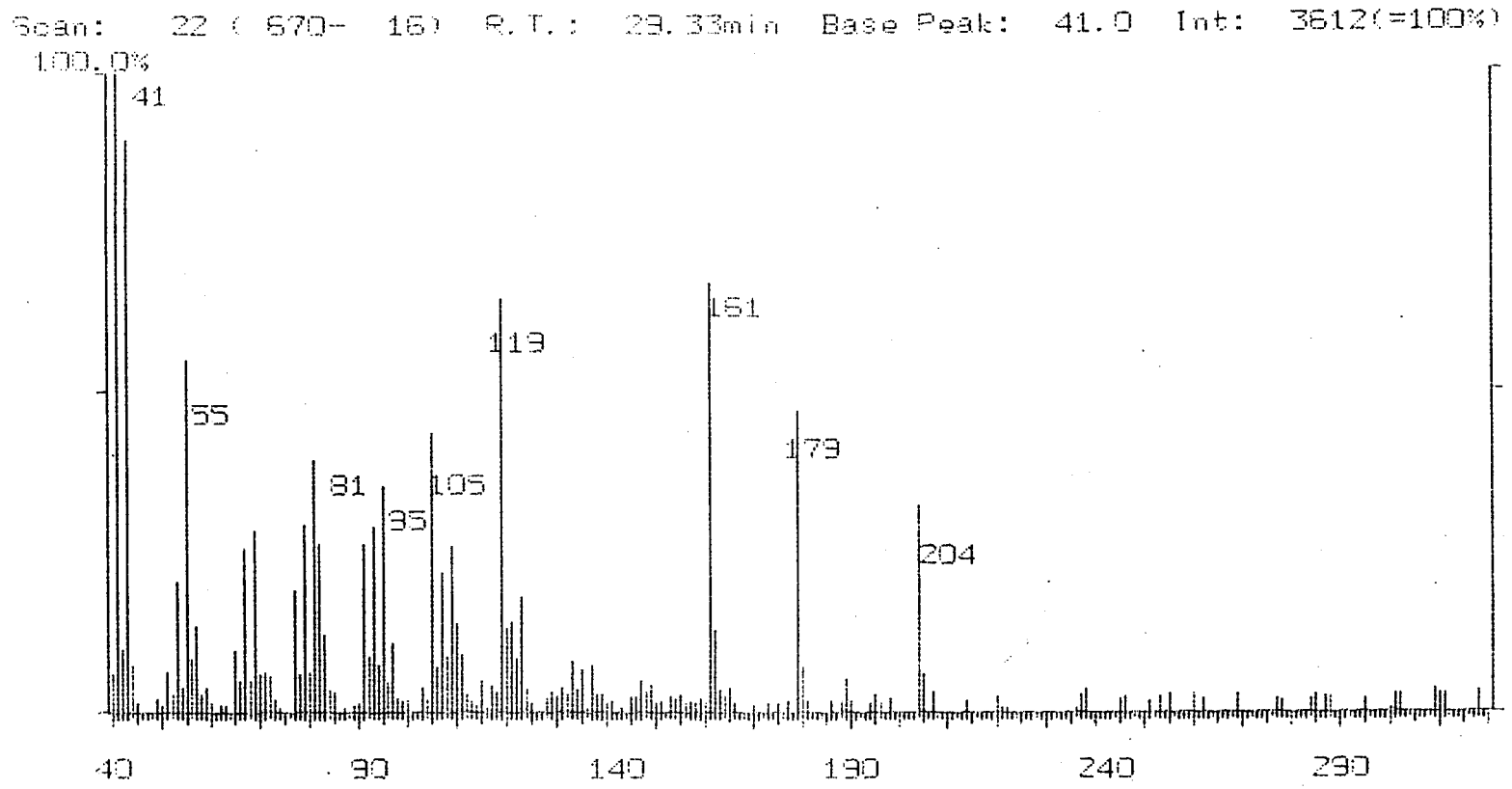


Fig. 4.1.22 Mass spectrum of β-terpineol

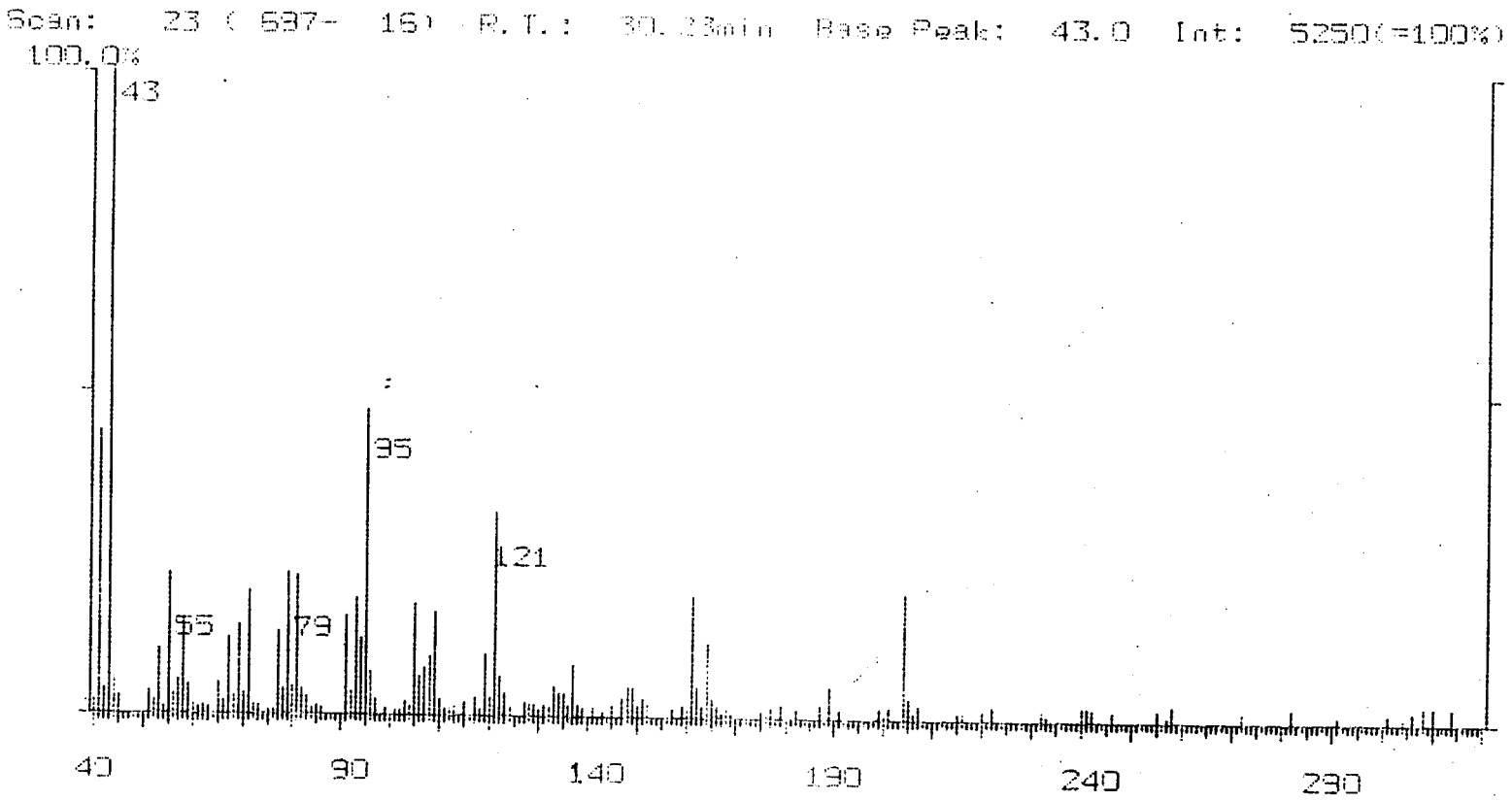


Fig. 4.2.1 Gas chromatogram of the essential oil of *Mentha rotundifolia* (L.) Huds. Somaclonal variant

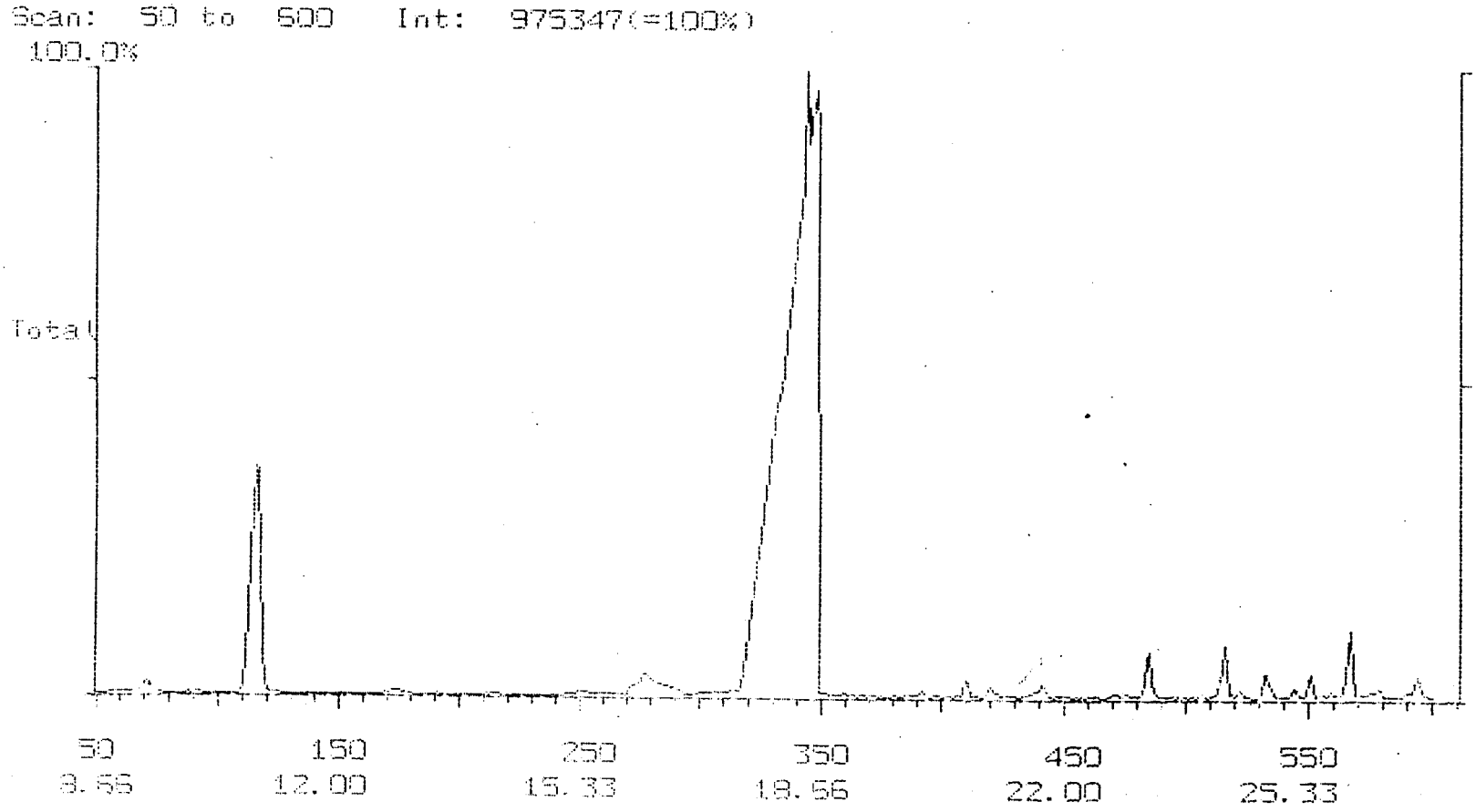


Fig. 4.2.2 Mass spectrum of myrcene

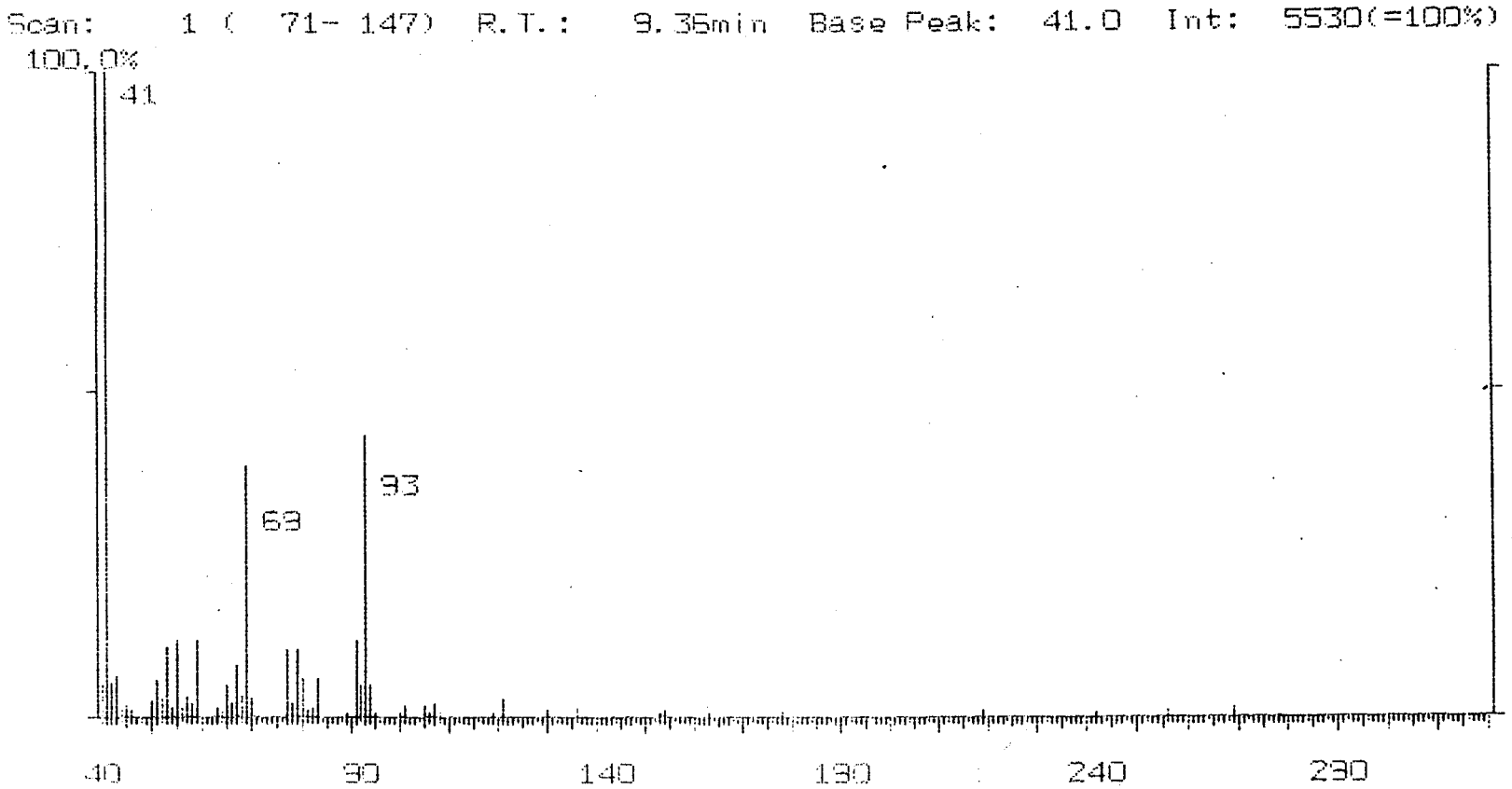


Fig. 4.2.3 Mass spectrum of limonene

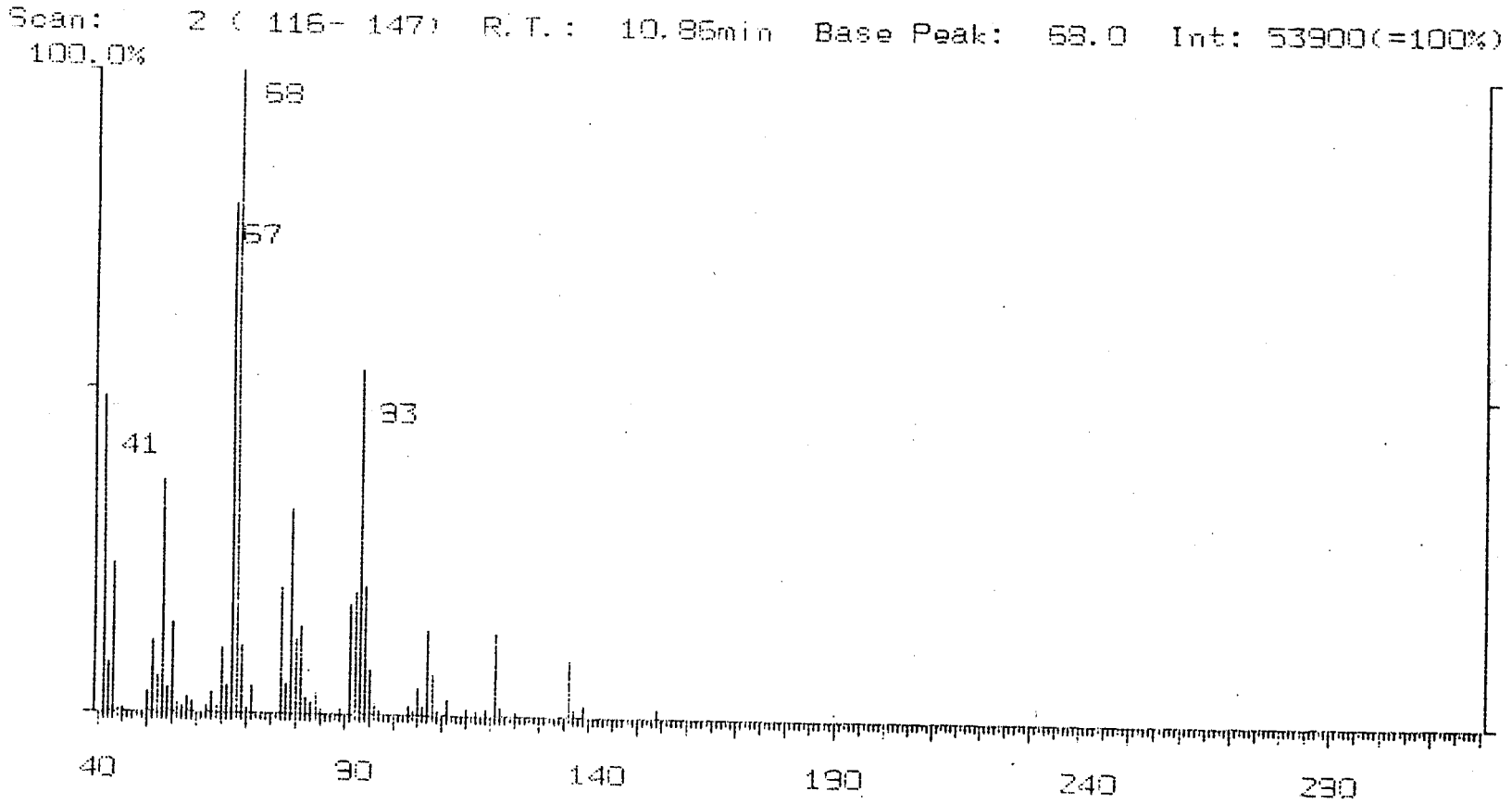


Fig. 4.2.4 Mass spectrum of methyl chavicol

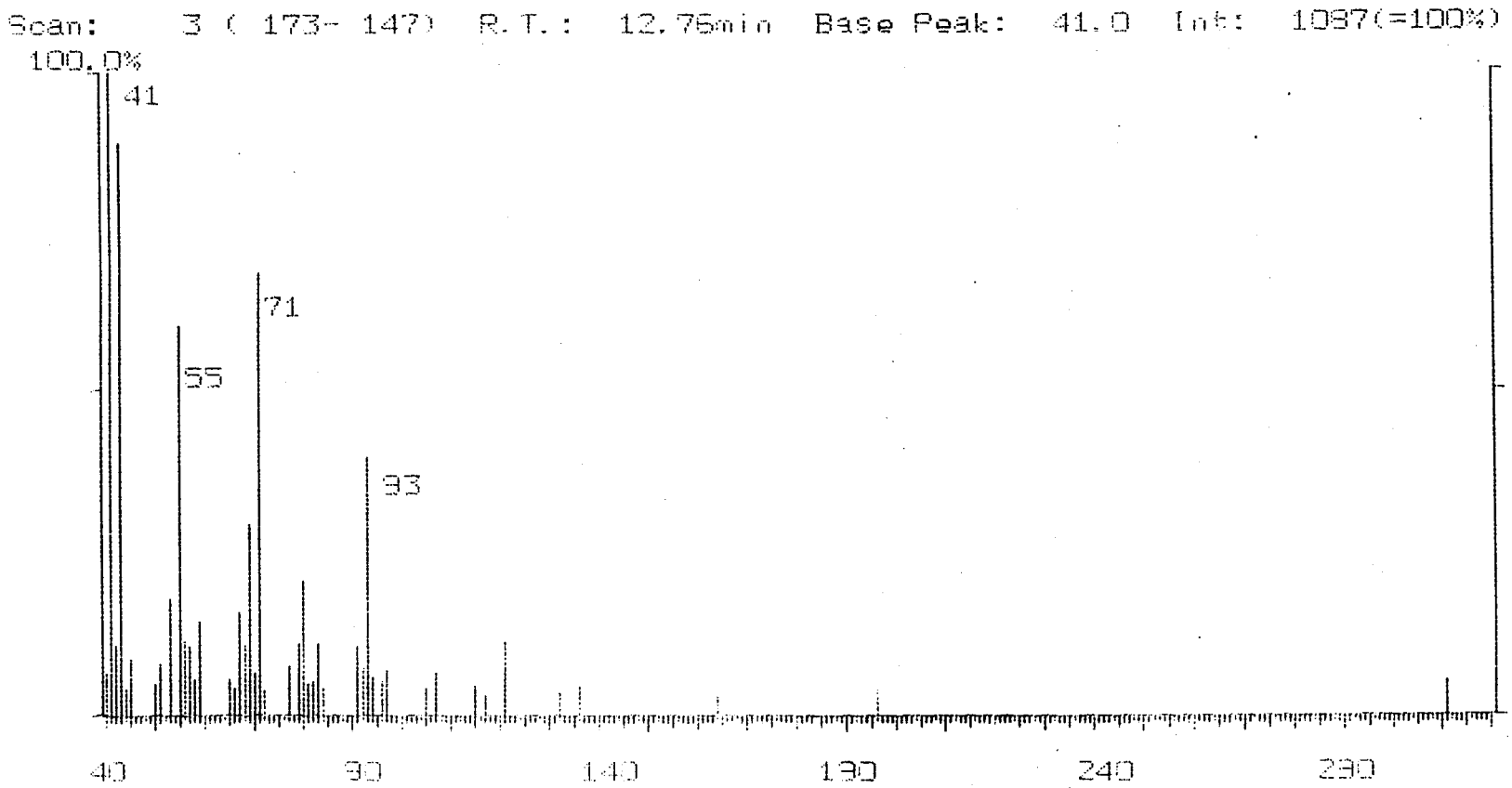


Fig. 4.2.5 Mass spectrum of isoborneol

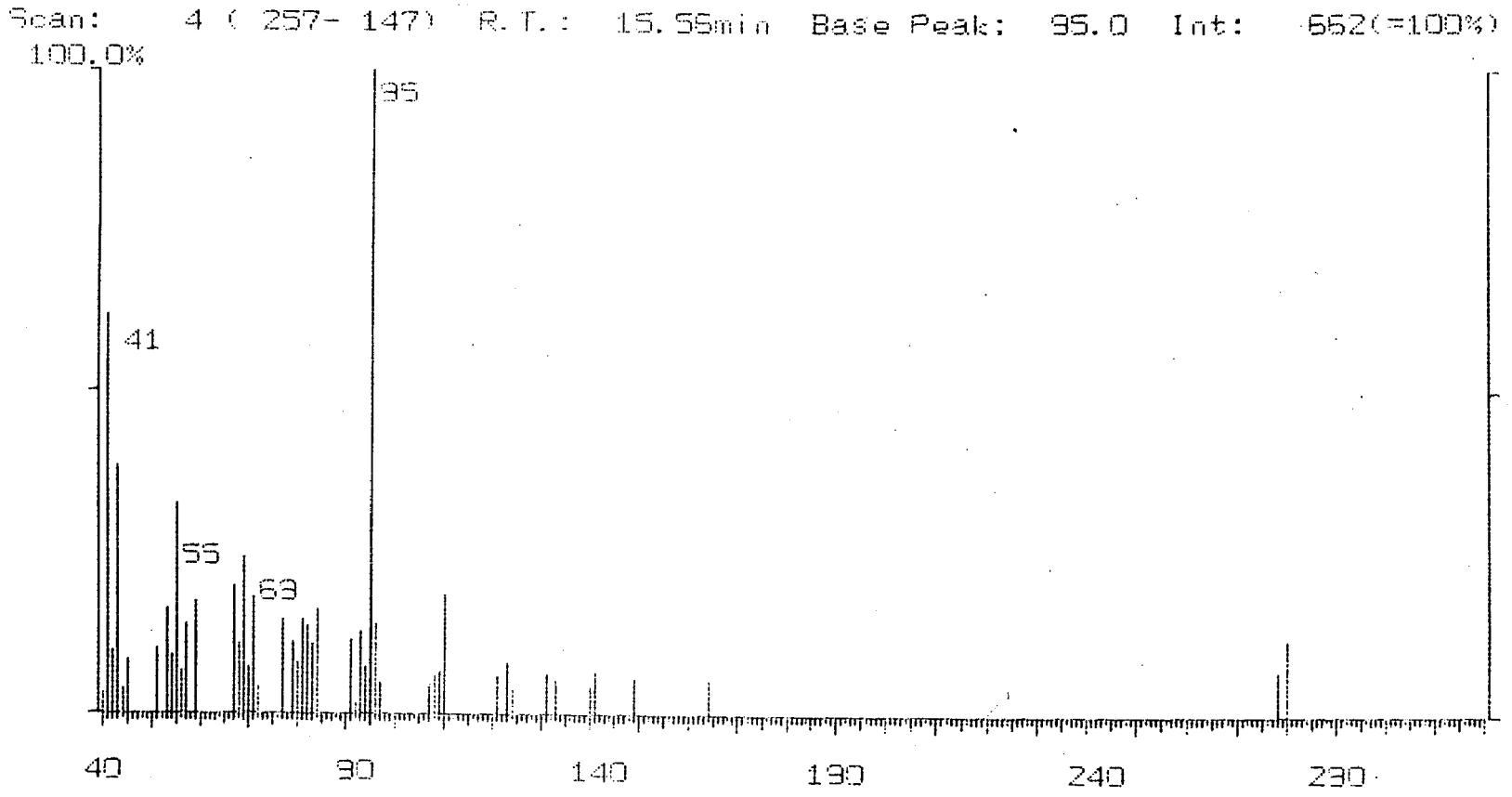


Fig. 4.2.6 Mass spectrum of cis-6-nonenal

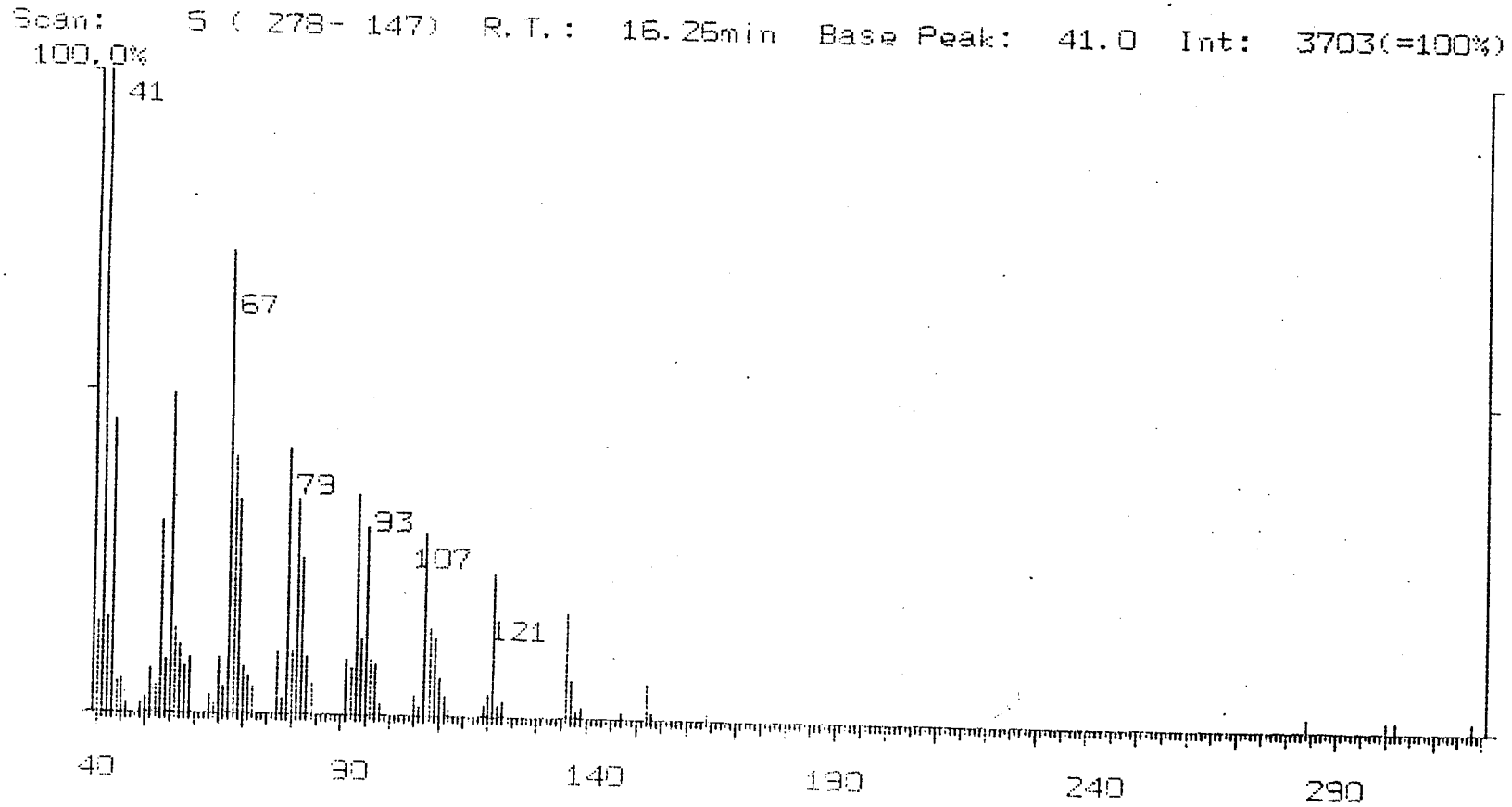


Fig. 4.2.7 Mass spectrum of carvone

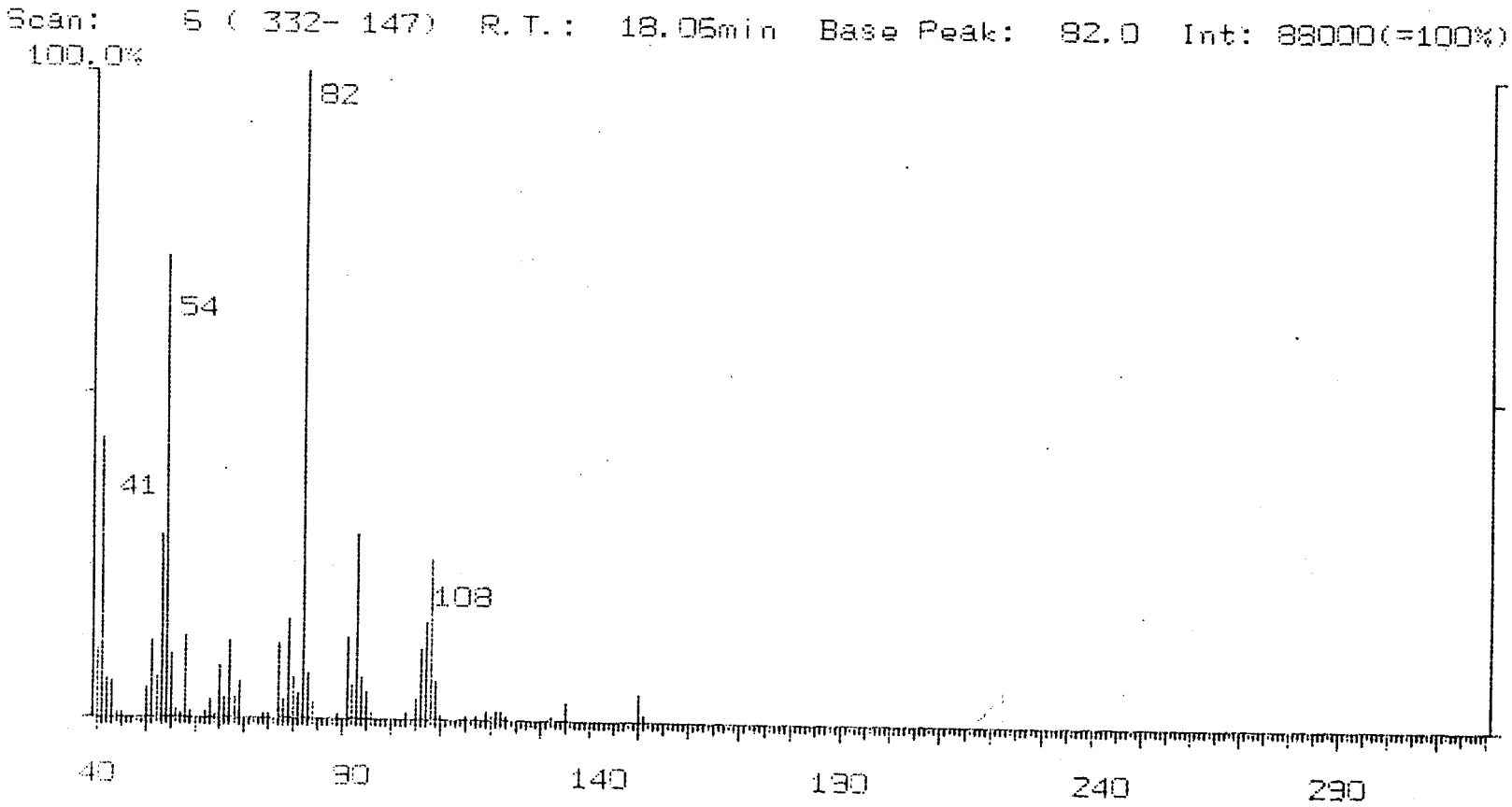


Fig. 4.2.8 Mass spectrum of Unidentified component

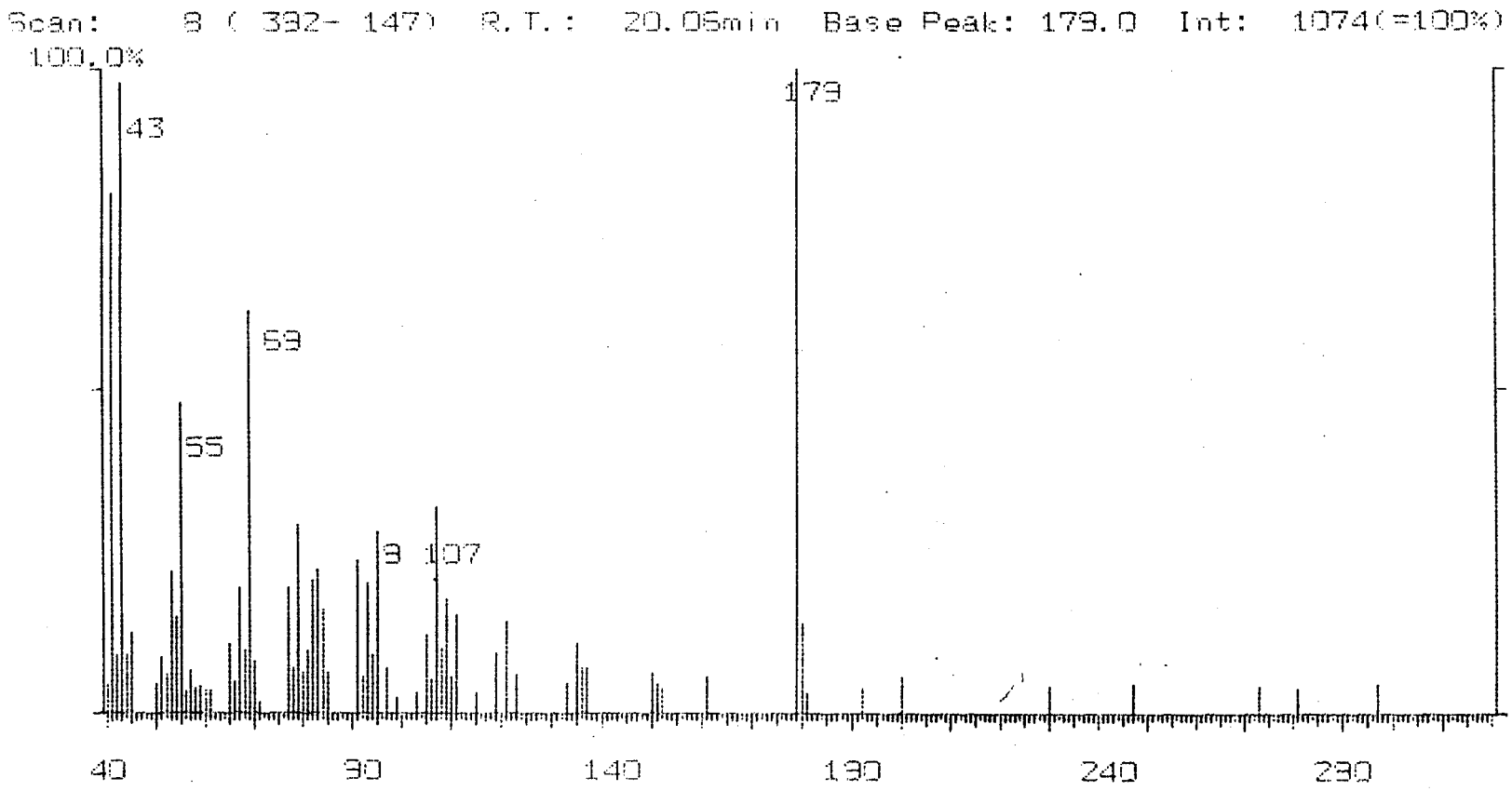


Fig. 4.2.9 Mass spectrum of α-terpinyl acetate

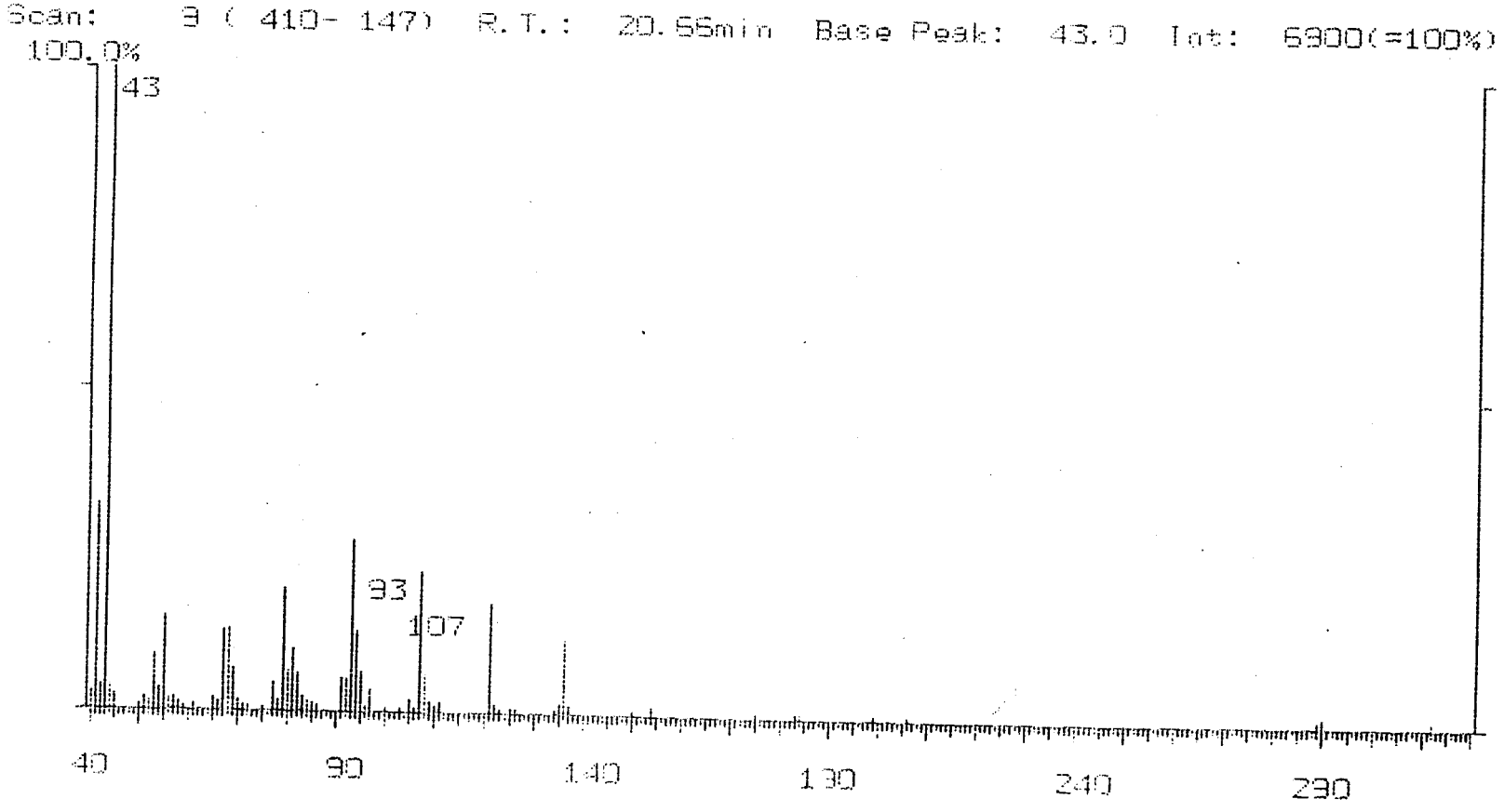


Fig. 4.2.10 Mass spectrum of 2,3 diethyl-6-methyl pyrazine

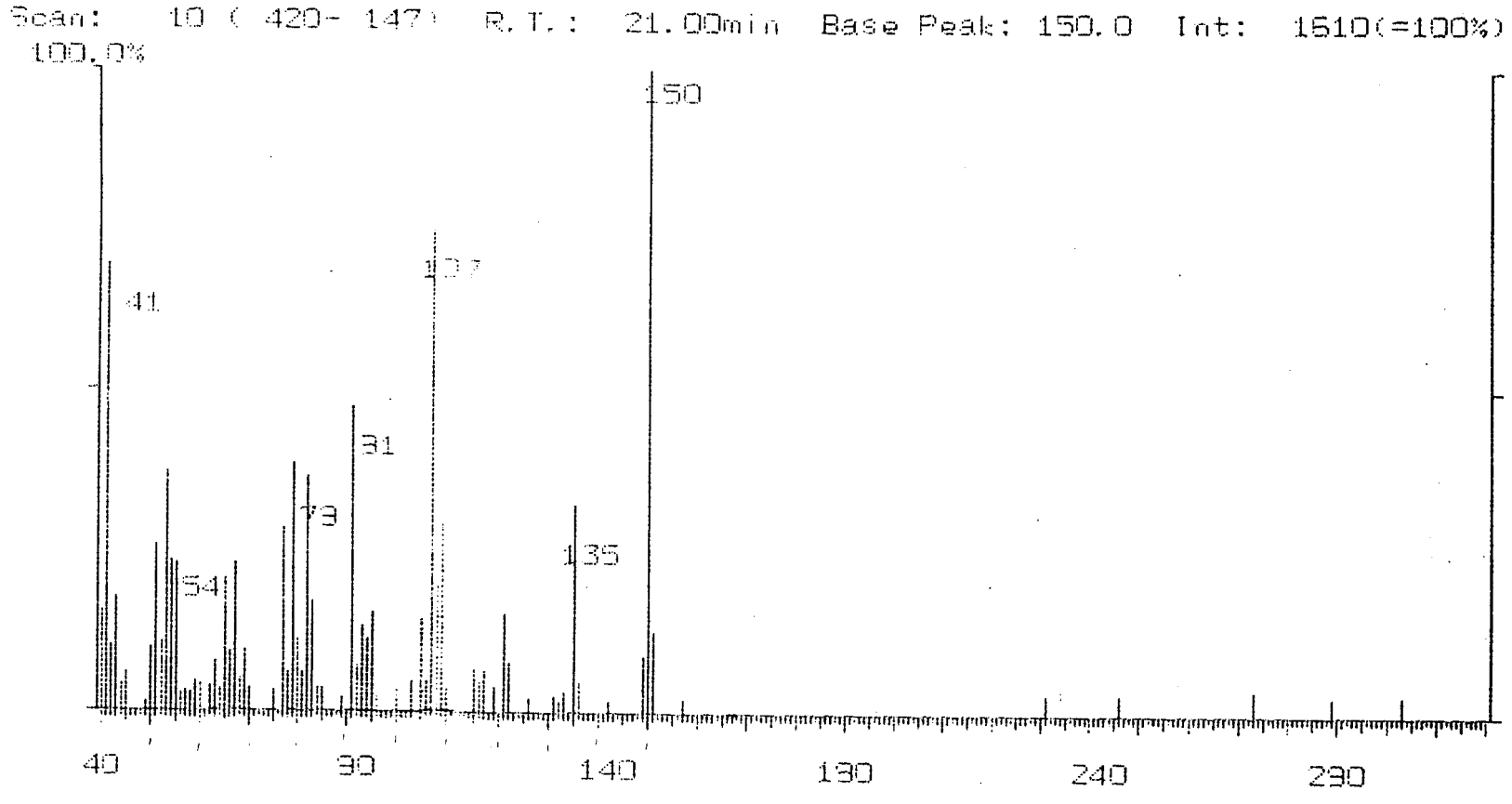


Fig. 4.2.11 Mass spectrum of p-mentha trans 2,8 dien-1-ol

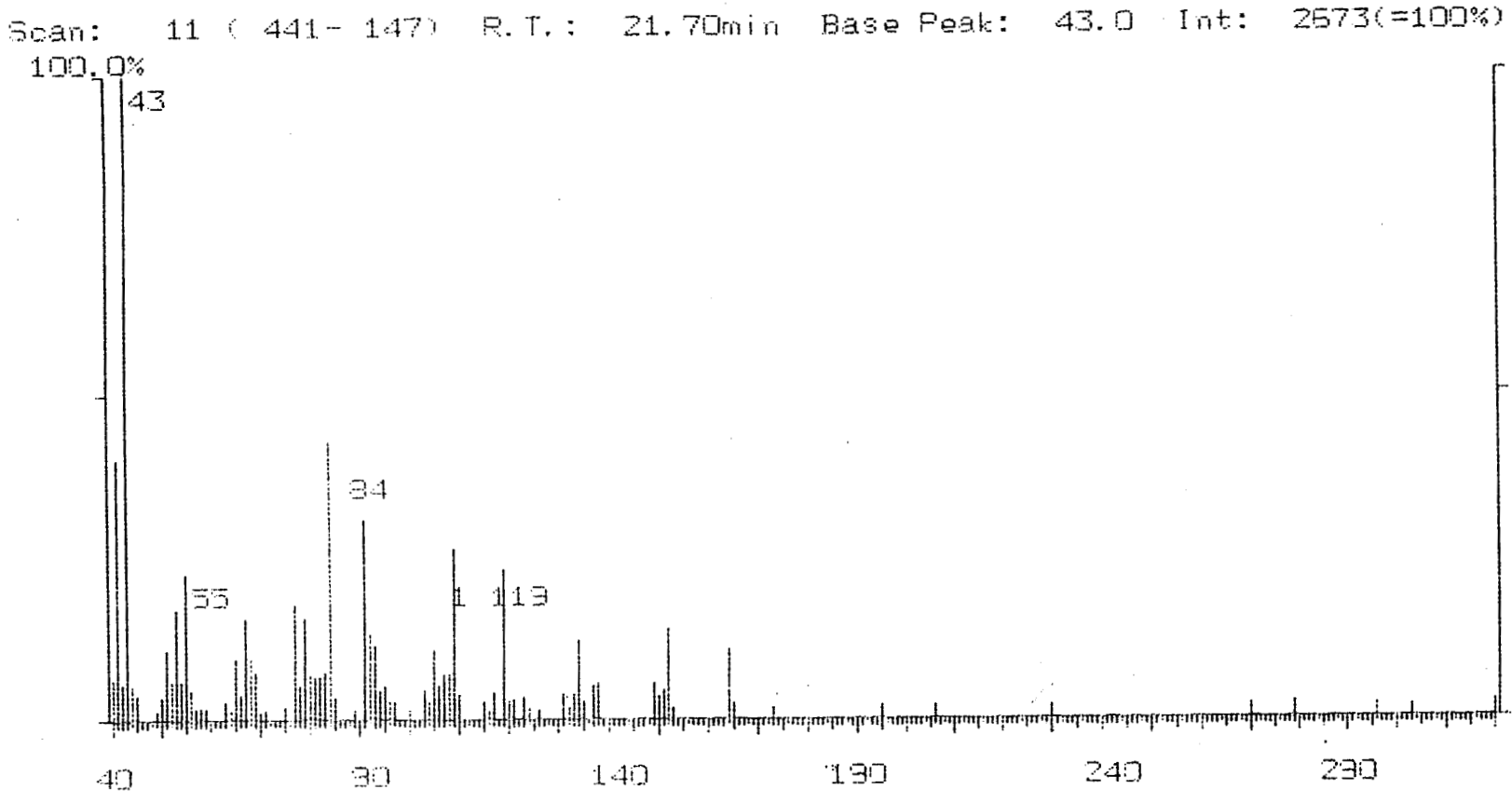


Fig. 4.2.12 Mass spectrum of β-elemene

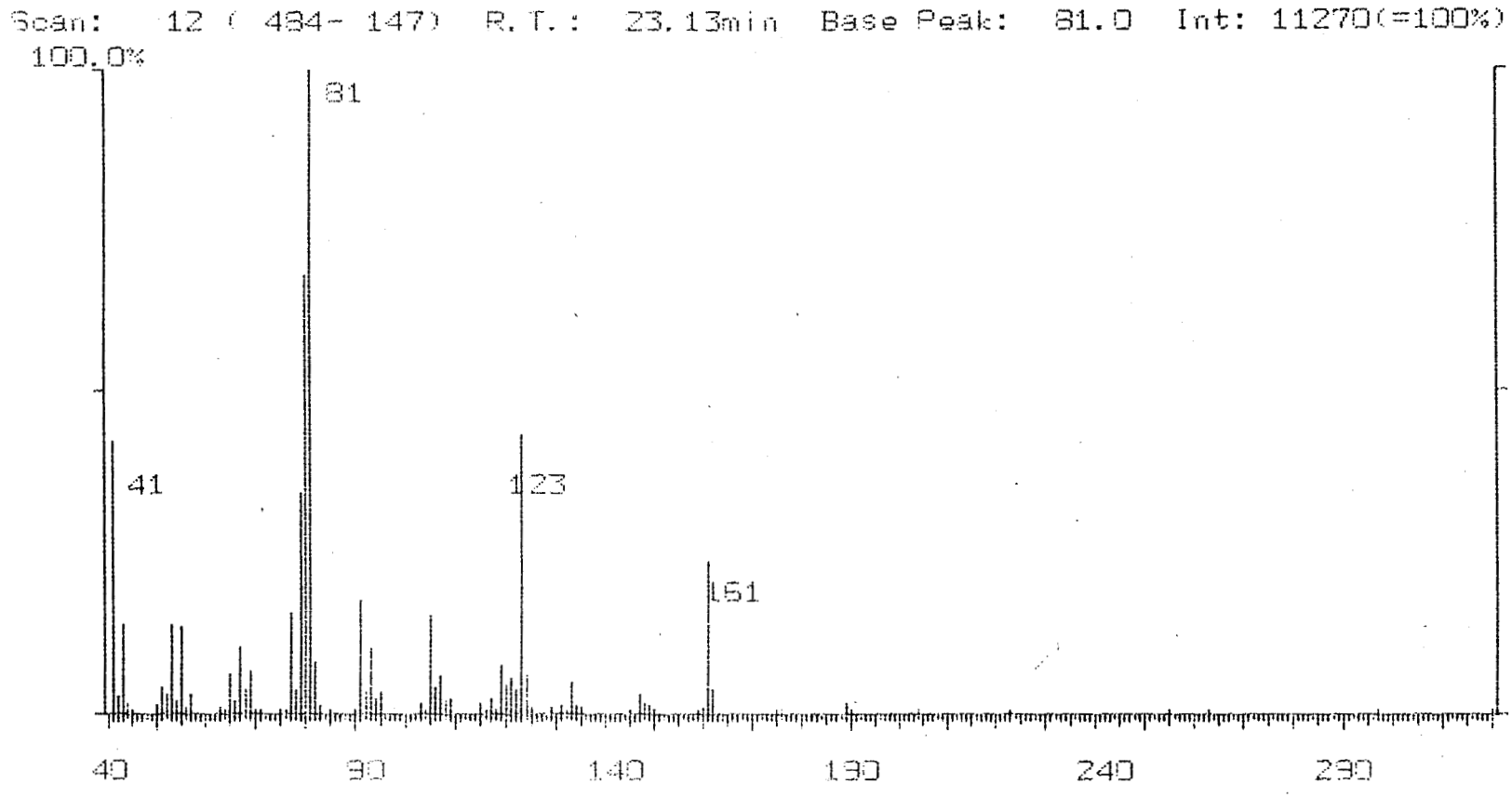


Fig. 4.2.13 Mass spectrum of γ-caryophyllene

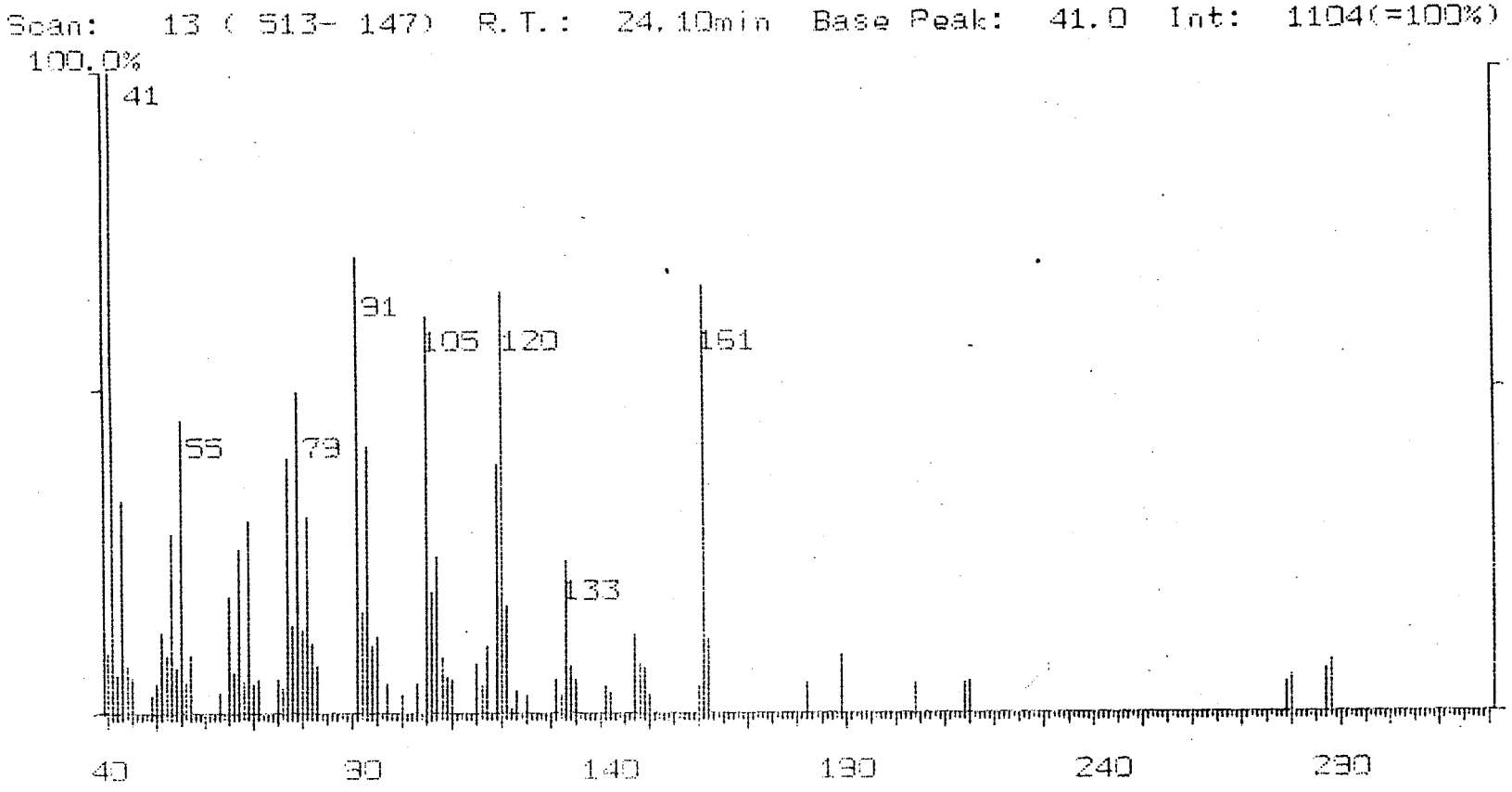


Fig. 4.2.14 Mass spectrum of trans-2-cis-6-nonadien–1-ol

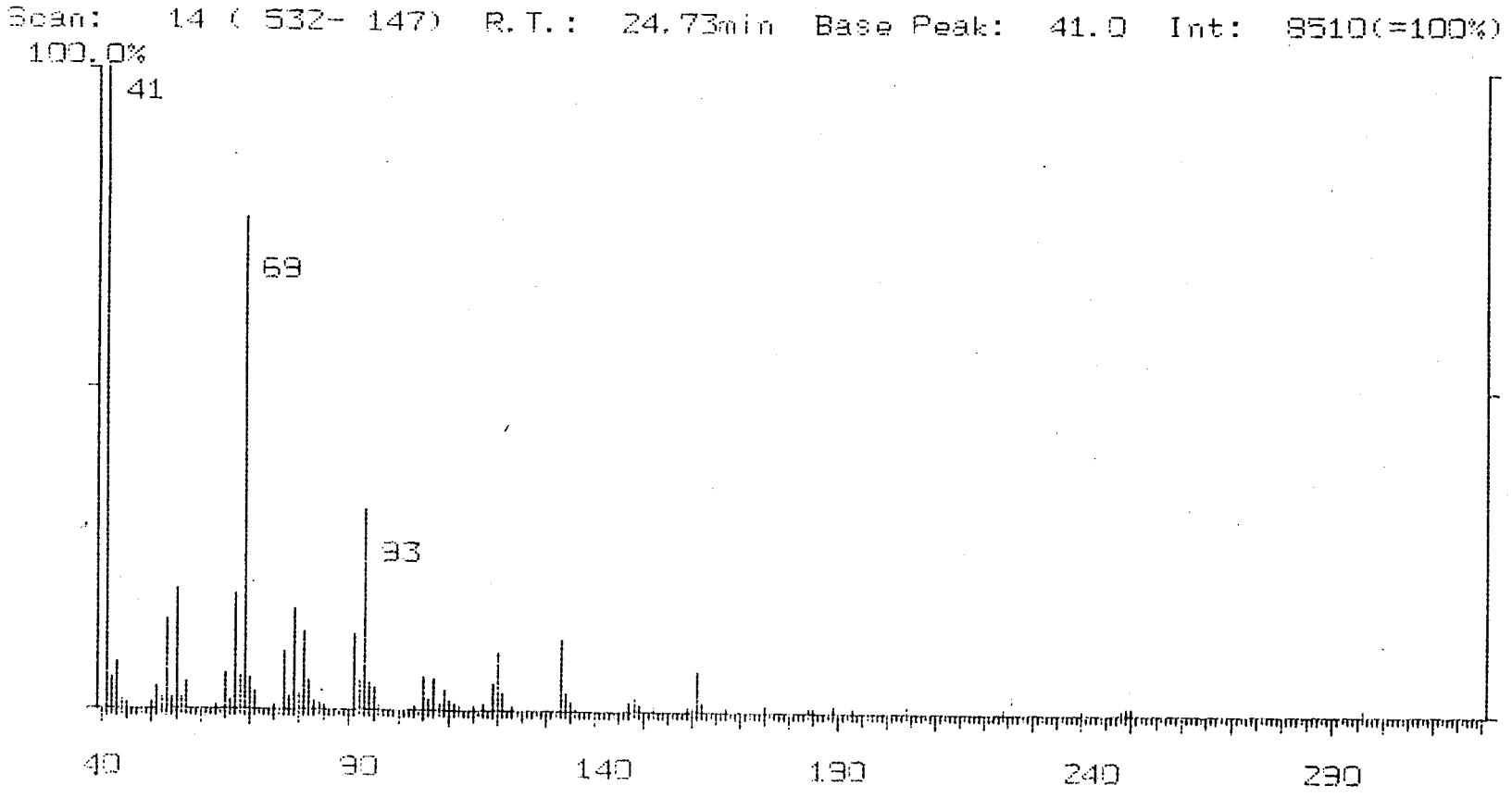


Fig. 4.2.15 Mass spectrum of α-cububene

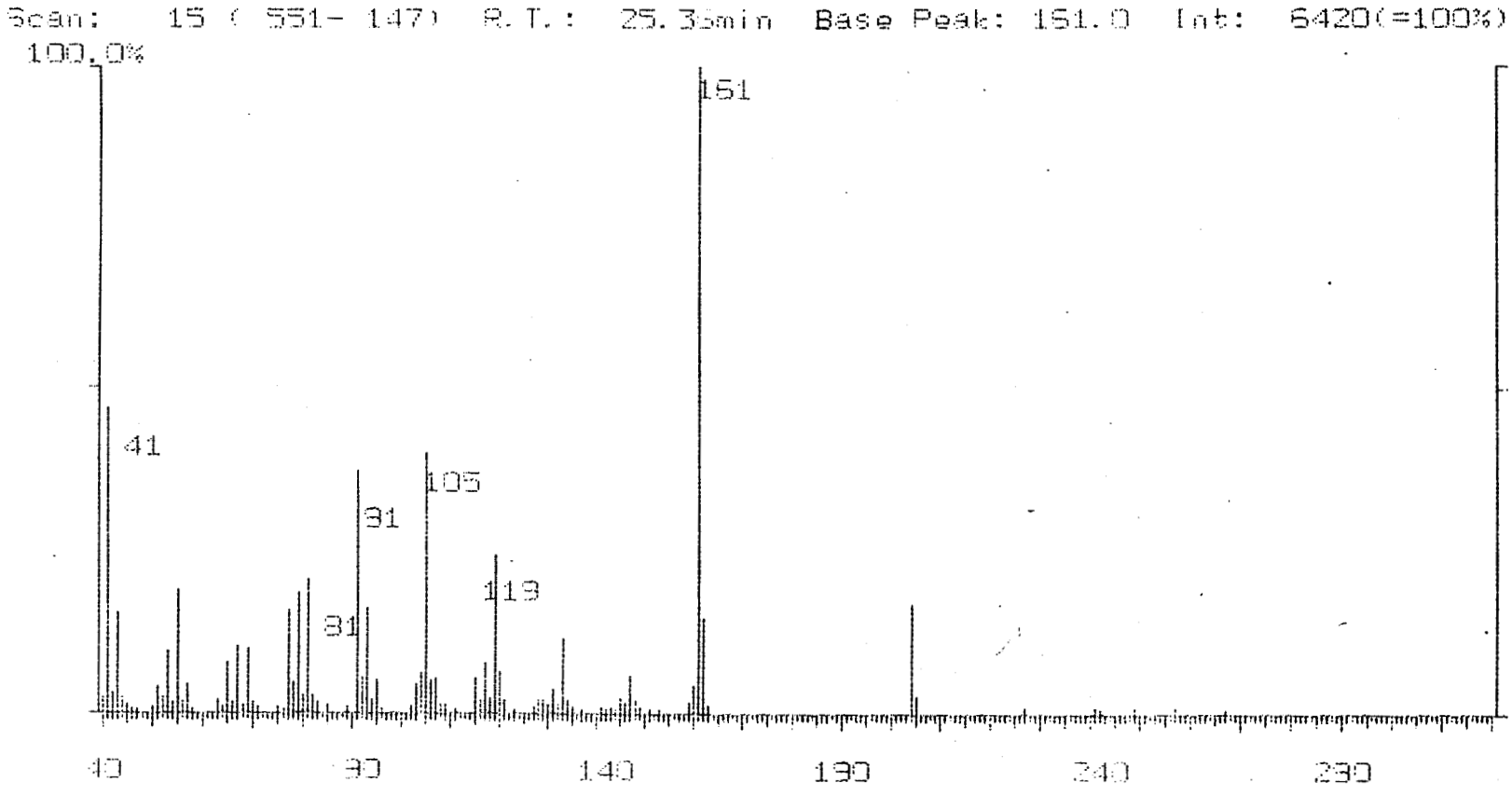


Fig. 4.2.16 Mass spectrum of β-bisabolene

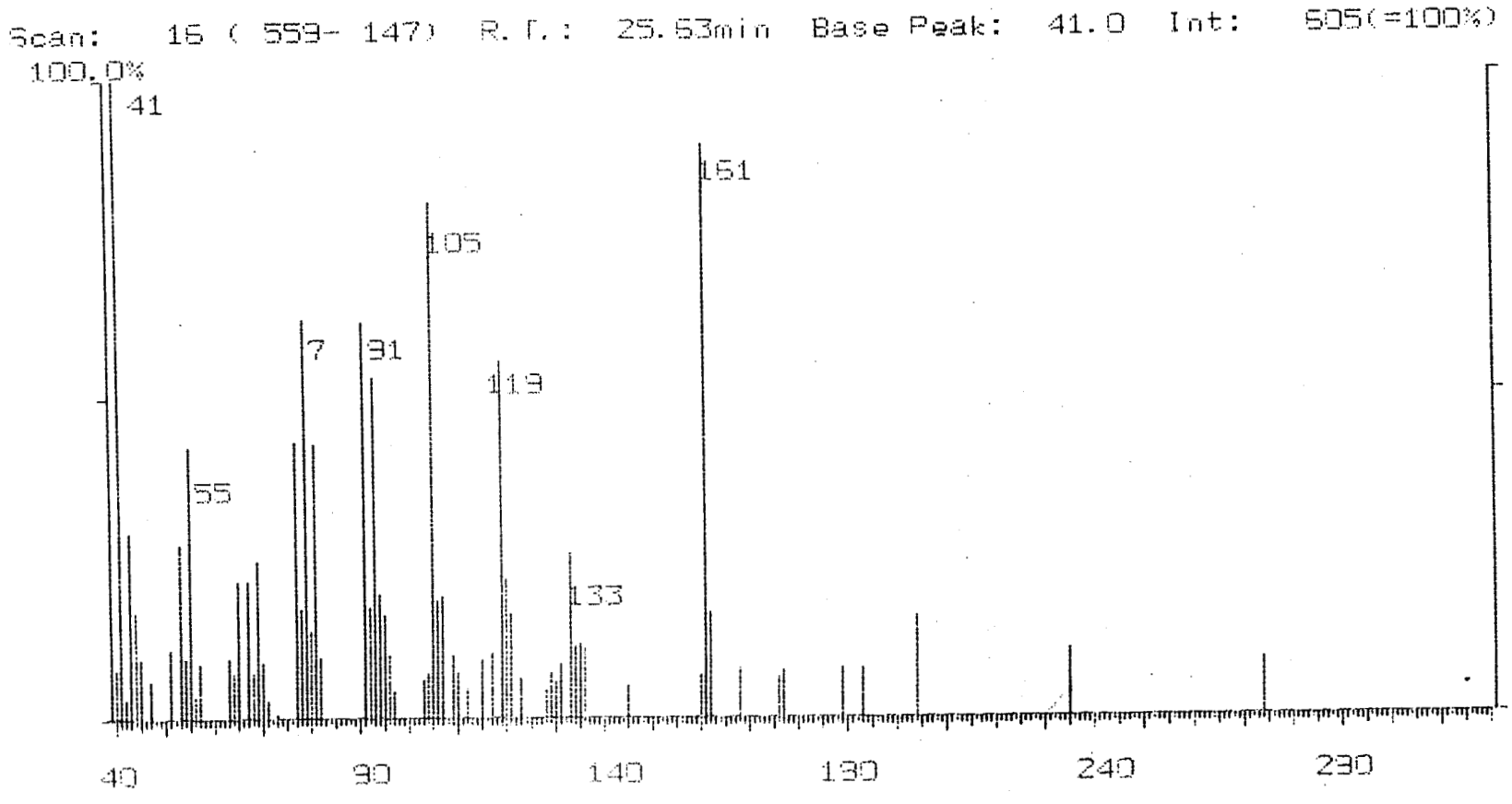


Fig. 4.2.17 Mass spectrum of 1 ethynyl-2-trimethylsilyl benzene

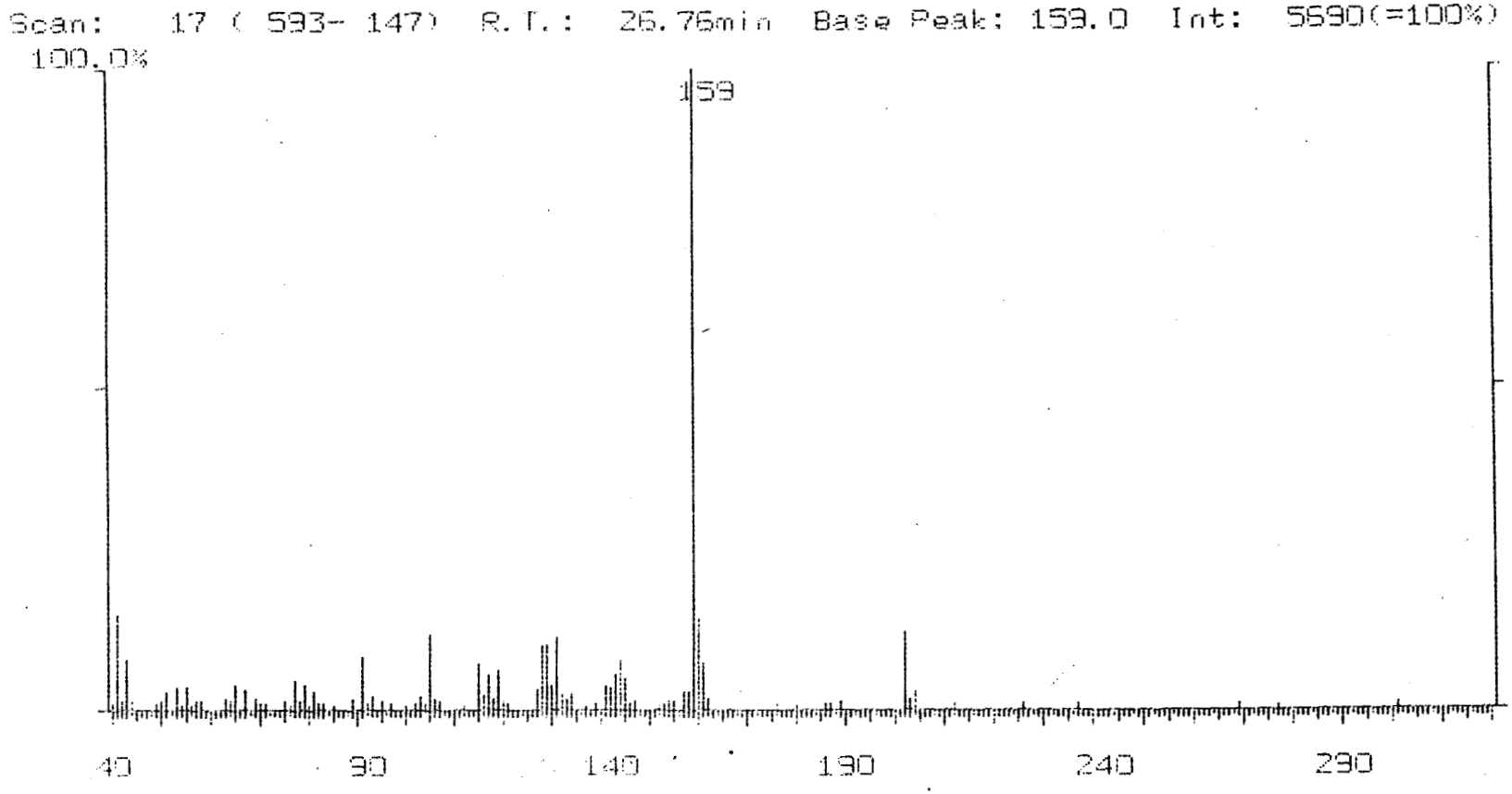


Fig. 4.2.18 Mass spectrum of α-amorphene

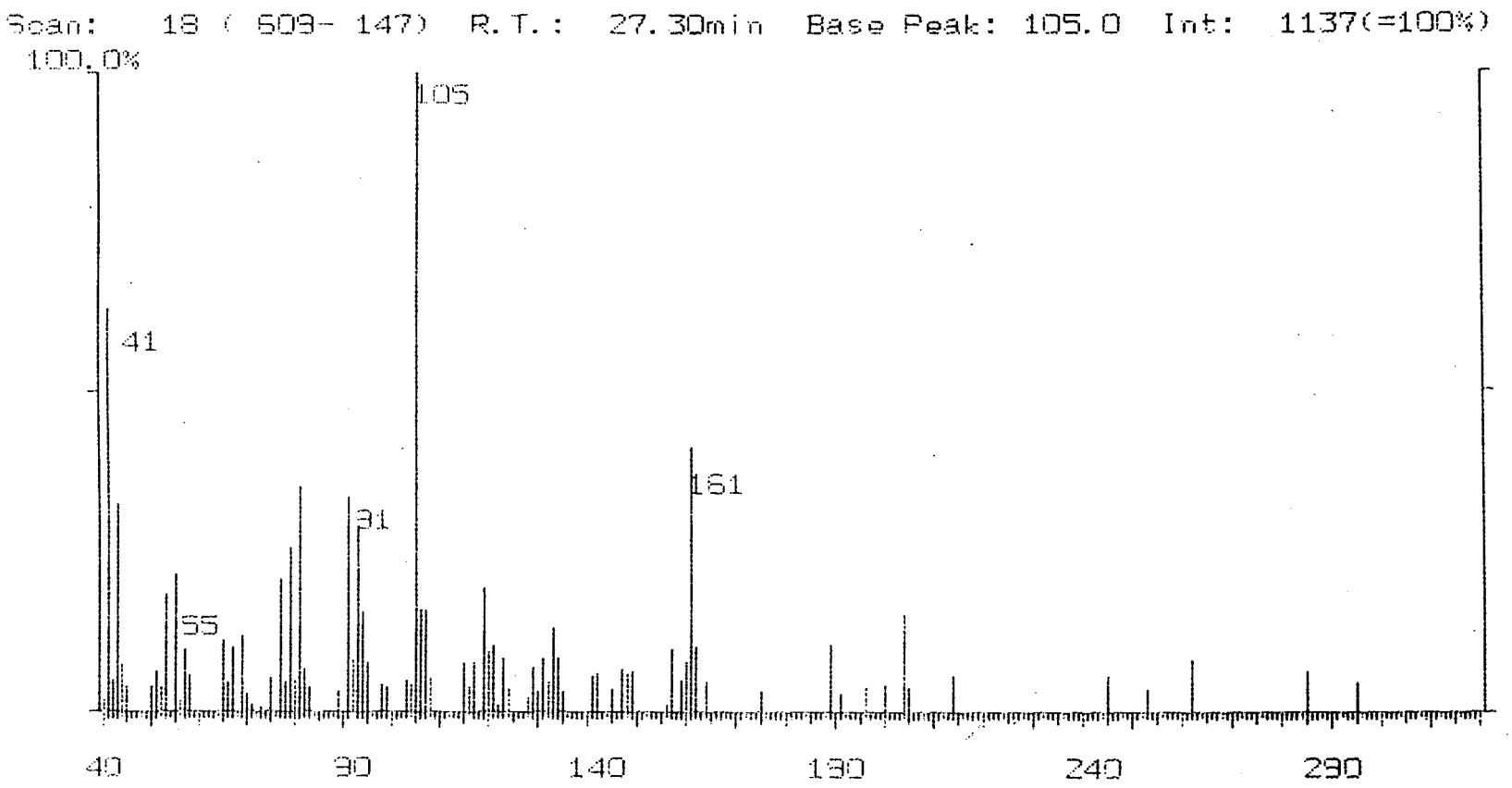


Fig. 4.2.19 Mass spectrum of aromadendrene

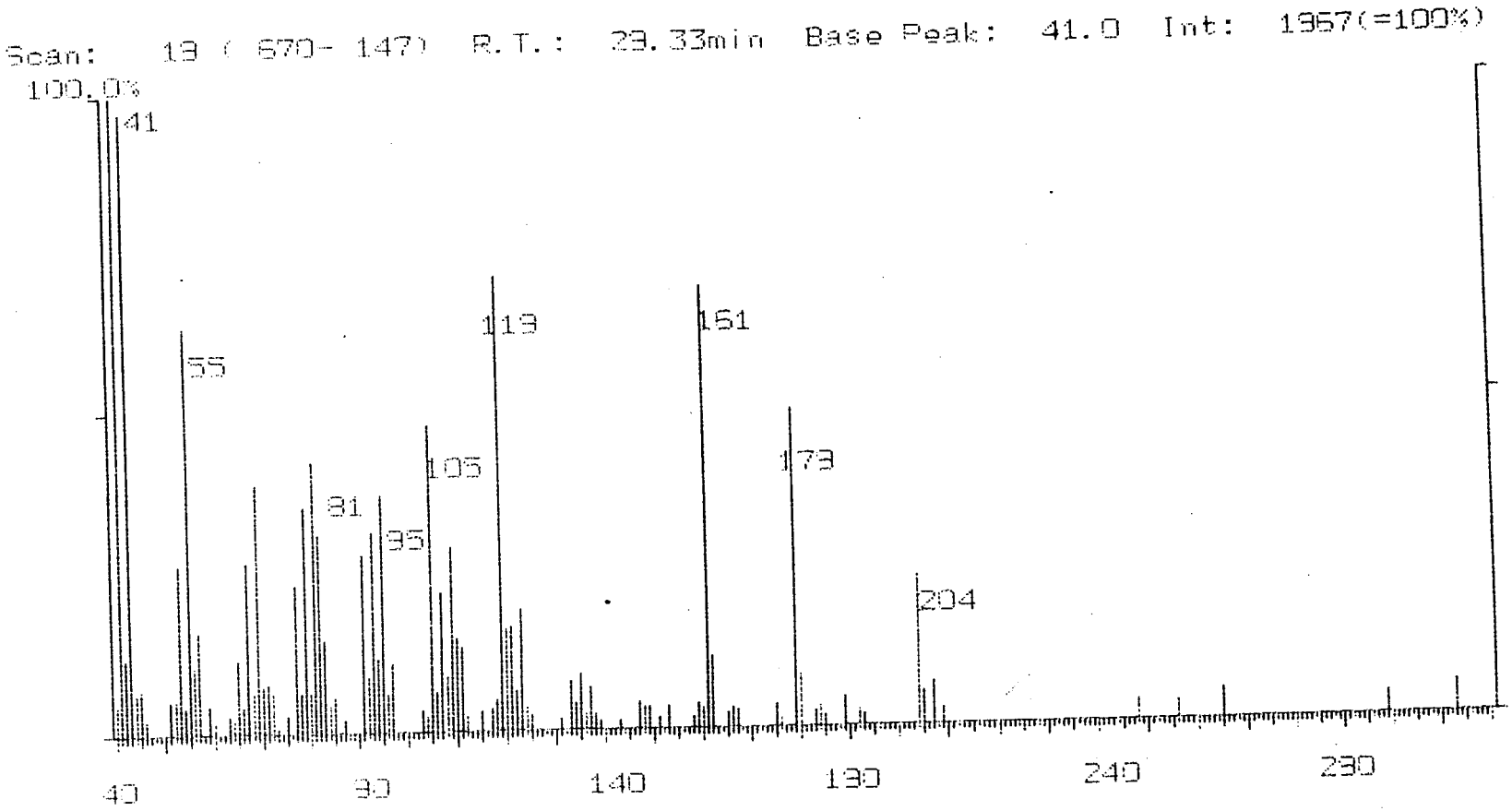


Fig. 4.2.20 Mass spectrum of juniper camphor

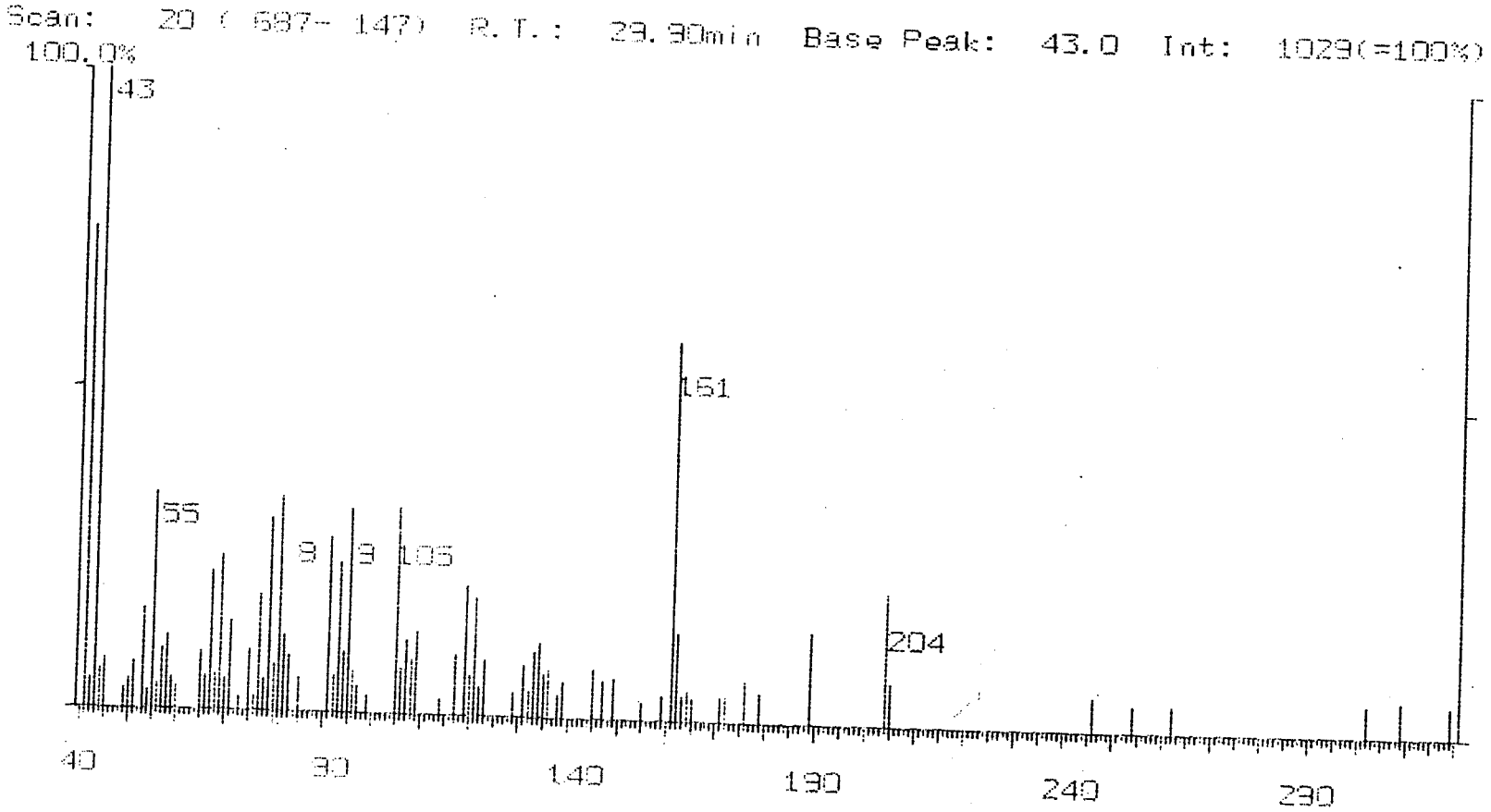


Fig. 4.2.21 Mass spectrum of β-terpineol

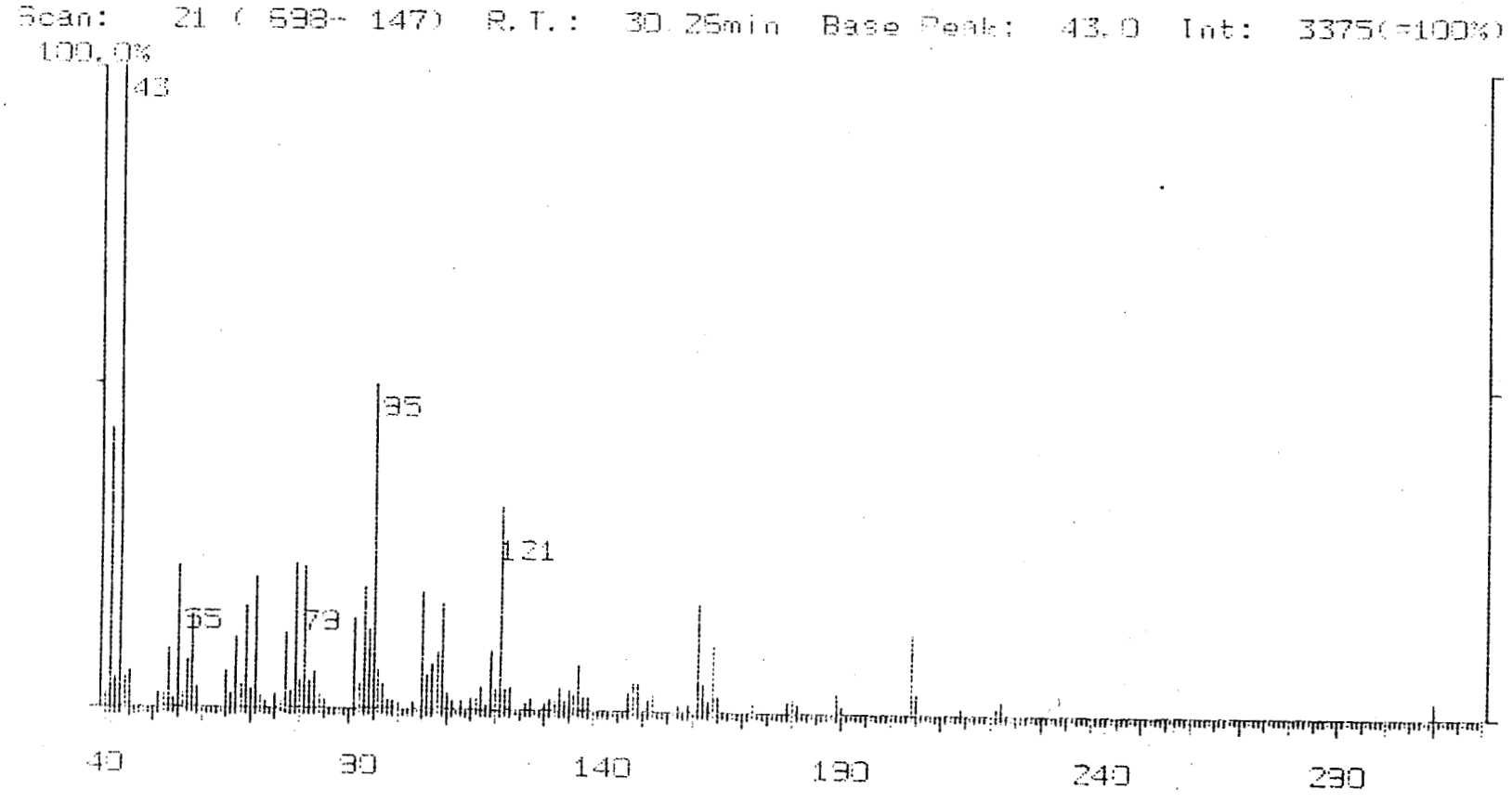
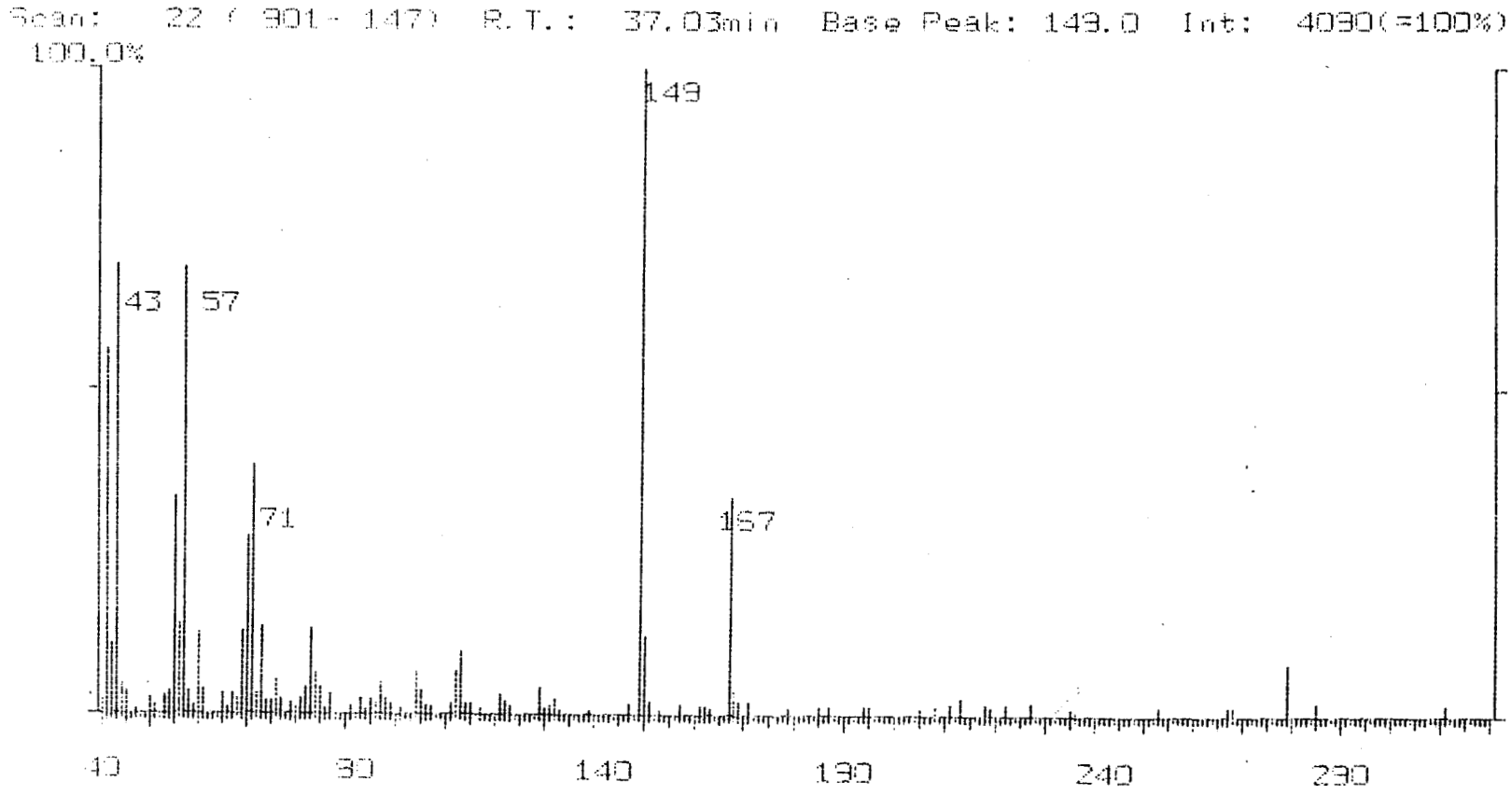


Fig. 4.2.22 Mass spectrum of di isobutyl phtalate



DISCUSSION

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

DISCUSSION

1. Micropropagation

Plant cell and tissue culture techniques are being widely used in a variety of basic research programmes. The potential impact of this novel technique on the genetic improvement of mints has generated considerable interest in the scientific community. Conventional breeding techniques are not suitable for improving commercial mints due to strict essential oil profiles required by mint oil industries.

Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable (Breiman *et al.,* 1987). Other reports claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al.*, 1989). Shenoy & Vasil (1992) reported that micropropagation through meristem culture is generally associated with a low risk of genetic instability because the organized meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions. A number of physiological and morphological changes have been reported in unorganized callus tissue including habituation, changes in biochemical sensitivity and requirements, alteration of growth habits and modification of cellular constituents.

Plant tissue culture has the potential to introduce genetic variability through somaclonal variation (Jullien *et al.*, 1998). If simple multiplication is the objective, any system that significantly reduces or eliminates the tissue culture generated variation can be of much practical utility. Variations may be due to several factors such as genotypes used, pathways of regeneration etc. (Breiman *et al.*, 1987; Vasil, 1987; 1988). Several parameters such as gross morphology (Swedlund & Vasil, 1985), field assessment, molecular studies (Breiman *et al.*, 1989; Shenoy & Vasil, 1992; Choudhury *et al.*, 1994) etc. have been employed for assessing the effect of *in vitro* culture.

The morphogenetic response of the explant is mainly based on the type and concentration of the hormone used. In the present study it was observed that a combination of BAP and IAA was most effective in inducing multiple shoots from nodal explants (Figs. 1.11-1.15). Lower concentrations of BAP induced axillary shoot elongation

from nodal explants, but higher concentrations produced some abnormal tissue growth that later caused some degeneration and death of tissue. KIN also was found to be ineffective in supporting shoot regeneration and it resulted in callusing in most cases. With 2,4-D alone or in combination with KIN, caulogenesis was highly reduced and explants always developed only callus. On the contrary, with IAA & BAP, shoots developed readily without apparent callus (Figs. 1.11-1.15). Among the auxins used, neither NAA nor 2,4-D supported shoot regeneration.

For the present study, nodal segments of *M. rotundifolia* were mainly used. This method is considered to be a low risk method for genetic instability (Pierick, 1991; Schoofs, 1992). But there are several reports on the occurrence of somaclonal variation among micropropagated plants. (Evans & Sharp 1985; Mathur *et al.,* 1988).

In the present study, variation among micropropagated plants was visible at the morphological level (Fig. 1.16-1.23). This type of morphological variation was also reported earlier (Tiwari *et al.*, 1995). Phenotypic variations observed among micropropagated plants may be due to several mechanisms of somaclonal variations like change in chromosome number and structure, dominant and recessive mutations and changes in chloroplasts and mitochondria (Bingham & McCoy, 1986).

According to Larkin *et al.* (1989), the presence or absence of variation depends upon the source of explant and method of regeneration. Stress induced by tissue culture process (hormone effects, nucleotide pool imbalance etc.) can cause alterations in DNA. These alterations could affect the expression of specific genes (Kaeppler & Phillips, 1993).

It has already been reported that the nature of growth regulators used in the medium may result in the occurrence of somaclonal variation (Patel & Berlyn, 1982). The stimulatory effect of BAP on multiple shoot formation is also well established (Shahzad & Siddiqui, 2000). Tender coconut water contains regeneration inducers and that from mature coconut lack these factors (Tuleke *et al.*, 1961) or contain regeneration inhibitors (George & Sherrington, 1984). Regeneration can be affected by explant age (Welander, 1988), explant origin and culture maintenance conditions (Pierik, 1987).

One of the possible mechanisms suggested to explain somaclonal variation is the activation of different classes of mobile genetic elements, such as those reported during tissue cultures of different plants (Peschke & Phillips, 1991; Hirochika, 1993; Hirochika *et al.*, 1996).

Epigenetic variation is another important cause of somaclonal variation in plants. This aspect of somaclonal variation involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change (Kaeppler *et al.*, 2000).

The culture-generated plants may vary from parent plant for its morphological characters as a result of somaclonal variation induced by culture stresses (Patnaik *et al.*, 1999). In the present investigation, a few morphological variants with 1-4 leaves at each whirl (Figs. 1.16-1.23) were developed but these plants could not withstand the hardening process and failed to establish successfully in the soil. Genomic changes appear to be the basis for the phenotypic alterations (Phillips *et al.*, 1994).

2. Cytological Analysis

In the present study, chromosome number variation was not observed either in the callus (Fig. 2.2) or in the somaclonal variant (Fig. 2.3) when compared with its parent (Fig. 2.1). Chromosome number was found to be 2n=24 in all samples, which coincides with majority of the previous reports (Table:1). Basic chromosome number is one of the most widely used characters in biosystematic studies and there has been a vast amount of phylogenetic speculation whether this value can be used as a dependable and stable marker of the direction of evolution (Jones, 1970; 1974; 1978).

Plant cells growing in an artificial culture environment show numerous genetic changes. These alterations are manifested as increased frequencies of gene mutations, chromosome breakage, transposable element activation, quantitative trait variation and modifications of normal DNA methylation patterns (Kaeppler & Phillips, 1993). The possibility of minor genetic reshuffles in the form of minor structural changes in chromosomes without affecting their original chromosome number as the origin of

variants can not be ruled out. Thus the callus culture can be proposed as a potential source of regenerants bearing structural changes of the corresponding degrees and this in turn might result in the generation of somaclonal variants (Mohanty *et al.*, 1991).

Genetic heterogeneity in cultures arises mainly due to factors like i) expression of chromosomal mosaicism or genetic disorders in cells of the initial explants and ii) new irregularities brought about by culture conditions through spontaneous mutations. Cell or tissue cultures undergo frequent genetic changes such as polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplification and mutation and these are expressed at biochemical or molecular levels (Ignacimuthu, 1997).

In the present study, the average chromosome length and total chromosome length of the somaclonal variant was found to be slightly higher than that of the parent plant. Disparity Index and Variation Coefficient was lesser in the somaclonal variant. Chromosomal breakage and deletion may lead to slight changes in the size of the chromosomes. Robertsonian translocations can also lead to changes in the size of the chromosomes. The number of chomosomes with nearly median primary constriction was higher in the parent plant. The somaclonal variant was characterized by a greater number of chromosomes with nearly submedian primary constriction (Tables: 10-12). The reduction in the total chromosome length in the callus may be probably due to the altered enviroment provided for the development of the callus.

Excessive number of submetacentric chromosomes reveals an advanced evolutionary status (Levitsky, 1931). High Disparity Index denotes an advanced heterogeneous nature of the karyotype (*Mohanty et al.*, 1991). Change of the centromere from median to submedian and increased size difference between different chromosomes of the same set are two basic processes responsible for karyotype speciation (Levitsky, 1931) and this is often considered as a potential factor in the evolution of species, especially in the diploid levels (Stebbins, 1970; Mathew & Thomas, 1974).

Cultured cells in metaphase carried structurally altered chromosomes in them (Fig. 2.2). Moreover, callus cells show a decrease in the average chromosome length and total chromosome length. Conditions in the artificial environment of cell culture may

enhance mutation rate, which may act as a genetic shock. The mutation rate may also be enhanced by leakage of toxic by-products and exudates from the calli into the surrounding medium (Oihoft & Phillips, 1999). The decrease in chromatin content may also be due to transposable element activation. Transposable element activation has been shown to be induced by genomic shock (Mc Clintok, 1984). These aberrant cells have a positive selective value in *in vitro* culture systems. Such a type of aberrant cell selection may be comparable to genetic drift. Regenerants from such cultures (somaclonal variants) might be useful for the selection of useful genetic variants (Mohanty *et al.*, 1991), since they may have a rejuvenated genetic content.

The increase in total chromosome content of the somaclonal variant may be probably due to the genetic stability achieved by the variant after regeneration. Minute and cryptic structural differences and gene alterations and rearrangements are therefore responsible for the origin of new species. Such structural changes might have also contributed to the origin of different cytotypes (Stebbins, 1971). The number and form of chromosomes in single cells and the frequencies of primary structural change are the features by which a proliferating cell population can be characterized in cytogenetic terms (Ford, 1964).

Chromosomal breakage and subsequent alterations in chromosomes were reported in many plants. The changes include cytological aberrations, which are primarily the result of chromosome breakage, single base changes, changes in the copy number of repeated sequences and alterations in DNA methylation pattern (Benzion *et al.*, 1986). Mutations involve loss or gain of a defined enzyme function. Every deleterious change in the cistron controlling this enzyme should result in the mutational event (Szybalski *et al.*, 1964).

The chemical composition of the culture medium has been shown to affect the cytogenetic behavior of plant cells *in vitro* (Bennici *et al.*, 1970; Karp, 1992). Mineral deficiencies, chelating agents and some heavy metal ions have been reported to have some role in inducing chromosomal breakage and rearrangements in plants (Steffensen, 1961). The hormone itself can potentially be toxic to the cell thereby directly leading to chromosomal aberrations. The concentration and type of hormone in the culture medium

also influence the pattern of methylation (LoSchiavo *et al.*, 1989). Singh (1986) reported a few chromosomal variations in callus cultures of crops, which are produced due to the effect of media components. According to Singh (1976), KIN can cause chromosomal change. Effect of hormones like 2,4-D, IAA, NAA and KIN on chromosome aberration in cultured plants was already reported (Singh, 1993).

High concentration of growth regulators result in karyotypic alterations in cultured cells. Several phytohormones have shown to induce chromosomal variability in cultured cells, leading to the formation of somaclones. Variants may arise due to single gene mutations in cultured cells. Another aspect of single gene mutation responsible for somaclonal variation relates to transposable elements. Variations have been reported as a result of insertion of plasmid like DNA in the mitochondrial genome of cell cultures of some other plants (Ignacimuthu, 1997).

Another reason for somaclonal variation may be the molecular changes caused by mitotic crossing over in regenerated plants. Such small changes in the structure of chromosomes could alter expression and genetic transmission of specific genes, such as deletion or duplication of a copy or copies of a gene, or gene conversions during the repair process. Further recombination or chromosome breakage at a preferential region or 'hot spots' of a particular chromosome affect the genome in a disproportionately high frequency, resulting in altered phenotypic expressions (Ignacimuthu, 1997).

3. Random Amplified Polymorphic DNA (RAPD) Analysis

The use of PCR amplification to detect target DNA sequences has many applications in plant genotyping, gene mapping, diagnosis and diversity assessment (Kreader *et al.*, 2001). In the present study, the total DNA content of the somaclonal variant (~75ng) was found to be higher than that of the parent plant (~50ng). An increase in the chromosome length of the somaclonal variant may be probably responsible for the increase in the DNA content. It is evident from the electrophoretic gels (Figs. 3.6-3.7) that somaclonal variation at DNA level is present in the hitherto mentioned variant of *M. rotundifolia* (TC 1). A few bands were found missing in the somaclonal variant TC 1when two primers (OPE 14 and OPF 05) were used (Fig. 3.6-3.7). No additional bands could

be detected in this variant by marker screening. Similar results were already reported in several other plants (Brown *et al.*, 1993; Wang *et al.*, 1993; Munthali *et al.*, 1996; Hashmi *et al.*, 1997) using RAPDs. RAPD profiles were unambiguously used to establish the distinct identity as different from the parent plant in many new varieties of *M. arvensis* (Khanuja *et al.*, 2001a; Khanuja *et al.*, 2001b), *M. spicata* var. *viridis* (Khanuja *et al.*, 2001c), *M. piperita* (Dwivedi *et al.*, 2001a), *M. gracilis* var. cardiaca (Dwivedi *et al.*, 2001b), and a few other medicinal plants (Dwivedi *et al.*, 2001c; Patra *et al.*, 2001b). In *M. rotundifolia* there is no report on this type of analysis.

According to Rani *et al.* (1995), polymorphism in amplification products represent 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 & 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). The efficacy of RAPD markers in detecting genetic changes after *in vitro* culture is well understood (Piccioni *et al.*, 1997; Olhoft & Phillips, 1999; Kaeppler *et al.*, 2000). This method is reported to be an efficient tool in detecting somaclonal variation (Al-Zahim *et al.*, 1999).

Failure of amplification of different lines may be due to a single base change or completely different sequences (Vierling & Nguyen, 1992). Presence of RAPD markers in two genotypes indicates a high level of homology at that site. The sequence difference between the two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint (Williams *et al.*, 1990).

In the present study certain intensity differences in the bands were also noticed in the amplification products of all the six primers tested. The same type of results were also reported in other plants also (Yang & Quiros, 1993; Hashmi *et al.*, 1997).

4. Essential Oil Analysis

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 cultured 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). Variations for four major constituents of essential oil were recorded in somaclonal variants of mints (Kukreja *et al.*, 1991a). Over the years, *de novo* synthesis of many commercially important chemical compounds have been reported (Nair, *et al.*, 1986; Calleboutet *et al.*, 1990).

The reports on somaclonal variants of essential oil yielding plants such as *M. piperita* (Holm *et al.*, 1989; Nadaska *et al.*, 1990) and *M. arvensis* (Kukreja *et al.*, 1992) have rendered considerable variation in their oil content. A significant genetic variation was reported in accordance with variation in physiological and biochemical traits in different somaclones of other plants also (Tiwari *et al.*, 1995).

In the present study, GC-MS analyses revealed 21 components each in the parent plant (Figs. 4.1.1 – 4.1.22) and in the somaclonal variant ((Figs. 4.2.1- 4.2.22; Table 14). There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant).

The parent plant was characterized by components like α-pinene, sabinene hydrate, ethyl 9,12 octa decadienoate, trans-2-octenal, isopulegol, citronellol, alloaromadendrene, β-caryophyllene, and β-gurgunene. The unique components in the somaclonal variant were methyl chavicol, isoborneol, cis-6-nonenal, 2,3 diethyl-6-methyl pyrazine, p-mentha trans 2,8 dien-1-ol, γ-caryophyllene, β-bisabolene, 1-ethynyl-2-trimethylsilyl benzene, α-amorphene, juniper camphor and di isobutyl phtalate. The

common compounds in both were myrcene, limonene, carvone, α-terpinyl acetate, β-elemene, α-cububene, aromadendrene, trans-2-cis-6-nonadien-1-ol, and β-terpineol. Among these common essential oil constituents, the amount of carvone and limonene was found to be higher in the somaclonal variant (87.4% and 8.4% respectively) than the parent plant (84% and 6.2% respectively), whereas in the case of limonene, β-elemene, trans 2 cis 6 nonadien-1-ol and α-cububene, it was vice versa (Table:14).

The lesser value of coefficient of similitude (27.27) obtained when the essential oils of both the parent plant and the somaclonal variant (TC 1) was compared, shows the dissimilar nature of the essential oil composition of these oils (Table: 14). This dissimilarity may be probably due to the variations in the biosynthetic pathways of essential oils which are genetically controlled.

Plant cell cultures have produced (i) new compounds previously not known in the intact plant, (ii) new derivatives of known compounds and (iii) new compounds by biotransformation of molecules incorporated in the medium. It is presumed that production of new compounds or derivatives might be due to altered gene function in cultured cells when compared to the mother plant (Merillon & Ramawat, 1999). Previous reports prove that in *M. rotundifolia,* the products synthesized *in vitro* were more oxidized than the natural forms (Kukreja *et al.*, 2000). The type of growth regulators in the culture medium can affect the production of secondary metabolites in cultured cells quite dramatically (Cline & Coscia, 1988). Changes in the constituents of *in vivo* and *in vitro* grown plants are influenced by various genetic and non-genetic factors (Gerhardt, 1972). But there are also reports on tissue cultured plants that match the parent plant in their biosynthetic capacities (Kireeva *et al.*, 1978; Charlwood & Charlwood, 1983). However, in the present investigation, the marked differences observed in the essential oil constitution of the parent plant and the somaclonal variant may be due to their respective biosynthetic pathways (Tetenyi, 1973).

The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. The nutritional component of various culture media have been known to affect secondary product formation. The form in which nitrogen is provided and the concentration supplied have effects on the products 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 the secondary metabolism on phytohormone content. High doses of growth promoters can increase the content of secondary metabolites (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 pathway is operated *in vitro* via a change in the organization of the 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). Illumination also is reported to be a controlling factor in the biosynthesis and accumulation of secondary metabolites (Luckner & Diettrich, 1987).

Quantitative and qualitative production of mint essential oils is clearly controlled by simple genetic systems (Lincon *et al.*, 1986). A number of reports on correct decipherence of the biosynthetic pathways for the terpenoids have already been appeared (Croteau *et al.*, 1991; Akhila *et al.*, 1991; Lange *et al.*, 2000). Biosynthetic data demonstrate that three different dominant genes are involved in the formation of the main ketones in mint essential oils. Among these, a dominant gene 'C' is responsible for the formation of C-2 oxygenated compounds like carvone. In the present investigation, carvone is the major component detected from both the parent plant and the somaclonal variant. So this gene may be present in the dominant form in both these plants. A dominant gene 'R' is responsible for the formation of alcohols from the corresponding ketones (Hefendehl & Murray, 1972; Murray, 1960a) and finally a dominant gene 'E' controls the formation of monoterpene acetates from all monoterpene alcohols (Hefendehl & Murray, 1976).

Reitsema (1958) and Lawrence (1978) reported carvone or dihydrocarvone as the main compounds in *M. rotundifolia* oils. *M. rotundifolia* is a natural sterile hybrid between *M. longifolia* and *M. suaveolens* (Harley & Brighton, 1977). Hendriks *et al.* (1976) reported that the essential oils of the artificial hybrids between *M. suaveolens* and *M. longifolia* were characterized by the main compound piperitone oxide or dihydrocarvone.

The metabolism of monoterpenes is strongly influenced by environmental factors. It has been shown that the diurnal changes in temperature is an important factor of influence regarding the oil composition (Burbott & Loomis, 1967). It is generally accepted that the definition of interspecific chemical races may concern the presence or absence of a particular compound in the secondary metabolism (Tetenyi, 1973; Harbone & Turner, 1984).

Limonene is a key component of *Mentha* essential oils as it was shown to be the precursor of two groups of cyclic p-menthane monoterpenes (Kjonaas & Croteau, 1983; Croteau *et al.*, 1991). A blocking gene L_m causing accumulation of limonene was reported from mints (Hefendehl & Murray, 1973). Isopentenyl pyrophosphate (IPP) is the immediate precursor leading to the formation of over 22000 known terpenoids (Connolly & Hill, 1992).

Chemical investigation on the essential oils of *M. rotundifolia* parent plant and its somaclonal variant revealed that the analyzed plant material may probably belong to a carvone chemotype. In both the samples, the major component remained unchanged even if there is marked variation in the other components. This indicates that the genetic changes due to culture stresses or hormones used did not affect the biosynthetic pathway of major components. The absence of some constituents in the somaclonal variant may be due to some hindrance to the biosynthesis of these components. The appearance of certain new components in the essential oil of somaclonal variant may be due to the triggering of certain diverged biosynthetic pathways. Almost all the secondary metabolites - the monoterpenes, sesquiterpenes and phenyl propenes arise from one of the three biosynthetic pathways or from a combination of two or more of these pathways. These are known as the acetate, mevalonate and shikhimate pathways (Waterman, 1993). Changes in these pathways lead to variation in the chemical composition of the essential oils. Volatile oils are complex mixtures often containing more than 100 individual components. In the present investigation, there is a marked change in the minor components of the essential oil of the somaclonal variant. Most oils have one to several major components, which impart characteristic odour and taste, but many minor constituents also play their part in the final product.

Since there is a close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al.*, 1989). It is clearly evident that the biosynthesis of secondary plant products is controlled by genetic factors (Franz, 1989). The genetic bases of biosynthesis of monoterpenoids and sesquiterpenoids have already been reported (Lincon *et al.*, 1986). The marked deviation in the essential oil composition of the somaclonal variant from the parent plant may be due to the genetic changes revealed by chromosome studies and RAPD analysis.

Today the modification of oil composition in aromatic plants by 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 an approach to understand control mechanisms regulating the flux of monoterpenic compounds will be of great use to the essential oil industry. In such a study, mint could be considered as a model plant because monoterpene pathways are particularly well known in this plant. Such manipulation experiments were still hampered in *M. rotundifolia* by the lack of suitable and reproducible *in vitro* shoot regeneration system. 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.

SUMMARY

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

SUMMARY

Lamiaceae is a family of medicinal and aromatic herbs. The genus *Mentha* is one of the most important taxa of this family. Mint is the most important commercial essential oil bearing plant from the standpoint of worldwide production. The economic importance of mints is due to the production of mint oil as a raw material for confectionary, pharmaceutical and cosmetic industries as well as for flavouring.

M. rotundifolia or apple mint is grown mainly for culinary purposes. It is very important medicinally also. It is used in the preparation of toothpastes, mouthwash, soaps and perfumes. It has been found that our mint products are rated poor in quality and fetch lesser price in the world market.

M. rotundifolia is a sterile diploid natural hybrid. This plant does not flower due to genetic reasons. It is vegetatively propagated through rhizomes. An alternative method for improving this plant is the production of somaclones through tissue culture. Plant tissue culture has the potential to induce genetic variability. In this work, efforts have been concentrated in revealing the cytogenetical aspects and the related phytochemical after-effects of somaclonal variation in *M. rotundifolia*.

1. Micropropagation

MS media with different hormonal concentrations of auxins and cytokinins and coconut water were used for multiple shoot induction. Best results were obtained with BAP 2mg/l & IAA 2.5mg/l from nodal explants. The frequency of shoot induction was very high. The field-transplanted plants having higher oil content were used for further analysis.

Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable. The morphogenetic response of the explant is mainly based on the type and concentration of the hormone used. In the present study it was observed that a combination of BAP and IAA was most effective in inducing multiple shoots from nodal explants. Stress induced by tissue culture process (hormone effects, nucleotide pool imbalance etc.) can cause alterations in DNA. These alterations could affect the

expression of specific genes. The nature of growth regulators used in the medium may result in the occurrence of somaclonal variation.

2. Cytological Analysis

The cytological preparations revealed that the ploidy level of the parent plant and the cultured plant was invariably the same. Both were diploid (2n=2x=24). Neither chimeral nor aneuploid variations were obtained. Average chromosome length of the parent plant (1.0017 μm) was slightly smaller than the somaclonal variant (1.1561 μm). Average chromosome length of the callus was still smaller (0.9379 μm). The total chromosome length of the somaclonal variant was 27.74 μm and that of the the parent plant was 24.04 μm. The total chromosome length of the callus was 22.52 μm. The disparity index of the parent plant was 35.48, somaclonal variant was 17.76, and that of the callus tissue was 31.37. the variation coefficient was higher in the case of callus (20.20), when compared to the parent plant (17.66) and the somaclonal variant (11.60). The Total Forma percentage of the parent plant was 37.60, the somaclonal variant was 36.50 and the callus was 44.31. A single pair of chromosome with secondary constriction was detected. The karyotype formula of the parent plant was $A_2B_{12}C_8D_2$, of the callus was $A_2B_{20}C_2$ and of the somaclonal variant was $A_2B_{10}C_{12}$.

Plant cells growing in an artificial culture environment show numerous genetic changes. These alterations are manifested as increased frequencies of gene mutations, chromosome breakage, transposable element activation, quantitative trait variation and modifications of normal DNA methylation patterns. The chemical composition of the culture medium has been shown to affect the cytogenetic behavior of plant cells *in vitro*. The concentration and type of hormone in the culture medium also influence the pattern of methylation.

3. Random Amplified Polymorphic DNA Analysis

The total DNA extracted was quantified by comparing the bandwidth of the isolated genomic DNA with that of the weight marker after running in agarose gels. The amount of DNA was found to be greater (~75ng) in the somaclonal variant than in the

parent plant (~50ng). Six different decamer oligonucleotide primers (OPA 02, OPA 08, OPB 07, OPD 19, OPE 14 & OPF 05) were used for the present investigation. Of the six primers used, all successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from a minimum of 3 to a maximum of 11. The size of the amplification products also differed and ranged from approximately below 0.5 Kb to 3 Kb. The RAPD fingerprint of the somaclonal variant differed from the parent plant with 2 primers (OPE 14 and OPF 05) used. A few bands were found to be missing in the somaclonal variant when these two primers were used. No additional bands could be detected in this marker screening. Amplification products with OPE 14 generated no bands above 0.75 Kb length in the somaclonal variant but bands were clearly visible in the parent plant's amplification products in this region. When the primer OPF 05 was used for amplification, a fragment with nearly 1 Kb length was found missing in the somaclonal variant which was clearly present as a prominent band in the parent plant's finger print. In both these cases, no additional bands were present in the variant. The other primers used could not generate any polymorphism, but certain intensity differences in the bands were noticed in the amplification products of all the six primers tested.

The efficacy of RAPD markers in detecting genetic changes after *in vitro* culture is well understood. This method is reported to be an efficient tool in detecting somaclonal variation. The sequence difference between the two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint.

4. Essential oil Analysis

The oil yield of the parent plant was 1%. The somaclonal variant (TC 1) contained a higher quantity of oil (1.3%). GC-MS analyses revealed 21components each in the parent plant the somaclonal variant. There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant). The coefficient of similitude between the parent plant and the somaclonal variant

was 27.27. This low value indicates the dissimilarity of the biosynthetic pathways in both these plants.

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 to produce a new compound. It is presumed that production of new compounds or derivatives might be due to altered gene function in cultured cells compared to the mother plant. Quantitative and qualitative production of mint essential oils is clearly controlled by simple genetic systems. In both the samples, the major component remained unchanged even if there is marked variation in the other components. This indicates that the genetic changes due to culture stresses or hormone used did not affect the biosynthetic pathway of major components. The absence of some constituents in the somaclonal variant may be due to some hindrance to the biosynthesis of these components. The appearance of certain new components in the essential oil of somaclonal variant may be due to the triggering of certain diverged biosynthetic pathways.

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. This study reveals 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.

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CHROMOSOME IMAGING, RAPD AND GC-MS ANALYSES ON A SOMACLONAL VARIANT OF *MENTHA ROTUNDIFOLIA* (L.) HUDS.

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By

DEENA MERIA JOSE

Genetics and Plant Breeding Division
Department of Botany
UNIVERSITY OF CALICUT
Kerala, India

2002

Mentha rotundifolia (L.) Huds.

DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT

Calicut University P. O., Kerala - 673 635, India

Phone : (0494) 400275 PBX 0494 401144, exten. BOTANY Fax : 0494-400269 Grams : UNICAL

Date: 12-08-02

Dr. John E. Thoppil,
Lecturer (Sr. Grade),
Genetics & Plant Breeding Div.

CERTIFICATE

This is to certify that the thesis entitled 'Chromosome Imaging, RAPD and GC-MS Analyses on a somaclonal variant of Mentha rotundifolia (L.) Huds.' is an authentic record of work carried out by Miss Deena Meria Jose in the Department of Botany, University of Calicut during 1999-2002 under my supervision and guidance and that no part thereof has been presented earlier for any other degree or diploma.

12-8-2002
Dr. John E. Thoppil

DR. JOHN E. THOPPIL
Lecturer, Genetics &
Plant Breeding Division
Department of Botany
University of Calicut
673 635, Kerala, India

DECLARATION

I hereby declare that the thesis entitled 'Chromosome Imaging, RAPD and GC-MS Analyses on a somaclonal variant of Mentha rotundifolia (L.) Huds.' submitted for the Ph.D. Degree of the University of Calicut has not been submitted earlier for the award of any other degree or diploma and that it represents the original work carried out by me.

Date:12- 08-02

Deena Meria Jose

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CONTENTS

Abbreviations Used

2,4-D	2,4-Dichlorophenoxy acetic acid
ACE	Acetylcholineesterase
AP PCR	Arbitrarily Primed Polymerase Chain Reaction
BA	Benzyl Adenine
BAP	6- Benzyl Amino Purine
CNS	Central Nervous System
CS	Coefficient of Similitude
CTAB method	Cetyl Trimethyl Ammonium Bromide method
CW	Coconut Water
DI	Disparity Index
dNTP	deoxy Nucleotide Tri Phosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
FPP	Farnesyl Pyrophosphate
GA_3	Gibberellic Acid
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GGPP	Geranyl Geranyl Pyrophosphate
GPP	Geranyl Pyrophosphate
HPLC	High Performance Liquid Chromatography
IAA	Indole 3-Acetic Acid
IPP	Isopentenyl Pyrophosphate
KIN	Kinetin (6 furfurylaminopurine)
MS medium	Murashige & Skoog medium
NAA	α-Naphthyl Acetic Acid
NIR	Near Infra Red Spectroscopy
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TAE buffer	Tris Acetic Acid EDTA buffer
TE buffer	Tris HCl EDTA buffer
UI	Unidentified Component
VC	Variation Coefficient

INTRODUCTION

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

INTRODUCTION

Aromatic plants have been of great interest to mankind from the beginning of human civilization. Aromatic plants and their derivatives, particularly the essential oils are now becoming one of the most important export items from many developing countries in Asia. The main reason for such an upswing is the technological advances in the production and processing of the essential oils and their increased use in various medicines. There is great potential to improve the yield and quality of these plants, either by mere selection of existing species or varieties through plant breeding or by other novel methods of plant improvement.

In the dawn of human cultural evolution the art of curing was essentially magical (Naranjo, 1984). Herbal medicines can be viewed as the precursor of modern pharmacology. References to miracle herbs or wonder drugs are often found in old literature. In India, references to the curative properties of some herbs in *Rigveda* seem to be the earliest record of use of plants in medicine. Few plants, that were usually psychoactive, known as magic or psychedelic plants, were used by ancients. Later on empirical medicine arose, using many plants for the treatment of various afflictions. This tendency culminated in the Old World, in the famous work *Materia Medica* by Dioscorides, published in the first century AD. The ancients possessed an extensive vegetal pharmacopoeia. Some of the oldest pre-literature archaeological records in both the Old and New Worlds deal with the medicinal use of plants.

Medicinal plants are the source of various alkaloids, terpenoids and other chemical substances quite essential to mankind. Medicinal plants normally growing in wild habitat show an increasing depletion of natural resources (Tandon, 1994). Keeping this in view, there is an urgent need for the systematic cultivation of medicinal plants through novel techniques for their germplasm conservation, selection of desired genotypes and mass propagation of superior genotypes (Ahuja, 1994; Filippini *et al.*, 1994). Plants are the traditional source of many chemicals used as pharmaceuticals, biochemicals, fragrances, food colours and flavours (Leung, 1980). Most valuable phytochemicals are the products of plant secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult. Procurement, cultivation and regeneration of important medicinal and aromatic plants used in

indigenous systems of medicine are the other aspects of current scientific importance as pharmaceutical industry depends on it for raw material.

Edible materials, which are, used more for their aromatic flavour than for their food value are known as spices. Spices are fragrant or aromatic plant parts used as a whole, ground, paste or in liquid form for flavouring and seasoning foods. They have played a vital role in the world trade due to their varied properties and applications. The flavour and aroma are due to essential oils. Spices serve mainly three purposes: they do not let the food to be monotonous, they disguise the stale flavour of food materials and increase the pleasure of eating and they help to cool the body by increasing the rate of perspiration. Spices by themselves are not high in nutrient value. But spices and herbs can play an extremely valuable role in good nutrition by helping to increase the appeal and appreciation of foods that are nutritionally important to us. Aroma and flavour characteristics are important quality attributes of spices, which are based on the nature of essential oil in them. Spices were sometimes considered so important that they were used in beverages, medicine and even in lieu of money.

Mentha (mint), one of the most important taxa of the family Lamiaceae occurs in all the five continents, although its native occurrence in the New World is restricted to a single species in the North. It is frequent in the tropics (Tyagi & Ahmad, 1989; Tyagi *et al.*, 1992). The genus comprises of about 25 species (Willis, 1973) distributed in the temperate regions of the Old World. According to Bhattacharjee (2000), six species of this genus are distributed in India, majority of them being found in the Western and Eastern Himalayas. Several species are cultivated widely in different parts of the world for their content of menthol and its other important derivatives (Heywood, 1978). *Mentha* species were originally cultivated in Eastern Asia, mainly in Japan and China (Bersaghi, 1945). Regular cultivation of mints started around 1870 in Japan (Mehra, 1982).

The genus has been subdivided into a number of sections, of which by far the largest and taxonomically most complex is Section *Mentha* (=subgenus *Menthastrum* Cossom & Germain). This includes all the better known species of commerce, such as Japanese mint or corn mint or field mint (*M. arvensis*), peppermint (*M. piperita*), common or native spearmint (*M. spicata*), Scotch spearmint (*M. cardiaca*), Bergamot mint or

orange mint (*M. citrata*), apple mint (*M. rotundifolia*) etc. (Tyagi & Ahmad, 1989; Tyagi *et al.*, 1992). In modern taxonomic treatment of the genus, there are five sections (*Mentha* Sect. *Audibertia*, Sect. *Eriodontes*, Sect. *Mentha*, Sect. *Preslia* and Sect. *Pulegium*) containing 19 species and 13 named hybrids involving *M.* Sect. *Mentha.* Formation of hybrid species of the genus *Mentha*, and in particular with those of the subgenus *Menthastrum* Cossom & Germain is a very common phenomenon in wild populations, where more than one species occur (Harley & Brighton, 1977). Identification of mints is difficult due to great phenotypic and genetic variability caused by interspecific hybridization. *Mentha* species are extremely variable and specific limits are hard to define, with consequent unstable nomenclature (Santapau & Henry, 1973). Assumptions of type specimens and the consequential publication of illegitimate names have rendered the nomenclature of this polymorphic genus more difficult. Almost 2300 names have been published for essentially 20 species of this genus (Tucker *et al.*, 1980).

Mints are subdivided into three groups – culinary, medicinal and fragrant. The first group includes the species *M. viridis* (lamb or pea mint), *M. sylvestris* (wild mint, crisped or curled mint) and *M. rotundifolia*. The second group contains *M. piperita*, *M. gentilis* (gingermint) etc. and the third group includes *M. citrata*, *M. aquatica* (water mint) etc. (Genders, 1972).

Theophrastus in the fourth century was the first to use the name 'Minthe' in referring to the mint, *M. viridis*. According to Greek mythology, a nymph called 'Menthe', who was greatly loved by Pluto, was transformed into mint herb by Pluto's jealous wife (Macleod, 1968). The poets celebrate Minthe, a daughter of Cocytus, as being turned into mint by Proserpine in a fit of jealousy (Hereman, 1980). Mints have been used and valued for aromatic purposes for thousands of years. The ancient Greeks and Romans used them. Mints were the favourite herbs of the Roman scholar Pliny. He enjoyed them for their aromatic effect: "the very smell of mints reanimates the spirit" (Doyle, 1998). In the Middle Ages, mints were used for strewing purposes – they were scattered about castles to mask the dreadful odours and to repel insects. As the writer Gerald stated, "the smell rejoiceth the heart of man", for which they used to strew it in chambers and places of recreation, pleasure and repose where feasts and banquets were made (Macleod, 1968).

There are Biblical references also regarding this herb. In the New Testament, in chapter 23, verse 23, Mathew notes their value by quoting Jesus' condemnation of the Pharisees for requiring a tithe of mint, dill and cumin. The philosopher Seneca, though a stoic, died in a bath strewn with mint. The herb's culinary virtues were also known from classical times (Conway, 1973). Mint's ability to prevent milk from curdling was well known (Culpeper, 1999). In Italy, mint was spread on the floor of churches and hence called 'Erba Santa Maria' (Nayar, 1985). Shakespeare mentioned mint along with lavendar and marjoram in 'The Winter's Tale'. In France, mint was called 'Mente de Notre Dame'. Mints were employed much by Victorian ladies as they used bottles of smelling salts to revive anyone in danger of swooning. In Greece, ancients used to scent each part of their body with different perfumes and they reserved mint for their arms. Put into bath with balm and other herbs, mints help to comfort and strengthen the nerves and sinews (Macleod, 1968). They have been found in Egyptian graves and are described in old literature of China. In ancient Greece, the juice of mints was applied to arms as an after bath rubdown (Clarkson, 1972).

The economic importance of mints is due to the production of mint oil as raw material for confectionary, pharmaceutical and cosmetic industries as well as for flavouring food, beverages, tobacco (Rech & Pires, 1986), candies, medicines, tooth pastes, mouthwash, and chewing gums (Chambers & Hummer, 1994; Banthrope, 1996). *Mentha* species are known from time immemorial as kitchen herbs (Mehra, 1982). It is also used for flavouring meat, fish, sauces, soups, stews, vinegar, teas, tobacco and cordials. The fresh leaf tops of all the mints are used in beverages, apple sauce, ice creams, jellies, salads, sauces for fish and meat, chutneys etc. (Pruthi, 1976).

The herbs of mint are much esteemed in India as diaphoretic (Keys, 1976), diuretic (Watt & Breyer-Brandwijk, 1962), carminative, antiseptic, deodorant and stimulant (Dey & Bahadur, 1973; Nadkarni, 1976). In herbalism, mint tea is used to help digestion, to revive appetite and to alleviate rheumatism. The Arabs have always believed that mints increased virility and some modern herbalists still prescribe it in cases of impotence and decreased libido (Conway, 1973). According to Dioscorides, it has a healing, binding and drying quality and therefore the juice taken in vinegar stops bleeding. It stirs up venery or bodily lust, stops vomiting and allays cholera. Applied with salt, it helps to heal the wound

caused by the biting of mad dogs and pain in the ears. It is also used against the poison of venomous creatures, used to wash the heads of young children to cure sores or scabs and to cure the obstructions of liver. It is good against the gravel and stone in kidneys and the stranguary. The smell is comfortable for head and memory (Culpeper, 1999). The leaves are used as a contraceptive and to cure indigestion (Vedavathy *et al.*, 1997). Mints having pungent camphoraceous odour and taste are used to cure headache, rheumatism, neuralgia, cholera, colic, diarrhoea, flatulence, dysmenorrhoea, hiccup, palpitation of the heart, pruritus, diphtheria and toothache. It is also used in pharmaceutical preparations to disguise the taste of evil smelling and unpleasant drugs, and in antiseptic inhalations (Nadkarni, 1976).

Mints are extremely bad for wounds. If too much is taken, it makes the blood thin and wheyish (Culpeper, 1999). Mints are also reported to have antimicrobial (Mimica *et al.*, 1993; Lis Balchin, 1997; Thoppil *et al.,* 2001a), antibacterial (Singh *et al.*, 1992; Patnaik *et al.,* 1995; Sivropoulou *et al.*, 1995; Alippi *et al.,* 1996; Ela *et al.*, 1996; Patnaik *et al.*, 1996; Hassanein & Eldokseh, 1997; Shapiro, 1994; Carvalho *et al.*, 1999), antifungal (Mizutani *et al.*, 1989; Singh *et al.,* 1992; Patnaik *et al.*, 1996; Carvalho *et al.,* 1999), antiviral (Ismail, 1994), antiparasitic (Santana *et al.*, 1992), antinociceptive (Atta & Alkofahi, 1998), acaricidal (Mc Donald & Tovery, 1993; Perucci *et al.*, 1996), insecticidal (Franzios *et al.*, 1997), mosquito repellant (Thorsell *et al.,* 1998), larvicidal (Nadkarni, 1976), nematicidal (Oka *et al.*, 2000), herbicidal (Seidlova & Sarapalka, 1997) and genotoxic (Franzios *et al.*, 1997) activities.

M. rotundifolia (L.) Huds., a perennial pubescent herb otherwise called apple mint (CSIR, 1962) or wooly mint (Usher, 1984) or round leaved mint (CSIR, 1992) is grown for culinary purposes (Genders, 1972). It is a native of Europe and cultivated in Indian gardens (Chopra *et al.*, 1969). It is a diploid natural hybrid (2n=24) between the species *M. longifolia* (L.)(2n=24) and *M. suaveolens* Ehrh. (2n=24). *M. rotundifolia* auct. non (L.) Huds. and *M. suaveolens* Ehrh. are synonyms (Wiersema & Leon, 1999). *M. rotundifolia* is a delicious flavoured mint. It has digestive properties (Genders, 1972) and good for mint sauce (Bhattacharjee, 2000). Mint is used widely in Indian cuisine. The best-known examples are mint coriander chutney, green curries of meat, fish and poultry, mint raita, mint sweet chutney etc. Being a carminative, it is used for cooking peas, dals and other

dishes that are difficult to digest. It is also used for mint tea, mint sherbets etc. In Western cuisine it is used to sprinkle over fruit cups, melon balls, fruit aspic salads and as a garnish in hamburgers, sauces like cranberry, mint jelly etc. It is also mixed with cream cheese, wine punches, liqueurs, fruit juices etc. (Thangam, 1989).

In Vietnam, this plant is used for the treatment of headache, dyspepsia and fever. Pharmaceutical enterprises around the world have produced different balms such as Truong Son (Long Mountain), Sao Vang (Gold star) and Cao con ho (Tiger Balm). It is also used in food and perfume production industry and for making boudon, toothpastes and soaps (Dung & Thin, 1992). Leaves are used for flavouring (Usher, 1984). It helps to digest meat and used in confectionary trade for flavouring cakes (Genders, 1972). A mixture of *M. rotundifolia* (apple mint) and *M. citrata* (orange mint) is used in jellies to make it delicately fragrant (Clarkson, 1972). A hot infusion of apple mint can help at the start of a cold (Anonymous, 1996). *M. suaveolens* is used to cure hepatic complaints, as a tranquillizer and anticatarrhal agent (Gonzales Tejero *et al.*, 1992). *M. rotundifolia* is also reported to have nematicidal (Oka *et al.*, 2000) and cytotoxic (Minija *et al.*, 1999) properties.

Mints are considered today as the most important commercial essential oil bearing plants from the stand point of world wide production (Lawrence, 1985). Of the known flavouring materials in the world, mint is one of the most popular flavour and ranks probably third after *Vanilla* and *Citrus* (Singh *et al.*, 1998). Minty fragrances differ greatly in their organoleptic properties, going from fresh and cool long lasting tastes as those of spearmint to sweeter flavours and fragrances such as peppermint and cornmint (Sacco *et al.*, 1999). To the perfumer, anything that smells cool and fresh, would be classified as minty, and anything minty would fall into the mint category to be used to provide a cool, fresh, natural and clean top note to perfumes. For a perfumer, all mint notes are top note materials because they have high volatility and therefore they can be employed in the initial stage of a perfume's progression (Doyle, 1998). Such a history and economics make this plant a thrust area of growing research.

It has been found that our mint products are rated poor in quality and fetch lesser price in the world market as compared to Brazil, China and USA (Singh *et al.*, 1998).

Owing to their diversified uses, the demand of mint oils is ever increasing. Thus there is an urgent need to increase the production per unit area by developing effective package of practices for developing high oil yielding varieties as well as for developing high quality oil producing varieties. Selection of new clones is needed with improved terpene accumulation and desirable agronomic traits.

Decline of potential for the production of active principle or essential oil content is a common feature with cultivation of medicinal plants and a continuous selection is required to maintain the high yield of active constituents for commercially viable programmes. Therefore selection through conventional breeding as well as through tissue culture are exploited nowadays for obtaining high yielding cultivars. Development in the technology of plant tissue culture since its pioneering experiments by Laibach (1925; 1929), White (1934; 1937), Loo (1941), Skoog (1944) and Murashige & Skoog (1962) have contributed in establishing a strong foundation for the applications of this versatile technology.

M. rotundifolia does not flower due to genetic reasons and thus sexual reproduction and seed setting are lacking (Thoppil, 1993). It is vegetatively propagated through vigorous rhizome system. These vegetative clones fail to exhibit variability. In contrast to the conventional methods of vegetative propagation through rhizomes, the use of *in vitro* propagation enables the production of large number of variants. This is an alternative method for improving mint, which involves the use of tissue culture regeneration and the production of somaclonal variants (Larkin & Scowcroft, 1981; Constabel, 1990). Somaclonal variation is a term coined by Larkin & Scowcroft (1981) to cover all types of variations, which occur in plants regenerated from cultured tissues. Plant tissue culture has the potential to induce genetic variability in mint genotypes through somaclonal variants, somatic hybrids or transgenic plants. However a prerequisite to applied plant biotechnology is the development of a suitable and reproducible plant regeneration system (Jullien *et al.*, 1998). Potential use of cell culture (Drupeau *et al.*, 1987), multiple shoots (Constabel *et al.*, 1982; Endo *et al.*, 1987; Hirata *et al.*, 1987) and improvement of various cultivation conditions (Facchini & Dicosmo, 1991) have been attempted to scale up production of secondary metabolites. The exploitation of tissue culture technique in medicinal plants for the extraction of important

chemical compounds is indeed more advantageous (Tabata, 1977). A wide variety of compounds have been shown to be produced in shoot, callus or cell suspension cultures at levels equal to or higher than the levels in the intact plant sources (Brodelins, 1988; Dodds & Roberts, 1995).

Plant tissue culture has been extensively used to exploit the secondary metabolite it can produce. Growth of a cell in a totally controlled environment of physical and chemical factors provides an excellent system for studying changes in the production of secondary metabolites, which are always present in small quantities. The basic information has provided significant clues about genes and their functioning, leading to genetic manipulation of biosynthetic pathways so as to obtain desired products by either blocking a pathway or enhancing the metabolic reaction (Merillon & Ramawat, 1999).

Exploitation of possible somaclonal variation, which has been observed in *in vitro* cultures, could be used to widen the genetic pool from which to select desirable traits. Application of modern biotechnology can complement conventional breeding techniques and it helps in the development of improved varieties (Chomchalow & Sahavacharin, 1981; Vasil, 1988; Bajaj, 1991). *In vitro* propagation can yield a large number of clonal plants for continuous plant establishment. It is also important for germplasm conservation (Whithers, 1980; Kathiravan & Ignacimuthu, 1999; Kukreja & Dhawan, 2000). Variation is a ubiquitous phenomenon associated with tissue culture (Carlson & Polacco, 1975; Green, 1977). Induced variation is an alternative source to naturally occurring variability for crop improvement (Ansari & Siddiqui, 1995). Tissue culture induced variation is defined as the variation that arises *de novo* during the period of dedifferentiated cell proliferation that take place between culture of an explant and production of regenerants (Munthali *et al.*, 1996). Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin & Scowcroft, 1981), which is often heritable (Breiman *et al.*, 1987). Other reports claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al.*, 1989).

The genus *Mentha*, in view of its medicinal and commercial importance, has been the subject of cytological, genetic and phytochemical studies (Sobti, 1971; Murray *et al.*, 1972; Gill *et al.*, 1973; Sobti, 1975; Tucker & Fairbrothers, 1981; Harley & Brighton,

1992). It exhibits a variety of genetic forms with different sets of chromosomes. Chromosome number proved to be of important systematic value in many groups of Labiatae as in *Mentha* (Harley & Heywood, 1992). A major contribution to the cytology of the genus was the work of Harley & Brighton (1977) which tested chromosome counts of many accessions of almost all the taxa recognized today, with major emphasis on *M.* sect. *Mentha*. It is well known that the DNA content undergoes a multiplication with increase in genome sets (Kundu & Sharma, 1985). However there are certain confirmed reports (Dworick & El Bayoumi, 1969; Grant, 1969) indicating a relative decrease of the amount of DNA at higher ploidy levels. Based on a base number of x=12 (Ikeda & Udo, 1963; Harley & Brighton, 1977) somatic chromosome numbers in sect. *Mentha* range from 2n=24 to 120 (Morton, 1956; Harley & Brighton, 1977). Several workers (Nagao, 1941; Sharma & Bhattacharyya, 1959; Ouweneel, 1968; Tyagi & Naqvi, 1987) have also recorded aneuploid individuals. It is well known that an increase in chromosome number is reflective of an increase in cell size (Stebbins, 1971).

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). Chromosomal variation is a common feature of plant tissue culture. Changes can take place at the ploidy level like the production of aneuploids (Taliaferro *et al.*, 1989), polyploids (Mariotti *et al.*, 1984) and mixoploids (Mariotti *et al.*, 1984; Taliaferro *et al.*, 1989). Karyological studies can bring to light the variations in chromosome number and their size and suggest the direction of chromosomal evolution in specific taxa (Jones, 1978). Chromosomal differences may also cause changes in quality and composition of the essential oils (Guenther, 1949).

Computer aided chromosome image analysis system is a modern technique for karyomorphological analysis. Ordinary karyotype analysis has provided a limited success in chromosome identification. Possibility of making errors is much greater in the conventional method of measuring and characterizing by visual evaluation. These difficulties can be overcome by computer aided chromosome image analysis system. It allows an accurate chromosome pairing mainly in those cases where the chromosome size is very small (Fukui & Kakkeda, 1994). This technique gives a better knowledge of

the cytogenetic constitution of the material under study (Fukui & Kakkeda, 1990; 1994; Fukui & Iijima, 1992; Fukui & Kamisugi, 1995).

Genomic analysis is a prerequisite for establishing the genetic stability and uniformity of a desired clone (Ikeda & Ono, 1967). Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones but most of them have limitations. Karyological analysis can not reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al.*, 1993). The Polymerase Chain Reaction (PCR) (Saiki *et al.*, 1988) has been the basis of a growing range of newer techniques. PCR allows the specific amplification of DNA sequences, making it ideal for the identification of plant genotypes. Amplification of a genotype specific sequence can take advantage of some of the many features of PCR like speed, simplicity, specificity, sensitivity and cost (Henry, 1997). Molecular markers such as Restriction Fragment Length Polymorphism (RFLP) (Tanksley *et al.*, 1989) and Randomly Amplified Polymorphic DNA (RAPD) (Welsh & Mc Clelland, 1990; Williams *et al.*, 1990) appears to be good, but when compared to RFLP, RAPD appears to provide a better basis for genetic characterization because of simplicity of the necessary procedures (Baird *et al.*, 1992). The approach of using molecular markers including RAPD profiles is a powerful tool not only for the identification of genotypes but also to quantify the extent of genetic variation in any given population. While on one hand the approach of RAPD profiling has been useful in tissue culture methods for detection and selection of somaclonal variants (Munthali *et al.*, 1996), this molecular technique, at the same time with the same logic, is directly utilizable for assessing the population of micropropagated clones from any given explant for genetic uniformity.

Using PCR with short primers of arbitrary sequences, RAPD markers were recently shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991; Roy *et al.*, 1992). This is an alternative approach for finding new DNA based polymorphic markers among closely related genotypes (Welsh & Mc Clelland, 1990; Nymbom *et al.*, 1990; Lindhout *et al.,* 1999). RAPD analysis using PCR with arbitrary oligonucleotide primers (Williams *et al.*, 1990; 1992) has the advantage of being non radioactive, rapid and convenient assay of polymorphism and requires only a small amount of crude DNA. Today, RAPD technique has been adopted

most widely. The main issues associated with the use of these techniques are the problem of ensuring reproducibility of amplification profiles. The nature of the amplification process with short primers is such that many sites in the genome are potential templates and the profile obtained may be influenced by any variation in the method used to prepare the DNA template and the exact reaction composition and conditions used in the PCR (Muralidharan & Wakeland, 1993). Obtaining reliable results depends upon standardizing these conditions or identifying combinations of conditions that give consistent results, even when variations in the key variables are encountered. A key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparation and DNA isolation. Both the quantity and quality of the template DNA preparation have the potential to substantially influence the result.

Polymorphism result from either base changes at the primer binding site (point mutation) or chromosomal changes in the amplified regions (insertions, deletions or inversions) which alter the size or prevent the successful amplification of a target DNA. Southern hybridizations are not required and polymorphisms can also be detected in fragment containing highly repeated sequences, which are recalcitrant to RFLP analysis. The extent of polymorphism detected by RAPDs is therefore greater than that observed by RFLPs (Williams *et al.*, 1992).

Plants represent an unlimited source of phytochemicals such as the metabolites of primary and secondary metabolism. The secondary compounds are of major interest because of their different functions and a wide range of biological activities. Secondary metabolites are compounds that are biosynthetically derived from the primary metabolites and their distribution in the plant kingdom is restricted. These compounds are generally detected in lower volume compared to the primary metabolites and also possess significant biological activities. So they are also termed as the 'higher value – lower volume products' or 'speciality chemicals' (Roja & Rao, 1998).

Essential oils belong to the most vital constituents of many spices and medicinal plants. It is well known that plant derived natural products are extensively used as biologically active compounds. Among them, essential oils were the first preservatives used by man originally in its natural state within plant tissues and then as oils obtained by

distillation. Essential oils are distinguished from fatty oils in that they evaporate or volatilize in contact with air and possess a pleasant taste and strong aromatic odour. They can be removed from plant tissues without any change in composition (Hill, 1972). Most essential oils are very complex mixtures consisting of hundreds of compounds (Bicchi *et al.*, 1999). Essential oils are complex mixtures of odorous and steam volatile compounds which are deposited by plants in the subcuticular space of glandular hairs and in cell organelles like oil bodies, idioblasts, excretory cavities and canals or exceptionally in heartwoods.

Biochemical studies with isolated peltate glandular trichomes have revealed that the secretory cells are not only responsible for the secretion of monoterpenes into the oil storage space, but also serve as the actual site of monoterpene biosynthesis (Gershenson *et al.*, 1992; Mc Caskill *et al.*, 1992). The oil composition especially terpene components are important in chemotaxonomy. Hundreds of different compounds with open chain, cyclic and bicyclic skeletons are known. Most of them have a typical odour, so they are responsible for the aroma of many herbs and spices. Monoterpenes are characteristic plant ingredients. They are also important as raw material for flavouring agents (Faber *et al.*, 1997). The chemical constituents associated with the typical olfactory characteristics of mint oils are monoterpenes and to a lesser extent sesquiterpenes both of which belong to a structurally diverse group of natural products known as isoprenoids (Lange & Croteau, 1999).

The biogenetic origin of most of the *Mentha* oil components has been deciphered in the last 20 years (Murray *et al.*, 1980; Maffei, 1988). It is known that the chemical composition of the oil is greatly affected by extrinsic factors like light, water, temperature, soil and nutrients (Grahle & Hoeltzel, 1963; Burbott & Loomis, 1967; Clark & Menary, 1979a; 1979b; 1981; Murray *et al.,* 1988; Lawrence, 1989) and intrinsic factors like genotype and stage of development of leaves and glands (Lawrence, 1989; Clark & Menary, 1981; Murray *et al.,* 1988; Kokkini, 1991). Oil yield and quality are genotype dependent characteristics (Murray, 1960a; b; Hefendehl & Murray, 1976).

Chemically and biogenetically, the natural product essential oil is heterogeneous. The best known constituents of essential oils are terpenoids, the mono, hemi, sesqui and

diterpenes (C_5, C_{10}, C_{15} and C_{20} respectively), but they may also contain aliphatic and aromatic esters, phenolic compounds and substituted benzene hydrocarbons (Ramawat, 1999). Phenyl propanoids, alkane derivatives, alkanals, alkanoic acids etc. are by far the other ubiquitous essential oil components (Hegnauer, 1982). The volatile fraction of the essential oil, commonly called terpenes, contain a large number of hydrocarbons, all of the formula $C_{10}H_{16}$ (De Mayo, 1959). Some essential oils are attractive to certain animals and insects (Nicholas, 1973) and some are repellant. Sesquiterpenes regulate bud dormancy, abscission and are used as antifungals. Plants having considerable amount of oil are prevented from becoming too warm. Some oils help in wound healing (Guenther, 1949; Hegnauer, 1982).

A multiplicity of ecological roles has been ascribed to essential oils and their constituents. There is a general consensus that these compounds contribute a basic armament in the defensive potential of the plants against excessive water loss (Rhoades, 1977; Stephanou & Manetas, 1995) or biotic attack and act as antiherbivore, antibacterial and antifungal agents. Certain allelopathic functions have also been reported in many cases (Harbone, 1997; Fischer, 1991; Cole, 1992). In recent years essential oils have received much attention as resources of potentially useful bioactive components. They are plant secondary metabolites mainly composed of terpenoid compounds and play an important role in the interactions between plant and insects. The effect of essential oils on insects range from an attraction or repellence to that of toxicity or even lethality (Karpouhtsis *et al.*, 1998). Many of these crude mixtures have been found to have *in vitro* antifungal, antibacterial, cytostatic and insecticidal activities (Janssen *et al.*, 1987; Thompson, 1989; Konstantopoulou *et al.*, 1992; Sivropoulou *et al.*, 1995; Mehmood *et al.*, 1997; Deena & Thoppil, 2000; Thoppil *et al.*, 2001a, b). It is also used as a termiticide (Beckstrom-Sternberg & Duke, 1996). Today there is an increasing interest in the use of 'microbicidal' plants because of the necessity of finding safer insecticides and microbicides in combination with the need of preventing environmental degradation and pollution (Franzios *et al.*, 1997). The mint essential oils and their fractions rich in specific terpenes and semisynthetic products of individual terpenes have been found to possess a variety of antimicrobial, pesticidal and anticancer properties, on which new uses of mint oils are expected to be based (Patra *et al.*, 2001a).

Essential oils are effectively used in aromatherapy. They are believed to stimulate the olfactory nerves and exert influence on the brain centre that controls emotion (Mabey, 1988). Essential oils are used as natural rejuvenating and antiwrinkle agent in aromatherapy (Varshney, 1991). It is suitable in the treatment of pain, psychological disturbances, allergies, skin diseases, gastrointestinal disorders, cardiovascular problems, urinary disorders, gynaecological disturbances, cancer etc. (Jamil, 1997).

Menthol, the major component in mint oils is a crystalline stearopten obtained by cooling the distilled oil and freezing the distillate by ice and salt. It occurs in permanent prismatic or colourless acicular crystals resembling sulfate of magnesium and very slightly soluble in water (Boericke, 1991; Khory & Katrack, 1999). It is an antineuralgic and applied externally in alcoholic solution or in the form of the popular 'menthol care' (Dey & Bahadur, 1973). In India it is taken with betels providing a cooling sensation when chewed (Tyagi & Naqvi, 1987). It is also used against malaria, tape worm infection, giardiasis (Anonymous, 1973), acute nasal catarrh, pharyngitis, laryngitis and neuralgia (Boericke, 1991). Menthol is a stimulant of Central Nervous System (CNS) but it can be toxic when ingested with serious effects to the CNS (Benezra *et al.*, 1985; Der-Marderosian & Liberti, 1988). Because of its refreshing aroma and cooling effects, menthol is used in tooth pastes, mouthwashes, pan masala, hair oils, chewing gums, candies, pain relieving balms (Singh *et al.*, 1998), after shave lotions and medicinal and cosmetic preparations (Tyagi & Naqvi, 1987). It is also used widely in lotions, antiseptics, dentrifuges, cigarettes and certain foods (Schery, 1972).

In addition, the versatile use of several aromatic plants in food and cosmetic industries demands an extensive screening of essential oils and their components. Individual chemicals isolated from essential oils are more often used than the oils (Brud & Gora, 1989). So identification of trace components is very helpful to reveal the quality of the oil. Analysis of essential oils can be easily done using the technique of Gas Chromatography-Mass Spectrometry (GC-MS). GC is a tool for separating the volatile components while analysis depends upon retention characteristics under standard conditions. The mass spectrometer can be used as a detector for a gas chromatograph in which 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 the

GC-MS technique and the routine nature of much of the data obtained make the computer a very useful accessory to the GC-MS unit. With the help of GC-MS technique, it has now been possible to analyze directly the fragrances of natural or artificial materials without the use of heat or solvents and directly by the use of head space analysis (Thappa *et al.*, 1982). MS differs from other types of spectral analysis in that the sample does not absorb radiation from the electromagnetic spectrum. It is highly sensitive and only a small quantity of the sample is required. When coupled with separation techniques like GC or HPLC (High Performance Liquid Chromatography), it is a highly specific way to identify organic compounds (Smith & Busch, 1999).

With regard to mint oil chemical composition, discovery and selection of new landraces or chemotypes of *Mentha* species bring forth novel and unique flavour profiles. These are unique model systems for the study of essential oil biosynthetic pathway regulation and can be applied commercially, fulfilling some special market requirements. In view of great public concern about the safety of synthetic aroma chemicals and also formation of mixture of isomers by synthetic chemicals, essential oil bearing plants shall remain an ideal commercial source for aroma chemicals (Hussain, 1991). The quality and quantity of the aromatic compounds that can be extracted from a sample is a sensitive aspect that depends upon the species, variety, locality, time and method of collection, method of extraction, storage etc., making quality control a very intricate issue. The present study is an attempt to generate somaclonal variants of *M. rotundifolia* by *in vitro* techniques, that differ from the parent plant in quality and quantity of the essential oil and to reveal the genetic basis of variation in them by using the chromosome image analysis system and RAPD technique.

REVIEW OF LITERATURE

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

REVIEW OF LITERATURE

1. Micropropagation

Genetic variability, which forms the basis of the selection for crop improvement, can be generated through hybridisation, polyploidy and induced mutagenesis. Nowadays attention has been focussed on inducing genetic variability through tissue cultures and the plants regenerated from undifferentiated callus cultures have now become a new and useful source of genetic variation.

Tissue culture of peppermint using White's medium supplemented with 2,4-D and coconut water (CW) was attempted as early as in 1951 and by 1960, slow growing cultures of *M. piperita* had been established and maintained on semisolid agar medium containing 15% coconut water and 1 mg/l 2,4-D (Hove, 1951). Callus cultures of peppermint and spearmint were established on a high salt medium containing an extraordinary amount of myo inositol (5 g/l). In addition to this, a complex mixture of vitamins was also used to establish callus cultures of these mints (Lin & Staba, 1961). Tulecke *et al.* (1961) reported the presence of regeneration inducing components in tender coconut water.

Lamba & Staba (1963) investigated the effects of various growth regulators added in solid media on *M. spicata* cell suspension. George & Sherrington (1984) reported the presence of regeneration inhibitors in mature coconut water. Bayliss (1980) also generated genetic variability through cell cultures. Since then the application of plant tissue culture for the induction of stable and heritable variations had been demonstrated in a range of economic plant species (Evans & Sharp, 1985; Bajaj, 1986). Experimental conditions for rapid multiplication of various *Mentha* species (*M. arvensis, M. spicata, M. piperita, M. viridis* and *M. pulegium*) using single node stem segments had also been described (Rech & Pires, 1986). Axillary bud proliferation was observed on MS media supplemented with BAP and kinetin (KIN). Micropropagation of *M. piperita* was also demonstrated by Repcakova *et al.* (1986).

Codaccioni & Vescovi (1987) obtained *in vitro* cultures of *M. viridis* in presence of mannitol. Rodov & Davidova (1987) tried meristem culture in mint. They found that amending the proliferation medium with BA and rooting the microcuttings could increase

the propagation rates. Mariska *et al.* (1987) obtained bud multiplication through *in vitro* culture in *Mentha* spp. Stable and heritable variation was reported by Mathur *et al.* (1988) in plants derived through tissue cultures. Bhaumic & Datta (1988) standardised a new media composition to obtain menthol by tissue culture of Japanese mint. Axillary buds of *M. piperita* had been used as a test system for micropropagation (Ravisankar & Venkataraman, 1988). The role of hormones in tissue culture and micropropagation of mints was reported by Krikorian *et al.* (1988). The propagated plants were true to parent and produced 0.85% oil on fresh weight basis.

Geslot *et al.* (1989) proposed an *in vitro* multiplication method for mints, combining the advantages of apex culture and microcutting techniques. The study concerned six taxa of *Mentha* and the results were comparable in various taxa except for some quantitative differences. Shoot elongation of mints was found to be greatly affected by temperature and photosynthetic photon flux (Karlsson *et al.*, 1989). It was reported that the carbon source in the culture medium had a regulatory role in the *in vitro* shoot multiplication in many plants (Welander *et al.*, 1989).

Plant regeneration from callus cultures of mint depended on explant source, genotype and culture medium components. An exclusive study was made to determine the influence of explant source and medium components on shoot regeneration. Leaf discs from peppermint, lavender mint, orange mint, spearmint and Scotch spearmint were cultured on various MS based media (Van Eck & Kitto, 1992). BAP and CW supported significantly large number of shoots (Van Eck & Kitto, 1990). Banthorpe & Brown (1990) reported the growth and secondary metabolism in cell cultures of *Mentha* in buffered medium. Malek *et al.* (1992) and Moe *et al.* (1990) reported the effect of day and night temperatures on stem elongation of mints. With this background, efforts were initiated to develop high yielding somaclones possessing desired oil composition and resistance to various diseases (Kukreja *et al.*, 1991a; b; 1992; 1998).

Appelgren (1991) reported the correlation between light quality and stem elongation in micropropagation of mints. The effect of photosynthetic photon flux, photoperiod and photoperiod/dark period temperature on *in vitro* stem elongation and shoot length of mints was reported by Kozai *et al.* (1992; 1995; Tutty *et al.*, 1994).

According to Kozai *et al.* (1993), stem elongation of *in vitro* mints was also greatly affected by humidity in the culture vessel.

Micropropagation protocol of *Mentha* species using terminal and axillary buds with modified MS medium had been reported (Cellarova, 1992; Romallo & Gonzalez, 1994). Van Eck & Kitto (1992) obtained efficient regeneration of peppermint and orange mint from leaf disks. Reed *et al.* (1995) detected the presence of endophytic bacteria in mint plants. They have also reported eradication methods to make cultures bacteria free. Buckley *et al.* (1995) characterised and identified these bacteria using biochemical and morphological tests.

According to another report, size of culture chamber and medium volume had influenced the growth rate of shoot tips of spearmint (Tisserat, 1996). A regeneration protocol allowing 51% shooting frequency from microcuttings of peppermint was proposed by Caissard *et al.* (1996). According to Jeong (1996), photosynthetic photon flux and difference between day and night temperatures had greatly affected the stem elongation and growth of *M. rotundifolia.*

Kukreja (1996) had reported a two step procedure for micropropagation and *in vitro* shoot regeneration from leaf and nodal explants of a commercial cultivar of peppermint. Nodal explants cultured on MS medium containing KIN or BAP and IAA produced multiple axillary and adventitious shoots which easily rooted on IAA fortified MS medium.

Bandziuliene & Indrisiunaite (1996) also reported micropropagation of mint. Regenerated buds from *in vitro* cultured leaves of peppermint had been obtained on a ½ MS medium supplemented with saccharose and various concentrations of BAP. Medou *et al.* (1997) used young leaves from *in vitro* micropropagated plants cultivated in the dark for this purpose. Micropropagation was reported in spearmint and peppermint by Sajina *et al.* 1997. Berry *et al.* (1997) obtained regeneration from leaf disks and petioles of *M. citrata, M. piperita, M. spicata* and *M. gracilis.* Somaclones exceeding the control plants in oil and menthol content was produced by micropropagation (Xue *et al.*, 1998). Li *et al.* (1999) obtained efficient plant regeneration from native spearmint.

Tisserat & Silman (2000a) studied the interactions of culture vessels, media volume, culture density and CO_2 level on spearmint shoot growth *in vitro*. High positive correlation occurred between the culture vessel capacity and spearmint fresh weight, leaf number and root and shoot number. Tisserat & Silman (2000b) also reported the enhancement effect of ultra high CO_2 levels on *in vitro* shoot growth and morphogenesis in Labiatae members. Kukreja & Dhawan (2000) tried somaclonal breeding for improvement of herb and essential oil yield in mints.

Entry and establishment of pathogenic bacteria in plant tissues was reported by Billing (1982). Leifert *et al.* (1989; 1991a) found bacterial contamination in micropropagated plant tissues. Presence of endophytic bacteria in mint species is reported by Reed *et al.* (1995). Increasing attention has been directed to the problem of microbial contamination of plant tissue culture by workers seeking to ascertain sources of contamination and to develop procedures for eliminating them by avoidance, rigorous manipulation of environmental and nutritional factors or treatment with antibiotics (De Fossard & De Fossard, 1988; Cassells, 1991; Debergh & Zimmerman, 1991; Viss *et al.*, 1991; Kneifel & Leonhardt, 1992). The popular solution was to use antibacterial substances like antibiotics. This approach had met with varying degrees of success (Cornu & Mitchel, 1987; Falkiner, 1990; Leifert *et al.*, 1991b). In some cases, antibiotic treatment appeared bacteriostatic, resulting in reduced rather than eliminated contamination (Phillips *et al.*, 1981; Bastiaens *et al.*, 1983; Young *et al.*, 1984). In other cases, phytotoxicity of the antibiotic had precluded their use at a level high enough to destroy all contaminants (Falkiner, 1990; Leifert *et al.*, 1991a; 1992). Falkiner (1988) reviewed the desirable features of antimicrobial substances for use in plant tissue culture and outlined the establishment of antibiotic combinations and effective concentrations for treatment of infected tissues. Combinations of antibiotics to control microbial contamination in micropropagated plants were tried by Leifert *et al.* (1992).

2. Cytological Analysis

Regenerability of a callus was often ascribed to numerical and structural changes in the chromosomes (Muir, 1965; Murashige & Nakano, 1965; Torey, 1967).

Chromosomal instability of callus and cell suspension cultures had often been reported (D'Amato, 1977). Chromosomal instability in cells grown *in vitro* not only included numerical variations but also comprised changes in chromosome structure and basic karyotype (Sunderland, 1977).

Variation in callus culture had been observed to be expressed within the regenerants of several plants (Sacristan, 1971; Orton, 1980). Numerical changes had been examined most often among flowering plants (Evans & Reed, 1981; Mohanty, 1990) in tissue culture. Chromosomal variation and frequency of spontaneous mutation associated with *in vitro* culture and regeneration had been reported by Edallo *et al.* (1981). It had been observed that plant regeneration in callus culture was obtained mainly from cells with diploid chromosome number (Larkin & Scowcroft, 1981; Vasil, 1983). Variation within regenerated plants was often reported (Mc Coy *et al.*, 1982; Scowcroft, 1984). The possibility of minor genetic reshuffles in the form of minor structural changes in chromosomes without affecting their original chromosome number in regenerants could not be ruled out. Structural alterations of chromosomes in cultured cells were reported by Mohanty *et al.* (1991).

A detailed analysis of the mitotic and meiotic behaviour of chromosomes of different species and populations of mints (*M. spicata, M. arvensis* var. *javanica, M. arvensis, M. piperita*) was conducted by Kundu & Sharma (1985). The chromosomes were very small in size (0.89 to 2.5μ) with median to nearly median primary constrictions. Chromosomal interchanges had been described to be the basis for obtaining somaclonal variation (Karp & Bright, 1985; Pijnaker & Ferweda, 1987). No direct relationship with chromosome number variation to yield and quality of essential oil was found in *M. arvensis* (Tyagi & Naqvi, 1987). The chromosome number in an F_1 hybrid progeny between *M. spicata* and *M. piperita* ranged from 36-115 (Tyagi & Ahmad, 1989). The normally expected chromosome number (2n=96) was found to be the most frequent.

3. Random Amplified Polymorphic DNA (RAPD) Analysis

Molecular markers had already been used in 1989 to detect the genetic basis and breeding applications of somaclonal variation (Evans, 1989). Bousquet *et al.* (1990)

obtained DNA amplification from vegetative and sexual tissues of plants by PCR. It was reported that molecular markers could be used for differentiation of nearly identical germplasm accessions (Carlson *et al.,* 1991; Potter & Jones, 1991; Weycott & Fort, 1991). RAPDs were used for identification of germplasm (Fukuorka, 1992; Vidal *et al.*, 1999) and in other gene introgression breeding programmes (Frederic *et al.*, 1992).

Shenoy & Vasil (1992) reported that micropropagation through meristem culture was generally associated with low risk of genetic instability because the organised meristems were generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions. Bouman *et al.* (1992), Bouman & Kuijpers (1994) and Al-Zahim *et al.* (1999) also found intraclonal RAPD polymorphism among micropropagated plants.

Many authors using RAPD or RFLP, have failed to observe intraclonal variations in various plant species (Merkle *et al.*, 1988; Shenoy & Vasil, 1992; Isabel *et al.*, 1993; Valles *et al.*, 1993; Choudhury & Vasil, 1993; Rout *et al.*, 1998). Recently DNA markers were utilised effectively in plant improvement programmes (Paterson *et al.*, 1991; Waugh & Rowell, 1992; Rafalsky *et al.*, 1994).

Molecular analysis of plants regenerated from embryogenic cultures was conducted by Choudhary *et al.* (1994). Virk *et al.* (1995) used RAPD for the study of diversity within plant germplasm. Rani *et al.* (1995) found RAPD variations among 23 micropropagated plants originating from the same clone which were morphologically similar. The variation of monomorphic bands in micropropagated plants by using different primers had been reported earlier (Potter & Jones, 1991; Angel *et al.*, 1996).

Sato *et al.* (1996) carried out somatic hybridisation between peppermint and gingermint by protoplast fusion. Chromosome counts and RAPD analysis indicated that it was an interspecific somatic hybrid. Among the reports on the use of RAPD analysis for the molecular analysis in the micropropagated clones, emphasis has been laid on the confirmation of genetic homogeneity of the raised plantlets. Wallner *et al.* (1996) had applied this technique to compare the field grown and *in vitro* micropropagated plants.

RAPD markers were generated and used in plant improvement programmes (Rafalsky *et al.*, 1996) and in assessment of botanical diversity (Karp *et al.*, 1996). Identification of plants using sequence characterised amplified region DNA markers were also reported (Xong & Bakalinsky 1996; Zheu *et al.* 1996; Shoyama *et al.* 1997). Similarly from the conservation point of view, Parani *et al.* (1997) used RAPD approach to compare the mother plant and micropropagated progenies to maintain the fidelity of elite genotypes that were to be conserved.

Kumar *et al.* (1997) developed a high menthol yielding hybrid clone of *M. arvensis*. The uniqueness of its genotype was evident at molecular level from its RAPD profile generated by a set of 12 primers. Recently a procedure that permits rapid isolation of somaclones of cultured genotypes in *M. arvensis* had been developed (Khanuja *et al.*, 1998). RAPD analysis of the phenotypically differing somaclones confirmed alteration at genetic levels also.

PCR amplification technique was used to detect the transgenic peppermint plants regenerated after cocultivation with *Agrobacterium* (Lin *et al.*, 1998). Shasany *et al.* (1998) have defined media and incubation conditions for highly efficient regeneration of shoots from internodal explants of slow and fast growing cultivars of *M. arvensis*. The genetic homogeneity of regenerated plants was ascertained with the use of RAPD technique.

Krasnyanski *et al.* (1998) used RAPD technique to identify the somatic hybrids between *M. piperita* and *M. spicata* produced by protoplast fusion. Diemer *et al.* (1999) produced *Agrobacterium tumefaciens* mediated transgenic *M. spicata* and *M. arvensis*. The presence and structure of transgenes were studied by the use of PCR analysis and Southern blot hybridisation. Direct sequencing of RAPD products was used as a practical tool for marker assisted selection of wheat (Hernandez *et al.*, 1999).

Krasnyanski *et al.* (1999) tried *Agrobacterium tumefaciens* mediated and direct gene transfer into protoplasts to produce transformed peppermint plants with limonene synthase gene. Both type of transformation resulted in transgenic plants, which were detected using PCR, and confirmed by Southern blot hybridisation. RAPD profiles were

unambiguously used to establish the distinct identity as different from the parent plant in many new varieties of *M. arvensis* (Khanuja *et al.*, 2001a; Khanuja *et al.*, 2001b), *M. spicata* var. *viridis* (Khanuja *et al.*, 2001c), *M. piperita* (Dwivedi *et al.*, 2001a) and *M. gracilis* var. *cardiaca* (Dwivedi *et al.*, 2001b). RAPD analysis had also been used for the confirmation of hybridity (Patra *et al.*, 2001c). Molecular characterization of several other plants have been done successfully by RAPD technique (Ayana *et al.*, 2000; Pradeepkumar *et al.*, 2001; Dwivedi *et al.*, 2001c; Patra *et al.*, 2001b).

4. Essential Oil Analysis

Accumulation of monoterpenes has been found to vary in *Mentha* cultures both between the taxa and between the different cell lines from the same species. A number of reports described that *in vitro* cultures of *M. spicata, M. piperita,* and *M. pulegium* failed to produce volatiles (Lin & Staba, 1961; Wang & Staba, 1963; Staba *et al.*, 1965; Becker, 1970) Monoterpene interconversions in a cell free system from *M. piperita* was reported by Bhattaile *et al.* (1968).

Bricout & Poupardin (1975) and Bricout *et al.* (1978) studied the biosynthesis of monoterpenes in six *Mentha* species. Monoterpene formation was lower in plant cultured *in vitro* than in control plants. In *M. rotundifolia* and *M. piperita* the products synthesized *in vitro* were more oxidised than the natural forms. *M. rotundifolia* produced epoxy piperitone in place of menthone and *M. piperita* elaborated menthofuran and pulegone in cultures instead of menthol and menthone. Terpenes did not accumulate in suspension cultures of six *Mentha* chemotypes (Aviv & Galun, 1978). None of the monoterpenes characteristic of essential oil of intact plant of *M. piperita* was found in the callus culture derived from this species.

Callus cultures established from the leaves of the allopolyploid form of *M. piperita* had the capacity to synthesize oil of intact plants (Kireeva *et al.*, 1978). The callus released the oil into the culture medium. They observed that under the normal conditions of cultivation, biosynthesis of terminal products (menthone and menthol) was inhibited and their precursors (piperitone and pulegone) were accumulated. Malnikov *et al.* (1979) also investigated formation of terpenes in callus cultures of genetically different lines of *M.*

spicata. Karasawa & Shimizu (1980) described the effect of KIN and NAA in the media on the composition of triterpenes between the callus tissue and intact plants of *M. rotundifolia, M. arvensis* and. *M. spicata*. There were some other cases where no terpenoid accumulation was reported in cultured tissue (Suga *et al.*, 1980).

Charlwood & Charlwood (1983) described the results of an investigation concerning the monoterpene production by tissue cultures of several *Mentha* species. They observed that many callus cultures accumulated only the early precursors of these compounds such as geraniol, linalool etc. But some other cultures synthesized monoterpenes characteristic of intact plants. Rodov & Reznikova (1982) used peppermint cell suspension cultures as model systems for studying the pathways of essential oil synthesis.

Essential oil composition of *in vitro* propagated *M. piperita* changed markedly during the culture period leading to the recognition of 3 phenotypes (Mucciarelli *et al.*, 1995). Chaput *et al.* (1996) developed a procedure to regenerate plants from leaf protoplasts of two micropropagated hybrid species of mint, *M. piperita* and *M. citrata* in order to determine whether the *in vitro* treatment could influence the monoterpene composition. In the field trial, a decrease in the amount of menthone and menthol and an increase of carvone levels were noticed in all protoplast-derived plants.

Jullien *et al.* (1997) applied biotechnological tools for the improvement of mints and essential oils. Karousou *et al.* (1998) tried *in vitro* propagation in two chemotypes of *M. spicata*. Abou-Mandour & Binder (1998) studied the effect of exogenous growth regulators on plant regeneration from tissue cultures of *M. spicata* and production of volatiles.

Essential oil obtained from shoot tip cultures of *M. spicata* by Hirata *et al.* (1990) showed that carvone and limonene were higher in the cultured plant than the parent plants. The production of these monoterpenoids was influenced by NAA and thiamine hydrochloride. New approaches directed to unravelling monoterpene metabolism and secretion and recent progress in transformation protocols had set the stages for the

systematic genetic engineering of essential oil production in mint (Lange & Croteau, 1999).

Volatile oil yield and composition of *M. spicata* obtained by solvent extraction and supercritical CO_2 extraction were compared by Pino *et al.* (1999a). Sensory profiles and GC-MS analysis of both extracts were performed and their results were compared with those obtained by steam distillation. The difference in aroma of the extracts was quite noticeable and was attributed to qualitative and quantitative differences in components.

The relation between oil yield and free menthol content (Ellis & Gaylard, 1944) and time of culturing and free menthol content (Ellis, 1945) were reported very earlier. Murray & Reitsema (1954) revealed genetic basis of the biosynthesis of ketones, carvone and menthone in *M. crispa*. Murray (1960a) reported genetic basis for conversion of menthone to menthol in Japanese mint.

Concerning the biosynthetic pathway leading to the formation of isomenthone, two possible precursors- piperitone (Burbott & Loomis, 1967) and pulegone (Katsuhara, 1966; Lawrence, 1978) were reported. Genetic basis for the reduction of piperitenone to the ketone pulegone, pulegone to menthone and menthone to menthol was revealed by Hendriks *et al.* (1976). Monogenic basis for the reduction of (+)-pulegone to (-)-menthone in *Mentha* oils was revealed by Lincon & Murray (1978).

According to Aviv & Galun (1978), biotransformation of pulegone to isomenthone occurred in *Mentha* cell lines. The relation between mint physiology and essential oil production was reported by Loomis & Croteau in 1979. According to Croteau & Martinkus (1979), (+)-neomenthyl β-D-glucoside was the major metabolite of (-)-menthone in peppermint. Multiple allele control of the biosynthesis of (-)-menthone and (+)-isomenthone stereoisomers in *Mentha* species was reported by Murray *et al.* (1980).

Limonene was the first cyclic intermediate in the biosynthesis of oxygenated p-menthane monoterpenes in *M. piperita* and other *Mentha* species (Kjonaas & Croteau, 1983). Kokkini (1983) reported a carvone chemotype of the diploid hybrid, *M. rotundifolia* found in a mixed population with its parental species *M. longifolia* and *M. suaveolens* growing in Greece.

Research published to date strongly suggested that the qualitative production of mint essential oils was clearly controlled by simple genetic systems (Lincon *et al.*, 1986).

Dmitrev *et al.* (1988) used GC-MS technique to reveal the chemical components of volatile substances released by plants. Bharadwaj (1989) made correlation studies on yield and quality characters of essential oil in peppermint.

Gershenzon *et al.* (1989) could localize the monoterpene biosynthesis in glandular trichomes of spearmint using biochemical and histochemical methods. Variation between the essential oil components in commercially exploited and wild populations of *M. spicata* was further reported by Kokkini & Vokou (1989). They characterised four different chemotypes in this species.

Isopentenyl diphosphate (IPP) was the intermediate essential oil precursor leading to the formation of over 22000 known isoprenoids (Connolly & Hill, 1992). Maat *et al.* (1992) analysed the essential oil composition of *M. mirennae* by GC-MS technique. The basic pathway and formation of monoterpenes, sesquiterpenes and diterpenes were revealed by Gershenzon & Croteau (1993). Genetic control of monoterpene biosynthesis in mints was reported by Croteau & Gershenzon (1994).

Biochemistry and molecular biology of isoprenoid biosynthetic pathway was revealed by Chappel (1995). Biosynthesis of monoterpenes and sesquiterpenes from plastid derived isopentenyl diphosphate was described by Mc Caskill & Croteau (1995). The identification of monoterpenes and sesquiterpene hydrocarbons in *M. piperita* using GC and GC-MS techniques was done by Zenkevich (1996).

Bohlman *et al.* (1998) revealed the molecular biology and phylogenetic analysis of plant terpenoid synthesis. Schulz *et al.* (1999) studied the chemical composition of secondary metabolites in the leaves of different *Mentha* species using Near Infra Red spectroscopy (NIR). Biosynthesis of monoterpenes in mint from Geranyl pyrophosphate (GPP) (Wise & Croteau, 1999), sesquiterpenes from Farnesyl pyrophosphate (FPP) (Cane, 1999) and diterpenes from Geranyl Geranyl pyrophosphate (GGPP) (MacMillan, 1999) were reported recently.

Table: 1

Previous reports on chromosome counts of *M. rotundifolia*

Name of taxa	Chromosome No.	Authority	Year
M. rotundifolia (L.) Huds.	2n=54	Schurhoff	1929
,,	2n=24	Ruttle	1931
,,	,,	Junell	1937
,,	2n=18	Heimans	1938
,,	2n=24	Nagao	1941
,,	,,	Junell	1942
,,	2n=54	Delay	1947
,,	2n=24	Suzuka & Koriba	1949
,,	,,	Morton	1956
,,	,,	Murray	1958
,,	2n=24, 36	Arora	1960
,,	,,	Murray	1960b
,,	2n=24	Love & Love	1961
,,	,,	Gadella & Kliphuis	1963
,,	,,	Sobti	1965
M. rotundifolia (L.) Huds. var. *variegata* Sole.	,,	,,	,,
M. rotundifolia (L.) Huds.	2n=24, 36	Ouweneel	1968
M. rotundifolia (L.) Huds. var. *nullata* Briq.	2n=36, 48	Sacco & Scannerini	,,
M. rotundifolia (L.) Huds.	2n=24, 36	Dahlgren *et al.*	1971
,,	2n=24	Sobti	1971

,,	,,	Harley & Brighton	1977
M. rotundifolia (L.) Huds.	,,	Gill	1981
	,,	Roy *et al.*	1983
	,,	Queiros	1985
	,,	Chambers & Hummer	1994
	,,	Thoppil	1993

Table: 2

Previous reports on chemical components of *M. rotundifolia* essential oil

Name of taxa	Part used	Chemical component	Authority	Year
M. rotundifolia (L.) Huds.	Whole plant	piperitenone oxide pinene limonene	CSIR	1962
M. rotundifolia var. *bullata*	Whole plant	piperitenone oxide	Sacco & Scannerini	1968
M. rotundifolia (L.) Huds.	Whole plant	rotundifolone neoisopulegol	Chopra *et al.*	1969
M. rotundifolia (L.) Huds.	Whole plant	piperitone piperitenone oxide	Hendriks	1971
M. rotundifolia (L.) Huds. Chemotype 1	Aerial parts	α-pinene camphene β-pinene sabinene myrcene limonene 1,8-cineole β-terpinene p-cymene terpinolene 3-octyl acetate 3-octanol nonanal hexylisovalerate menthone octyl acetate isomenthone β-bourbonene 1-terpinene-4-ol caryophyllene menthol germacrene D piperitone oxide δ-cadinene piperitenone piperitenone oxide α-pinene	Kokkini & Papageorgiou	1988
Chemotype 2	,,	camphene	,,	,,

M. rotundifolia (L.) Huds. Chemotype 2		β-pinene sabinene myrcene limonene 1,8-cineole cis-ocimene trans-ocimene γ-terpinene p-cymene terpinolene 3-octyl acetate 3-octanol nonanal hexylisovalerate menthone isomenthone linalool menthyl acetate neoiso menthyl acetate caryophyllene menthol pulegone α-terpineol germacrene D piperitone piperitenone	Kokkini & Papageorgiou	1988
M. rotundifolia (L.) Huds.	Whole plant	isopiperitenone carvone	Rastogi & Mehrotra	1990
,,	,,	piperitone oxide 1,2-epoxy menthyl acetate piperitenone piperitenone oxide limonene carvone pulegone menthone isomenthone cineole limonene o-cresol p-cresol	,,	1991
,,	,,	piperitenone oxide 1,2-epoxy neomenthol 1,2-epoxy neomenthol acetate 4-hydroxypiperitone		

M. rotundifolia (L.) Huds.		luteolin luteolinidin pelargonidin cyanidin delphinidin petunidin	Rastogi & Mehrotra "	1993 "
M. rotundifolia (L.) Huds.	Aerial parts	menthone isomenthone	Thoppil	1993
M. suaveolens Ehrh.	Fresh leaves	α-thujene α-pinene camphene sabinene β–pinene myrcene 3-octanol α-phellandrene α-terpinene p-cymene limonene 1,8-cineole γ-terpinene cis-linalool oxide trans-linalool oxide linalool camphor menthone isomenthone isopulegone menthofuran neomenthol menthol terpinen-4-ol isomenthol α-terpineole pulegone piperitone isopulegyl acetate neomenthyl acetate menthyl acetate piperitenone δ-elemene α-cububene α-copaene β-cububene β-bourbonene β-elemene β-caryophyllene	Velasco-Negueruela & Perez-Alonso	1996

M. suaveolens Ehrh.		aromadendrene β-humulene allo aromadendrene germacrene D viridiflorol α-cadineol	Velasco-Negueruela & Perez-Alonso	1996
M. rotundifolia (L.) Huds.	Aerial parts	piperitenone oxide pulegone carvone limonene 1,2-epoxy menthol acetate 1,2-epoxy neomenthol acetate allo-cymene α-phellandrene α-pinene apigenin β –pinene borneol acetate caffeic acid camphene cineole citral cyanidin delphinidinin dihydrocarvone diosphenol diosphenolene isoneopulegol isoneopulegol acetate isopiperitenone menthofuran menthol menthone neoisopulegol octan-3-ol octan-3-ol acetate p-cymene pelargonidin petunidin piperitenone piperitenone oxide piperitone piperitone oxide pulegone rotundifolone thujone	Beckstrom-Sternberg & Duke	1996

M. rotundifolia (L.) Huds	Air dried leaves	1-octen-3-ol myrcene p-cymene limonene (Z)-β-ocimene cis-sabinene hydrate trans-sabinene hydrate linalool isoamyl isovalerate 1-octen-3-yl-acetate veratrol menthone isomenthone borneol neomenthol menthol terpinen-4-ol α-terpineol 1-menthoxy-4-propyl benzene methyl chavicol carvone piperitone piperitine oxide bornyl acetate thymol piperitone oxide α-copaene 2,4(8),6-p-menthatrien-2,3-diol β–bourbonene cyperene β–caryophyllene β–gurgunene geranyl acetone α-humulene γ-muurolene germacrene D cis-β-gualene bicyclogermacrene α-muurolene γ-cadinene δ-cadinene germacren D-4-ol viridiflorol cubenol α-cadinol	Beckstrom-Sternberg & Duke	1996

M. rotundifolia (L.) Huds.		1,4-hydroxy-α-muurolene khusinol acetate hexadecanol hexadecanoic acid phytol	Pino *et al.*	1999b
M. rotundifolia (L.) Huds.	Fresh herb	pinene limonene	Bhattacharjee	2000

Table: 3

Previous reports on the biological activities of phytochemicals of *M. rotundifolia* essential oil detected in the present study.

Component	Biological activity	Authority	Year
α-pinene	allelochemic allergenic	Mitchell & Rook	1923
	irritant	Harbone & Baxter	1983
	cancer preventive	Stitt	1990
	insectifuge	Jacobson	1990
	herbicide	Keeler & Tu	1991
	coleoptiphile expectorant antiflu	Castleman	1991
	anti-inflammatory antiviral bactericide flavour insectiphile perfumery sedative tranquillizer	Beckstrom-Sternberg & Duke	1996
	bactericide	Srivastava *et al.*	2000
	insecticidal fumigant ovicidal	Singh *et al.*	2000
β-bisabolene	abortifacient	Pei-Gen & Nai-Gong	1991
	antiulcer	Yamahara *et al.*	1992
	antirhinoviral antiviral	Denyer *et al.*	1994
	perfumery	Beckstrom-Sternberg & Duke	1996

β-elemene	anticancer (cervix)	Leeuwenberg	1987
β-terpineol	insectifuge	Jacobson	1990
	perfumery	Beckstrom-Sternberg & Duke	1996
carvone	allergenic	Mitchell & Rook	1923
	antiseptic	Wagner & Wolf	1977
	perfumery	Harbone & Baxter	1983
	insectifuge insecticide	Jacobson	1990
	CNS stimulant cancer preventive	Stitt	1990
	carminative	Yamamoto *et al.*	1993
	flavour motor depressant nematicide sedative vermicide	Beckstrom-Sternberg & Duke	1996
	antibacterial antifungal	Hassanein & Eldokseh Oka *et al.* Aggarwal *et al.*	1997 2000 2002
limonene	nematicide	Beckstrom-Sternberg & Duke	1996
	larvicidal insecticidal	Singh *et al.*	2000
	allergenic antialzheimeran	Mitchell & Rook	1923
	sedative	Wagner & Wolf	1977
	expectorant	Harbone & Baxter	1983
	ACE inhibitor Insecticide	Grundy & Still	1985

	cancer preventive	Stitt	1990
	insectifuge	Jacobson	1990
	herbicide	Keeler & Tu	1991
	candidistat fungistat	Kang *et al.*	1992
	nematicide	Nigg & Seigler	1992
	antitumour	Yu *et al.*	1995
	viricide spasmolytic irritant fungiphilic enterocontractant bactericide antiviral anticancer antiflu antilithic antimutagenic	Beckstrom-Sternberg & Duke	1996
	ovicidal insecticidal	Singh *et al.*	2000
	antibacterial antifungal	Aggarwal *et al.*	2002
methyl chavicol	hepato carcinogenic	Bisset	1994
	insecticide	Beckstrom-Sternberg & Duke Singh *et al.*	1996 2000
myrcene	allergenic	Mitchell & Rook	1923
	insectifuge perfumery	Harbone & Baxter	1983
	antinociceptive	Rao *et al.*	1990
	fungicide	Keeler & Tu	1991
	analgesic antimutagenic	Kauderer *et al.*	1991

citronellol	antioxidant bactericide spasmolytic insecticide repellant	Beckstrom-Sternberg & Duke Singh *et al.*	1996 2000

MATERIALS AND METHODS

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

MATERIALS AND METHODS

1. Micropropagation

M. rotundifolia plants cultivated in the Nilgiris were collected and grown in the net house of Botany Department, University of Calicut. The plant was authenticated at the Herbarium of Botany Department, University of Calicut where a voucher specimen was deposited (CALI 86003). Nodal cuttings (1-1.5 cm) from three months old potted plants were used as explant to initiate the cultures. Nodal cuttings were collected in water. Expanded leaves were removed and the stem cuttings were washed with labolene detergent for 15 min. The tissue was again thoroughly washed in running water followed by a quick rinse (30 seconds) in 70% alcohol. This material was again washed in double distilled water. Surface sterilization was done using 0.1% mercuric chloride for 6 min. The stem cuttings were then washed 5-8 times in double distilled water to remove the traces of $HgCl_2$ The tissue was trimmed again and dipped in 15% streptomycin for 5 min. to eliminate the endophytic bacteria found commonly in *Mentha* species (Reed *et al.*, 1995) and implanted on to the culture medium. 1-2 week old leaves collected from the potted plants were used as explants for callus induction. The leaves were surface sterilized in 0.1% mercuric chloride for 4 min. 1 cm^2 pieces cut from the basal portion of the leaves were dipped in 15% streptomycin for 5 min. and inoculated on to the surface of the medium.

Murashige & Skoog (1962) basal medium (Table 4) with 3% sucrose, 100mg/l myoinositol and 0.8% agar was used. MS basal medium was supplemented with different concentrations of auxins, cytokinins, different combinations of both these and also 15%-50% coconut water alone or in combination with BAP. The pH of the medium was adjusted to 5.8. The media were sterilised at 120^0C for 20 min. 10-15 replicates of each hormonal combination were tried. The cultures were grown at 25 ± 3^0C with 55%-60% humidity under fluorescent day light tubes emitting 2000 lux for 16/8 h light/dark period and were subcultured every 5-7 weeks.

Table: 4

Murashige & Skoog Basal Medium

Stock Chemical	mg/l	Stock Concentration	Stock g/l
I NH_4NO_3	1650.00	50 X	82.50
KNO_3	1900.00		95.00
KH_2PO_4	170.00		8.50
$MgSO_4.7H_2O$	370.00		18.50
II $CaCl_2.2H_2O$	440.00	50 X	22.00
III Na_2EDTA	37.30	100 X	3.70
$FeSO_4.7H_2O$	27.80		2.80
IV $MnSO4.4H_2O$	22.30	100 X	2.23
$ZnSO4.7H_2O$	8.60		0.860
H_3BO_3	6.20		0.620
KI	0.83		0.083
$Na_2MoO_4.2H_2O$	0.25		0.025
$CoCl_2.6H_2O$	0.025		0.0025
$CuSO_4.5H_2O$	0.025		0.0025
V VITAMINS		100 X	
Glycine	2.00		0.200
Nicotinic acid	0.50		0.050
Pyridoxine-HCl	0.50		0.050
Thiamine-HCl	0.10		0.010

Establishment of Plants in Soil

Four to six weeks old micropropagated plants were subcultured in ½ MS medium for rooting. Root system with 5-10 roots (2-6 cm long) was developed after 3 week's growth in ½ MS medium for 7-8 days. The rooted plants were taken out from the culture tubes and planted in a sterilised mixture of sand and soil (1:1) in plastic cups and initially covered with polythene bag to control humidity (Das, 1993). These plants were initially irrigated with ½ strength liquid MS medium for 7-8 days. Well-established plants

were transplanted to earthen pots (Figs. 1.26-1.29) kept in the net house and watered regularly (Figs. 1.26-1.29).

2. Cytological Analysis

(i) Squash Preparation

Cytological preparations were made using improved techniques (Sharma & Sharma, 1990). Young healthy root tips were collected at the time of peak mitotic activity (10-11 AM) from both the parent plant and the field transplanted micropropagated plant. Two weeks old white friable callus was also used for squash preparation. The root tips and callus were washed thoroughly with distilled water and pre treated in cytostatic chemicals. Chilled saturated solution of para dichlorobenzene with a trace of aesculin was used as pre-treatment chemical. Small quantity of saponin was added to remove the oil content from the cells. The pre-treatment solution was initially chilled at 0-5^{0}C for 5 min. and root tips and callus were dipped in it. This was kept at 12-15^{0}C for 3 h. After this, the root tips and callus were washed thoroughly with distilled water and fixed in Carnoy's modified fluid (1 acetic acid : 2 ethanol) for 2 h.

The fixed root tips and callus were washed in distilled water and hydrolysed with 1N HCl for 20 min. and 30 min. respectively at room temperature. Traces of acid were removed by thorough washing in distilled water. The hydrolysed root tips were stained in 2% aceto orcein (Sharma & Sharma, 1990) for 3-4 h. The stained root tips and callus were washed in 45% acetic acid to remove excess stain and squashed in 45% acetic acid. Slides were scanned under LEICA GALLEN III microscope and photographs were taken with a Pentax camera system attached to it.

(ii) Karyomorphological Analysis

Karyograms were prepared from the microphotographs using computer based programs such as Adobe Photoshop, Auto CAD and data based analysing system (Microsoft Excel). Photographs were scanned and stored as digital images. These digital images were converted to grayscale images using Photoshop program. Identification

numbers were allotted to each chromosome and then loaded to Auto CAD for karyomorphometrical analyses. After determining the centromeric position, arm lengths of each chromosome were measured and centromeric indices were calculated. On the basis of arm ratio and centromeric indices, homologous chromosomes were identified and were classified (Table 5) according to Abraham & Prasad (1983). The images were loaded to Photoshop and karyograms were generated.

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

Disparity Index (DI) of the chromosomes were calculated with the method of Mohanty *et al.* (1991) by using the formula

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

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

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

Table: 5

Details of 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 to 0.61	1.01 to 1.63	49.99 to 38.01	50.01 to 61. 99
Nearly Submedian	Nsm(-)	0.60 to 0.34	1.64 to 2.99	38.00 to 25.01	62.00 to 74.99
Submedian	SM	0.33	3.00	25.00	75.00
Nearly Submedian	Nsm(+)	0.32 to 0. 23	3.01 to 4.26	24.99 to 18.20	75.01 to 81.80
Nearly Subterminal	Nst(-)	0.22 to 0.15	4.27 to 6.99	18.19 to 12.51	81.81 to 87.49
Sub Terminal	ST	0.14	7.00	12.50	87.50
Nearly Subterminal	Nst(+)	0.13 to 0.01	7.01 to 14.38	12.49 to 5.01	87.51 to 94.99
Nearly Terminal	Nt	0.06 to 0.01	14.39 to 19.99	5.00 to 0.01	95.00 to 99.99
Terminal	T	0	α	0	100.00

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

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

3. Random Amplified Polymorphic DNA (RAPD) Analysis

RAPD is an improved technique used to reveal sequence polymorphism between template DNAs based on selective amplification of DNA sequences. The template DNA (genomic DNA) can be prepared with any purification protocol appropriate for the biological sample under study.

(i) DNA Extraction

Total DNA was extracted from the parent plant and the cultured plants using CTAB method of Doyle & Doyle (1987) with minor modifications (Ausubel *et al.*, 1995). The steps involved are described below.

- Fresh young leaves of the parent and cultured plants of *M. rotundifolia* were collected in ice boxes and ground immediately in liquid nitrogen with ice cold mortar and pestle. The powder obtained was mixed with 16 ml of extraction buffer (extraction buffer was prepared by mixing 120 ml of 2% CTAB buffer [100 mM Tris HCl pH 8, 1.4 M NaCl, 20 mM EDTA pH 8, 2% CTAB] with 240 µl of 0.2% β mercapto ethanol) taken in oakridge tubes.

- The mixture was incubated at 65^{0}C for 1 h. in a water bath. After this an equal volume (16 ml) of chloroform: isoamyl alcohol (24:1) was added to it and mixed thoroughly. This mixture was centrifuged at 1000 rpm for 10 min. at 4^{0}C in a refrigerated centrifuge (Hitachi, Himac CR 21, Japan). The two phases seemed to separate.

- The clear upper aqueous phase was transferred into a fresh oakridge tube. 2/3rd volume of ice cold isopropanol was added into it and mixed gently. The DNA threads were visible.

- This mixture was kept at –20^{0}C for 15 min. for better precipitation and centrifuged at 1000 rpm for 10 min. at 4^{0}C.

- The supernatant was discarded and the tubes were inverted on paper towels for 5 min. Then the pellet was washed with 70% alcohol twice by centrifugation.

- The pellet was kept overnight for air-drying. The dried pellet was rehydrated again in TE buffer (10 mM Tris HCl pH 8, 0.1 mM EDTA pH 8).

(ii) Purification of Isolated DNA (Removal of RNA & Protein)

- RNase (0.8 µl) was added to 200 µl of dissolved unpurified DNA and the mixture was incubated overnight (~ 15 h) at 37^0C.

- An equal volume (200 µl) of a mixture of phenol, $CHCl_3$ & IAA (25:24:1) was added to the DNA & RNase mixture and centrifuged at 1000 rpm for 10 min. at 4^0C.

- The supernatant was transferred to a fresh tube and added equal volume of $CHCl_3$: IAA (24:1) mixture and centrifuged at the same conditions above.

- The upper aqueous phase was taken and $1/10^{th}$ volume (20 µl) of 3M CHCOONa (pH 5.2) was added into it. The DNA was precipitated with $2/3^{rd}$ volume of cold isopropanol. All the tubes showed transparent gel like precipitate. This DNA was pelleted by centrifugation under the same conditions above.

- The pellets were washed twice with 70% ethanol and kept for air-drying.

- The dried pure DNA was dissolved in 250-µl TE buffer at room temperature. This pure DNA was quantified by running in agarose gel. The quantity was assumed by comparing bandwidth of the sample with that of the weight marker (Fig. 3.1).

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

Arbitrarily Primed Polymerase Chain Reaction (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. Six different decamer oligonucleotide primers (OPA 02, OPA 08, OPB 07, OPD 19, OPE 14 & OPF 05) were used for the present investigation. The sequences of these primers are given in Table: 13.

PCR was performed using 25 µl reaction mixture. The reaction mixture contained 2.5 µl assay buffer (10 nM 3-Tris (hydroxymethyl) methyl aminopropane

sulphonic acid pH 8, 50 mM KCl), 0.3 µl dNTPs, 0.8 µl Taq polymerase, 1.25 µl $MgCl_2$, 2 µl primer and 1 µl genomic DNA. This mixture was made upto 25 µl using 17.15 µl autoclaved double distilled water. Amplification was carried out in a BIO RAD Gene Cycler (Japan) programmed for 35 cycles as follows: I^{st} cycle of 2 min. at 94°C, 1 min. at 40°C, 2 min. at 72°C; followed by 34 cycles each of 1 min. at 94°C, 1 min. at 40°C, 1 min. at 72°C and one final extension cycle of 15 min. at 72°C. The amplification products were size separated by electrophoresis (75 V for 4 h) in 2% agarose gels with 1 X TAE (Tris Acetate EDTA) buffer stained with 3 µl of bromophenol blue. The gels were scanned under UV light in an UV transilluminator. In all cases λ phage DNA digested with EcoRI & Hind III was used as size marker.

(iv) Amplified DNA Marker Scoring

Amplified DNA markers were scored as present or absent in each micropropagated plant. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

4. Essential Oil Analysis

(i) Essential Oil Extraction

Shade dried aerial plant parts of both the parent and the somaclonal variant were hydrodistilled separately in a Clevanger (Clevanger, 1928) apparatus at 100°C for 4 h as prolonged extraction normally increases the yield (Gildemeister & Hoffman, 1961). 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°C. The percentage of essential oil was calculated on a dry weight basis to avoid faulty estimation that may arise due to different water content of the tissues analysed each time (Von Rudloff, 1972).

(ii) Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS was carried out on a Shimadzu QP-2000 instrument at 70 eV and 250^{0}C. GC Column: ULBON HR-1 equivalent to OV-1, fused silica capillary – 0.25 mm x 50 m with film thickness 0.25 μ. The other conditions were: carrier gas – Helium, flow rate 2 ml/min., temperature programme: initial temperature – 100^{0}C for 6 min. and then heated at the rate of 10^{0}C per min. to 250^{0}C. Mass spectral identification was based on published spectra (de Brauw *et al.*, 1979-1988).

(iii) 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

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RESULTS

1. Micropropagation

Murashige & Skoog (MS) basal medium (Table: 4) with different hormonal combinations was used for micropropagation. The two types of explants – leaves and nodal cuttings – responded positively, but the response was extremely different in both cases. For callus induction and multiple shoot regeneration, medium with auxins (2,4-D, IAA and NAA) alone, cytokinins (BAP and KIN) alone and different combinations of both these were used. Besides these, MS medium with 15-50% coconut water alone or in combination with BAP was also tried (Tables: 6-9).

Of the two types of explants, the leaf explants produced white friable callus in medium supplemented with 2,4-D 1mg/l + KIN 0.2mg/l (Fig. 1.6)and greenish hard callus (Fig. 1.4) with 2,4-D 1.5mg/l + KIN 0.2 mg/l after a period of 4-5 weeks in culture. Both these leaf-derived calli did not respond to the shoot regeneration trials with different hormonal combinations. Leaf explants inoculated on media with 2,4-D 0.5mg/l produced only swelling and crumbling of the tissue and with 1.5 mg/ml produced a little white callus at periphery. Medium with BAP 0.2 mg/l + NAA 1 mg/l could help only in the rooting of the explant and no further development occurred in this leaf tissue. BAP 0.2 mg/l + IAA 0.5 mg/l combination resulted in swelling and crumbling of leaf tissue. A combination of BAP 1 mg/l + NAA 1.5 mg/l also gave the same result. Medium with BAP 1 mg/l + 2,4-D 1.5 mg/l produced very little callus at the cut ends of the leaf tissue. Combinations like BAP 1 mg/l + IAA 1.5 mg/l; BAP 2 mg/l + IAA 2 mg/l; KIN 0.5 mg/l + IAA 1 mg/l and KIN 1 mg/l + NAA 1.5 mg/l gave only swelling and crumbling of the leaf tissue (Fig. 1.8). 30% of the explants produced single shoots when a medium with BAP 2 mg/l + IAA 2.5 mg/l was used (Table: 6).

Small whole leaves collected from the *in vitro* elongated axillary buds were also used as explants. They induced direct multiple shoots in a medium with 25% coconut water, but the frequency of shoot induction was very low (3-5 shoots/leaf)(Fig 1.9). Combinations like CW 15% + BAP 0.5 mg/l; CW 35% + BAP 1 mg/l; CW 40% + BAP 1.5 mg/l; CW 45% + BAP 1.5 mg/l and CW 30% alone resulted only in swelling of the explant. Medium with CW 50% + BAP 1.5 mg/l produced callusing at periphery of the leaf tissue.

CW 20% + BAP 1 mg/l combination could produce single shoot from 40% of the leaf explants (Fig. 1.10; Table: 7).

The nodal cuttings also produced white friable callus in a medium with 2,4-D 1mg/l + KIN 0.2 mg/l (Fig. 1.1). The stem-derived callus showed a low frequency of shoot regeneration when subcultured into medium with BAP 0.5 mg/l (Fig. 1.2). The regeneration frequency in this case was very low (2-3 shoots). Nodal explants taken from this callus regenerated plants produced a large number of multiple shoots when inoculated in a medium with BAP 2mg/l + IAA 2.5mg/l (Fig. 1.24-1.25). A few calli produced large number of small hairy roots but no shoots in medium with NAA 0.5mg/l (Fig. 1.3). Direct multiple shoot induction was obtained from the nodal cuttings on medium having a hormonal combination of BAP 2mg/l + IAA 2.5mg/l after 3-4 weeks culture period (Fig. 1.11-1.15). Frequency of shoot induction was very high. About 20-40 shoots arose from each node. 95% of the explants responded positively in this medium. Medium having a hormonal combination of BAP 0.2 mg/l + NAA 1 mg/l produced greenish swelling of the explant. A combination of BAP 0.2 mg/l + IAA 0.5 mg/l produced yellow hard callus (Fig. 1.15). Media with BAP 0.5 mg/l + IAA 1 mg/l and BAP 1 mg/l + IAA 1.5 mg/l produced single shoots from the explants. A combination of BAP 2 mg/l + 2,4-D 2 mg/l resulted in rooting of 80% of the explants. White hard callus was produced on combinations like KIN 0.2 mg/l + 2,4-D 1.5 mg/l and KIN 0.2 mg/l + 2,4-D 2 mg/l (Fig. 1.7). The *in vitro* developed white friable calli produced small roots when subcultured on to a medium with KIN 0.5 mg/l + 2,4-D 2.5 mg/l. A combination of KIN 0.5 mg/l + NAA 1 mg/l resulted in bulging of the explant. Combinations like KIN 1 mg/l + NAA 1.5 mg/l and KIN 1 mg/l + IAA 1 mg/l produced calli at cut ends of the nodal explant. Low concentrations of BAP alone could only bring forth the elongation of axillary buds, but higher concentrations produced some abnormal tissue growth (Table: 8) .

A comparatively low frequency of shoot induction (5-10 shoots from each node) was obtained after 2-3 weeks time when 25% coconut water was used instead of hormones. Medium with CW 35% produced only 1-2 shoots from 50% of the explants. Combinations like CW 20% + BAP 0.5 mg/l; CW 25% + BAP 1mg/l; CW 30% + BAP 1 mg/l; CW 40% + BAP 1 mg/l; CW 45% + BAP 1 mg/l and CW 50% + BAP 1 mg/l resulted

in axillary bud elongation from the nodal explants. A combination of CW 15% + BAP 0.5 mg/l resulted in swelling of the nodal region and callusing at the cut ends (Table: 9).

Nodal cultures in both the above mentioned multiple shoot induction media (BAP 2mg/l + IAA 2.5mg/l and 25% CW) produced morphologically variant plants with varied number of leaves at the nodes, ranging from 1-4 leaves at each node (Fig. 1.16-1.23). The frequency of production of morphological variants was 10-20%.

The cluster of multiple shoots was separated and subcultured for rooting on ½ MS medium (Fig. 1.26). A cluster of 3-7 roots was developed after 2-3 weeks. Rooted plantlets were transferred to a sterilized mixture of sand and soil (1:1) (Figs. 1.27-1.28). The field survival frequency was 80%. Only those plants, which were morphologically similar to the parent plant, could establish successfully in the soil. The field-transplanted plants produced a little larger sized leaves compared to the parent plant. The plantlets obtained full maturity after a growth period of 2 months in the pots (Fig. 1.29). The cultured plants also did not flower.

Vigorous vegetative propagation by rhizome cutting was noticed in the cultured plant also. Since no remarkable morphological variation was noticed among the field established plants, further analyses at cytological, molecular and phytochemical levels were conducted to search for the possible somaclonal variation.

Table: 6

Effect of hormones on shoot multiplication and callus induction from leaf explants

Cytokinins		Auxins			% frequency of response		Nature of response	% of morpho-logical variants
BAP mg/l	KIN mg/l	NAA mg/l	IAA mg/l	2,4-D mg/l	shoot	callus		
0.2	-	1	-	-	-	40	Rooting of explant	-
0.2	-	-	0.5	-	-	80	Swelling & crumbling	-
1.0	-	1.5	-	-	-	50	Rooting of explant	-
1.0	-	-	-	1.5	-	10	Very little callus at cut ends	-
1.0	-	-	1.5	-	-	70	Swelling	-
2.0	-	-	2.0	-	-	70	Swelling	-
2.0	-	-	2.5	-	30	-	Single shoot	-
-	0.2	-	-	1	-	90	White friable callus	
-	0.2	-	-	1.5	-	80	Greenish hard callus	-
-	0.5	-	1	-	-	70	Swelling	-
-	1.0	1.5	-	-	-	60	Swelling	-
-	-	-	-	0.5	-	90	Swelling & crumbling	-
-	-	-	-	1.5	-	80	Little white callus at periphery	-

Table: 7

Effect of coconut water alone and in combination with BAP on shoot multiplication from leaf explants

CW (%)	BAP mg/l	Nature of response	% frequency of response		% of morpho-logical variants
			shoot	callus	
15	0.5	Swelling	-	50	-
20	1.0	Single shoot	40	-	-
25	-	3-5 multiple shoots	60	-	10
30	-	Swelling	-	70	-
35	1.0	Swelling	-	60	-
40	1.5	Swelling	-	40	-
45	1.5	Swelling	-	10	-
50	1.5	Swelling & callusing at periphery	-	10	-

Table: 8

Effect of hormones on shoot multiplication and callus induction from nodal explants

Cytokinins		Auxins			% frequency of response		Nature of response	% of morpho-logical variants
BAP mg/l	KIN mg/l	NAA mg/l	IAA mg/l	2,4-D mg/l	shoot	callus		
0.2	-	0.5	-	-	-	65	Callus with numerous small hairy roots	-
0.2	-	1	-	-	-	55	Greenish swelling	-
0.2	-	-	0.5	-	-	50	Yellow hard callus	-
0.5	-	-	-	-	10	-	2-3 shoots from callus on subculture	-
0.5	-	-	-	-	20	-	Axillary bud elongation	-
0.5	-	-	1	-	40	-	Single shoot	-
1.0	-	-	-	-	45	-	Axillary bud elongation	-
1.0	-	-	1.5	-	60	-	Single shoot	-
1.5	-	-	-	-	30	-	Axillary bud elongation	-
2	-	-	2.5	-	95	-	20-40 shoots	30
2	-	-	-	2	-	80	Rooting of explant	-
2.5	-	-	-	-	-	60	Abnormal tissue growth	-
3	-	-	-	-	-	20	Abnormal tissue growth	-
-	0.2	-	-	1	-	90	White friable callus	-
-	0.2	-	-	1.5	-	70	White hard callus	-
-	0.2	-	-	2	-	30	White hard callus	-
-	0.5	1	-	-	-	50	Bulging	-
-	0.5	-	-	2	-	80	Rooting of callus	-
-	1	1.5	-	-	-	60	Callus at cut ends	-
-	1	-	1	-	-	40	Very little callus at cut ends	-

Table: 9

Effect of coconut water alone and in combination with BAP on shoot multiplication from nodal explants

CW (%)	BAP mg/l	Nature of response	% frequency of response		% of morpho-logical variants
			shoot	callus	
15	0.5	Swelling and callusing at cut ends	-	50	-
20	0.5	Axillary bud elongation	20	-	-
25	1	Rapid axillary bud elongation	30	-	-
25	-	5-10 multiple shoots	40	-	10
30	1	Axillary bud elongation	40	-	-
35	-	1-2 shoots	50	-	-
40	1	Axillary bud elongation	10	-	-
45	1	Axillary bud elongation	20	-	-
50	1	Axillary bud elongation	10	-	-

2. Cytological Analysis

The cultured plant was analysed for any karyomorphological changes and to detect the cytological basis of any variation, if present. The ploidy level of the parent plant, callus and the cultured plant was invariably the same. They were found to be diploid (2n=2x=24). Neither chimeral nor aneuploid variations were obtained. Average chromosome length of the parent plant (1.0017 μm) was slightly smaller than that of the somaclonal variant (1.1561 μm). Average chromosome length of the callus was still smaller (0.9379 μm). The total chromosome length of the somaclonal variant was 27.74 μm and that of the parent plant was 24.04 μm. The total chromosome length of the callus was 22.52 μm. A single pair of chromosomes with secondary constriction was detected in all the samples analyzed (Figs. 2.1.A1-2.3.A3). The disparity index of the parent plant

1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
1.10

Fig. 1.11-1.15: Different stages of direct multiple shoot development from nodal Explant (BAP 2mg/l + IAA 2.5 mg/l)

Fig. 1.16: Morphological variant with single leaf at each node

Fig. 1.17: Morphological variant with 4 leaves at basal nodes and 2 leaves at upper nodes

Fig. 1.18-1.20: Morphological variants with 3 leaves at each node

1.11
1.12
1.13
1.14
1.15
1.16
1.17
1.18
1.19
1.20

Fig. 1.21-1.23: Morphological variants with 3 leaves at each node

Fig. 1.24- 1.25: Multiple shoots developed from nodal explants of callus regenerated plants

Fig. 1.26: Isolated plantlet in ½ MS medium for rooting

Fig. 1.27: Transplantation of the rooted plant to sand : soil (1:1) mixture

Fig. 1.28: Development of the plant in plastic cup

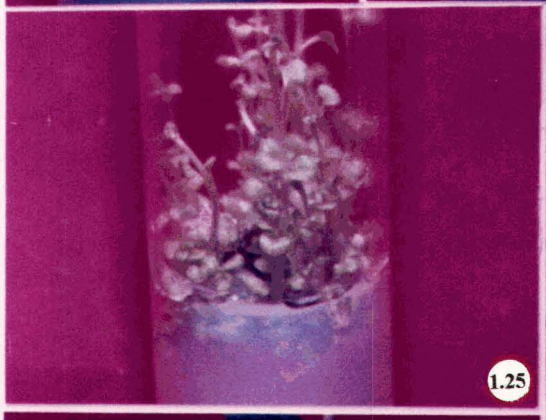
Fig. 1.29: Field established somaclonal variant


1.21


1.22

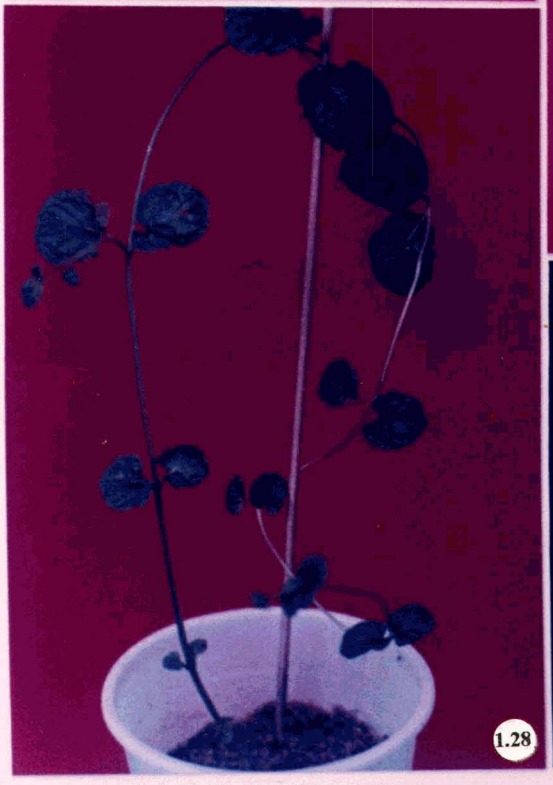

1.23


1.24


1.25


1.26


1.27


1.28


1.29

was 35.48, somaclonal variant was 17.76, and that of the callus tissue was 31.37. The variation coefficient was higher in the case of callus (20.20), when compared to the parent plant (17.36) and the somaclonal variant (11.60). The Total Forma percentage of the parent plant was 37.60, the somaclonal variant was 36.50 and the callus was 44.31. A general description of the common chromosome types found in the parent plant, somaclonal variant and callus is given below.

Type A : Chromosomes with secondary constriction ranging from 1.4019 μm to1.2582 μm with nearly submedian/ nearly median primary construction

Type B : Chromosome ranging from 1.3783 μm to 0.5991 μm with nearly median primary constriction.

Type C : Chromosome ranging from 1.2236 μm to 0.9525 μm with nearly submedian (-) primary constriction.

Type D : Chromosome having a length of 0.8388 μm with nearly submedian (+) primary constriction.

The karyotype formula of the parent plant was $A_2B_{12}C_8D_2$, of the callus was $A_2B_{20}C_2$ and of the somaclonal variant was $A_2B_{10}C_{12}$. Diagrammatic representation of the different chromosome types observed in the present investigation is shown in Fig. 2.4). Detailed karyotype description (Tables. 10-12), microphotographs of mitotic metaphase stages (Fig. 2.1-2.3), computer scanned images of karyotypes (Figs. 2.1.A1-2.3.A3), karyograms (Fig. 2.1.B-2.3.B) and idiograms (Figs. 2.1.C-2.3.C) of the parent plant, somaclonal variant and the callus are given below.

3. Random Amplified Polymorphic DNA Analysis

DNA was isolated from the parent plant and three cultured plants, of which TC 1 is the somaclonal variant mentioned hitherto. 20-30 ng of template DNA is required to amplify a specific DNA segment by PCR. The total DNA extracted was quantified by comparing the bandwidth of the isolated genomic DNA with that of the weight marker after running in agarose gels. The amount of DNA was found to be greater (~75ng) in the

Figs 2.1-2.3: Mitotic metaphases

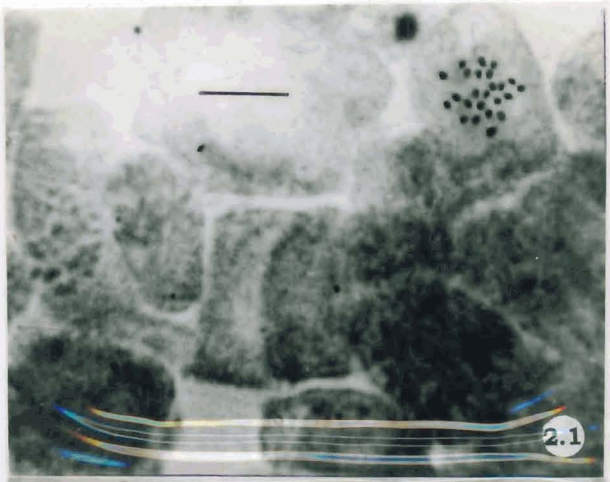
Fig. 2.1: *Mentha rotundifolia* (L.) Huds. Parent plant (2n=24)

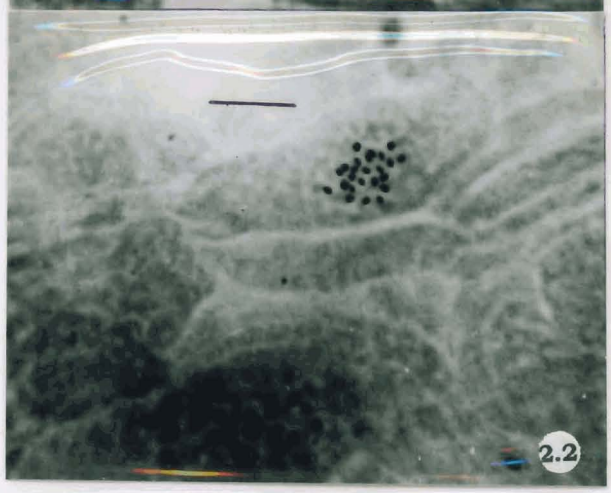
Fig. 2.2: M*entha rotundifolia* (L.) Huds. Callus (2n=24)

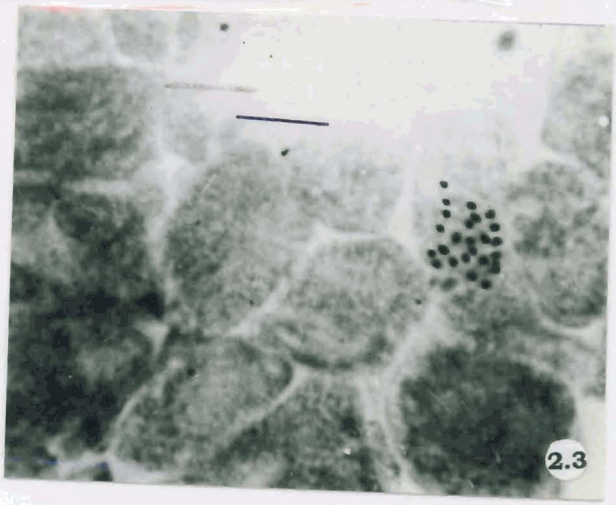
Fig. 2.3: *Mentha rotundifolia* (L.) Huds. Somaclonal variant (2n=24)

Bar = 10 μm

55B

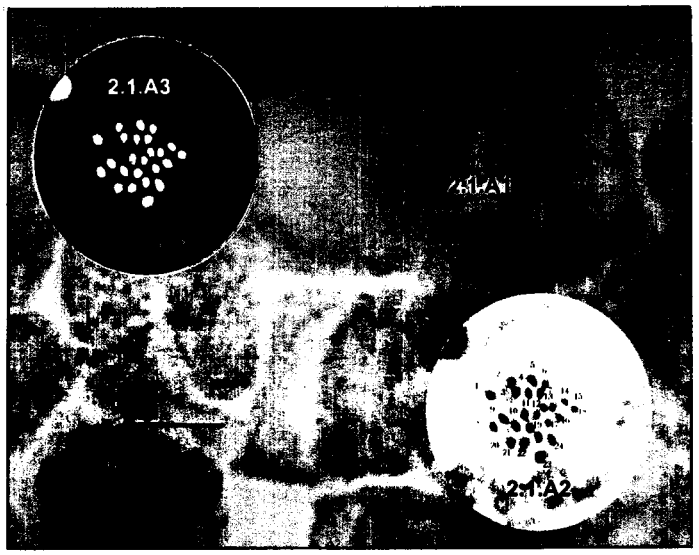




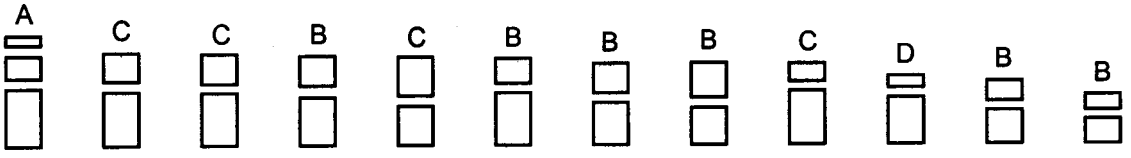


19

Mentha rotundifolia (L.) Huds.
Parent Plant



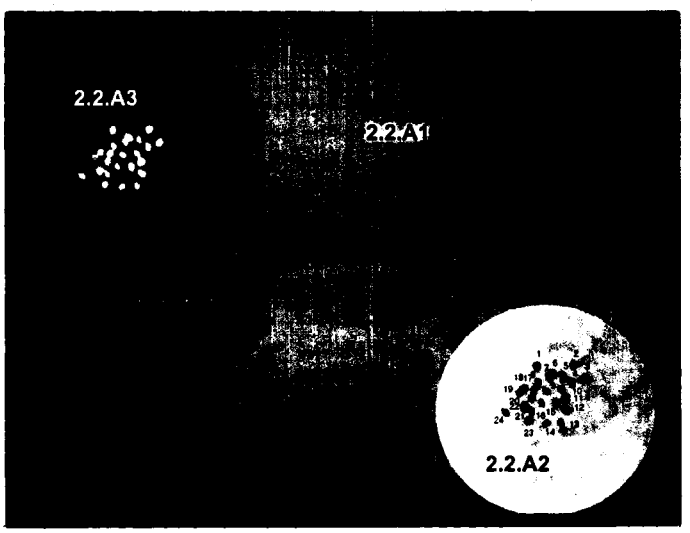
2.1.B Karyogram



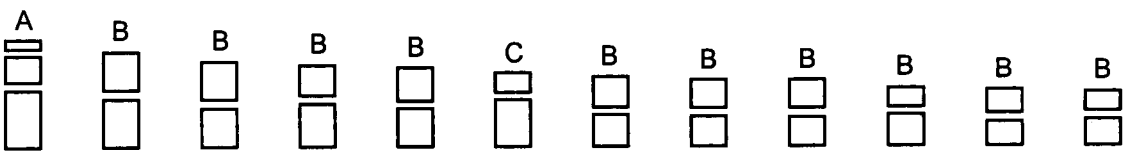
2.1.C Idiogram

Fig. **2.1.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.1.A2**: Resolved image
Fig. **2.1.A3**: Inverted image

Mentha rotundifolia (L.) Huds.
Callus



2.2.B Karyogram



2.2.C Idiogram

Fig. **2.2.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.2.A2**: Resolved image
Fig. **2.2.A3**: Inverted image

55F

Mentha rotundifolia (L.) Huds. ($2n = 2x = 24 = A_2B_{20}C_2$)

Callus

Normal somatic chromosome number	: 24
Chromosome pair with secondary constriction	: 1
Total chromosome length	: 22.52 μm
Range of chromosome length	: 1.3098 μm-0.6842 μm
Average chromosome length	: 0.9379 μm
Disparity Index	: 31.37
Variation Coefficient	: 20.20
TF value	: 44.31

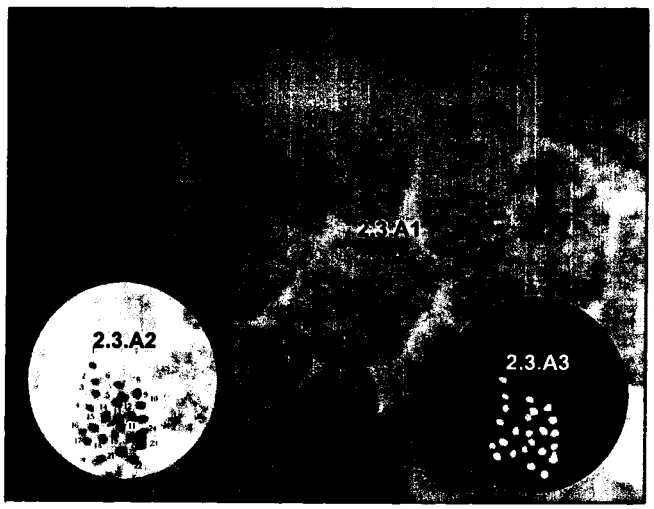
Table: 11

Detailed karyomorphometrical data

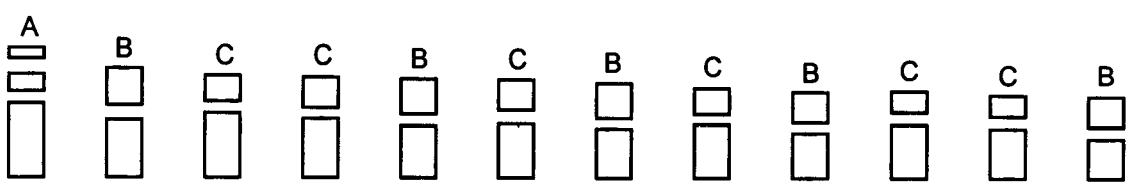
Chromosome type	No. of pairs	Total length (μm)	s (μm)	l (μm)	R_1 (s/l)	R_2 (l/s)	I_1 (s/c%)	I_2 (l/c%)	Nature of primary constriction
A	1	1.3098	0.5358	0.7740	0.6922	1.4445	40.90	59.09	Nm
B	1	1.1906	0.5332	0.6574	0.8110	1.2329	44.78	55.21	Nm
B	1	1.0716	0.5313	0.5403	0.9833	1.0169	49.58	50.41	Nm
B	1	1.0248	0.4295	0.5953	0.7214	1.3860	41.91	58.08	Nm
B	1	1.0121	0.4763	0.5358	0.8906	1.1249	57.06	52.94	Nm
C	1	0.9525	0.2976	0.6549	0.4544	2.2006	31.24	68.75	Nsm(-)
B	1	0.8929	0.4378	0.4551	0.9619	1.0395	49.03	50.96	Nm
B	1	0.8355	0.4062	0.4293	0.9462	1.0567	48.61	51.38	Nm
B	1	0.8335	0.4067	0.4268	0.9529	1.0494	48.79	51.20	Nm
B	1	0.7438	0.2893	0.4545	0.6365	1.5710	38.89	61.11	Nm
B	1	0.7054	0.3482	0.3572	0.9748	1.0258	49.36	50.63	Nm
B	1	0.6842	0.2976	0.3866	0.7697	1.2990	43.49	56.50	Nm

S : short arm
L : long arm
R_1 & R_2 : arm ratios
I_1 & I_2 : centromeric indices

Mentha rotundifolia (L.) Huds.
Somaclonal variant



2.3.B Karyogram



2.3.C Idiogram

Fig. **2.3.A1**: Computer scanned original image (Bar=10 μm)
Fig. **2.3.A2**: Resolved image
Fig. **2.3.A3**: Inverted image

Mentha rotundifolia (L.) Huds. ($2n = 2x = 24 = A_2B_{10}C_{12}$)

Somaclonal variant

Normal somatic chromosome number	: 24
Chromosome pair with secondary constriction	: 1
Total chromosome length	: 27.74 µm
Range of chromosome length	: 1.4019 µm - 0.9790 µm
Average chromosome length	: 1.1561 µm
Disparity Index	: 17.76
Variation Coefficient	: 11.60
TF value	: 36.50

Table: 12

Detailed karyomorphometrical data

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	1.4019	0.4268	0.9751	0.4376	2.2846	30.44	69.55	Nsm(-)
B	1	1.3783	0.6098	0.7685	0.7934	1.2602	44.24	55.75	Nm
C	1	1.2236	0.3659	0.8577	0.4266	2.3440	29.90	70.09	Nsm(-)
C	1	1.2196	0.4268	0.7928	0.5383	1.8575	34.99	65.00	Nsm(-)
B	1	1.1823	0.4878	0.6945	0.7023	1.4237	41.25	58.74	Nm
C	1	1.1586	0.4269	0.7317	0.5834	1.7139	36.84	63.15	Nsm(-)
B	1	1.1560	0.4852	0.6708	0.7233	1.3825	41.97	58.02	Nm
C	1	1.0977	0.3659	0.7318	0.5000	2.000	33.33	66.66	Nsm(-)
B	1	1.0505	0.4295	0.6210	0.6916	1.4458	40.88	59.11	Nm
C	1	1.0367	0.3049	0.7318	0.4166	2.4001	29.41	70.58	Nsm(-)
C	1	0.9895	0.3055	0.6840	0.4466	2.2389	30.87	69.12	Nsm(-)
B	1	0.9790	0.4302	0.5488	0.7838	1.2756	43.94	56.06	Nm

s : short arm
l : long arm
R_1 & R_2 : arm ratios
I_1 & I_2 : centromeric indices

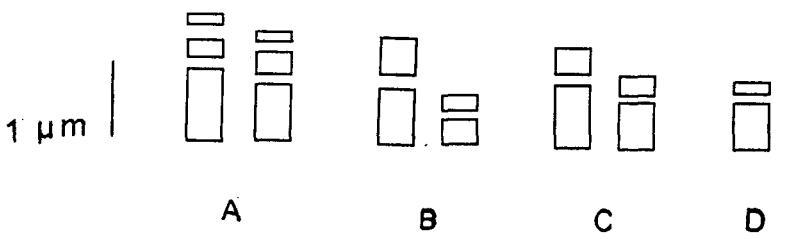


Fig. 2.4 Diagrammatic representation of the chromosome types observed in *Mentha rotundifolia* (L.) Huds. (Parent plant, callus and somaclonal variant).

somaclonal variant than in the parent plant (~50ng) (Fig. 3.1). 1µl of the template DNA was used for the preparation of reaction mixture. To detect the somaclonal variation at the molecular level, RAPD analysis was carried out using six primers of arbitrary sequences (Figs: 3.2-3.7). Of the six primers used, all successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from a minimum of 3 to a maximum of 11. The size of the amplification products also differed and ranged from approximately below 0.5 Kb to 3 Kb. The primers and the characterization of consistent bands are listed in Table: 13.

The RAPD fingerprint of the somaclonal variant (TC 1) differed from the parent plant with 2 primers (OPE 14 and OPF 05) used (Figs. 3.6-3.7). A few bands were found to be missing in the somaclonal variant when these two primers were used. No additional bands could be detected in the variant by this marker screening. Amplification products with OPE 14 generated no bands above 0.75 Kb length in the somaclonal variant but bands were clearly visible in the parent plant's amplification products in this region. When the primer OPF 05 was used for amplification, a fragment with nearly 1 Kb length was found missing in the somaclonal variant which was clearly present as a prominent band in the parent plant's finger print. In both these cases, no additional bands were present in the variant. The other primers used could not generate any polymorphism, but certain intensity differences in the bands were noticed in the amplification products of all the six primers tested.

In the case of TC 2 and TC 3, certain band differences were noticed when the primer OPE 14 was used. TC 2 showed minor band differences when the primer OPF 05 was used for amplification. The reproducibility of these genomic DNA bands were consistent in the case od TC 1 alone. Though TC 2 and TC 3 showed band differences, they were inconsistent in successive repetitions.

Fig. 3.1

P: Parent plant

TC 1: Tissue Cultured plant 1 (Somaclonal variant)

TC 2: Tissue Cultured plant 2

TC 3: Tissue Cultured plant 3

Fig. 3.1

Agarose gel electrophoresis of pure genomic DNA for quantification

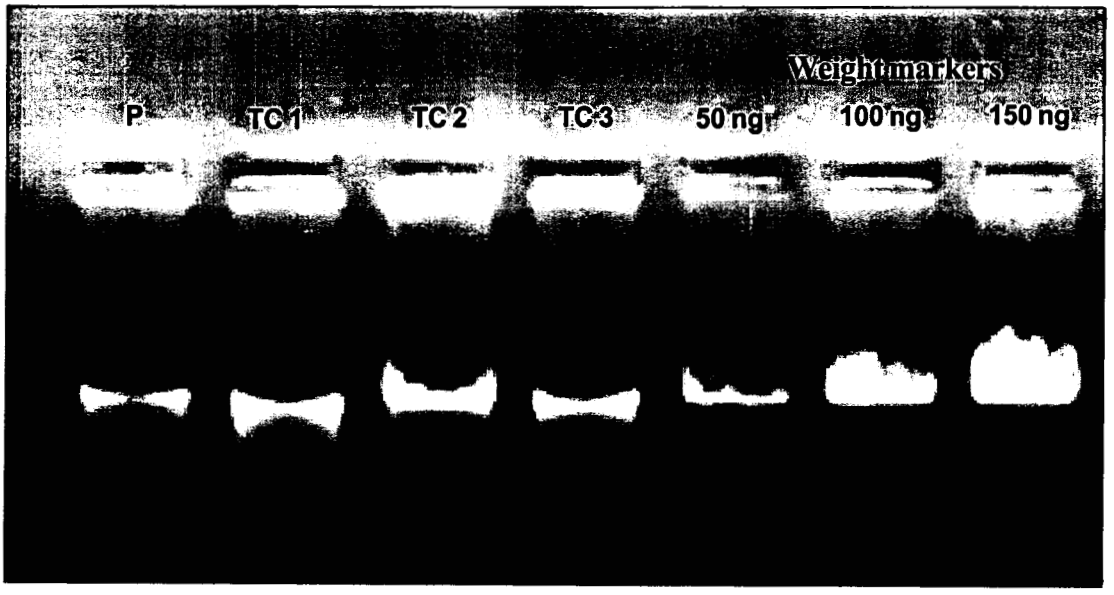


Fig. 3.2

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.2

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPA 02 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

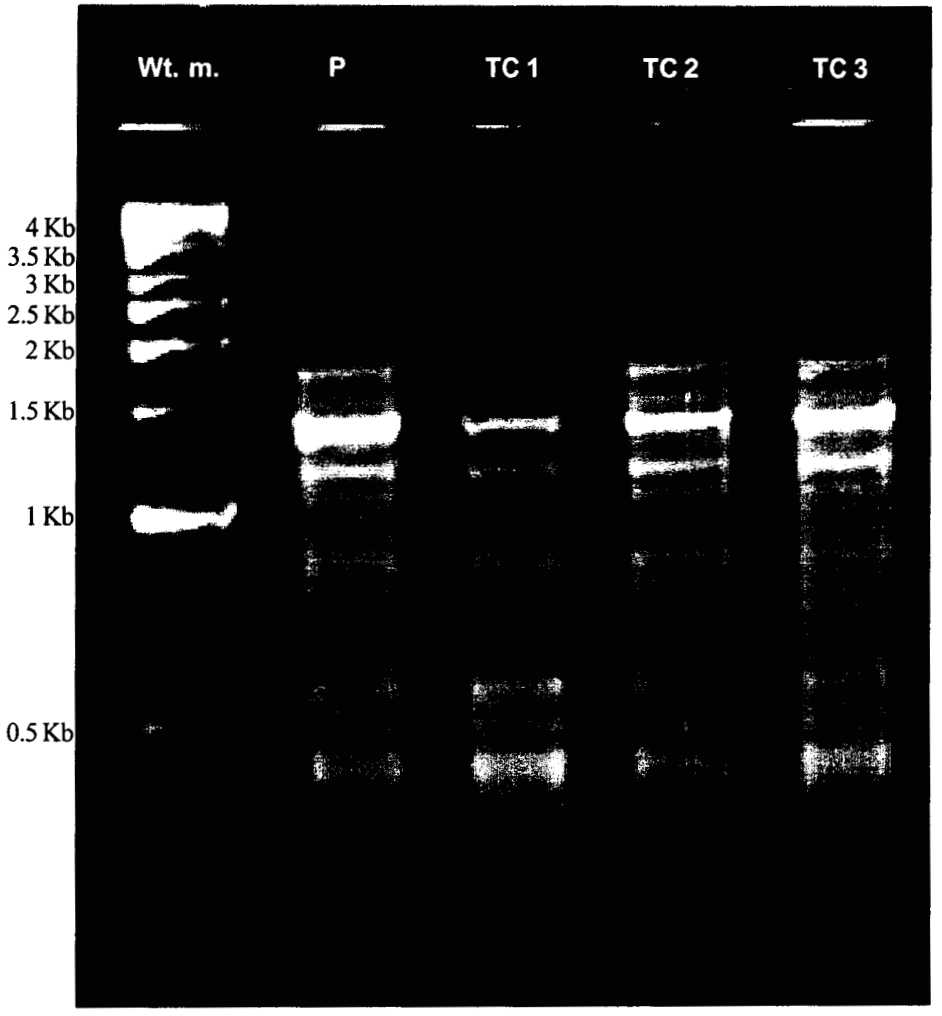


Fig. 3.3

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.3

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPA 08 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

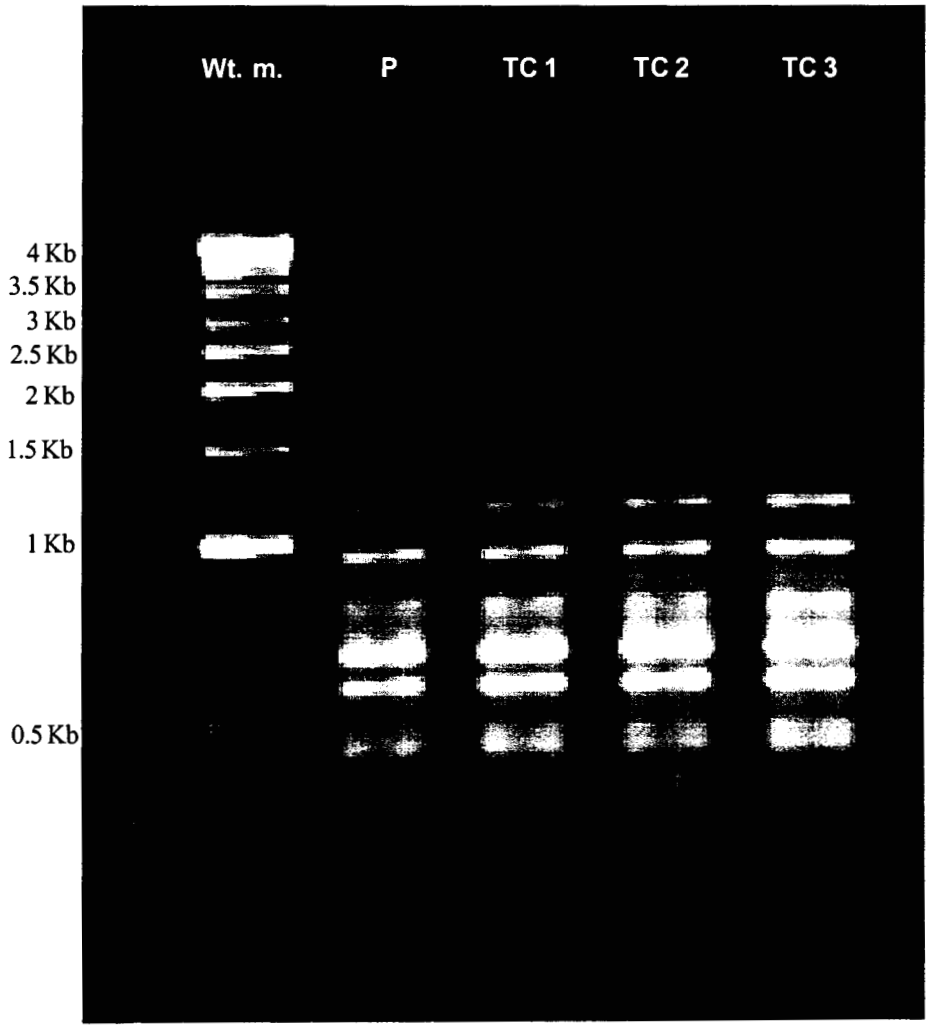


Fig. 3.4

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.4

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPB 07 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

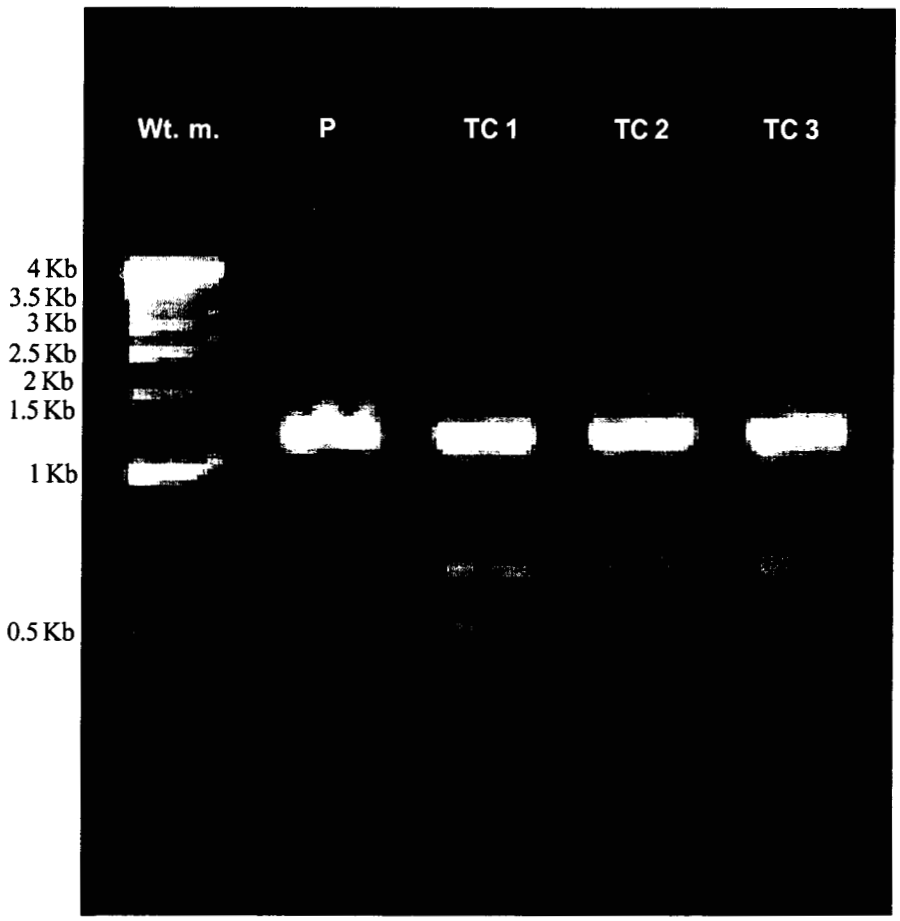


Fig. 3.5

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Fig. 3.5

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPD 19 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

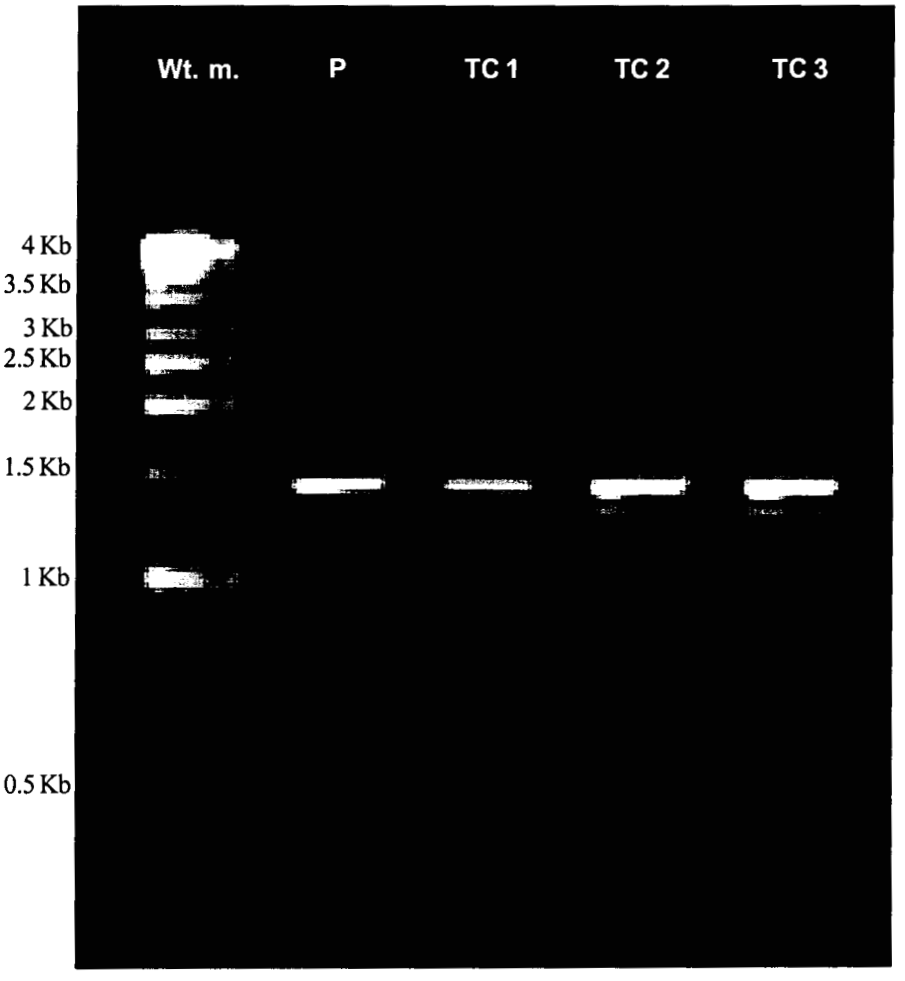


Fig. 3.6

Wt. m.: Weight marker

P: Parent plant

TC 1: Tissue Cultured plant 1 (Somaclonal variant)

TC 2: Tissue Cultured plant 2

TC 3: Tissue Cultured plant 3

Arrow represents absence of a prominent band in the somaclonal variant (TC 1) when compared to the parent plant, TC 2 and TC 3.

Fig. 3.6

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPE 14 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

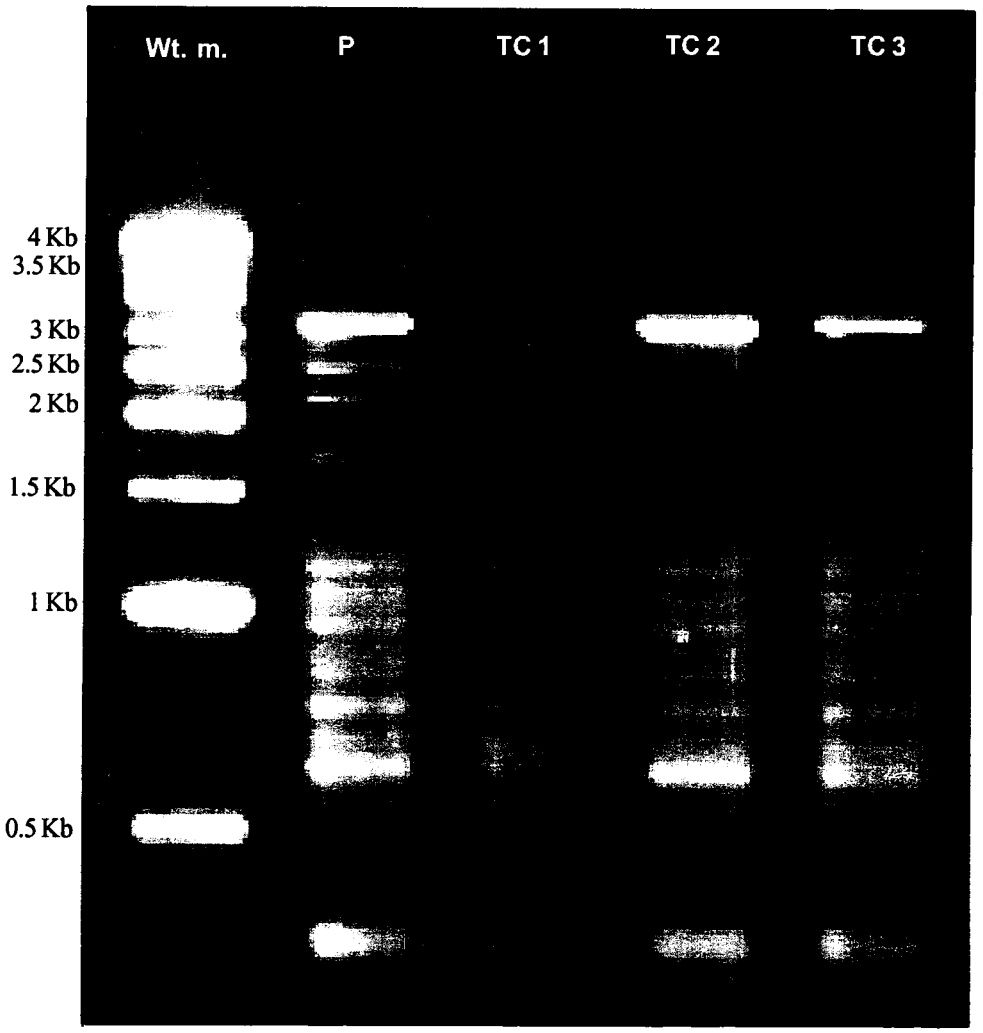


Fig. 3.7

Wt. m.:	Weight marker
P:	Parent plant
TC 1:	Tissue Cultured plant 1 (Somaclonal variant)
TC 2:	Tissue Cultured plant 2
TC 3:	Tissue Cultured plant 3

Arrow represents absence of a band in the somaclonal variant (TC 1)

Fig. 3.7

Agarose gel electrophoresis of amplified sequence from a RAPD reaction using primer OPF 05 of *Mentha rotundifolia* (L.) Huds. and molecular weight marker (Wt. m.) Eco R1 + Hind III double digest λ phage DNA

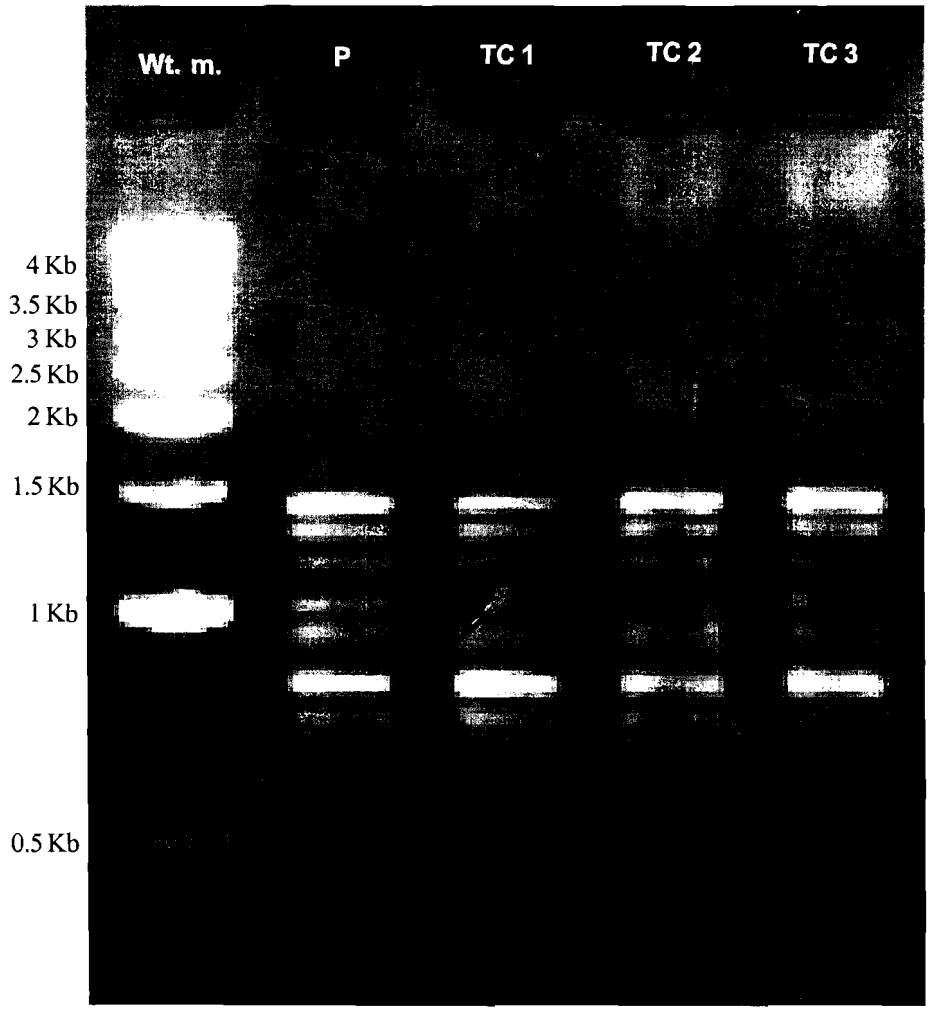


Table: 13

Primers and characterization of consistent bands in parent and three tissue cultured plants of *M. rotundifolia* (L.) Huds..

Primer	Sequence	Number of markers				Size Range in Kb (kilo base)
		Parent	TC 1*	TC 2	TC 3	
OPA 02	5′ TGCCGAGCTC 3′	7	7	7	7	~0.5-1.75
OPA 08	5′ GTGACGTAGG 3′	6	6	6	6	~0.5-1.25
OPB 07	5′ GGTGACGCAG 3′	3	3	3	3	~0.5-1.25
OPD 19	5′ CTGGGGACTT 3′	8	8	8	8	~0.5-1.5
OPE 14	5′ TGCGCCTGAG 3′	8	3	6	6	~0.25-3.25
OPF 05	5′ CCGAAT TCCC 3′	7	5	5	7	~0.75-1.5

* Somaclonal variant hitherto mentioned.

4. Essential oil Analysis

RAPD analysis revealed a notable deviation in the genetic make up of the somaclonal variant, TC 1 (Figs. 3.6-3.7) than TC 2 and TC 3. Moreover, repeatability was consistent only in the case of TC 1. So the essential oils of the parent plant and the somaclonal variant TC 1 were analyzed qualitatively and quantitatively to search for any biochemical variations in the secondary metabolism.

The oil yield of the parent plant was 1%. The somaclonal variant (TC 1) contained a higher quantity of oil (1.3%). The oil yield of TC 2 and TC 3 were almost equal to that of the parent. The oil of the parent plant was light yellow in colour and that of the somaclonal variant, TC 1was almost colourless. The essential oils of the parent as well as the somaclonal variant possess a warm herbaceous odour with a minty topnote.

(i) Gas Chromatography-Mass Spectrometry Analysis

The results of Gas Chromatography-Mass Spectrometry analyses of the parent plant and the somaclonal variant are listed in Table: 14. GC-MS analyses revealed 21 components each in the parent plant (Figs. 4.1.1 - 4.1.22) and in the somaclonal variant (Figs. 4.2.1 - 4.2.22). There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant).

α-pinene (tr.), sabinene hydrate (0.081%), ethyl 9,12 octa decadienoate (tr.), trans-2-octenal (tr.), isopulegol (tr.), citronellol (tr.), alloaromadendrene (tr.), β-caryophyllene (1.1), and β-gurgunene (0.7%) were the unique components detected from the parent plant. The somaclonal variant was characterized by a set of new components like methyl chavicol (tr.), isoborneol (tr.), cis 6 nonenal (0.176%), 2,3 diethyl 6 methyl pyrazine (0.031 %), p-mentha trans 2,8 dien 1 ol (tr.), γ-caryophyllene (0.4 %), β-bisabolene (1.0 %), 1 ethynyl-2-trimethylsilyl benzene (tr.), α-amorphene (0.091%), juniper camphor (0.081%) and di isobutyl phtalate (tr.). Components like myrcene, limonene, carvone, α-terpinyl acetate, β-elemene, α-cububene, aromadendrene, trans-2-cis-6-nonadien-1-ol, and β-terpineol were found in both *in vivo* and *in vitro* developed plants. In addition to these, three unidentified components in the parent plant and one unidentified component in the somaclonal variant were also noticed.

(ii) Chemotaxonomic Evaluation

The total number of chemical components detected by GC-MS in both the *in vivo* and *in vitro* grown plants was found to be 33. However the number of similar components which occur both in the parent and the somaclonal variant (TC 1) was found to be 9. Coefficient of similitude between the parent plant and the somaclonal variant was found to be 27.27.

Table:14

List of essential oil components detected in the present investigation

Sl.No	Components	Class	Composition %	
			A	B
1	α-pinene	Monoterpene	tr.	-
2	myrcene	,,	0.4	tr.
3	limonene	,,	6.2	8.4
4	sabinene hydrate	,,	0.081	-
5	ethyl 9,12 octa deca dienoate	Ester	tr.	-
6	trans-2-octenal	Aldehyde	tr.	-
7	isopulegol	Monoterpene	tr.	-
8	citronellol	,,	tr.	-
9	carvone	,,	82	87.4
10	UI		1.282	-
11	α-terpinyl acetate	,,	0.3	0.2
12	UI		tr.	-
13	alloaromadendrene	Sesquiterpene	tr.	-
14	β-elemene	,,	2.6	1.0
15	β-caryophyllene	,,	1.1	-
16	trans-2-cis-6-nonadien–1-ol	Unsaturated alcohol	1.0	0.3
17	α-cububene	Sesquiterpene	0.9	0.4
18	UI		2.9	-
19	β-gurgunene	Sesquiterpene	0.7	-
20	β-terpineol	Monoterpene	tr	tr.
21	methyl chavicol	Phenol	-	tr.
22	isoborneol	Monoterpene	-	tr.
23	cis-6-nonenal	Unsaturated aldehyde	-	0.176
24	UI		-	tr.
25	2,3 diethyl-6-methyl pyrazine	Heterocyclic compound	-	0.031
26	p-mentha trans 2,8 dien-1-ol	Monoterpene	-	tr.
27	γ-caryophyllene	Sesquiterpene	-	0.4
28	aromadendrene	,,	tr	0.056
29	β-bisabolene	,,	-	1.0
30	1 ethynyl-2-trimethylsilyl benzene	Benzene derivative	-	tr.
31	α-amorphene	Sesquiterpene	-	0.091
32	juniper camphor	Monoterpene	-	0.081
33	di isobutyl phtalate	Ester	-	tr.

A Parent plant of *M. rotundifolia* (L.) Huds.
B Somaclonal variant of *M. rotundifolia* (L.) Huds.
UI Unidentified component
tr. Trace component

Fig. 4.1.1 Gas chromatogram of the essential oil of *Mentha rotundifolia* (L.) Huds. Parent plant

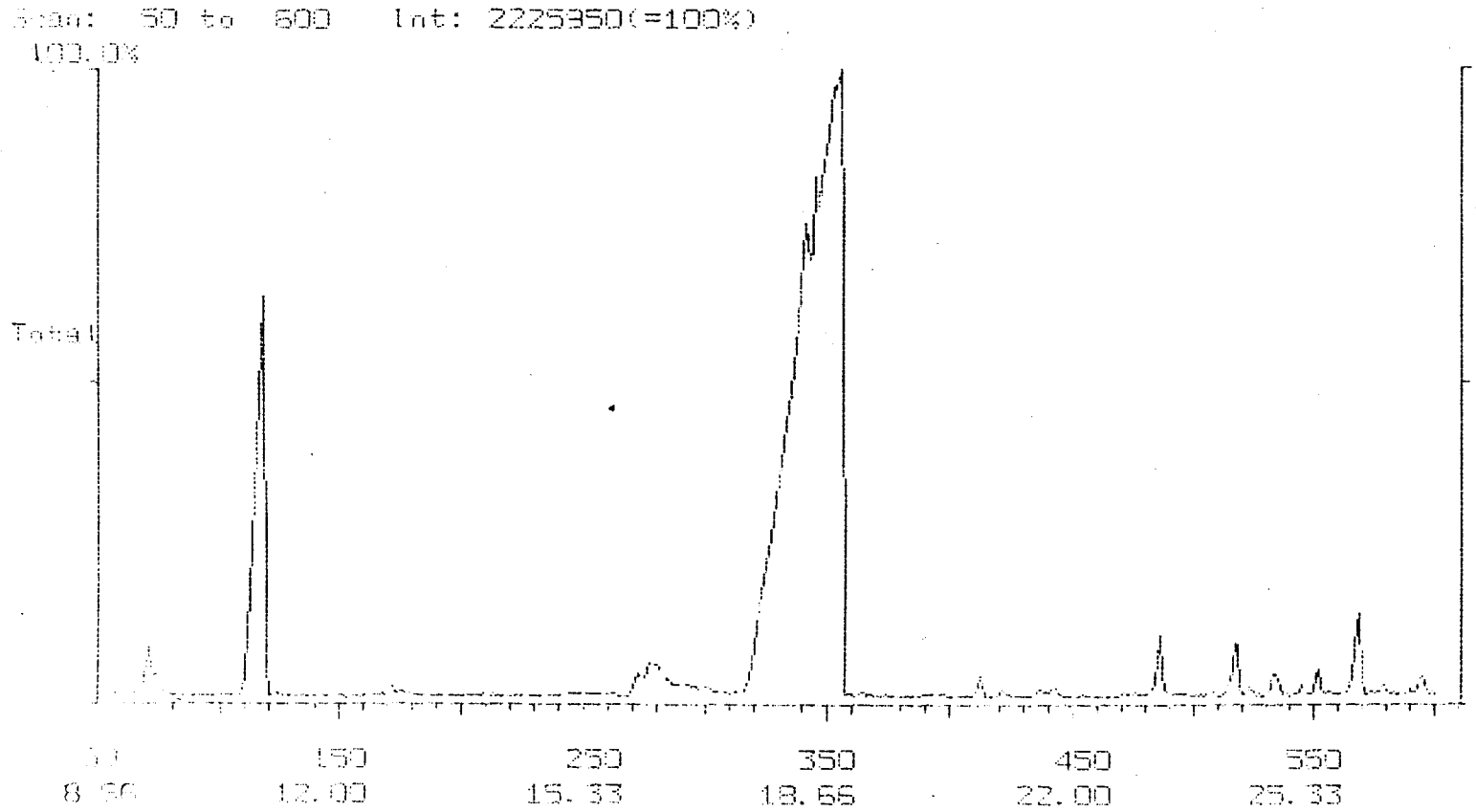


Fig. 4.1.2 Mass spectrum of α-pinene

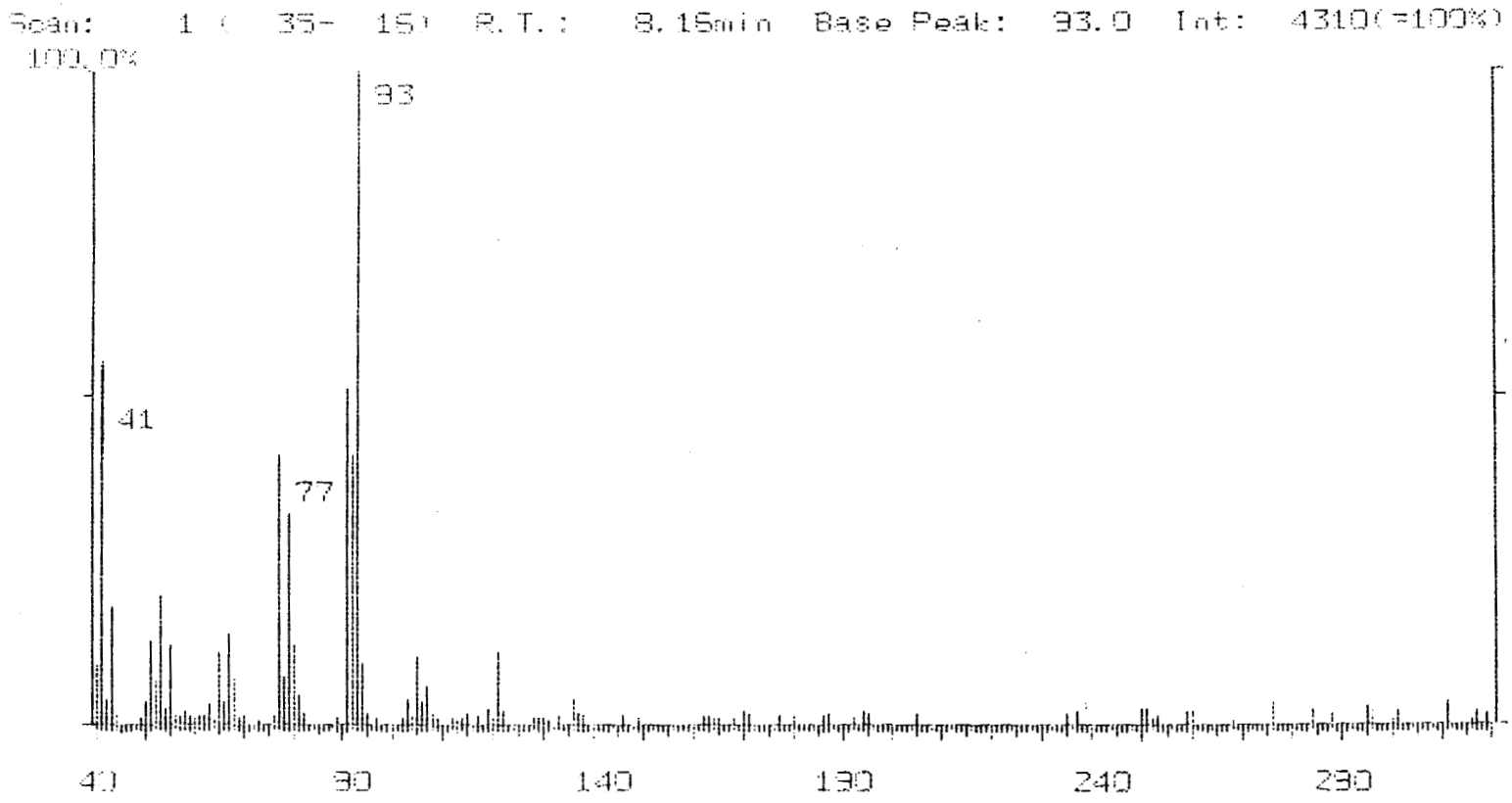


Fig. 4.1.3 Mass spectrum of myrcene

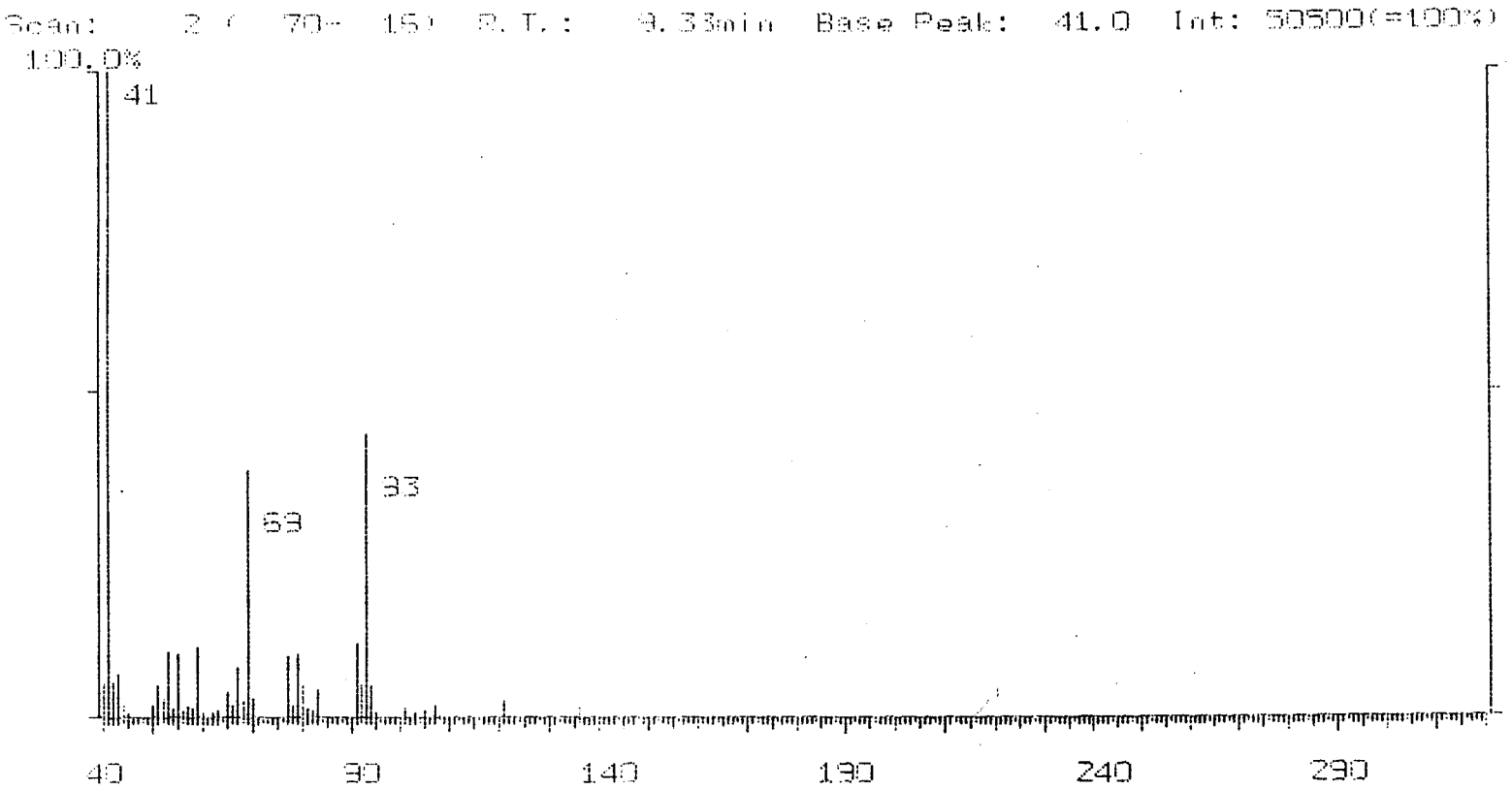


Fig. 4.1.4 Mass spectrum of limonene

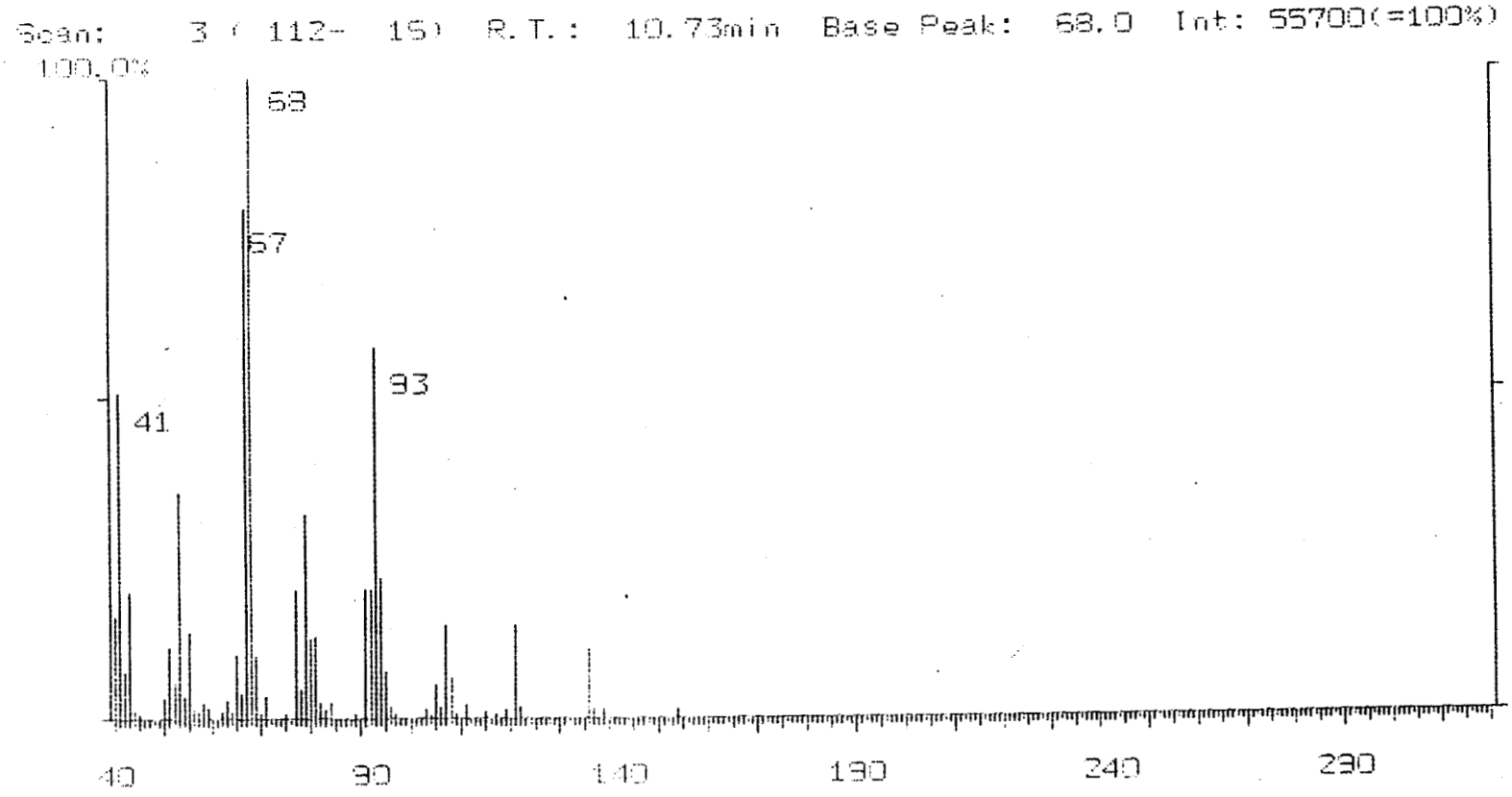


Fig. 4.1.5 Mass spectrum of sabinene hydrate

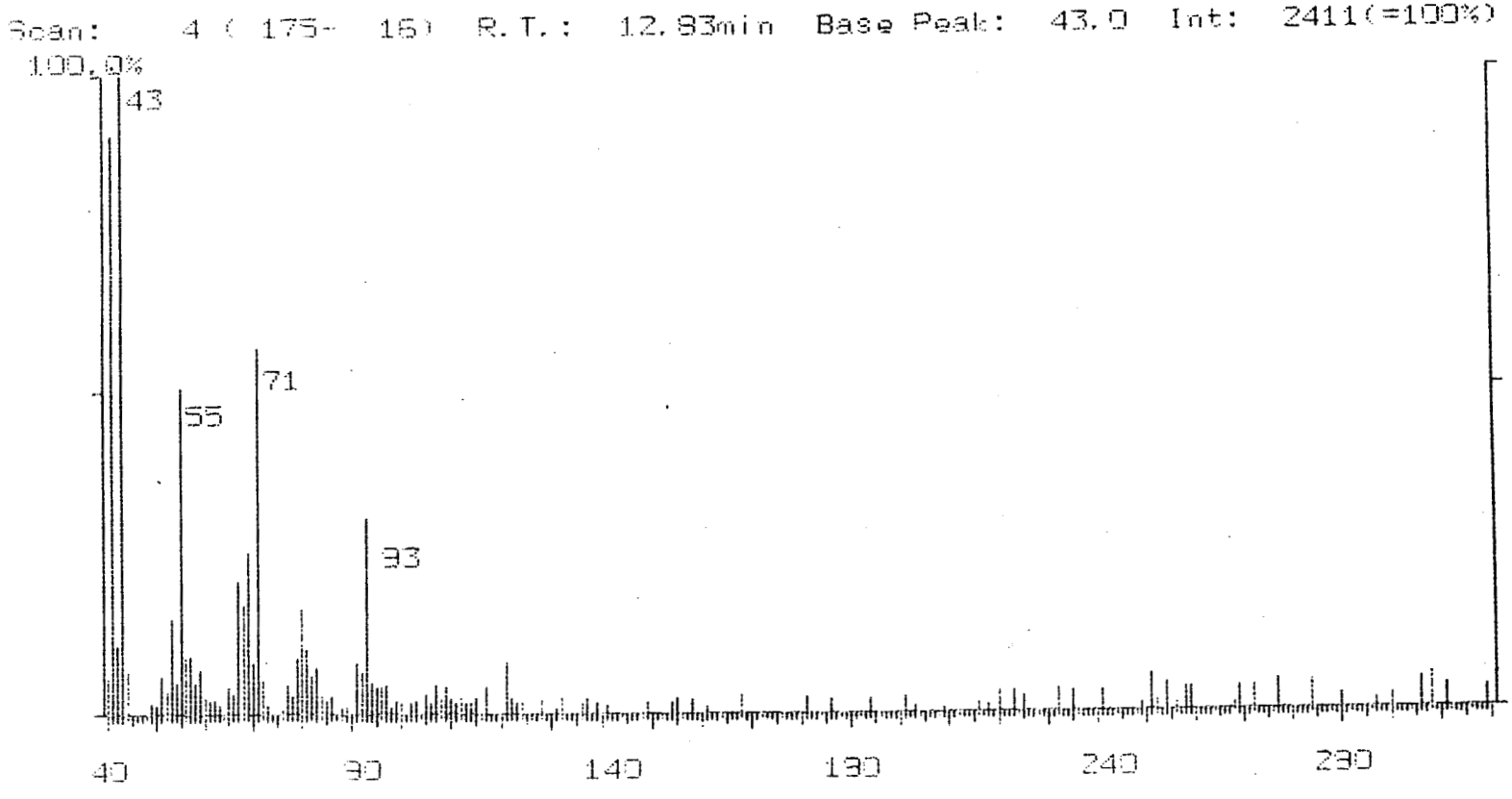


Fig. 4.1.6 Mass spectrum of ethyl 9,12 octa deca dienoate

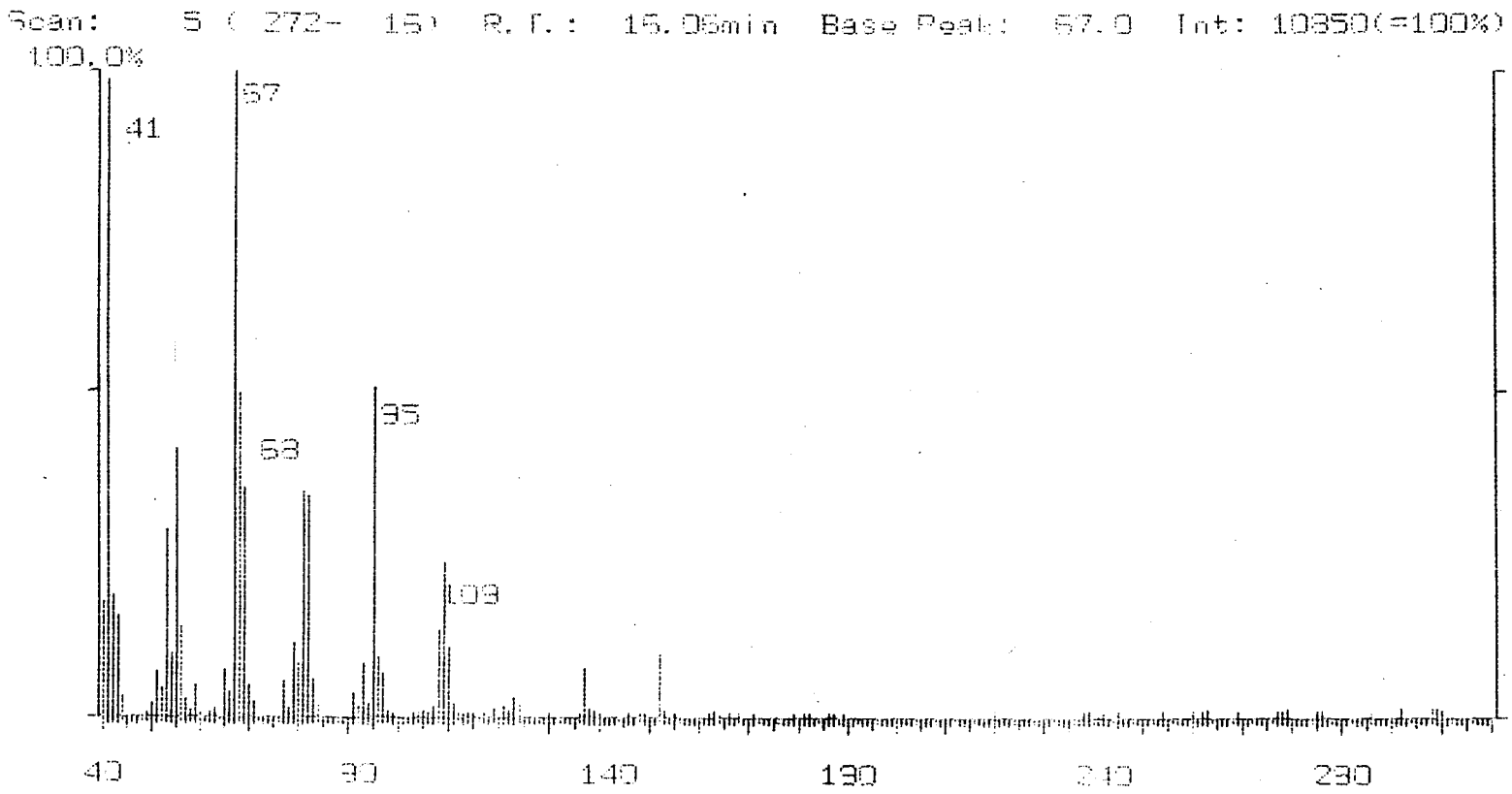


Fig. 4.1.7 Mass spectrum of trans-2-octenal

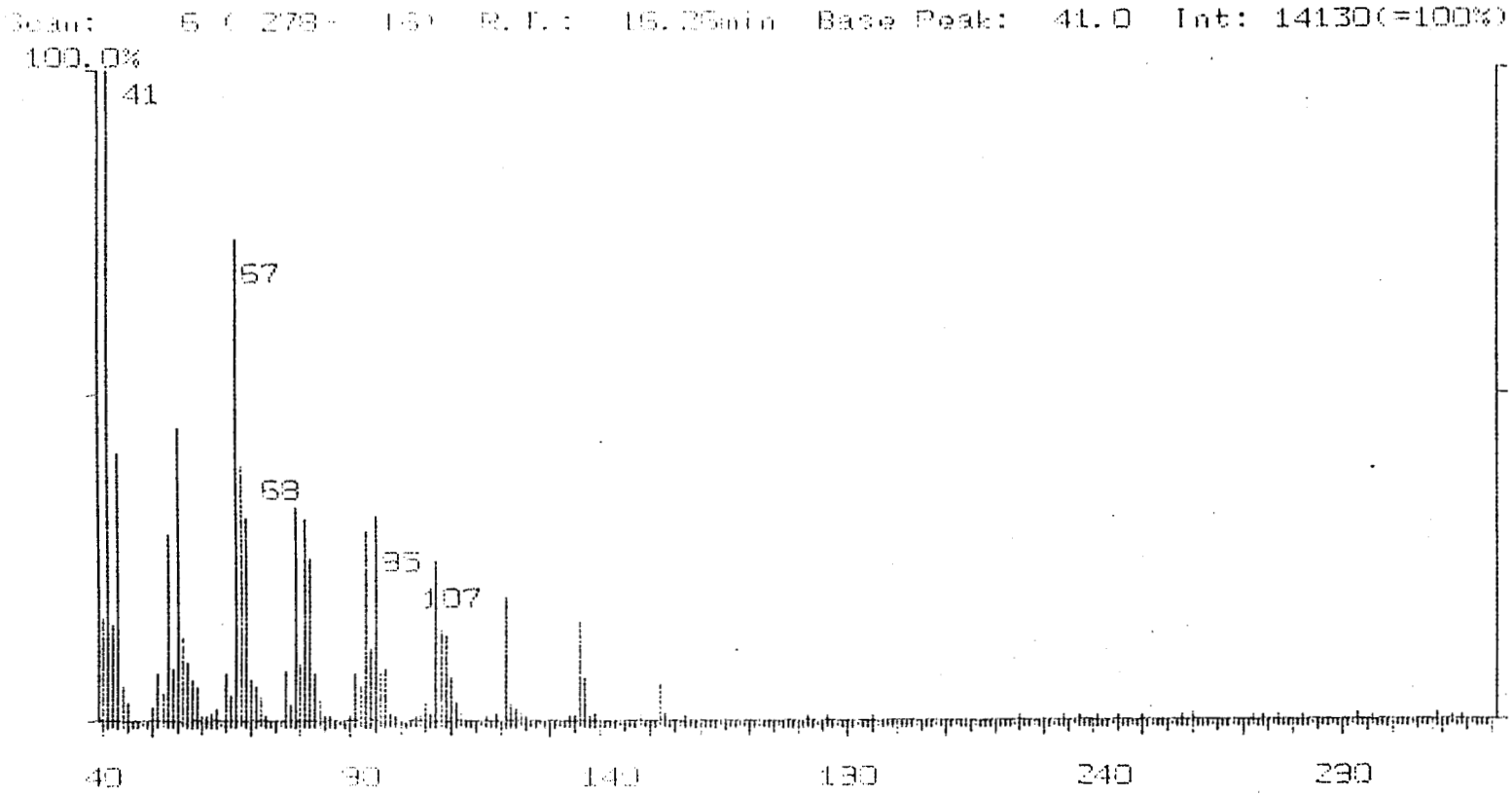


Fig. 4.1.8 Mass spectrum of isopulegol

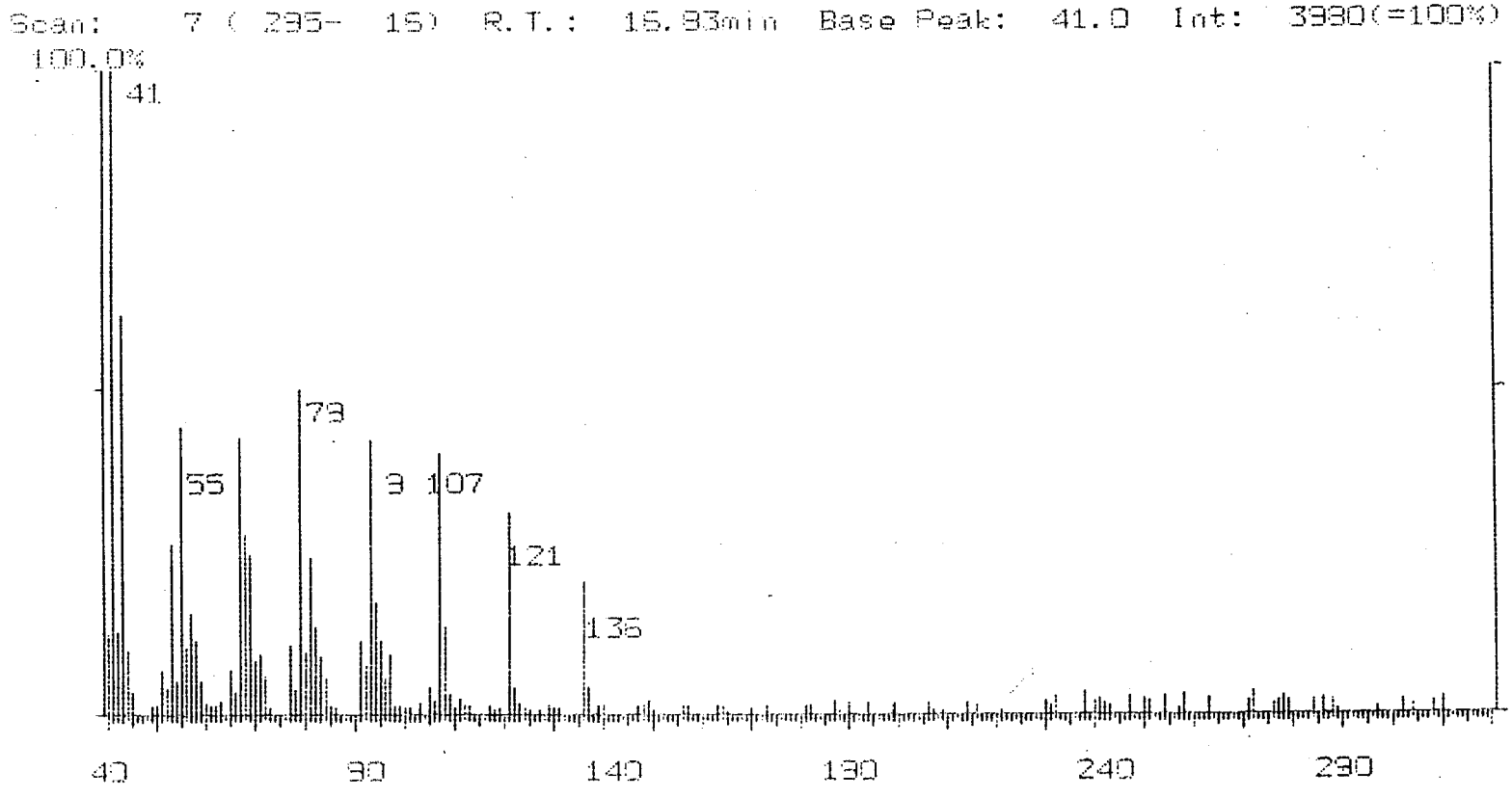


Fig. 4.1.9 Mass spectrum of citronellol

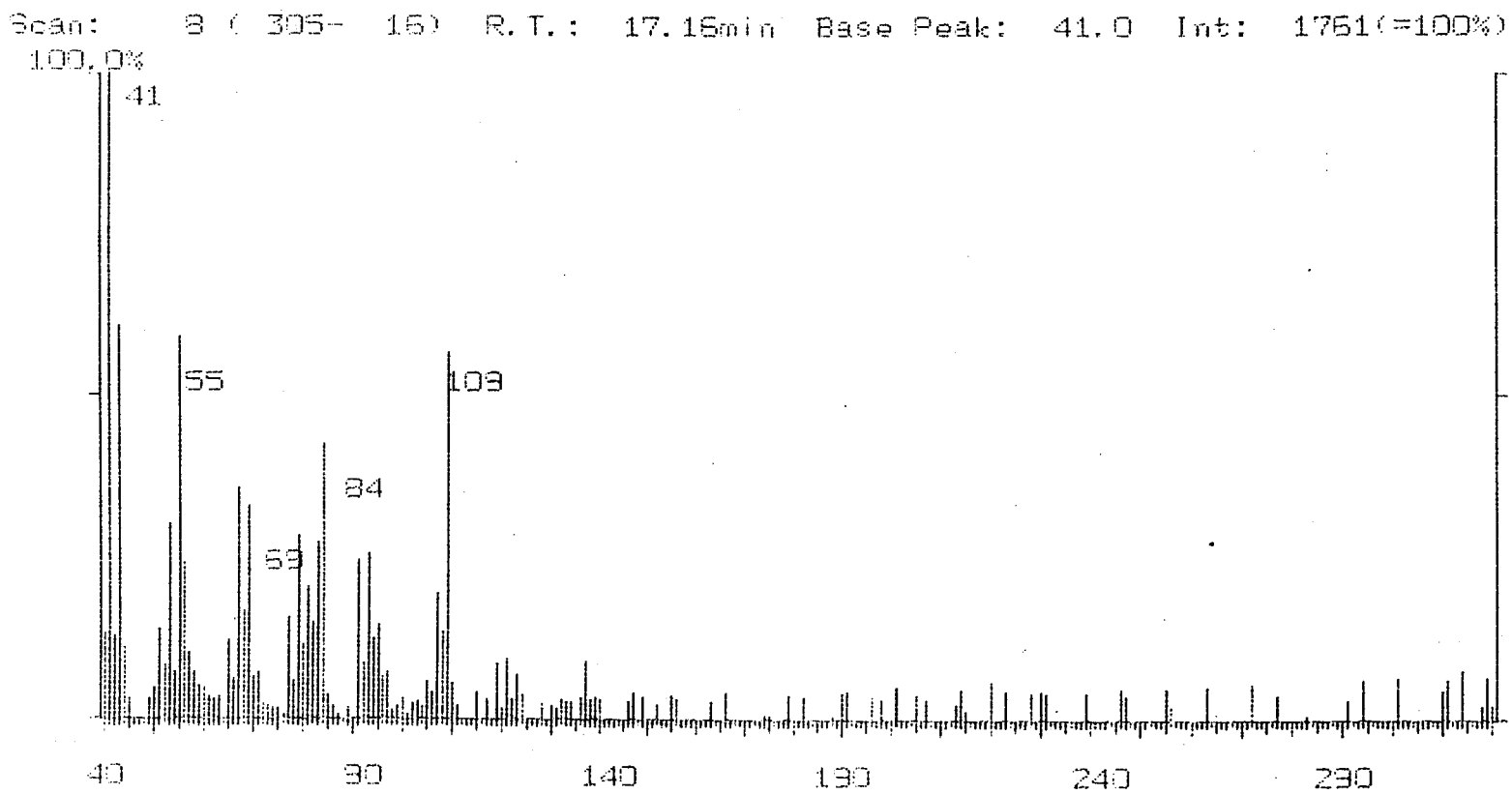


Fig. 4.1.10 Mass spectrum of carvone

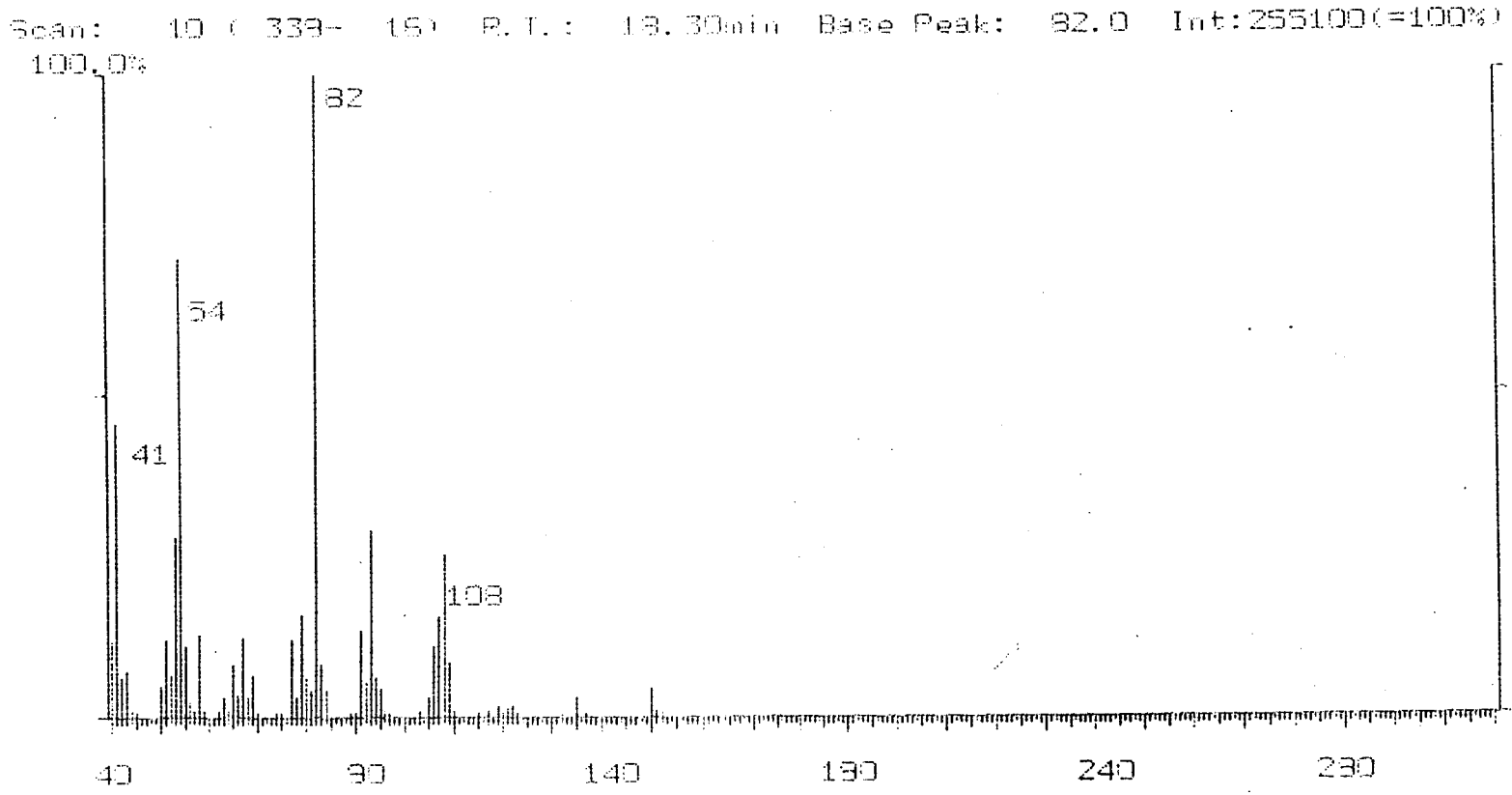


Fig. 4.1.11 Mass spectrum of Unidentified component

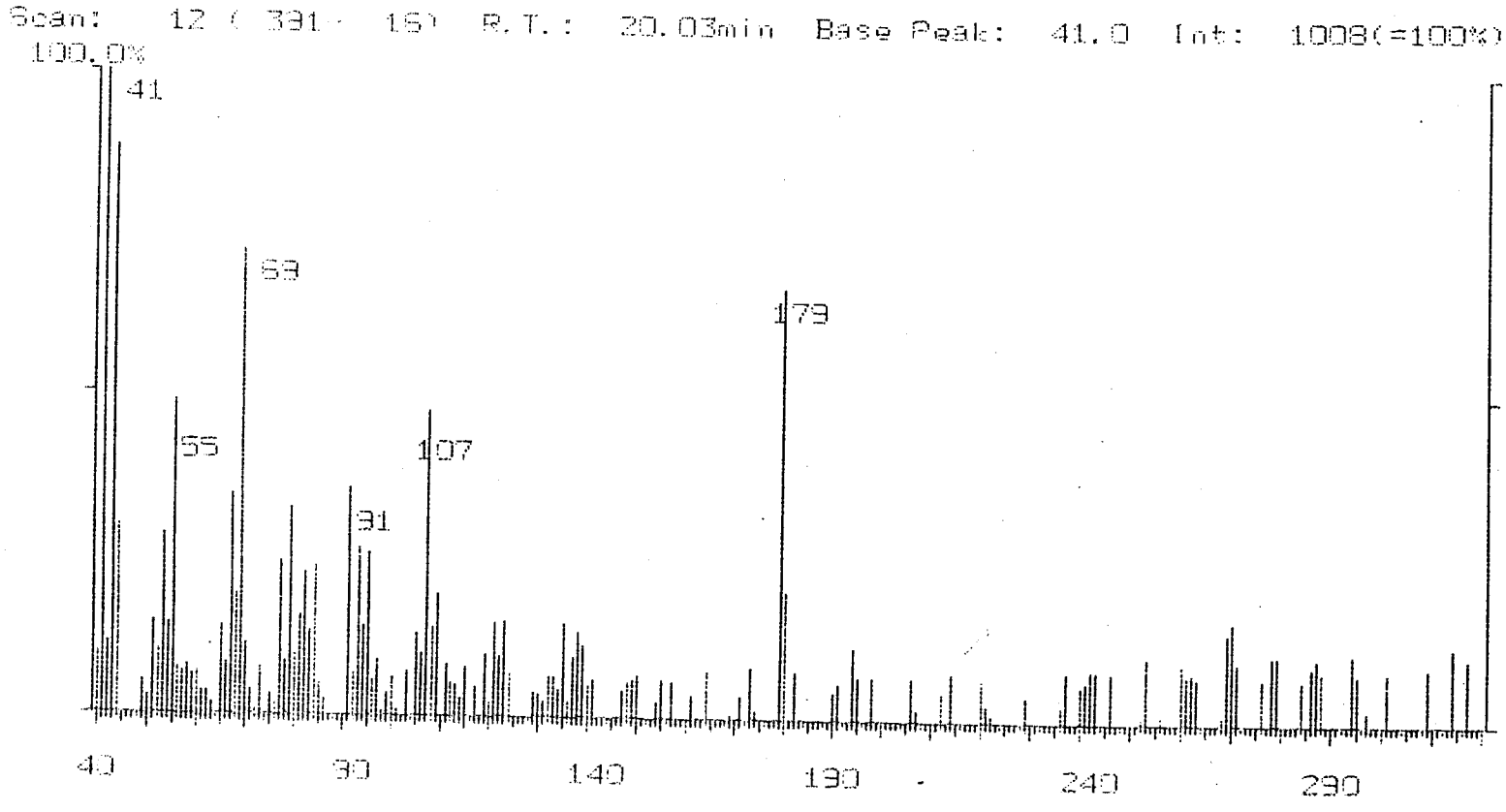


Fig. 4.1.12 Mass spectrum of α-terpinyl acetate

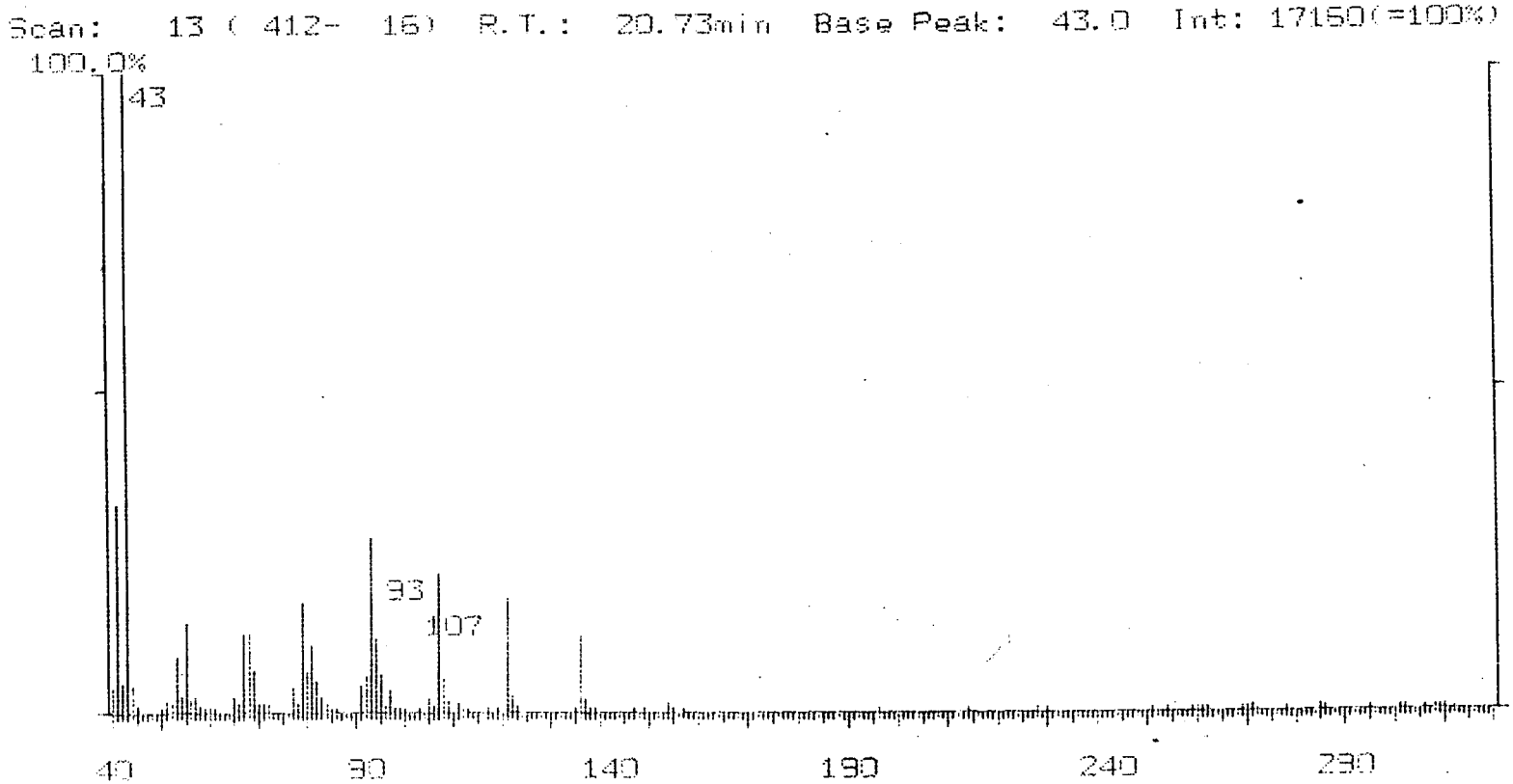


Fig. 4.1.13 Mass spectrum of Unidentified component

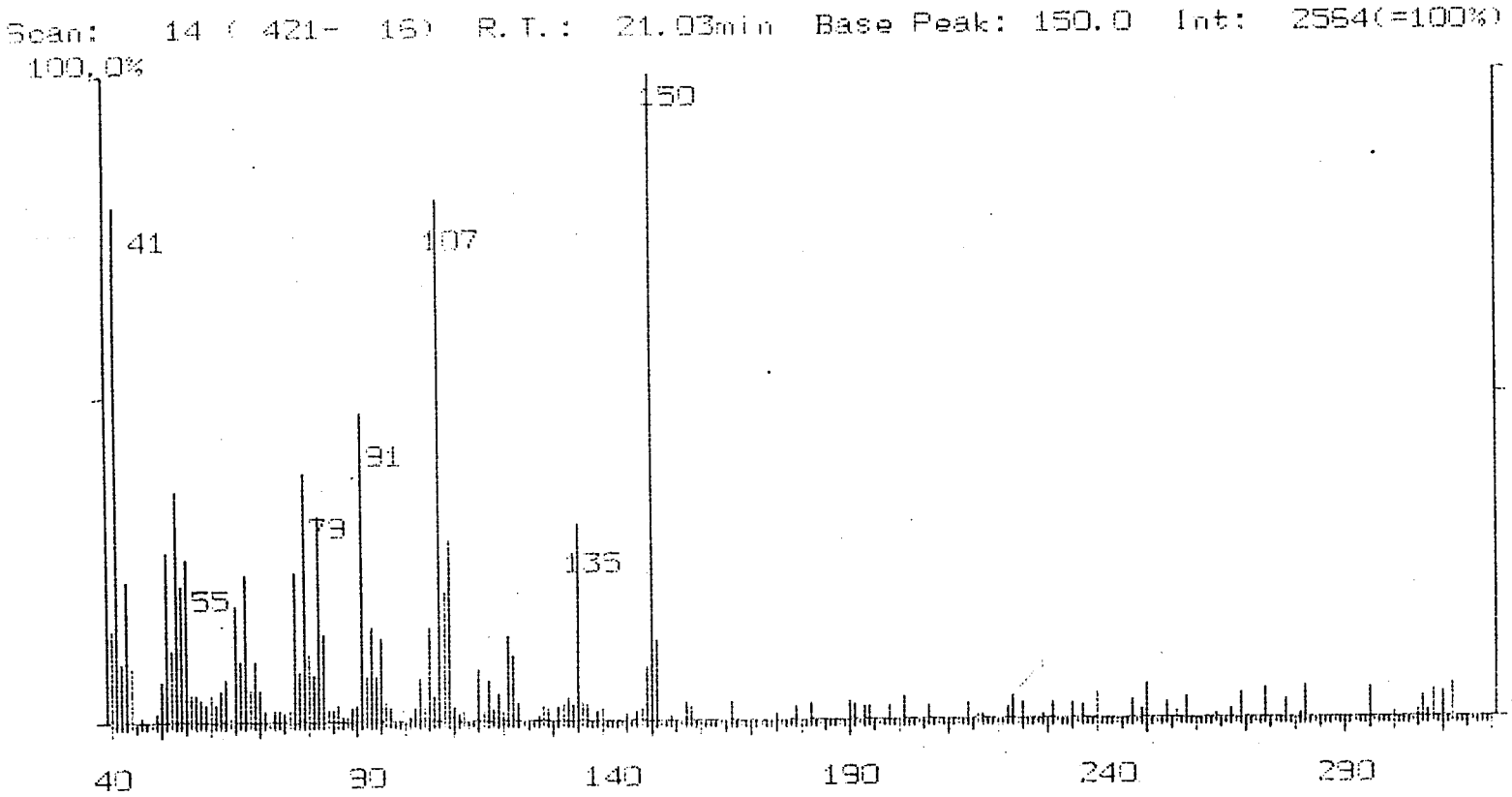


Fig. 4.1.14 Mass spectrum of alloaromadendrene

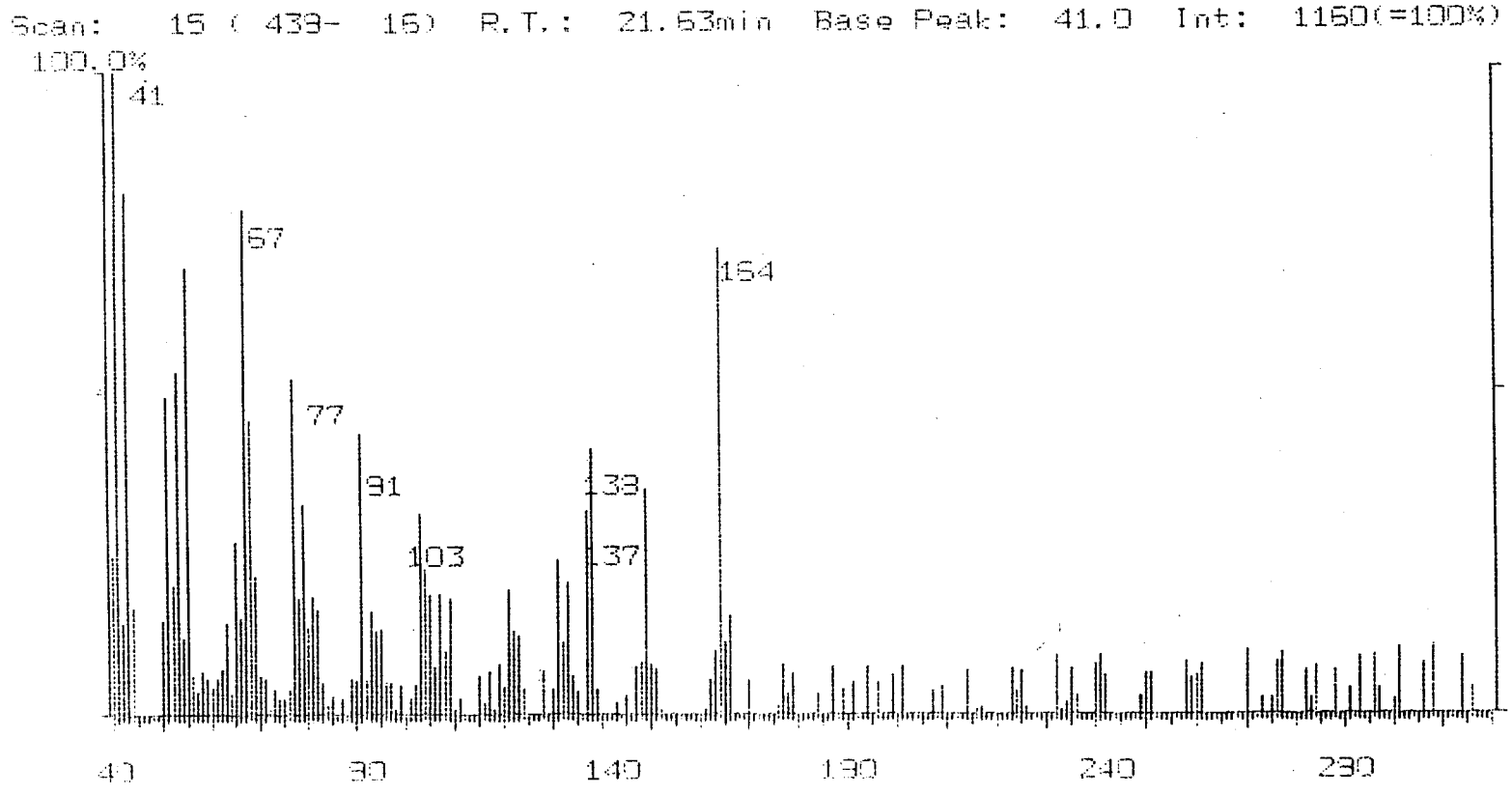


Fig. 4.1.15 Mass spectrum of β-elemene

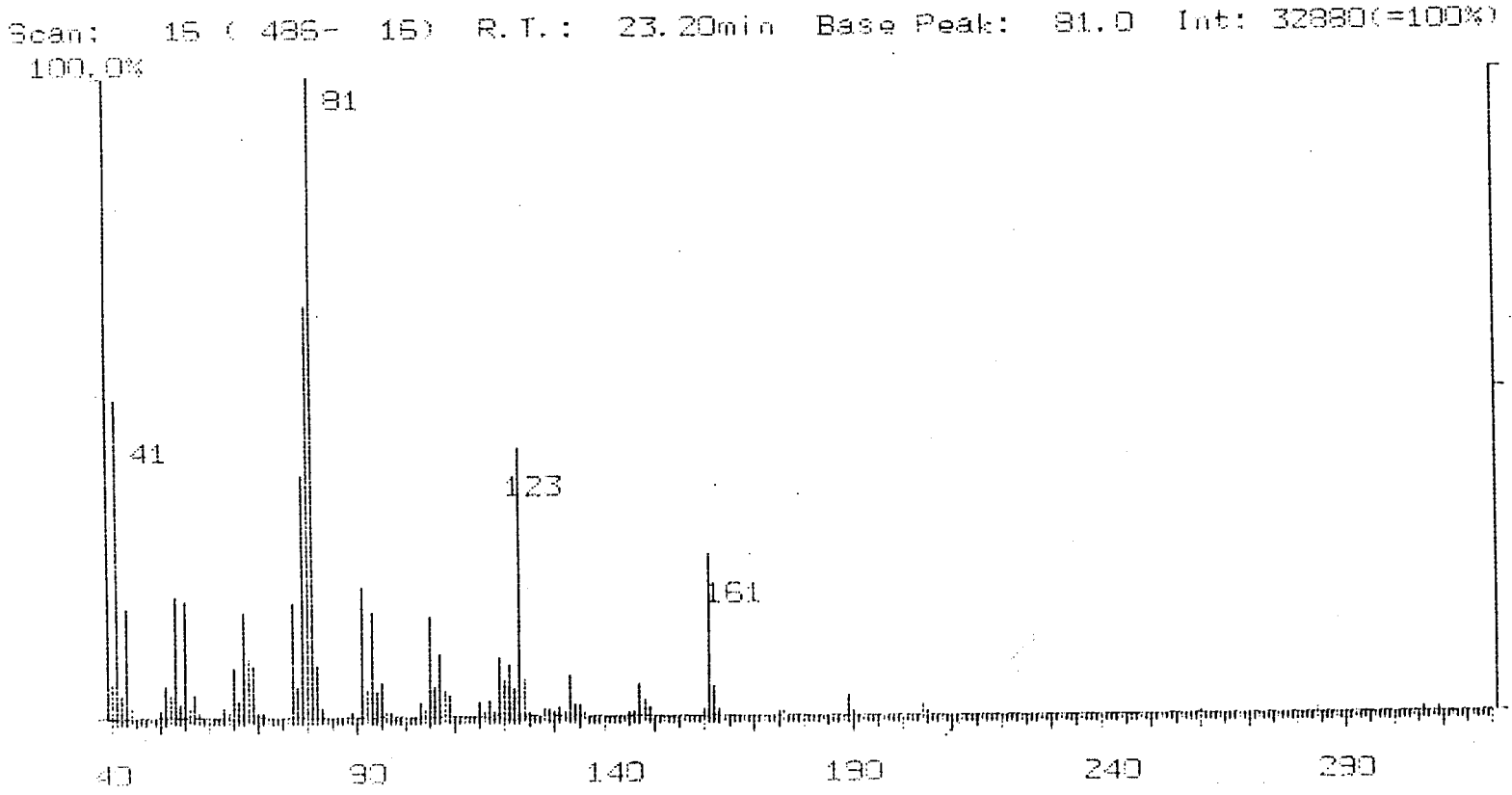


Fig. 4.1.16 Mass spectrum of β-caryophyllene

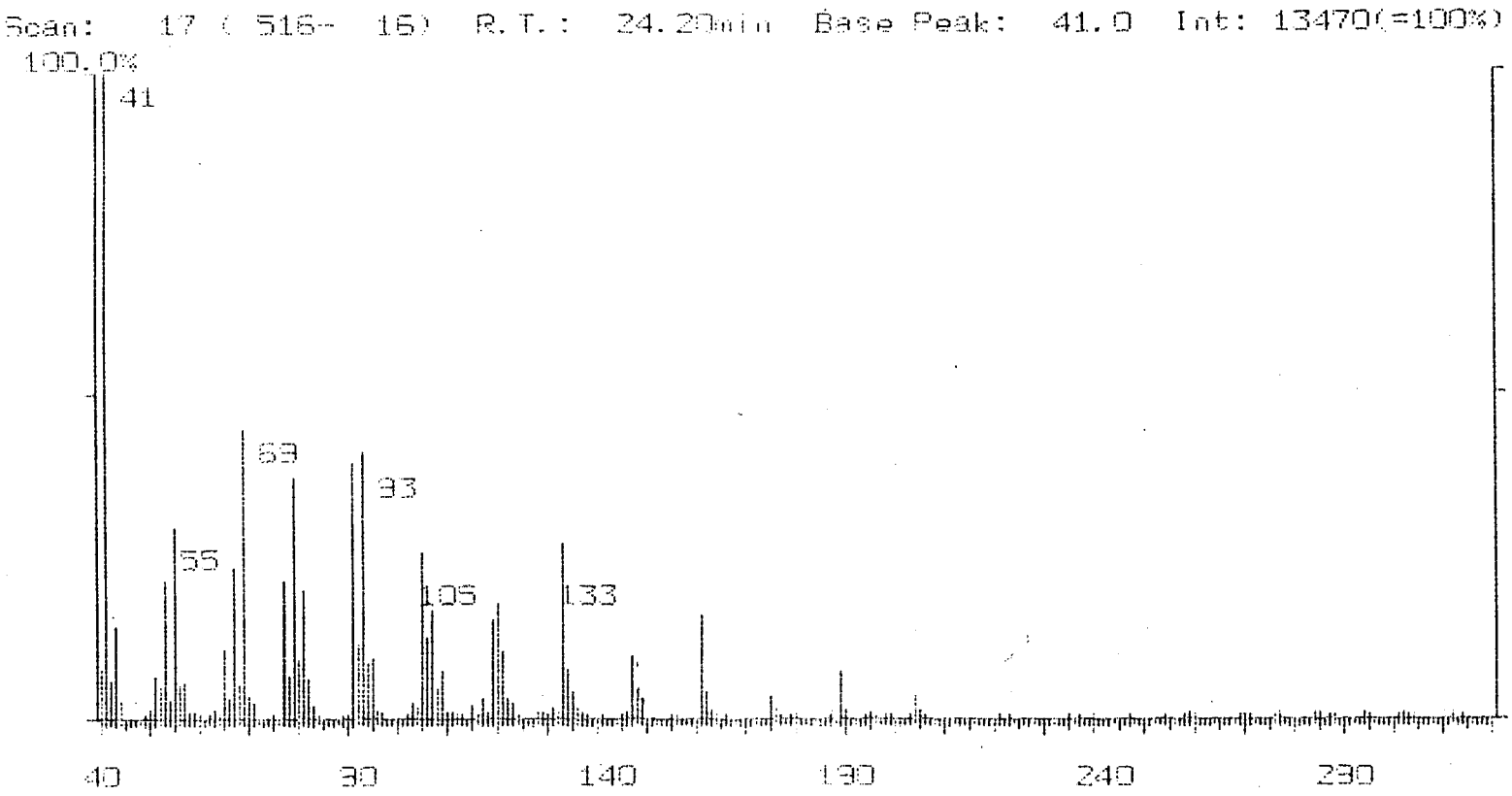


Fig. 4.1.17 Mass spectrum of trans-2-cis-6-nonadien–1-ol

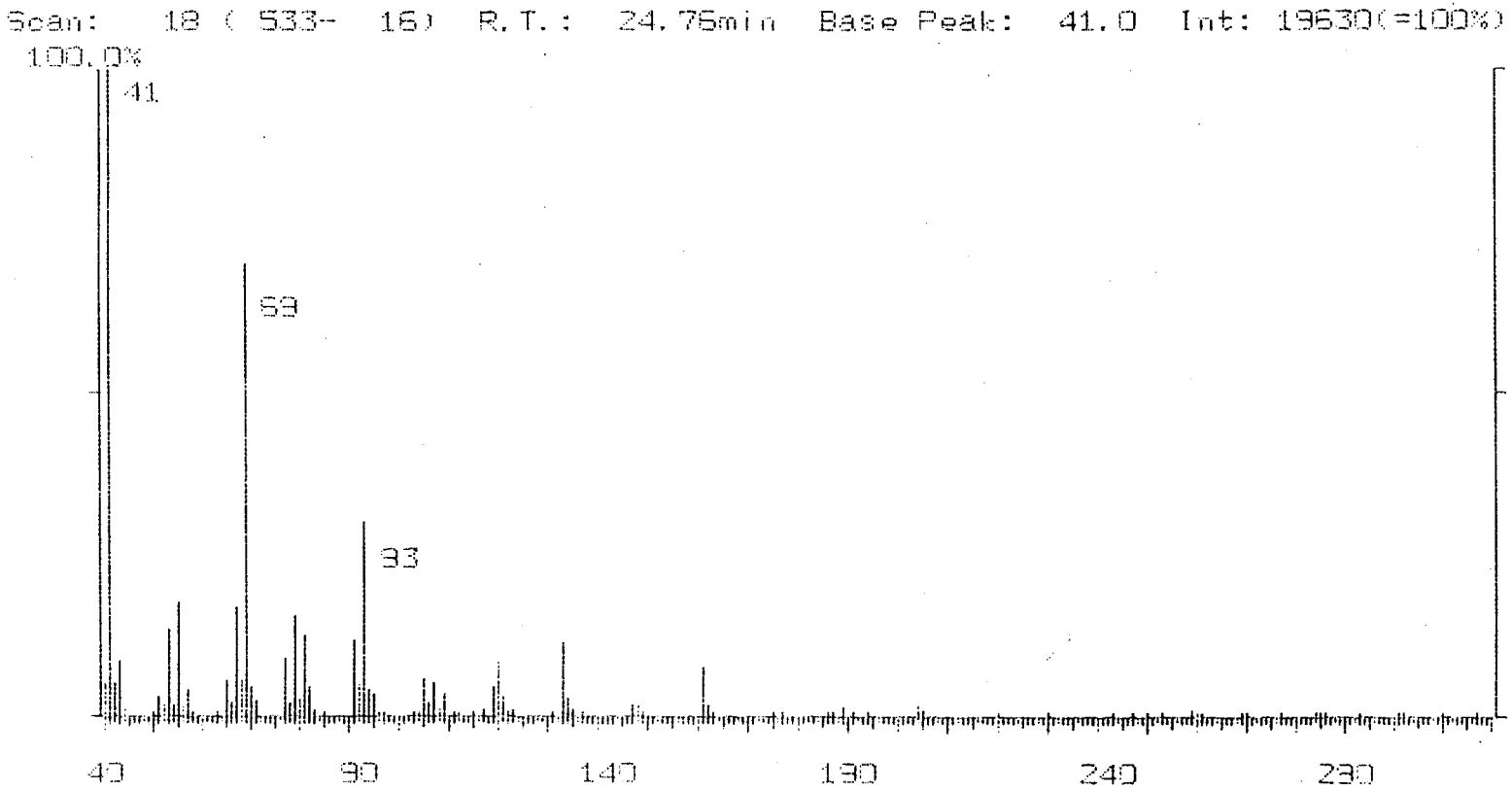


Fig. 4.1.18 Mass spectrum of α-cububene

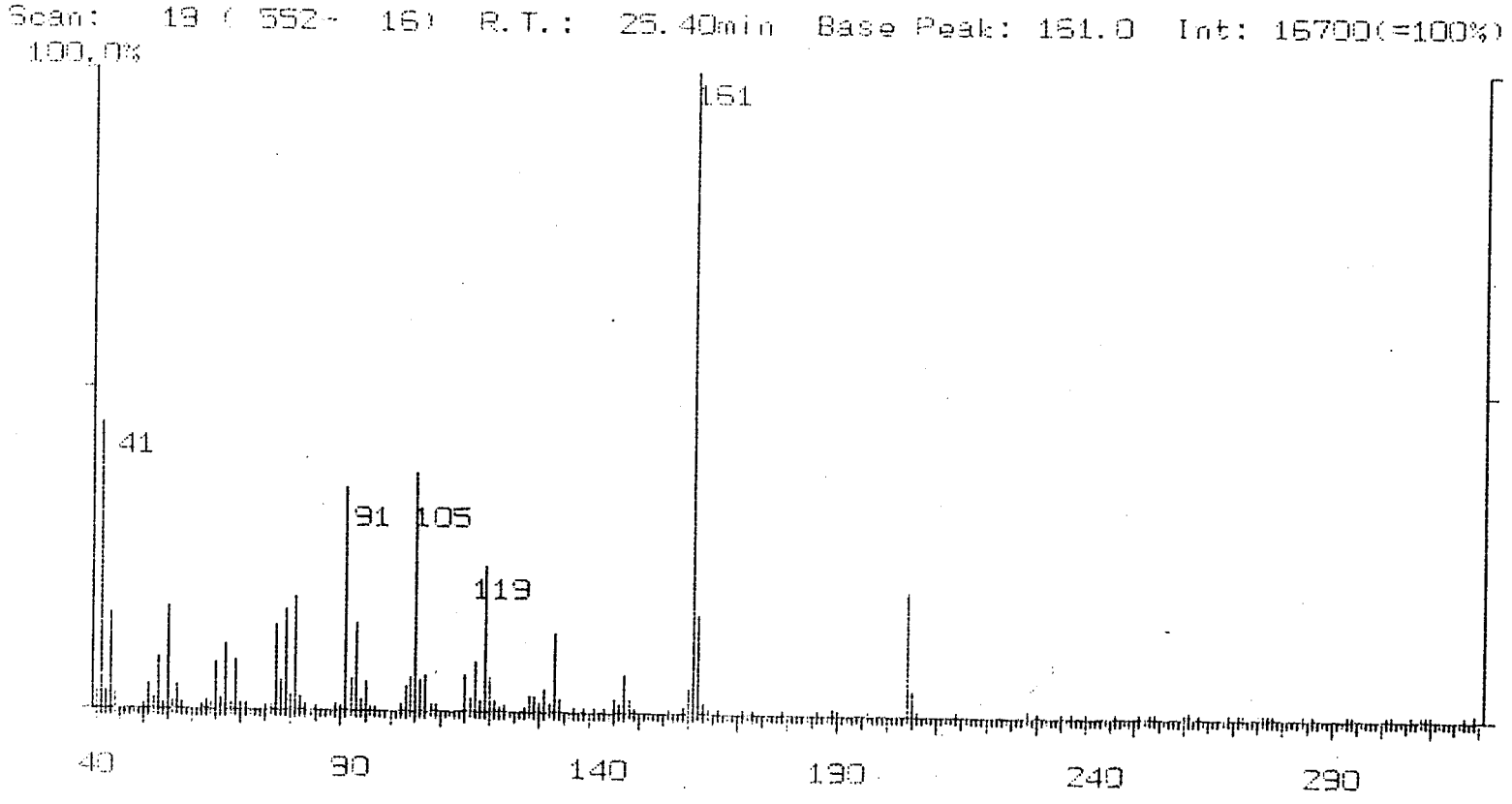


Fig. 4.1.19 Mass spectrum of Unidentified component

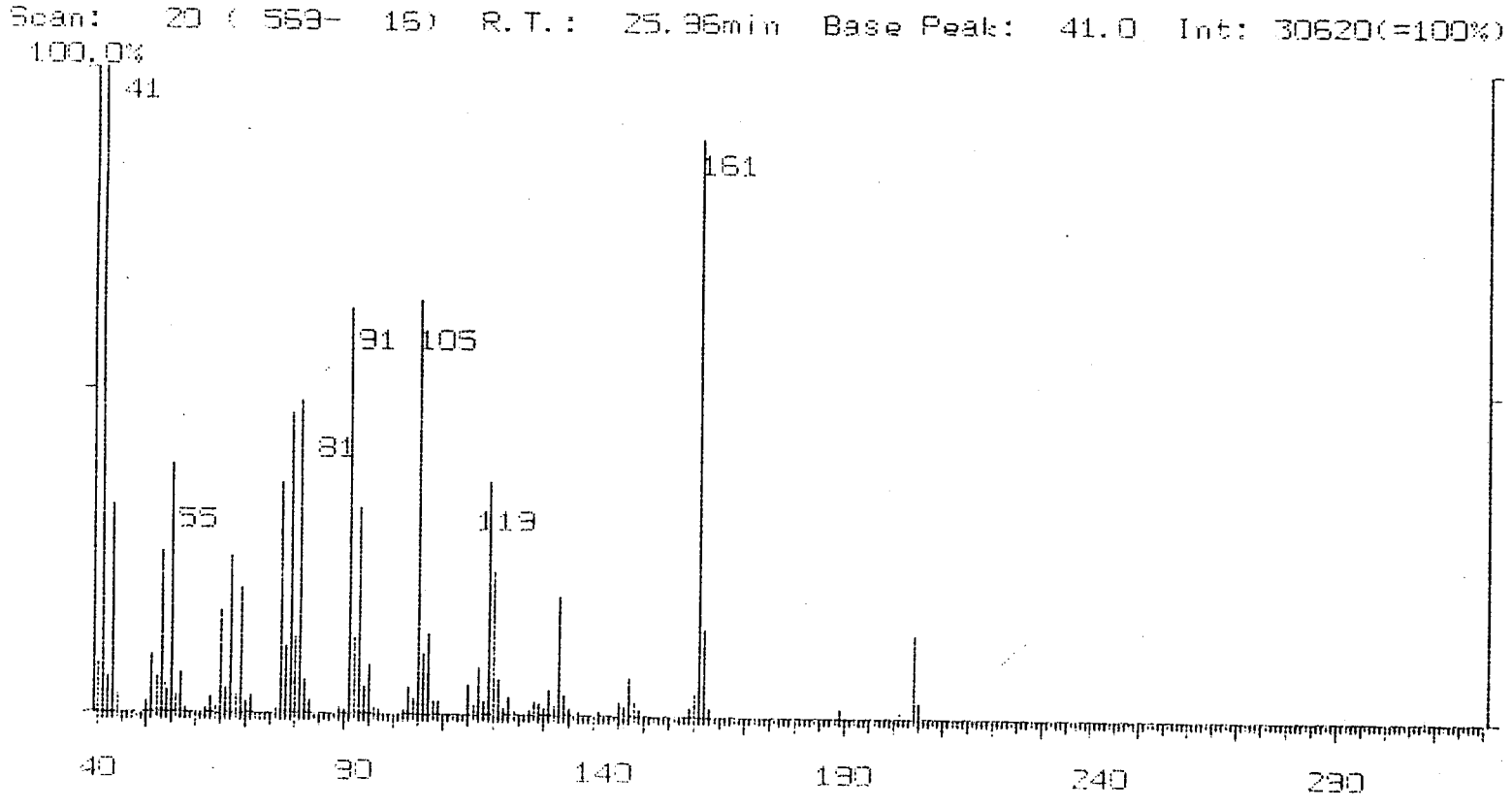


Fig. 4.1.20 Mass spectrum of β-gurgunene

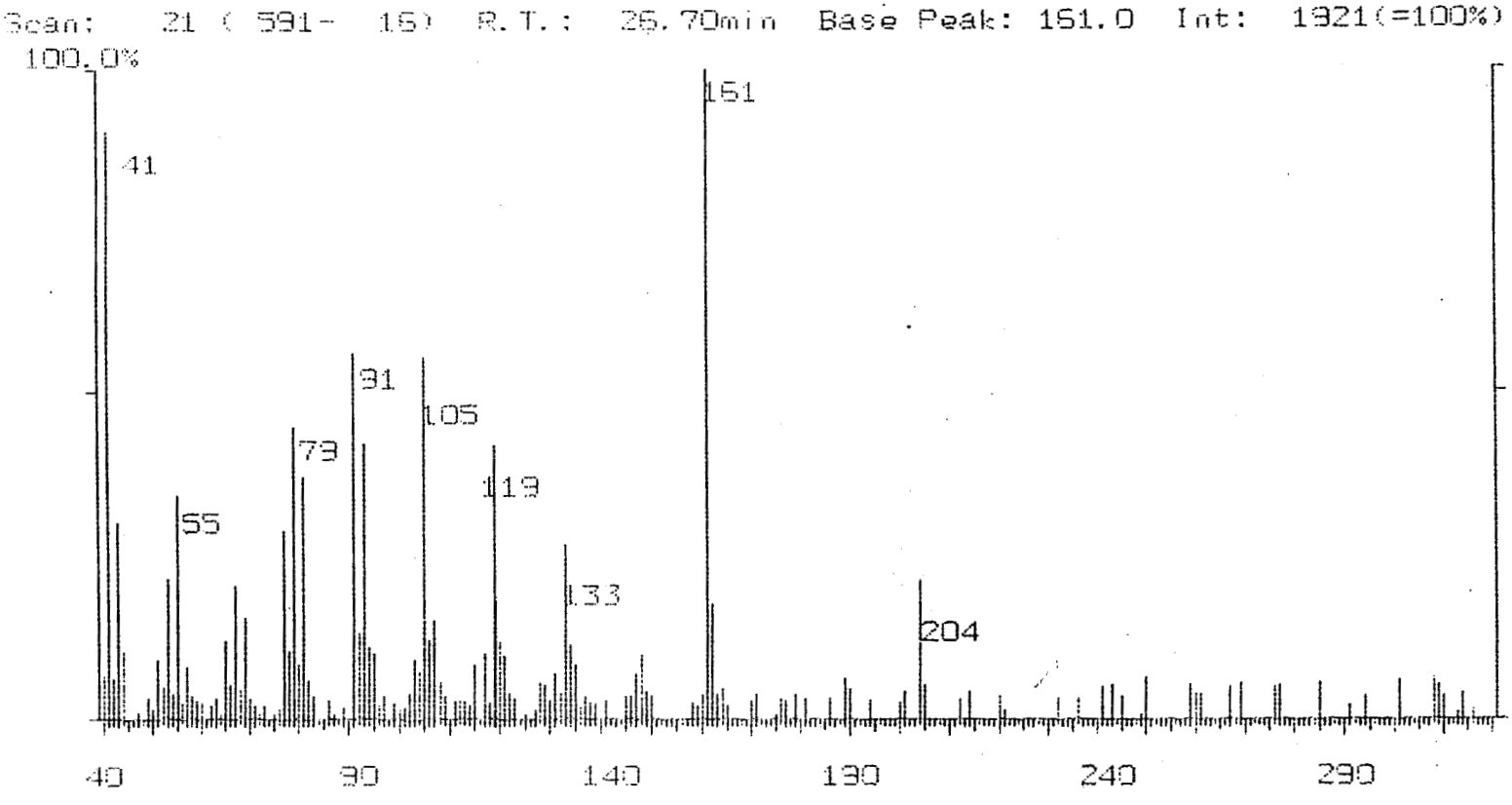


Fig. 4.1.21 Mass spectrum of aromadendrene

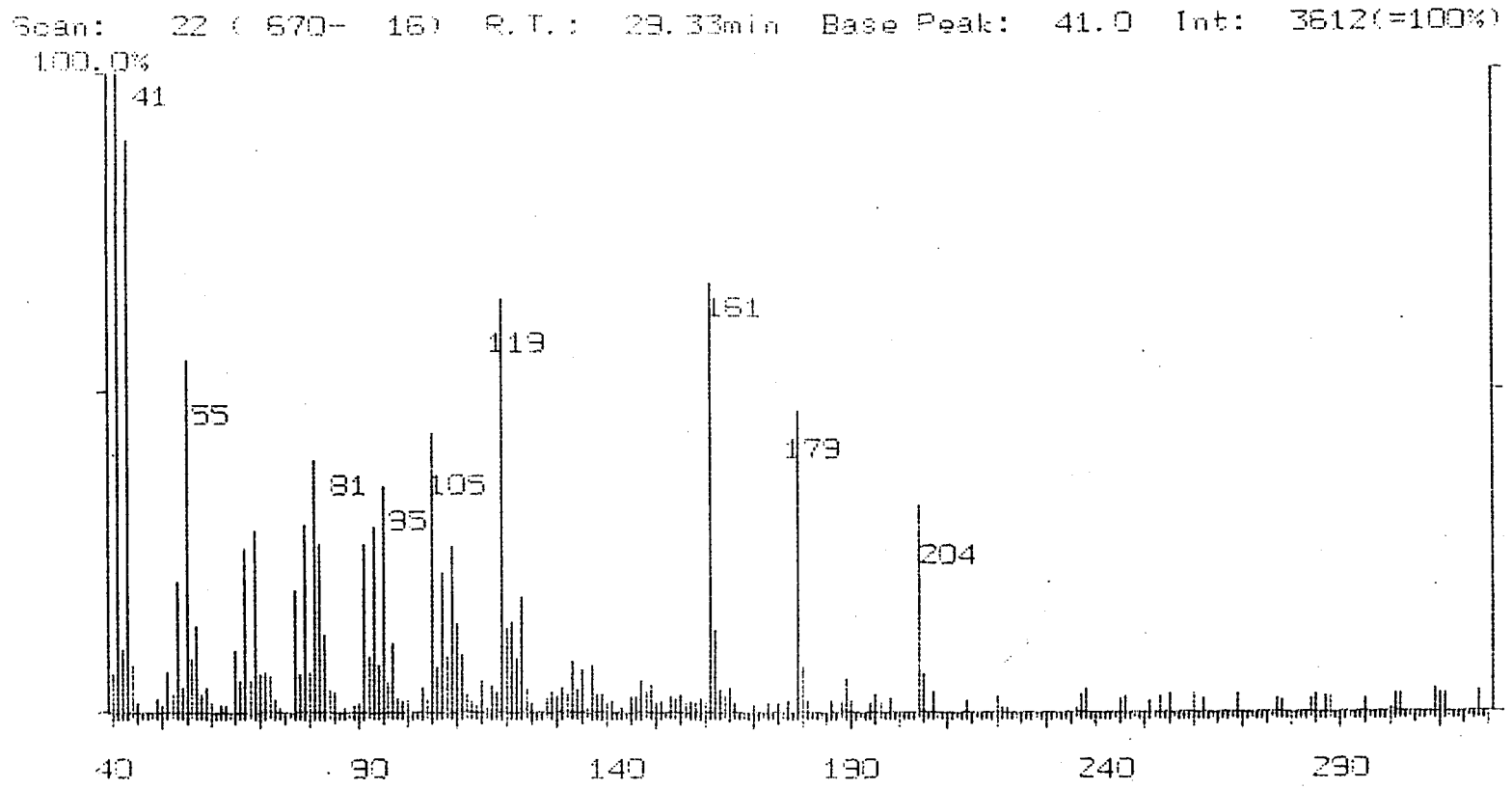


Fig. 4.1.22 Mass spectrum of β-terpineol

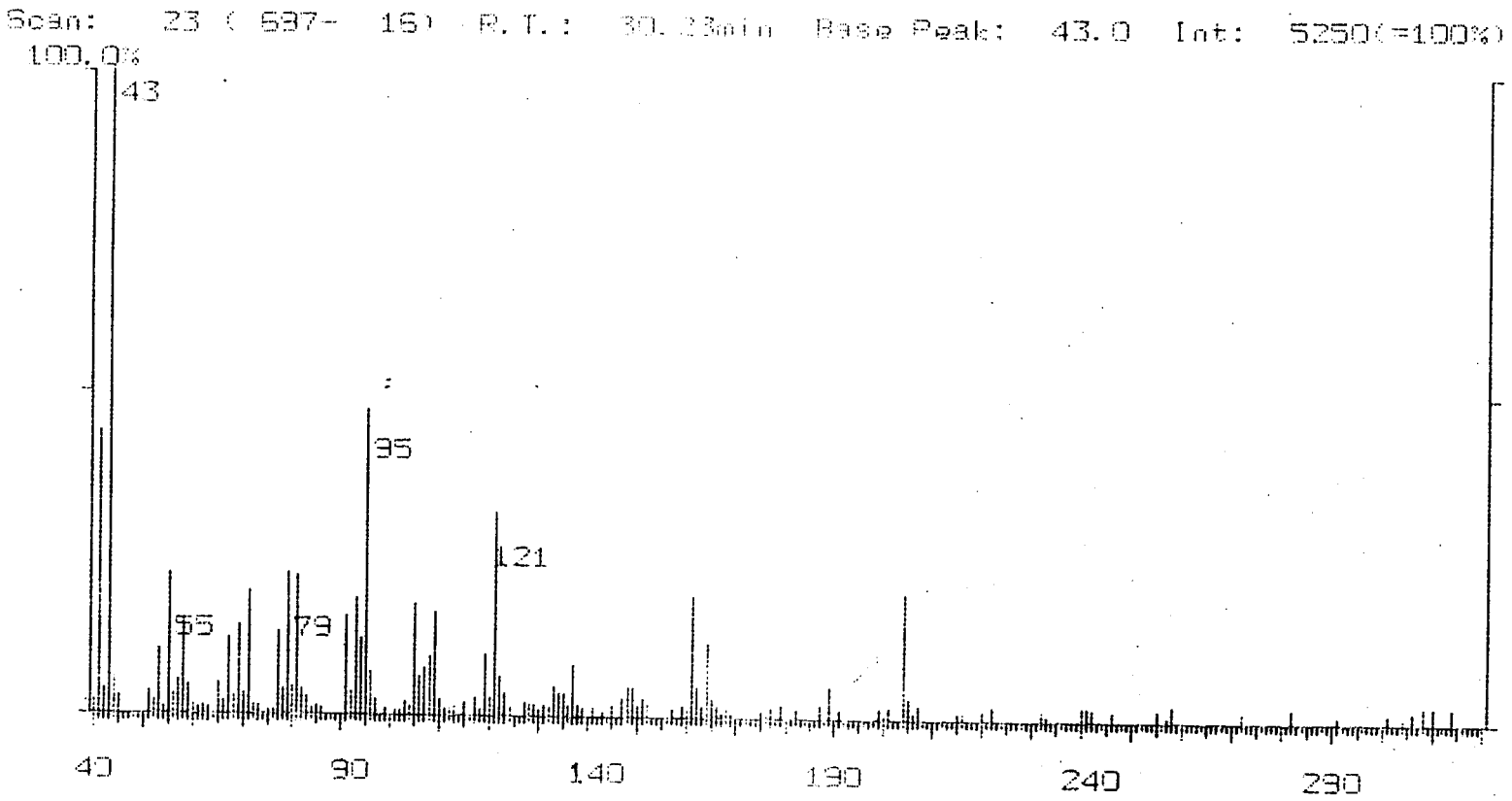


Fig. 4.2.1 Gas chromatogram of the essential oil of *Mentha rotundifolia* (L.) Huds. Somaclonal variant

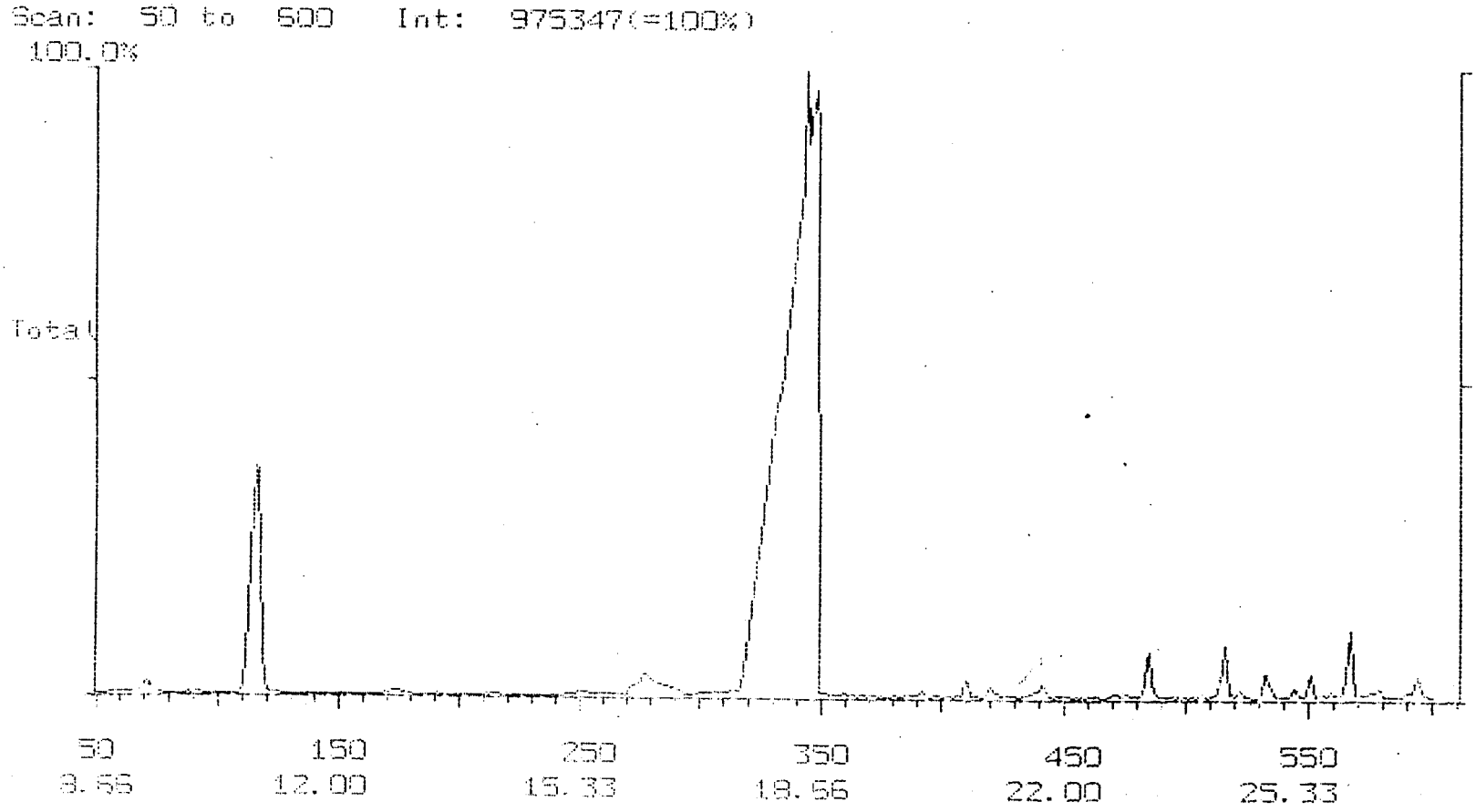


Fig. 4.2.2 Mass spectrum of myrcene

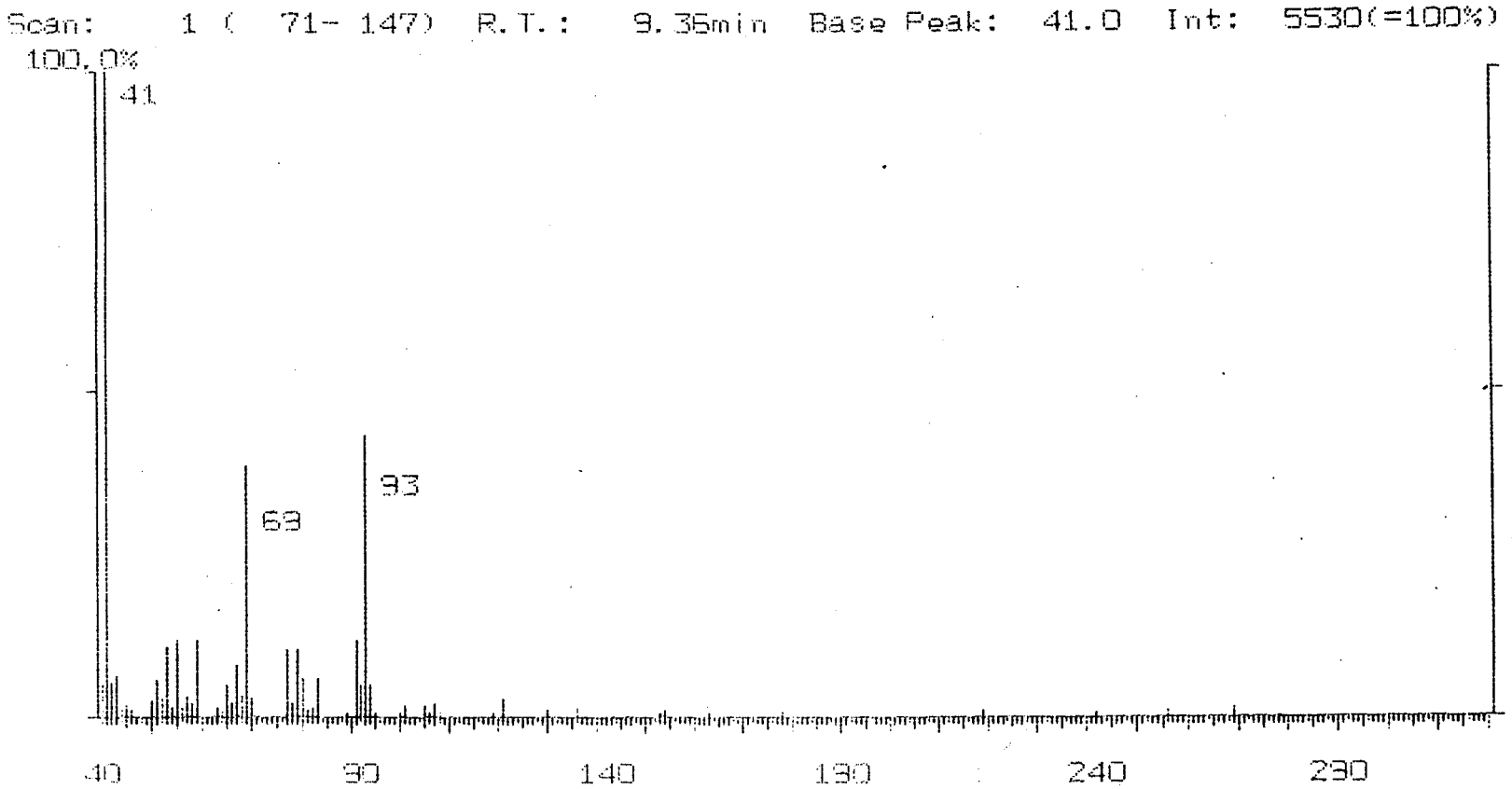


Fig. 4.2.3 Mass spectrum of limonene

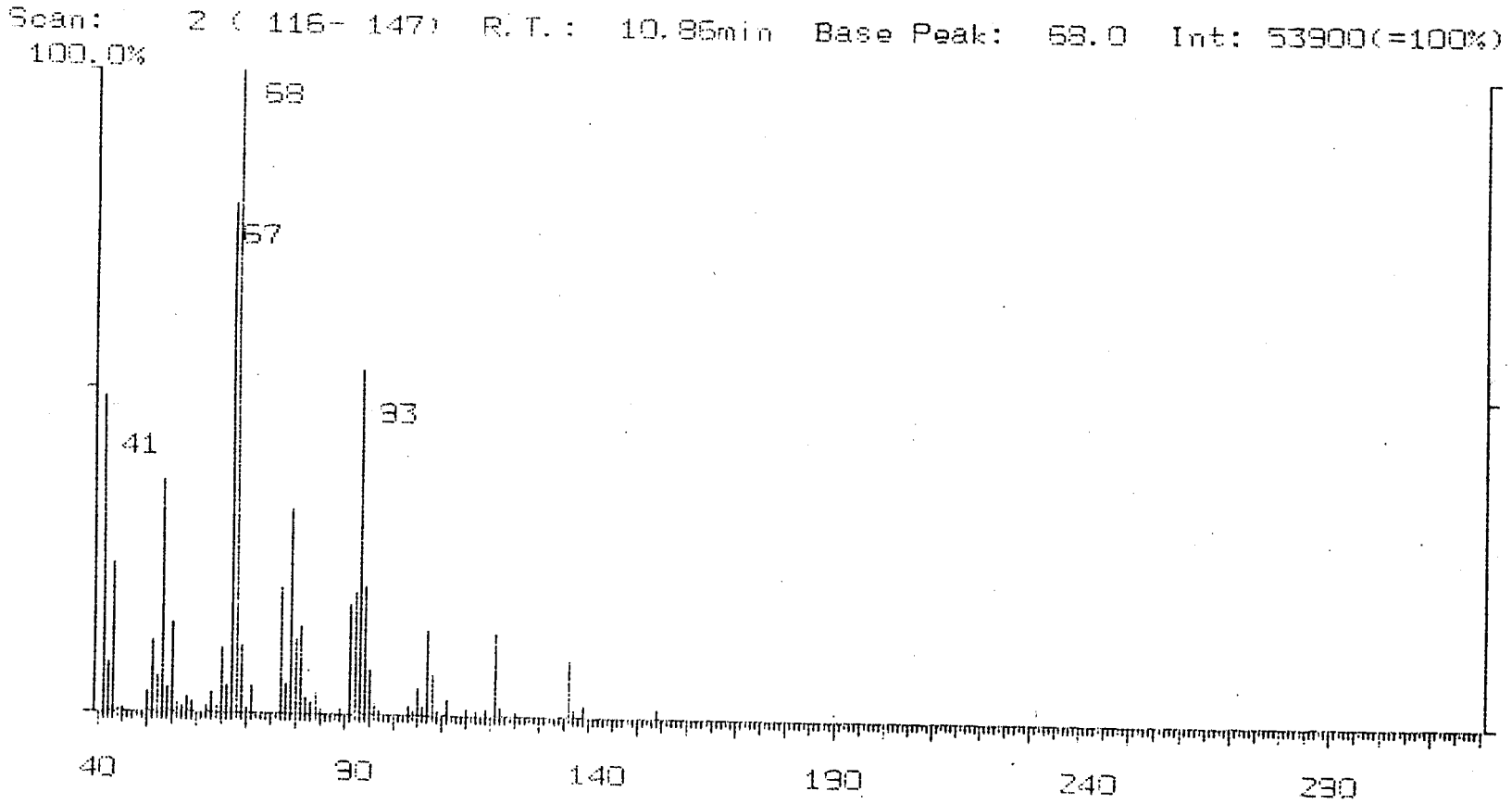


Fig. 4.2.4 Mass spectrum of methyl chavicol

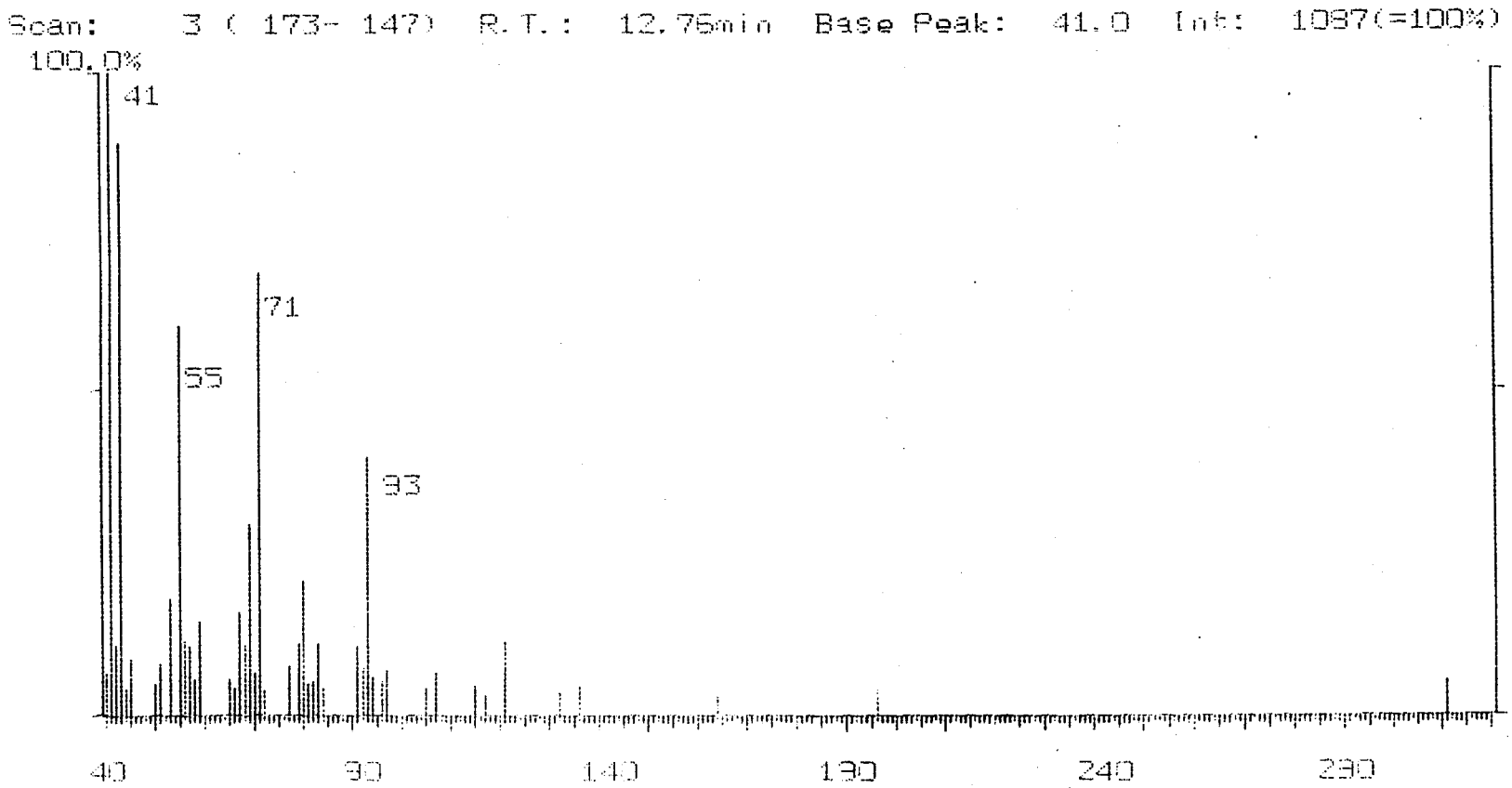


Fig. 4.2.5 Mass spectrum of isoborneol

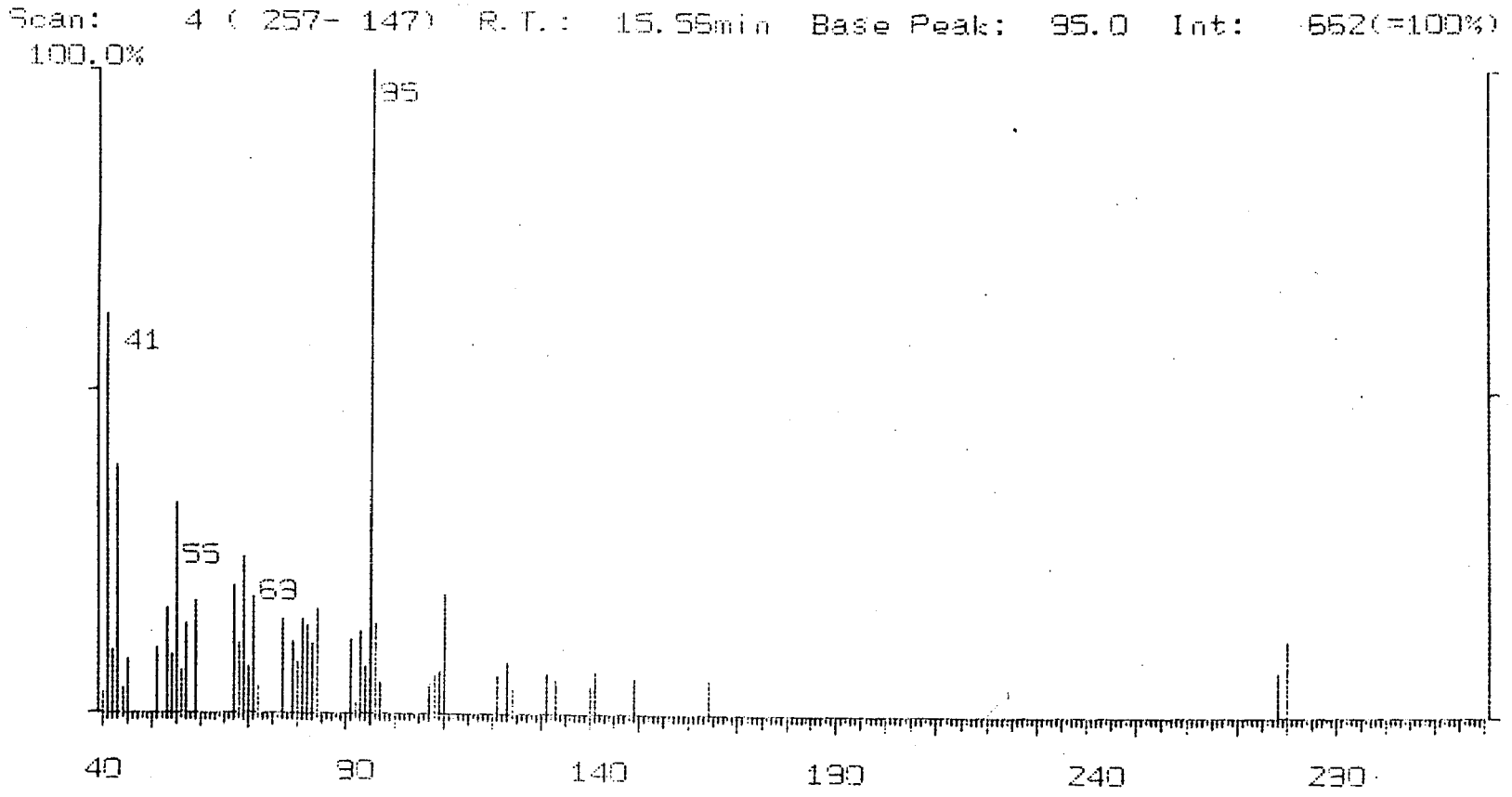


Fig. 4.2.6 Mass spectrum of cis-6-nonenal

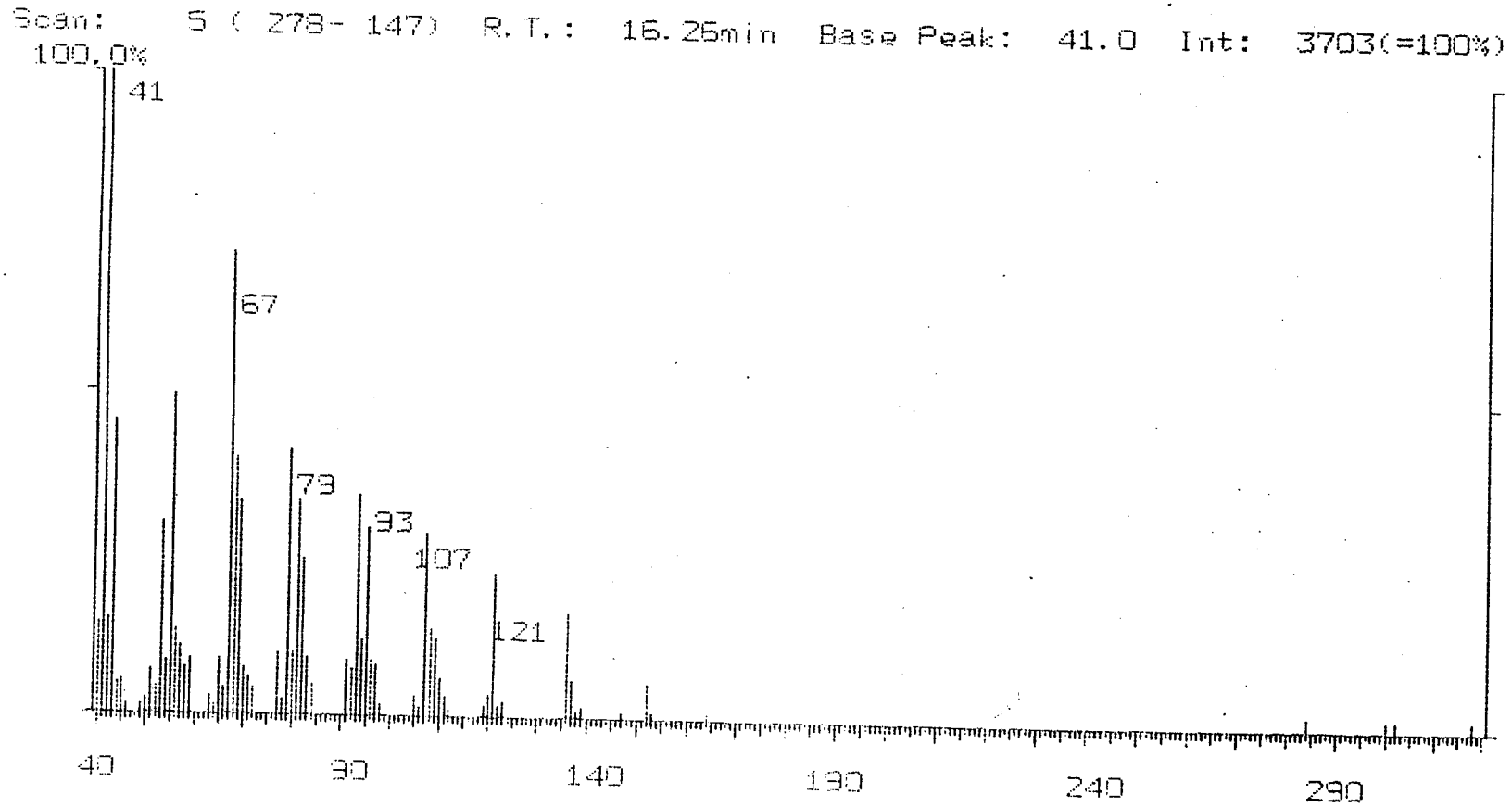


Fig. 4.2.7 Mass spectrum of carvone

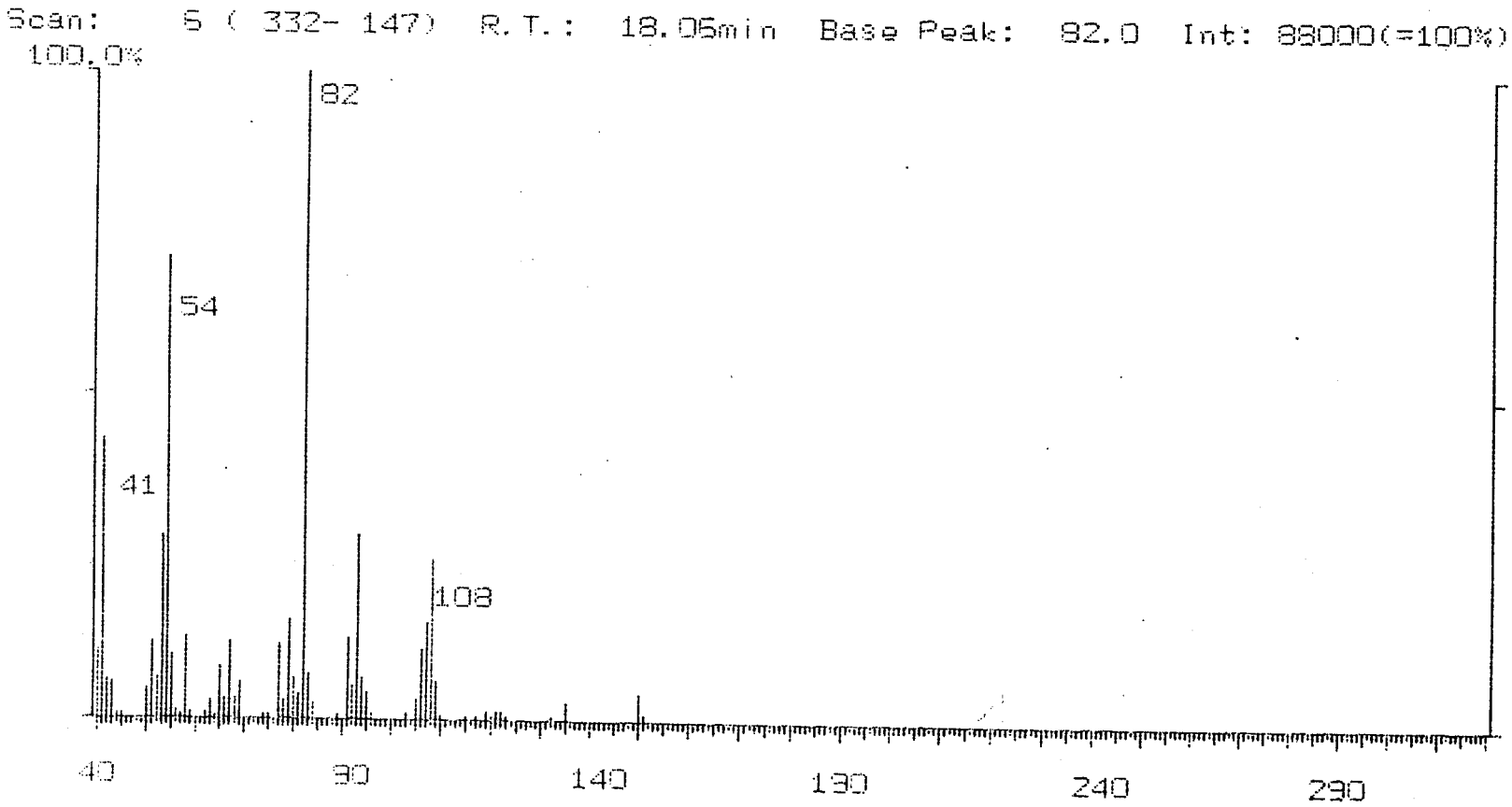


Fig. 4.2.8 Mass spectrum of Unidentified component

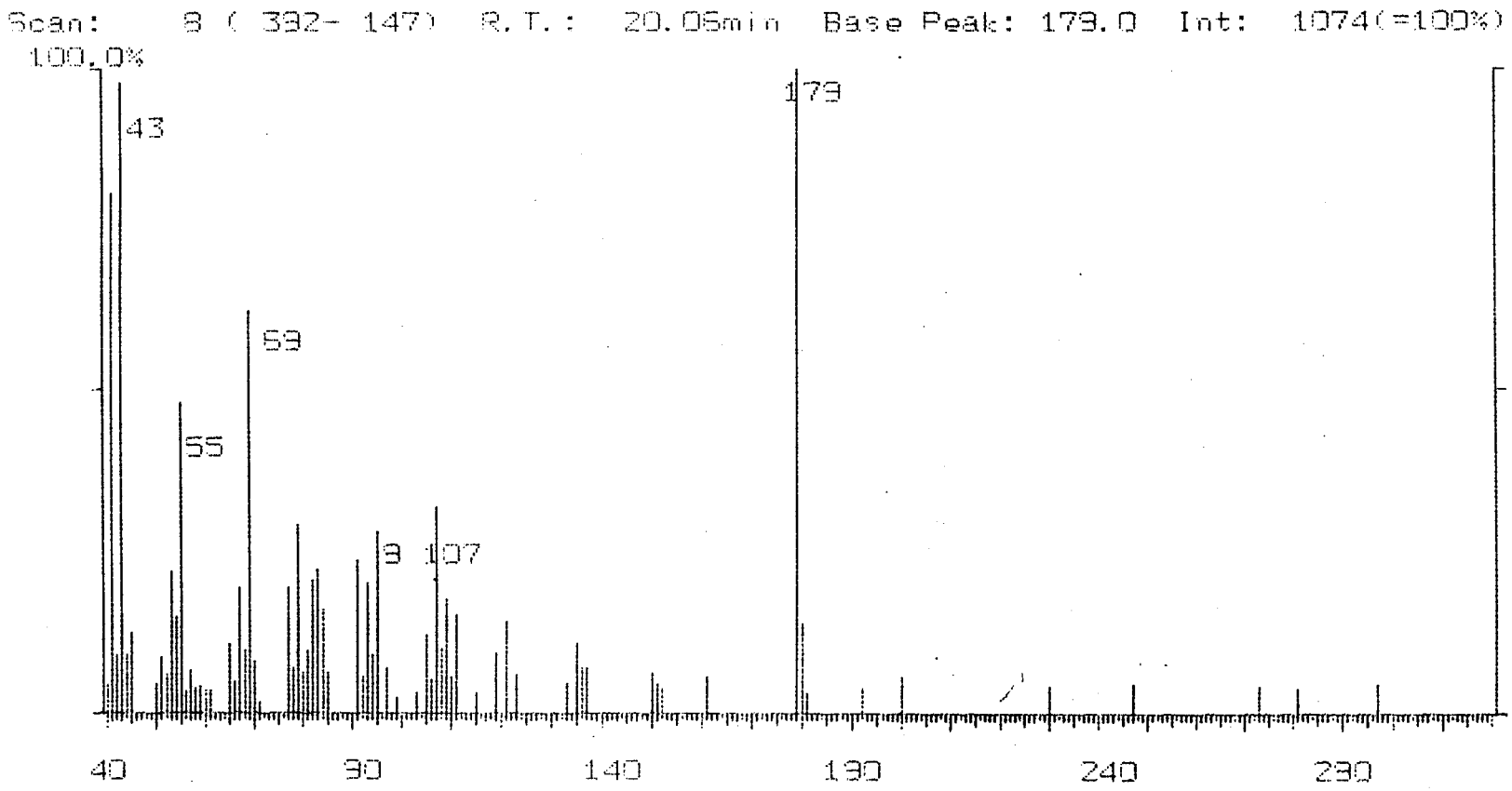


Fig. 4.2.9 Mass spectrum of α-terpinyl acetate

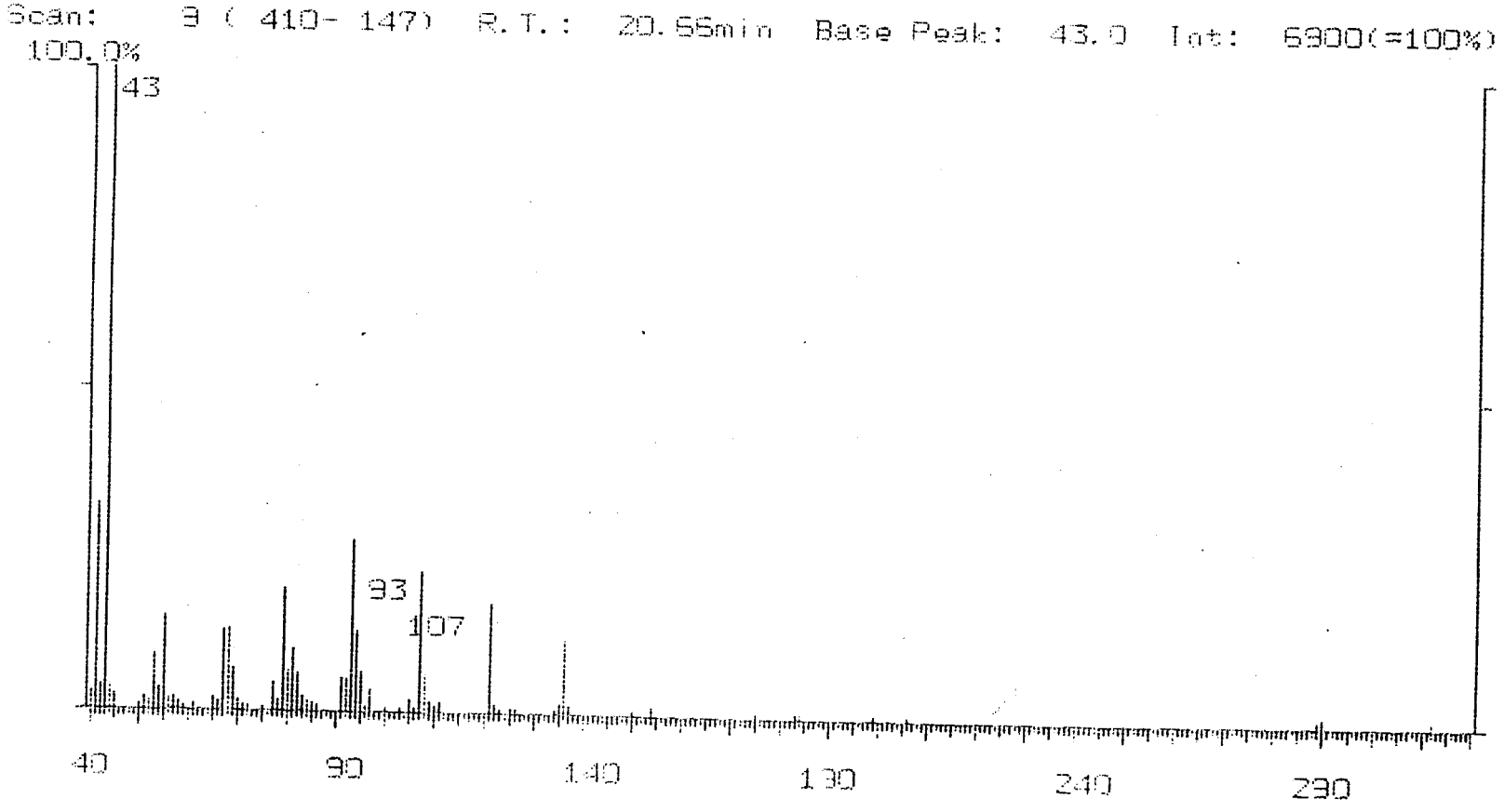


Fig. 4.2.10 Mass spectrum of 2,3 diethyl-6-methyl pyrazine

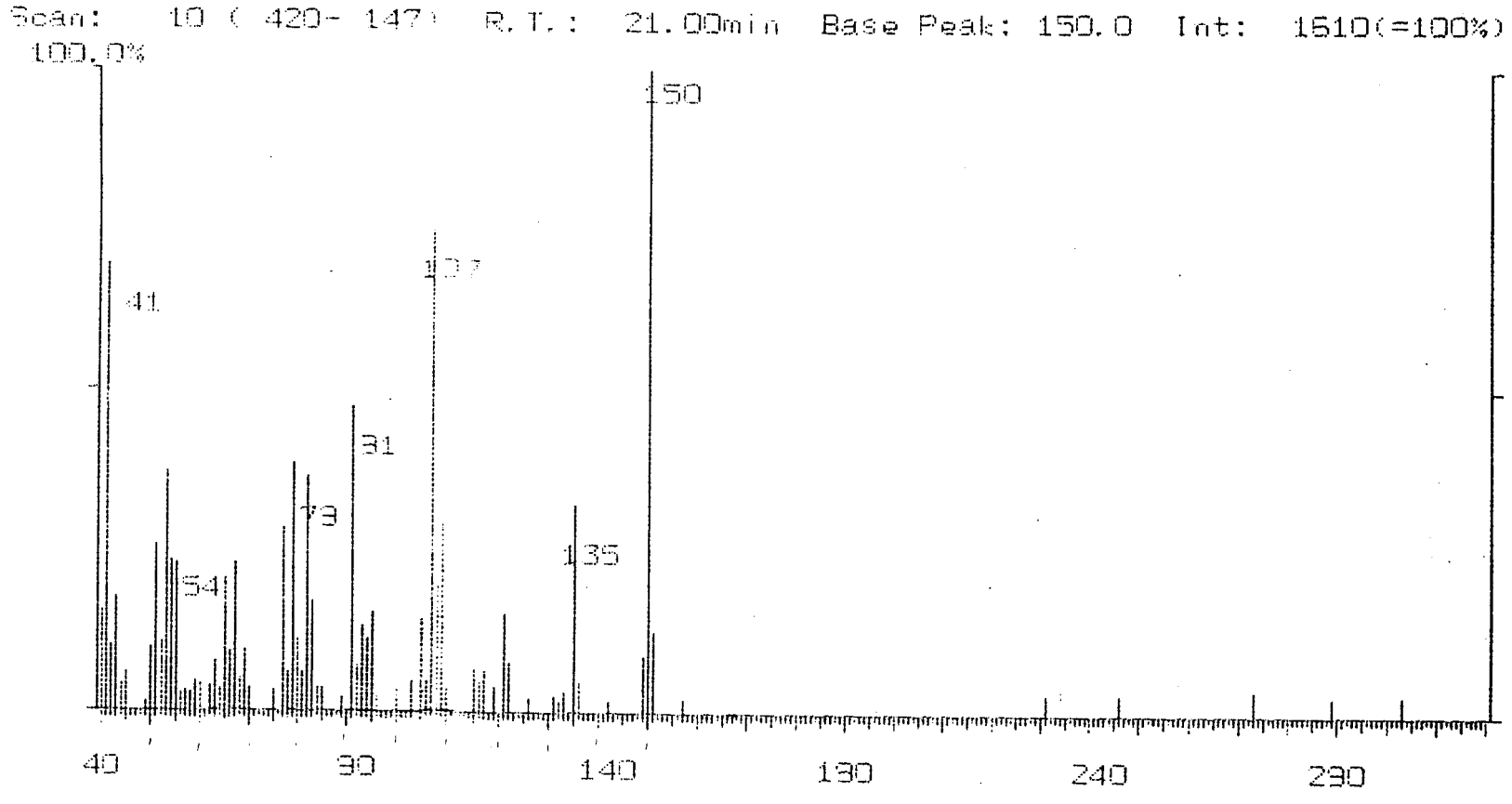


Fig. 4.2.11 Mass spectrum of p-mentha trans 2,8 dien-1-ol

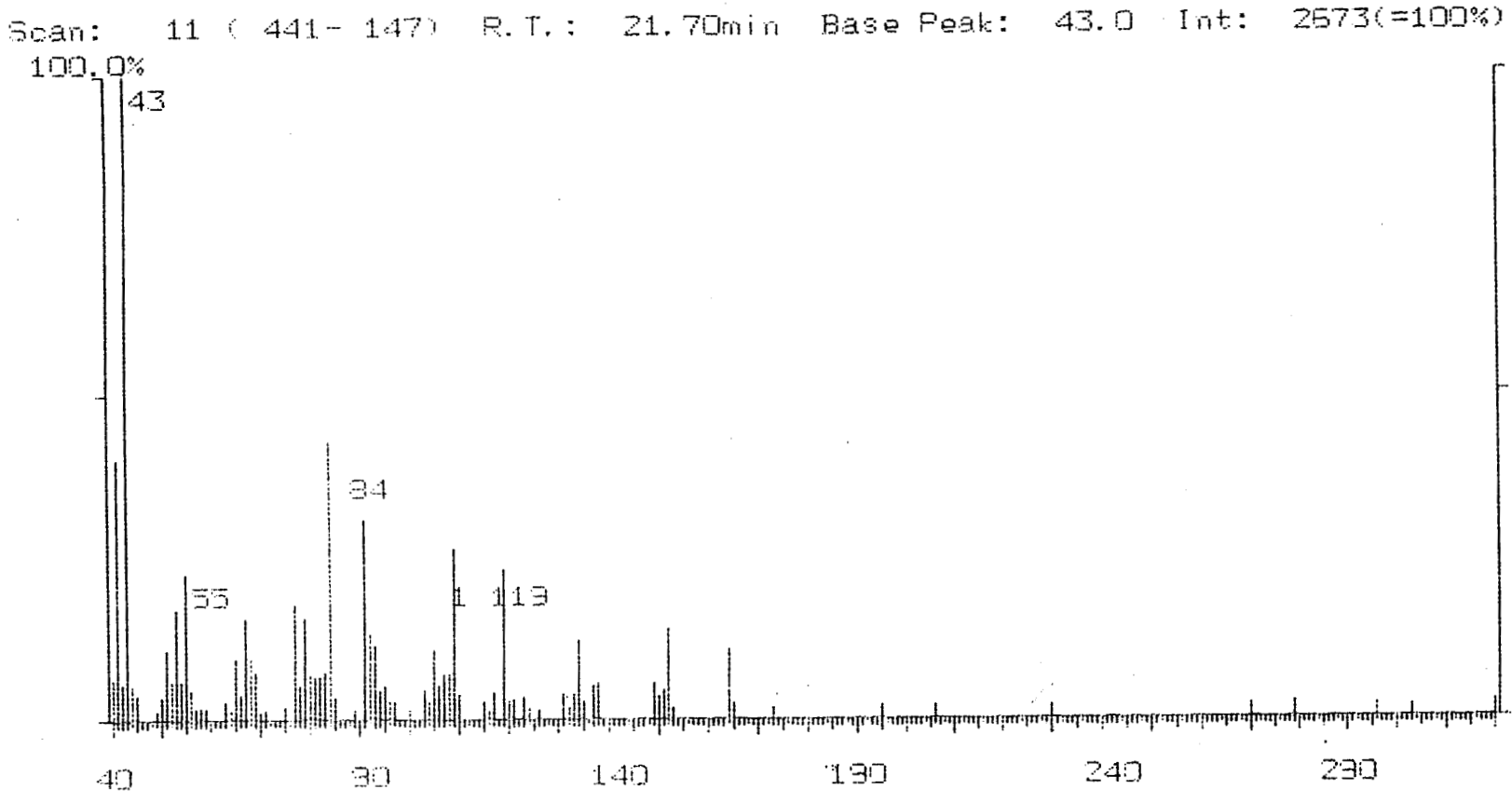


Fig. 4.2.12 Mass spectrum of β-elemene

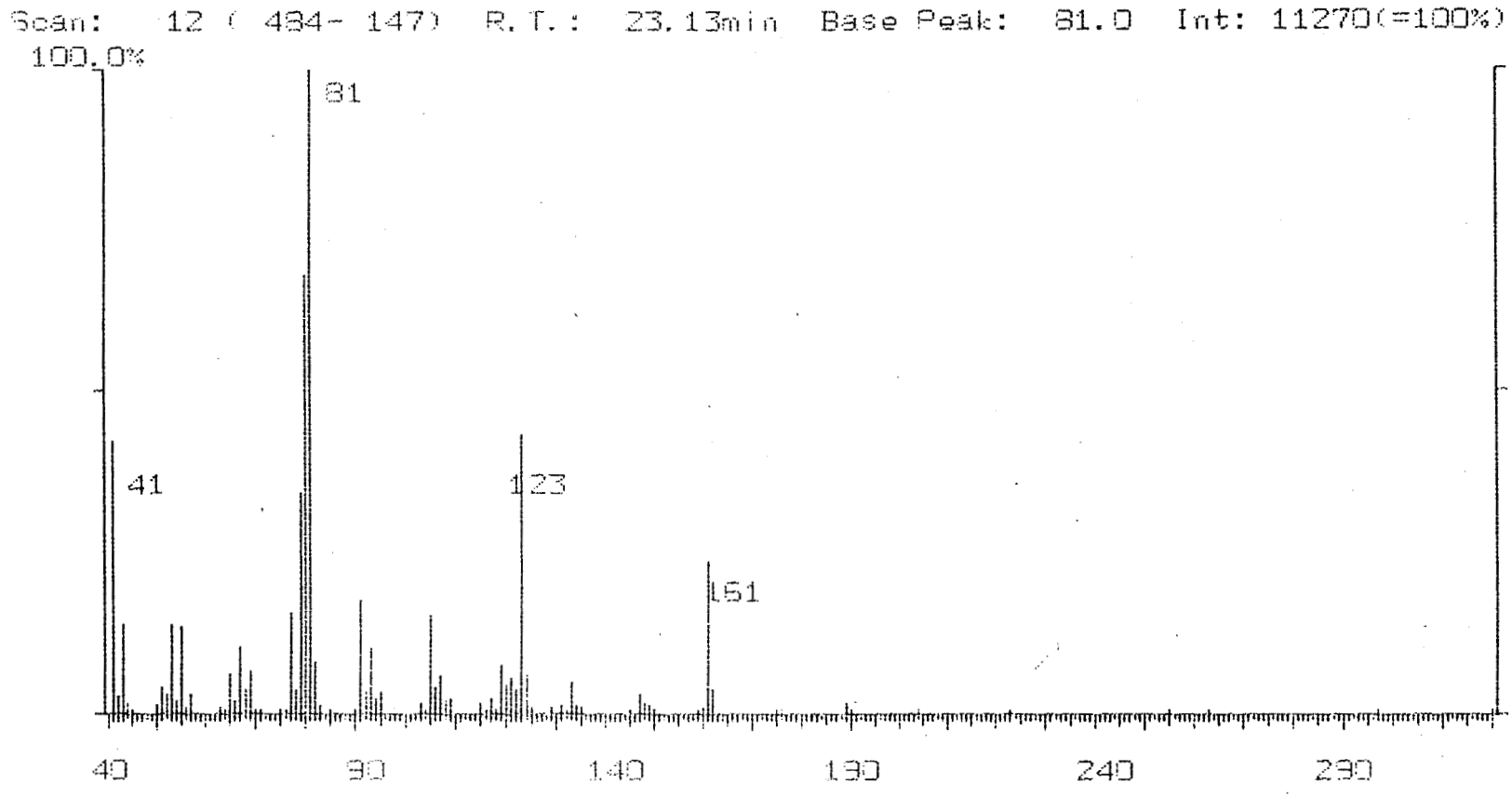


Fig. 4.2.13 Mass spectrum of γ-caryophyllene

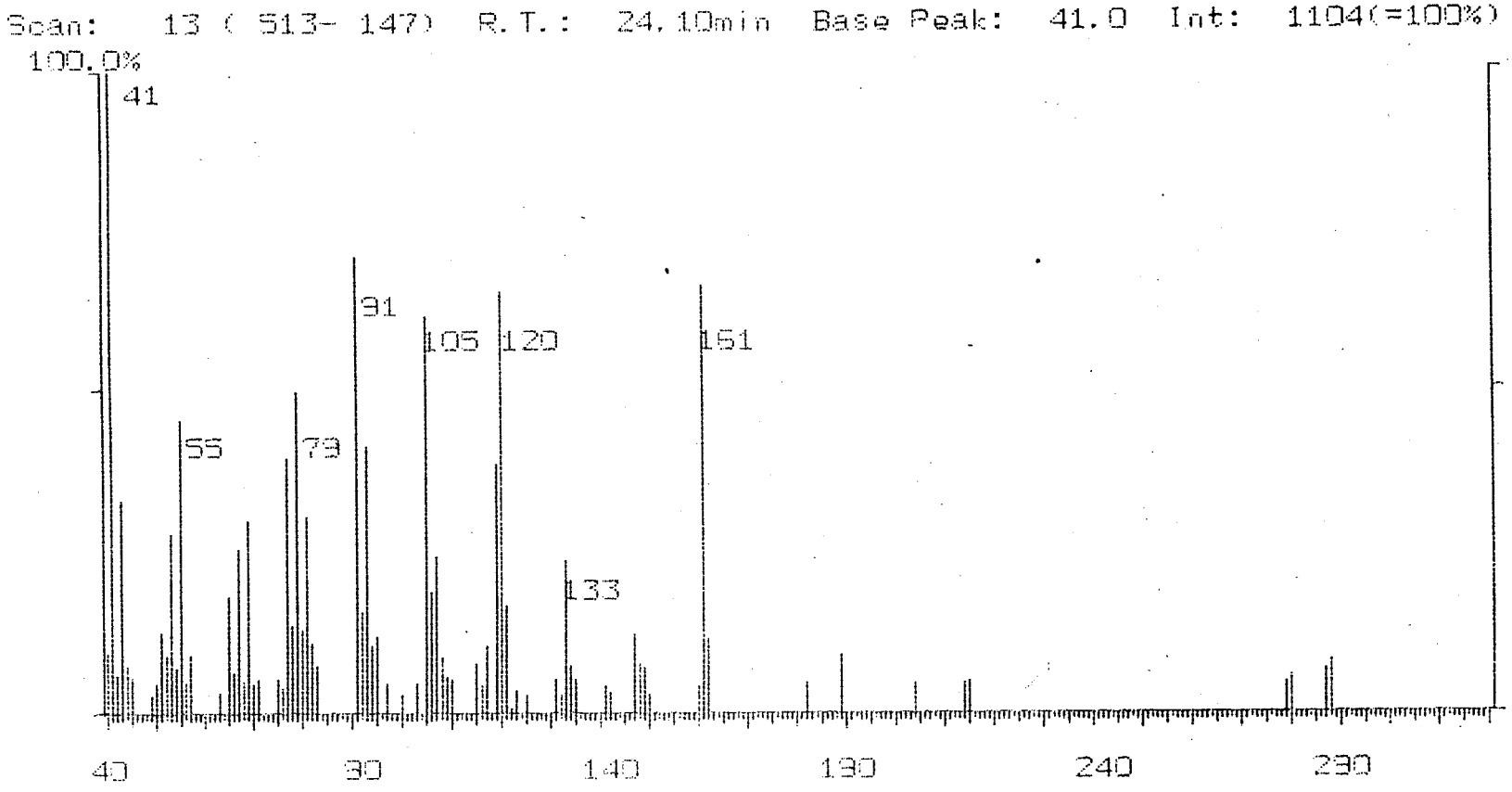


Fig. 4.2.14 Mass spectrum of trans-2-cis-6-nonadien–1-ol

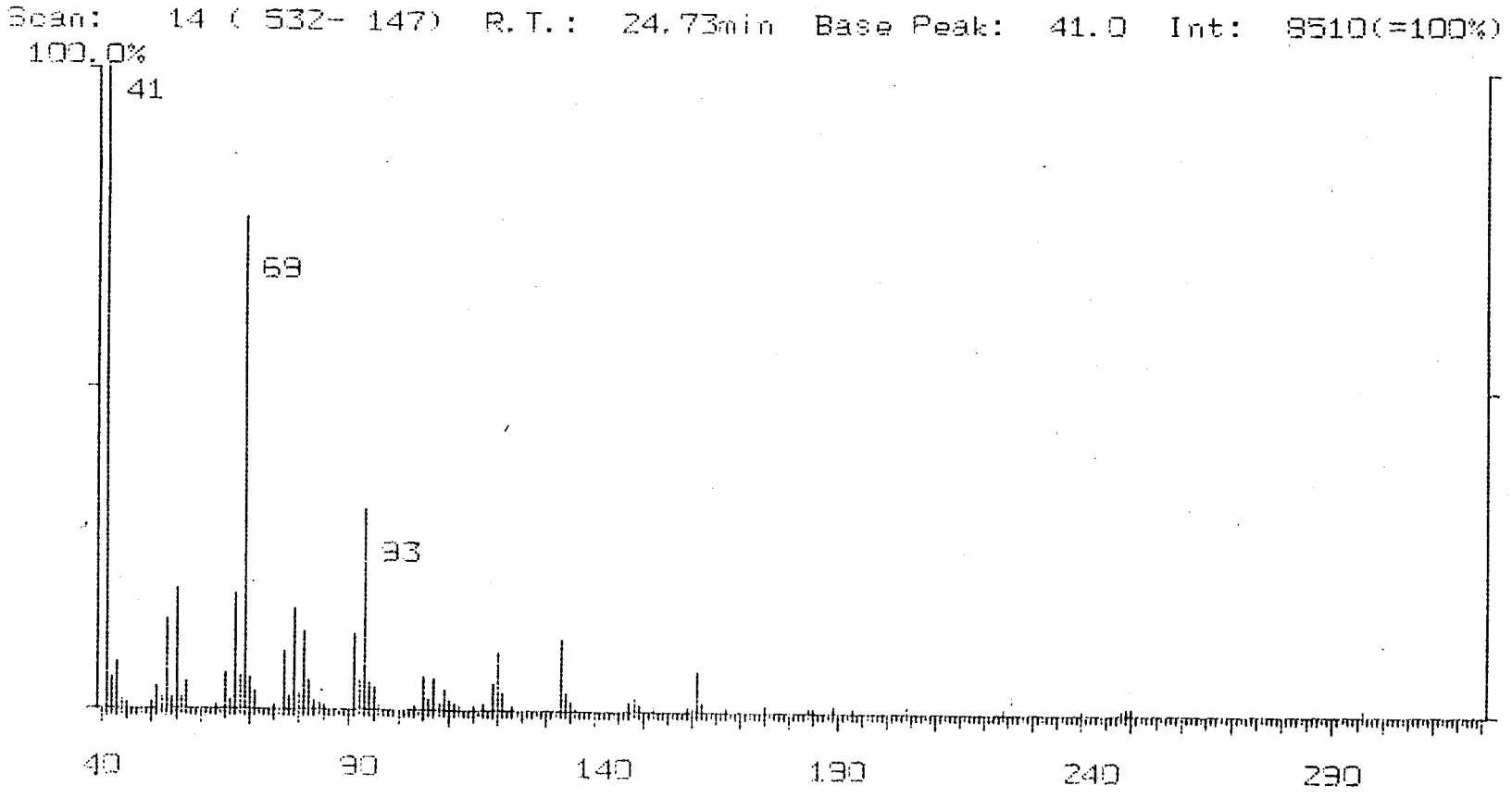


Fig. 4.2.15 Mass spectrum of α-cububene

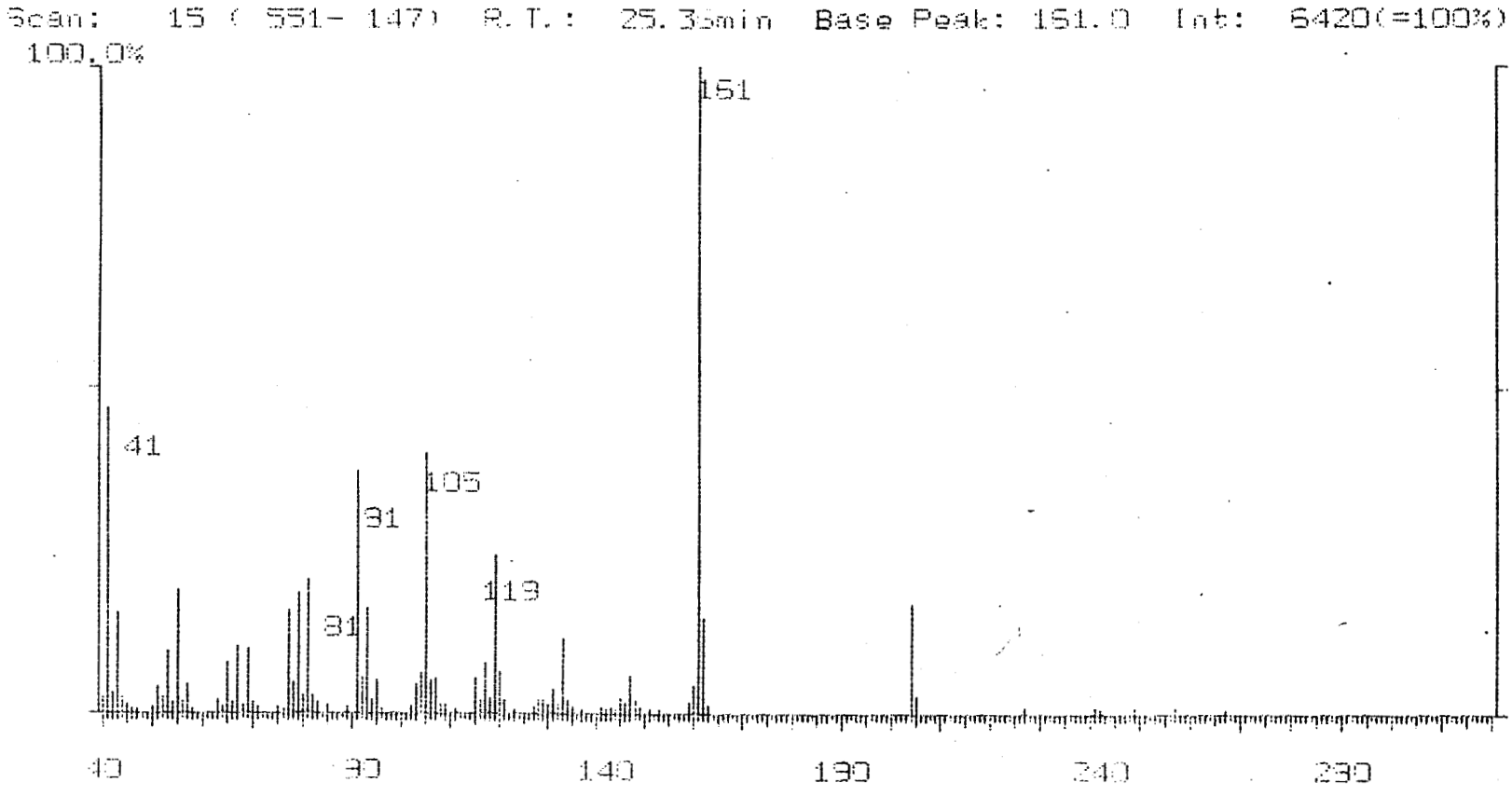


Fig. 4.2.16 Mass spectrum of β-bisabolene

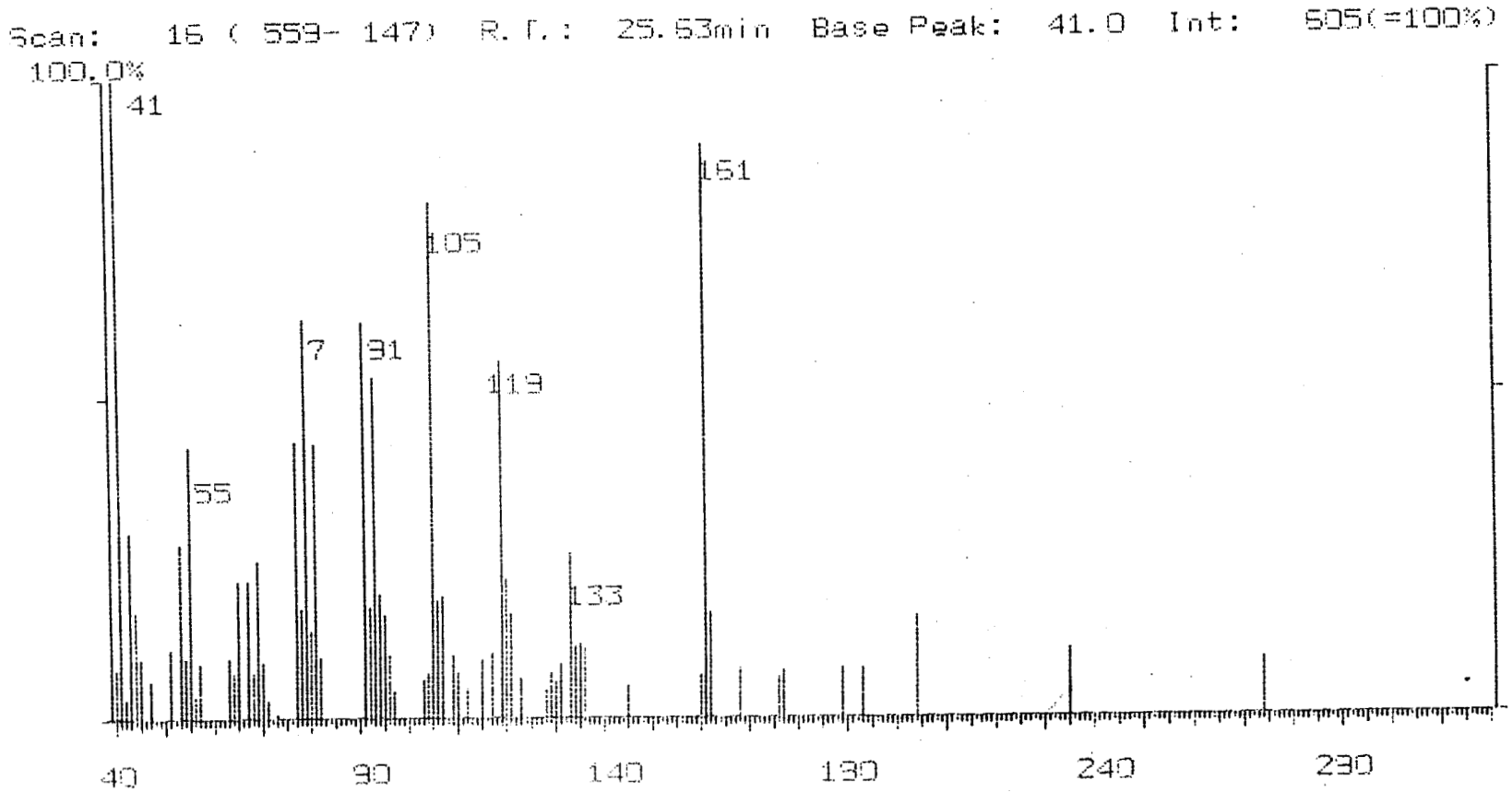


Fig. 4.2.17 Mass spectrum of 1 ethynyl-2-trimethylsilyl benzene

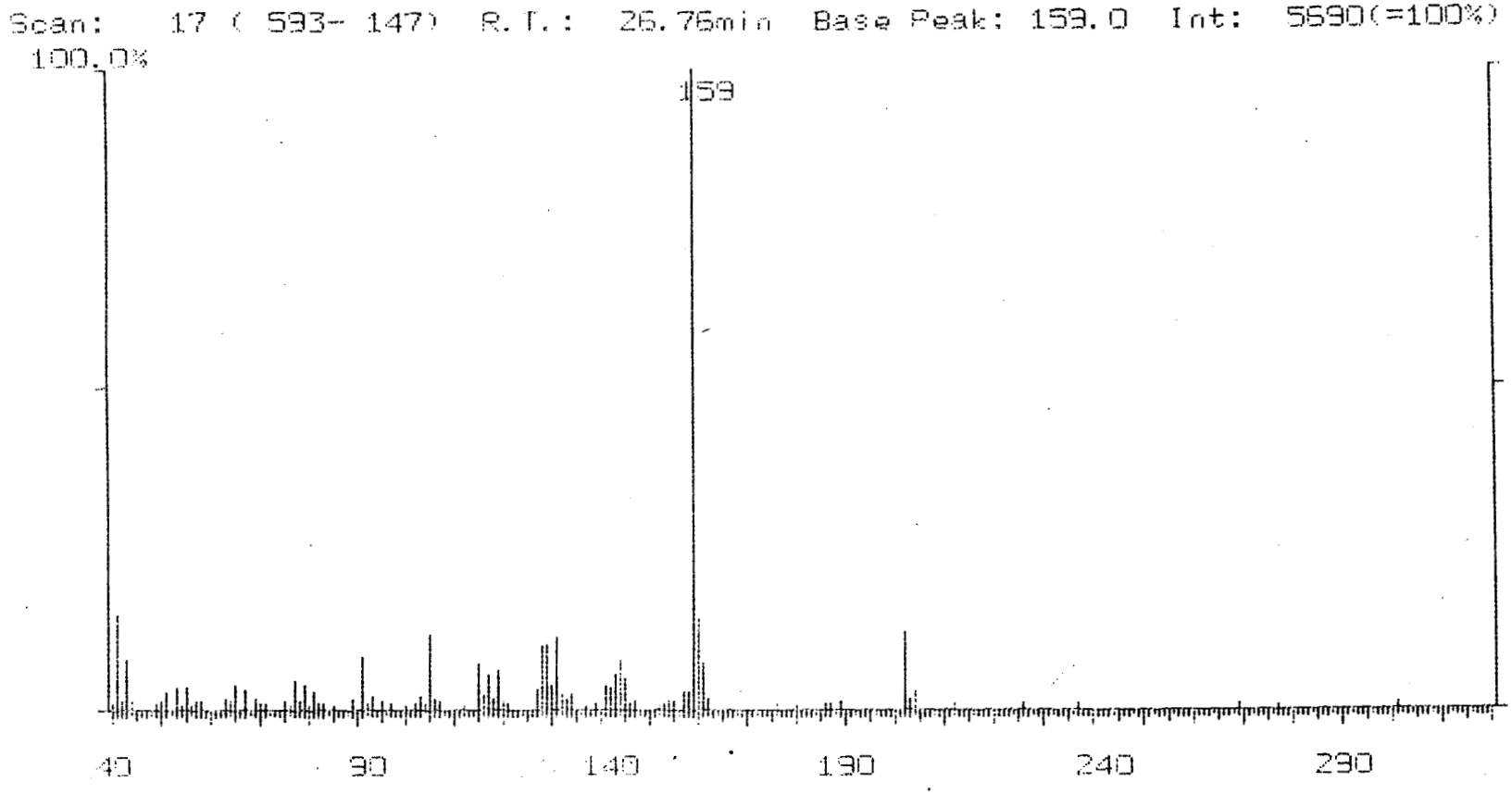


Fig. 4.2.18 Mass spectrum of α-amorphene

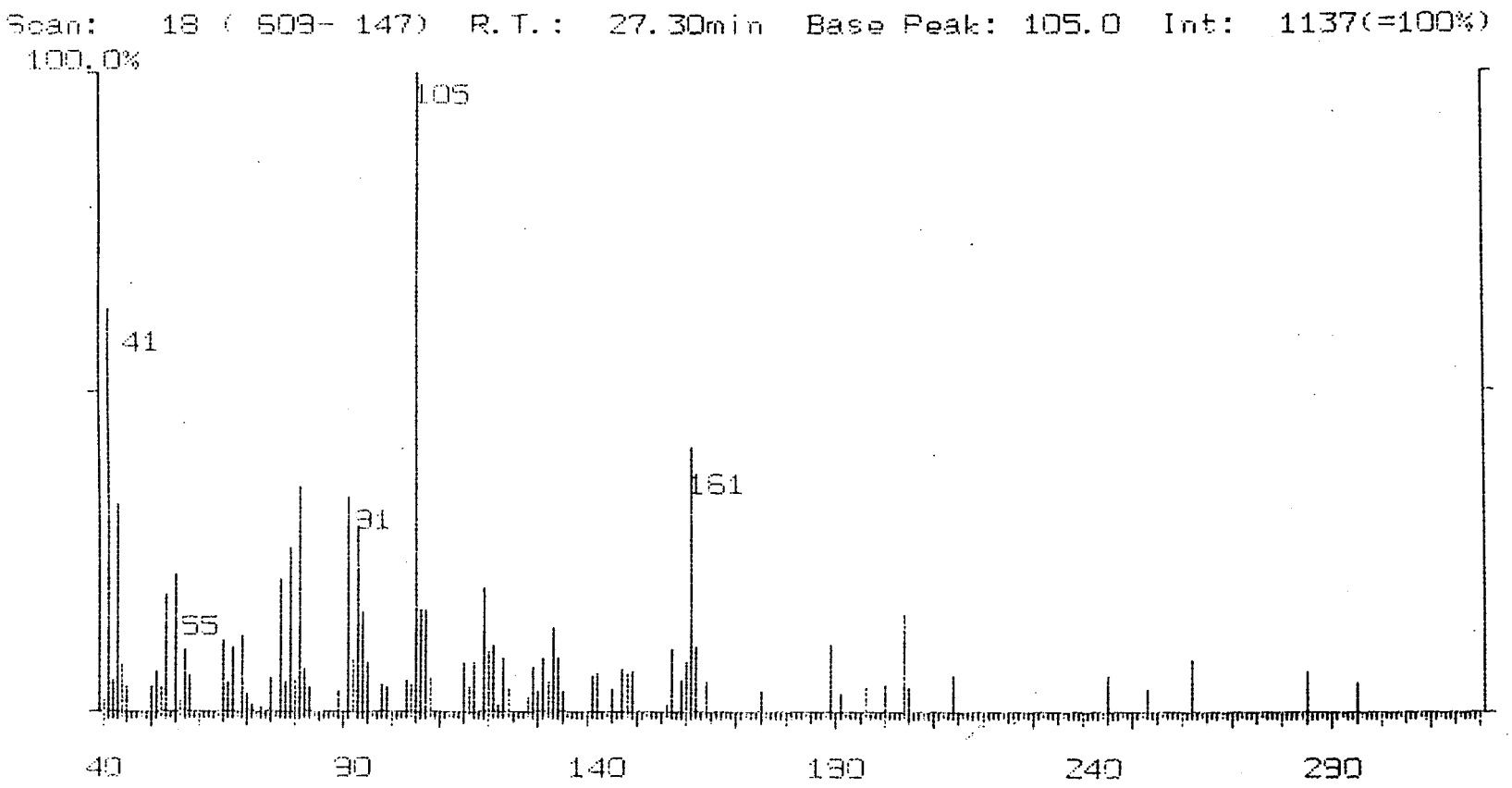


Fig. 4.2.19 Mass spectrum of aromadendrene

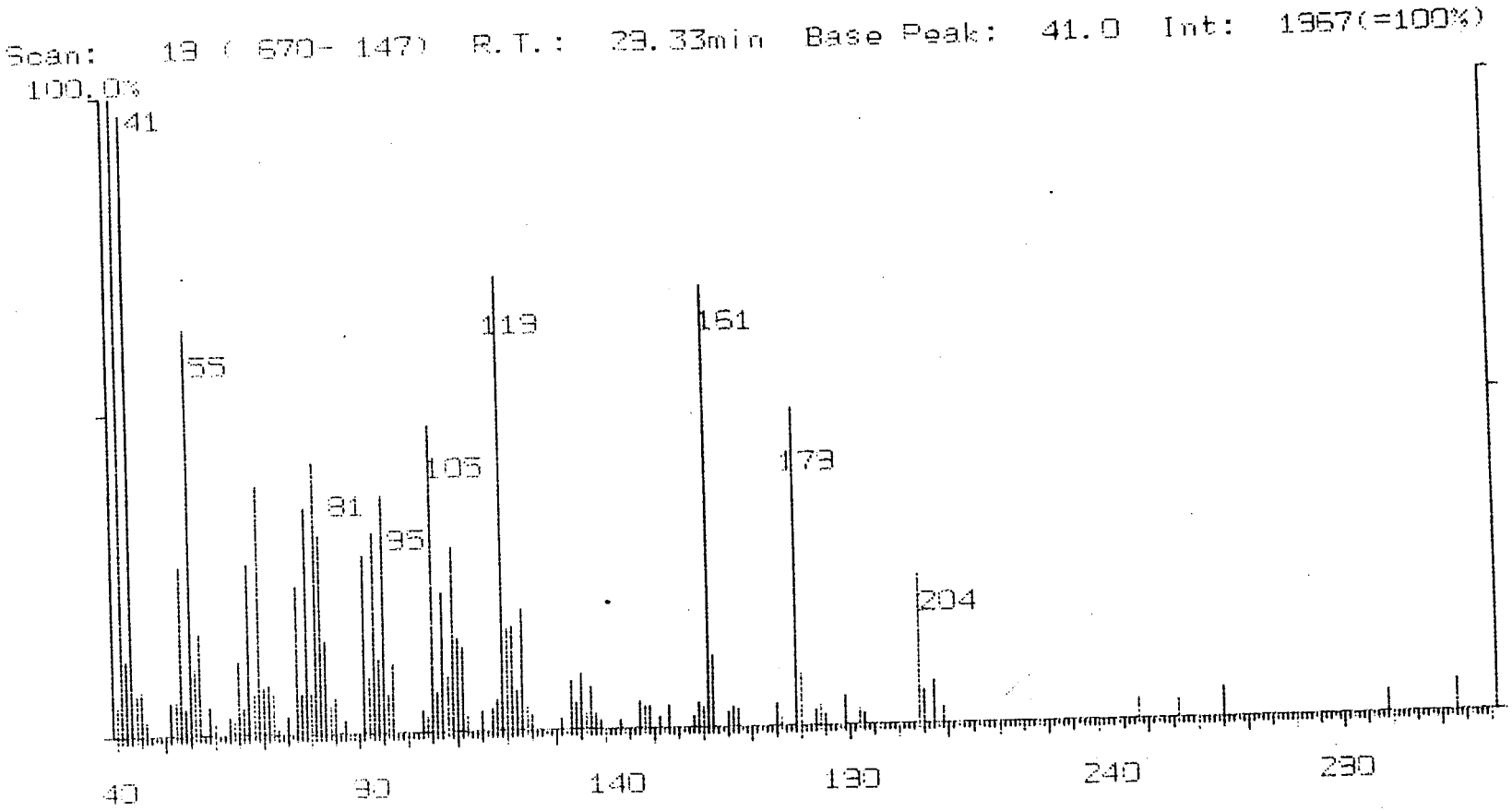


Fig. 4.2.20 Mass spectrum of juniper camphor

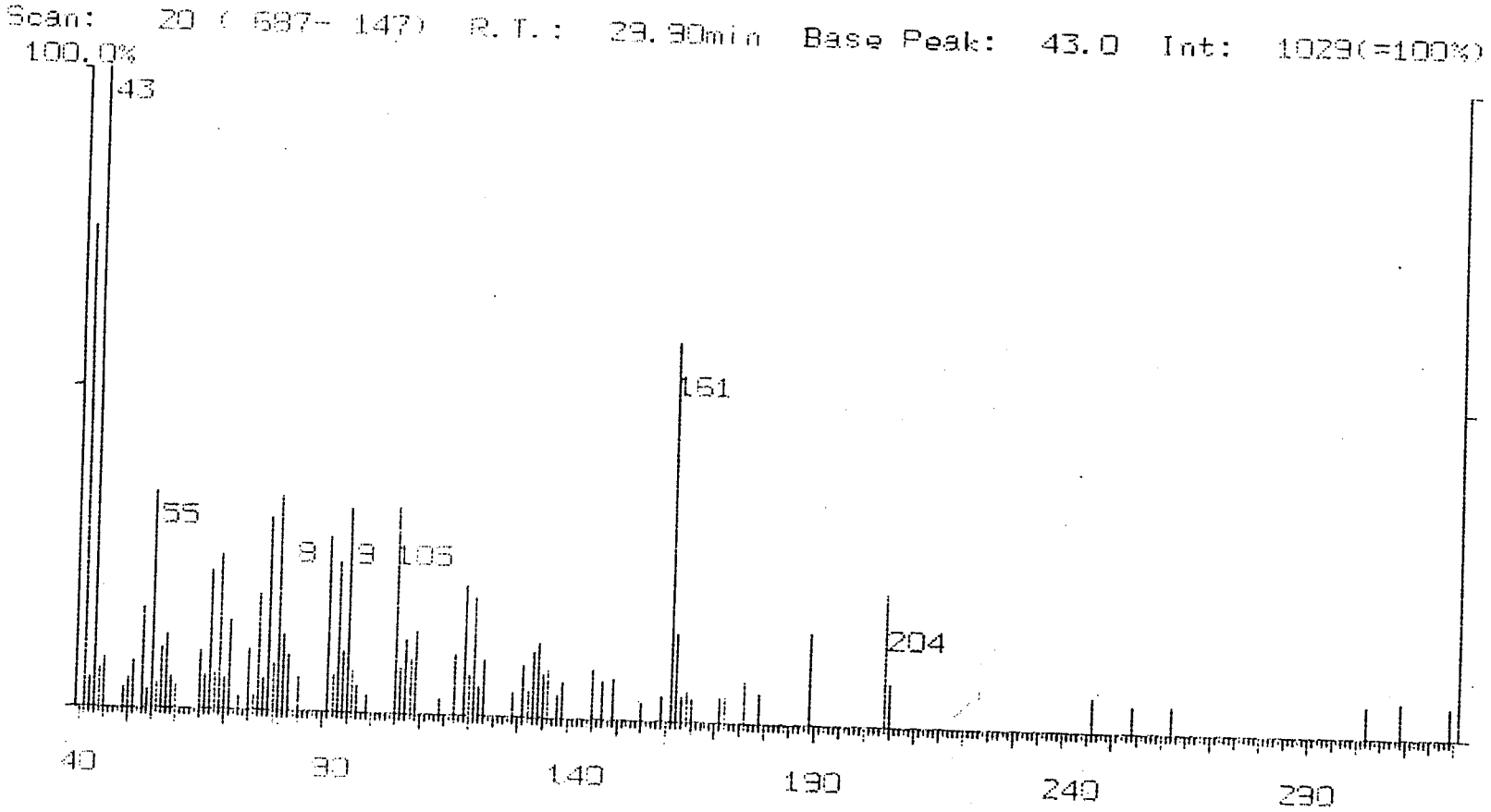


Fig. 4.2.21 Mass spectrum of β-terpineol

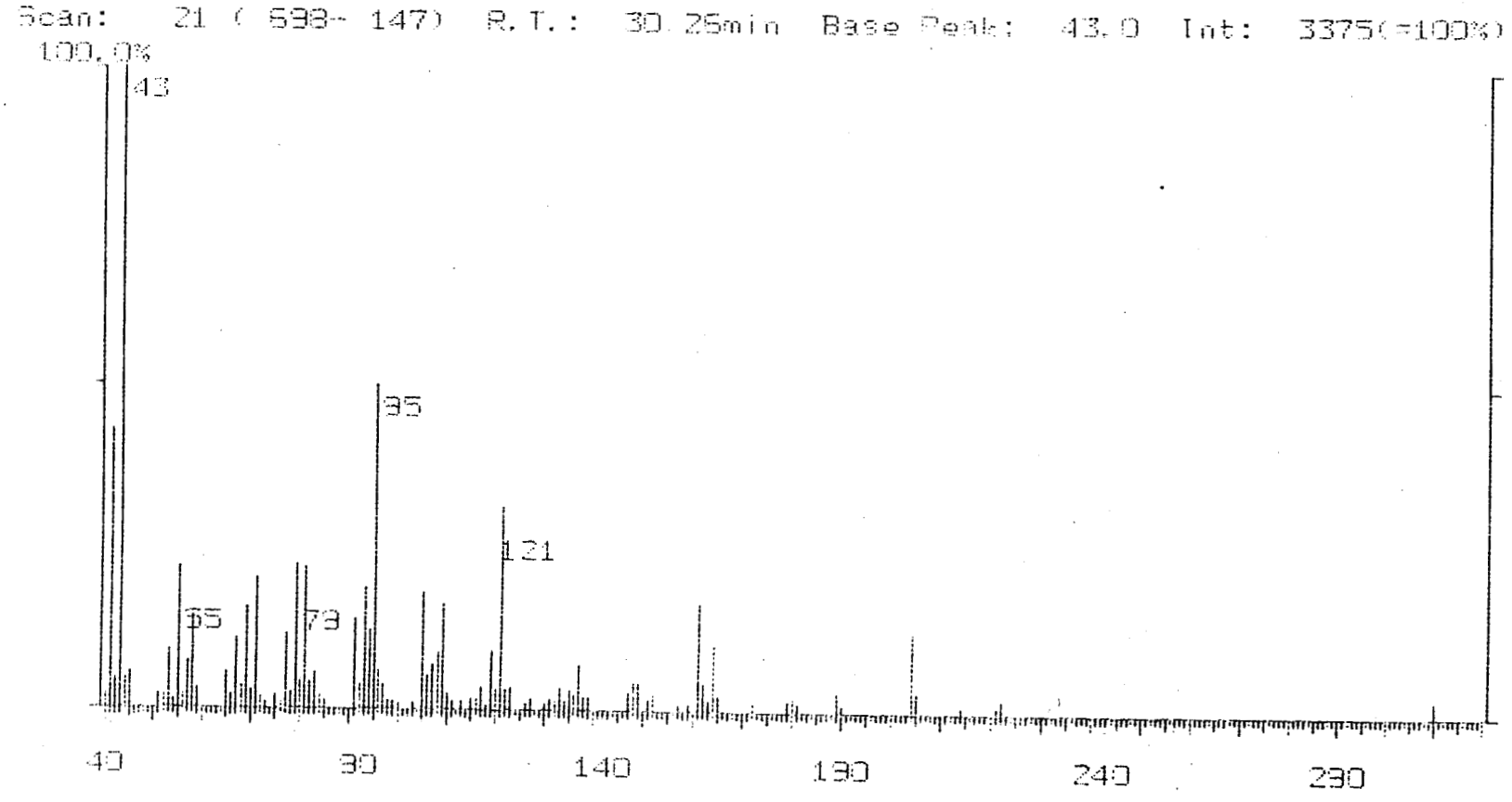
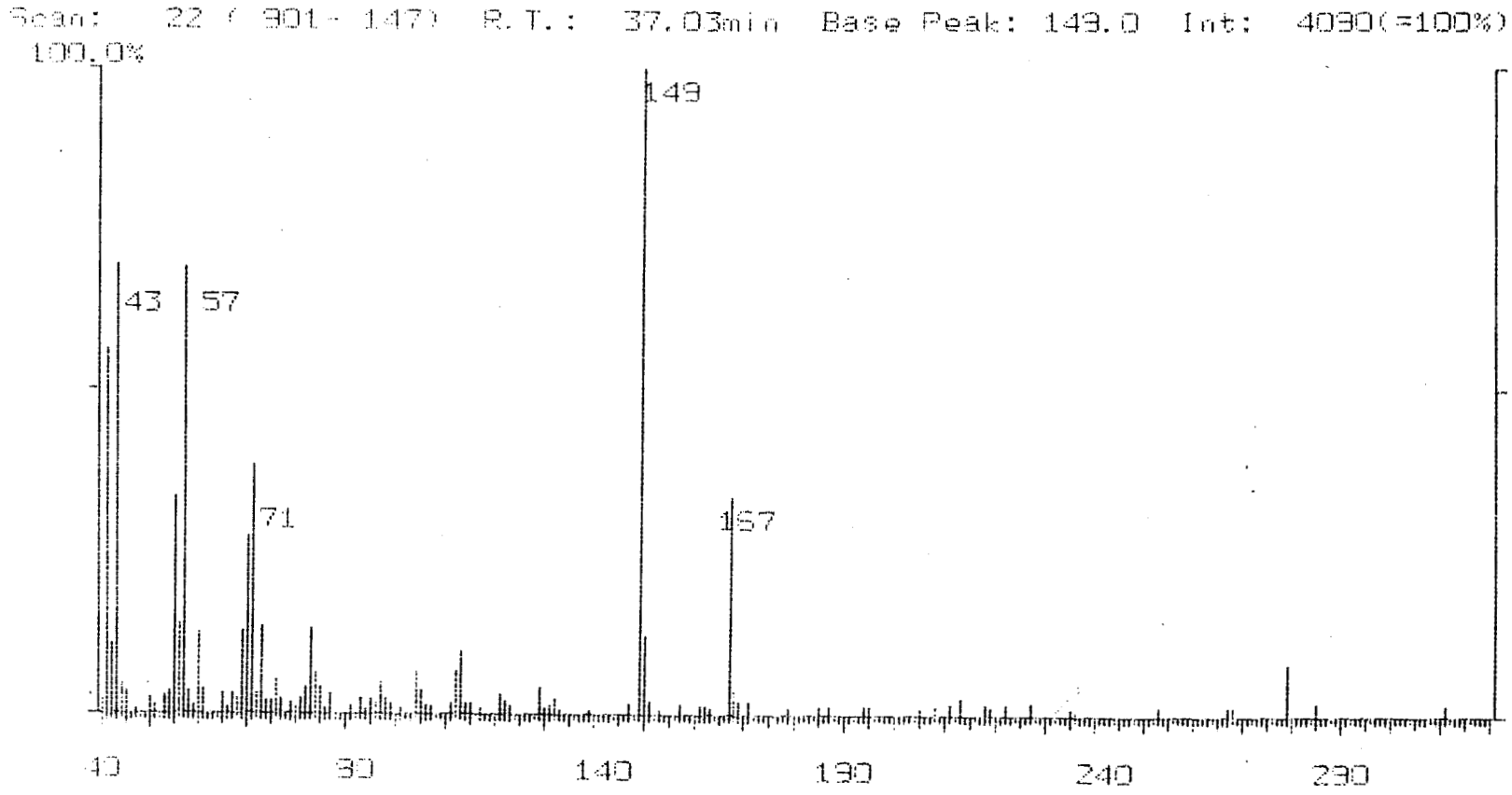


Fig. 4.2.22 Mass spectrum of di isobutyl phtalate



DISCUSSION

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

DISCUSSION

1. Micropropagation

Plant cell and tissue culture techniques are being widely used in a variety of basic research programmes. The potential impact of this novel technique on the genetic improvement of mints has generated considerable interest in the scientific community. Conventional breeding techniques are not suitable for improving commercial mints due to strict essential oil profiles required by mint oil industries.

Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable (Breiman *et al.,* 1987). Other reports claim that useful morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al.*, 1989). Shenoy & Vasil (1992) reported that micropropagation through meristem culture is generally associated with a low risk of genetic instability because the organized meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions. A number of physiological and morphological changes have been reported in unorganized callus tissue including habituation, changes in biochemical sensitivity and requirements, alteration of growth habits and modification of cellular constituents.

Plant tissue culture has the potential to introduce genetic variability through somaclonal variation (Jullien *et al.*, 1998). If simple multiplication is the objective, any system that significantly reduces or eliminates the tissue culture generated variation can be of much practical utility. Variations may be due to several factors such as genotypes used, pathways of regeneration etc. (Breiman *et al.*, 1987; Vasil, 1987; 1988). Several parameters such as gross morphology (Swedlund & Vasil, 1985), field assessment, molecular studies (Breiman *et al.*, 1989; Shenoy & Vasil, 1992; Choudhury *et al.*, 1994) etc. have been employed for assessing the effect of *in vitro* culture.

The morphogenetic response of the explant is mainly based on the type and concentration of the hormone used. In the present study it was observed that a combination of BAP and IAA was most effective in inducing multiple shoots from nodal explants (Figs. 1.11-1.15). Lower concentrations of BAP induced axillary shoot elongation

from nodal explants, but higher concentrations produced some abnormal tissue growth that later caused some degeneration and death of tissue. KIN also was found to be ineffective in supporting shoot regeneration and it resulted in callusing in most cases. With 2,4-D alone or in combination with KIN, caulogenesis was highly reduced and explants always developed only callus. On the contrary, with IAA & BAP, shoots developed readily without apparent callus (Figs. 1.11-1.15). Among the auxins used, neither NAA nor 2,4-D supported shoot regeneration.

For the present study, nodal segments of *M. rotundifolia* were mainly used. This method is considered to be a low risk method for genetic instability (Pierick, 1991; Schoofs, 1992). But there are several reports on the occurrence of somaclonal variation among micropropagated plants. (Evans & Sharp 1985; Mathur *et al.,* 1988).

In the present study, variation among micropropagated plants was visible at the morphological level (Fig. 1.16-1.23). This type of morphological variation was also reported earlier (Tiwari *et al.*, 1995). Phenotypic variations observed among micropropagated plants may be due to several mechanisms of somaclonal variations like change in chromosome number and structure, dominant and recessive mutations and changes in chloroplasts and mitochondria (Bingham & McCoy, 1986).

According to Larkin *et al.* (1989), the presence or absence of variation depends upon the source of explant and method of regeneration. Stress induced by tissue culture process (hormone effects, nucleotide pool imbalance etc.) can cause alterations in DNA. These alterations could affect the expression of specific genes (Kaeppler & Phillips, 1993).

It has already been reported that the nature of growth regulators used in the medium may result in the occurrence of somaclonal variation (Patel & Berlyn, 1982). The stimulatory effect of BAP on multiple shoot formation is also well established (Shahzad & Siddiqui, 2000). Tender coconut water contains regeneration inducers and that from mature coconut lack these factors (Tuleke *et al.*, 1961) or contain regeneration inhibitors (George & Sherrington, 1984). Regeneration can be affected by explant age (Welander, 1988), explant origin and culture maintenance conditions (Pierik, 1987).

One of the possible mechanisms suggested to explain somaclonal variation is the activation of different classes of mobile genetic elements, such as those reported during tissue cultures of different plants (Peschke & Phillips, 1991; Hirochika, 1993; Hirochika *et al.*, 1996).

Epigenetic variation is another important cause of somaclonal variation in plants. This aspect of somaclonal variation involve mechanisms of gene silencing or gene activation that were not due to chromosomal aberrations or sequence change (Kaeppler *et al.*, 2000).

The culture-generated plants may vary from parent plant for its morphological characters as a result of somaclonal variation induced by culture stresses (Patnaik *et al.*, 1999). In the present investigation, a few morphological variants with 1-4 leaves at each whirl (Figs. 1.16-1.23) were developed but these plants could not withstand the hardening process and failed to establish successfully in the soil. Genomic changes appear to be the basis for the phenotypic alterations (Phillips *et al.*, 1994).

2. Cytological Analysis

In the present study, chromosome number variation was not observed either in the callus (Fig. 2.2) or in the somaclonal variant (Fig. 2.3) when compared with its parent (Fig. 2.1). Chromosome number was found to be 2n=24 in all samples, which coincides with majority of the previous reports (Table:1). Basic chromosome number is one of the most widely used characters in biosystematic studies and there has been a vast amount of phylogenetic speculation whether this value can be used as a dependable and stable marker of the direction of evolution (Jones, 1970; 1974; 1978).

Plant cells growing in an artificial culture environment show numerous genetic changes. These alterations are manifested as increased frequencies of gene mutations, chromosome breakage, transposable element activation, quantitative trait variation and modifications of normal DNA methylation patterns (Kaeppler & Phillips, 1993). The possibility of minor genetic reshuffles in the form of minor structural changes in chromosomes without affecting their original chromosome number as the origin of

variants can not be ruled out. Thus the callus culture can be proposed as a potential source of regenerants bearing structural changes of the corresponding degrees and this in turn might result in the generation of somaclonal variants (Mohanty *et al.*, 1991).

Genetic heterogeneity in cultures arises mainly due to factors like i) expression of chromosomal mosaicism or genetic disorders in cells of the initial explants and ii) new irregularities brought about by culture conditions through spontaneous mutations. Cell or tissue cultures undergo frequent genetic changes such as polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplification and mutation and these are expressed at biochemical or molecular levels (Ignacimuthu, 1997).

In the present study, the average chromosome length and total chromosome length of the somaclonal variant was found to be slightly higher than that of the parent plant. Disparity Index and Variation Coefficient was lesser in the somaclonal variant. Chromosomal breakage and deletion may lead to slight changes in the size of the chromosomes. Robertsonian translocations can also lead to changes in the size of the chromosomes. The number of chomosomes with nearly median primary constriction was higher in the parent plant. The somaclonal variant was characterized by a greater number of chromosomes with nearly submedian primary constriction (Tables: 10-12). The reduction in the total chromosome length in the callus may be probably due to the altered enviroment provided for the development of the callus.

Excessive number of submetacentric chromosomes reveals an advanced evolutionary status (Levitsky, 1931). High Disparity Index denotes an advanced heterogeneous nature of the karyotype (*Mohanty et al.*, 1991). Change of the centromere from median to submedian and increased size difference between different chromosomes of the same set are two basic processes responsible for karyotype speciation (Levitsky, 1931) and this is often considered as a potential factor in the evolution of species, especially in the diploid levels (Stebbins, 1970; Mathew & Thomas, 1974).

Cultured cells in metaphase carried structurally altered chromosomes in them (Fig. 2.2). Moreover, callus cells show a decrease in the average chromosome length and total chromosome length. Conditions in the artificial environment of cell culture may

enhance mutation rate, which may act as a genetic shock. The mutation rate may also be enhanced by leakage of toxic by-products and exudates from the calli into the surrounding medium (Oihoft & Phillips, 1999). The decrease in chromatin content may also be due to transposable element activation. Transposable element activation has been shown to be induced by genomic shock (Mc Clintok, 1984). These aberrant cells have a positive selective value in *in vitro* culture systems. Such a type of aberrant cell selection may be comparable to genetic drift. Regenerants from such cultures (somaclonal variants) might be useful for the selection of useful genetic variants (Mohanty *et al.*, 1991), since they may have a rejuvenated genetic content.

The increase in total chromosome content of the somaclonal variant may be probably due to the genetic stability achieved by the variant after regeneration. Minute and cryptic structural differences and gene alterations and rearrangements are therefore responsible for the origin of new species. Such structural changes might have also contributed to the origin of different cytotypes (Stebbins, 1971). The number and form of chromosomes in single cells and the frequencies of primary structural change are the features by which a proliferating cell population can be characterized in cytogenetic terms (Ford, 1964).

Chromosomal breakage and subsequent alterations in chromosomes were reported in many plants. The changes include cytological aberrations, which are primarily the result of chromosome breakage, single base changes, changes in the copy number of repeated sequences and alterations in DNA methylation pattern (Benzion *et al.*, 1986). Mutations involve loss or gain of a defined enzyme function. Every deleterious change in the cistron controlling this enzyme should result in the mutational event (Szybalski *et al.*, 1964).

The chemical composition of the culture medium has been shown to affect the cytogenetic behavior of plant cells *in vitro* (Bennici *et al.*, 1970; Karp, 1992). Mineral deficiencies, chelating agents and some heavy metal ions have been reported to have some role in inducing chromosomal breakage and rearrangements in plants (Steffensen, 1961). The hormone itself can potentially be toxic to the cell thereby directly leading to chromosomal aberrations. The concentration and type of hormone in the culture medium

also influence the pattern of methylation (LoSchiavo *et al.*, 1989). Singh (1986) reported a few chromosomal variations in callus cultures of crops, which are produced due to the effect of media components. According to Singh (1976), KIN can cause chromosomal change. Effect of hormones like 2,4-D, IAA, NAA and KIN on chromosome aberration in cultured plants was already reported (Singh, 1993).

High concentration of growth regulators result in karyotypic alterations in cultured cells. Several phytohormones have shown to induce chromosomal variability in cultured cells, leading to the formation of somaclones. Variants may arise due to single gene mutations in cultured cells. Another aspect of single gene mutation responsible for somaclonal variation relates to transposable elements. Variations have been reported as a result of insertion of plasmid like DNA in the mitochondrial genome of cell cultures of some other plants (Ignacimuthu, 1997).

Another reason for somaclonal variation may be the molecular changes caused by mitotic crossing over in regenerated plants. Such small changes in the structure of chromosomes could alter expression and genetic transmission of specific genes, such as deletion or duplication of a copy or copies of a gene, or gene conversions during the repair process. Further recombination or chromosome breakage at a preferential region or 'hot spots' of a particular chromosome affect the genome in a disproportionately high frequency, resulting in altered phenotypic expressions (Ignacimuthu, 1997).

3. Random Amplified Polymorphic DNA (RAPD) Analysis

The use of PCR amplification to detect target DNA sequences has many applications in plant genotyping, gene mapping, diagnosis and diversity assessment (Kreader *et al.*, 2001). In the present study, the total DNA content of the somaclonal variant (~75ng) was found to be higher than that of the parent plant (~50ng). An increase in the chromosome length of the somaclonal variant may be probably responsible for the increase in the DNA content. It is evident from the electrophoretic gels (Figs. 3.6-3.7) that somaclonal variation at DNA level is present in the hitherto mentioned variant of *M. rotundifolia* (TC 1). A few bands were found missing in the somaclonal variant TC 1when two primers (OPE 14 and OPF 05) were used (Fig. 3.6-3.7). No additional bands could

be detected in this variant by marker screening. Similar results were already reported in several other plants (Brown *et al.*, 1993; Wang *et al.*, 1993; Munthali *et al.*, 1996; Hashmi *et al.*, 1997) using RAPDs. RAPD profiles were unambiguously used to establish the distinct identity as different from the parent plant in many new varieties of *M. arvensis* (Khanuja *et al.*, 2001a; Khanuja *et al.*, 2001b), *M. spicata* var. *viridis* (Khanuja *et al.*, 2001c), *M. piperita* (Dwivedi *et al.*, 2001a), *M. gracilis* var. cardiaca (Dwivedi *et al.*, 2001b), and a few other medicinal plants (Dwivedi *et al.*, 2001c; Patra *et al.*, 2001b). In *M. rotundifolia* there is no report on this type of analysis.

According to Rani *et al.* (1995), polymorphism in amplification products represent 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 & 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). The efficacy of RAPD markers in detecting genetic changes after *in vitro* culture is well understood (Piccioni *et al.*, 1997; Olhoft & Phillips, 1999; Kaeppler *et al.*, 2000). This method is reported to be an efficient tool in detecting somaclonal variation (Al-Zahim *et al.*, 1999).

Failure of amplification of different lines may be due to a single base change or completely different sequences (Vierling & Nguyen, 1992). Presence of RAPD markers in two genotypes indicates a high level of homology at that site. The sequence difference between the two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint (Williams *et al.*, 1990).

In the present study certain intensity differences in the bands were also noticed in the amplification products of all the six primers tested. The same type of results were also reported in other plants also (Yang & Quiros, 1993; Hashmi *et al.*, 1997).

4. Essential Oil Analysis

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 cultured 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). Variations for four major constituents of essential oil were recorded in somaclonal variants of mints (Kukreja *et al.*, 1991a). Over the years, *de novo* synthesis of many commercially important chemical compounds have been reported (Nair, *et al.*, 1986; Calleboutet *et al.*, 1990).

The reports on somaclonal variants of essential oil yielding plants such as *M. piperita* (Holm *et al.*, 1989; Nadaska *et al.*, 1990) and *M. arvensis* (Kukreja *et al.*, 1992) have rendered considerable variation in their oil content. A significant genetic variation was reported in accordance with variation in physiological and biochemical traits in different somaclones of other plants also (Tiwari *et al.*, 1995).

In the present study, GC-MS analyses revealed 21 components each in the parent plant (Figs. 4.1.1 – 4.1.22) and in the somaclonal variant ((Figs. 4.2.1- 4.2.22; Table 14). There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant).

The parent plant was characterized by components like α-pinene, sabinene hydrate, ethyl 9,12 octa decadienoate, trans-2-octenal, isopulegol, citronellol, alloaromadendrene, β-caryophyllene, and β-gurgunene. The unique components in the somaclonal variant were methyl chavicol, isoborneol, cis-6-nonenal, 2,3 diethyl-6-methyl pyrazine, p-mentha trans 2,8 dien-1-ol, γ-caryophyllene, β-bisabolene, 1-ethynyl-2-trimethylsilyl benzene, α-amorphene, juniper camphor and di isobutyl phtalate. The

common compounds in both were myrcene, limonene, carvone, α-terpinyl acetate, β-elemene, α-cububene, aromadendrene, trans-2-cis-6-nonadien-1-ol, and β-terpineol. Among these common essential oil constituents, the amount of carvone and limonene was found to be higher in the somaclonal variant (87.4% and 8.4% respectively) than the parent plant (84% and 6.2% respectively), whereas in the case of limonene, β-elemene, trans 2 cis 6 nonadien-1-ol and α-cububene, it was vice versa (Table:14).

The lesser value of coefficient of similitude (27.27) obtained when the essential oils of both the parent plant and the somaclonal variant (TC 1) was compared, shows the dissimilar nature of the essential oil composition of these oils (Table: 14). This dissimilarity may be probably due to the variations in the biosynthetic pathways of essential oils which are genetically controlled.

Plant cell cultures have produced (i) new compounds previously not known in the intact plant, (ii) new derivatives of known compounds and (iii) new compounds by biotransformation of molecules incorporated in the medium. It is presumed that production of new compounds or derivatives might be due to altered gene function in cultured cells when compared to the mother plant (Merillon & Ramawat, 1999). Previous reports prove that in *M. rotundifolia,* the products synthesized *in vitro* were more oxidized than the natural forms (Kukreja *et al.*, 2000). The type of growth regulators in the culture medium can affect the production of secondary metabolites in cultured cells quite dramatically (Cline & Coscia, 1988). Changes in the constituents of *in vivo* and *in vitro* grown plants are influenced by various genetic and non-genetic factors (Gerhardt, 1972). But there are also reports on tissue cultured plants that match the parent plant in their biosynthetic capacities (Kireeva *et al.*, 1978; Charlwood & Charlwood, 1983). However, in the present investigation, the marked differences observed in the essential oil constitution of the parent plant and the somaclonal variant may be due to their respective biosynthetic pathways (Tetenyi, 1973).

The production of secondary metabolites by plant cells *in vitro* is in part, dependent on the culture conditions employed. The nutritional component of various culture media have been known to affect secondary product formation. The form in which nitrogen is provided and the concentration supplied have effects on the products 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 the secondary metabolism on phytohormone content. High doses of growth promoters can increase the content of secondary metabolites (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 pathway is operated *in vitro* via a change in the organization of the 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). Illumination also is reported to be a controlling factor in the biosynthesis and accumulation of secondary metabolites (Luckner & Diettrich, 1987).

Quantitative and qualitative production of mint essential oils is clearly controlled by simple genetic systems (Lincon *et al.*, 1986). A number of reports on correct decipherence of the biosynthetic pathways for the terpenoids have already been appeared (Croteau *et al.*, 1991; Akhila *et al.*, 1991; Lange *et al.*, 2000). Biosynthetic data demonstrate that three different dominant genes are involved in the formation of the main ketones in mint essential oils. Among these, a dominant gene 'C' is responsible for the formation of C-2 oxygenated compounds like carvone. In the present investigation, carvone is the major component detected from both the parent plant and the somaclonal variant. So this gene may be present in the dominant form in both these plants. A dominant gene 'R' is responsible for the formation of alcohols from the corresponding ketones (Hefendehl & Murray, 1972; Murray, 1960a) and finally a dominant gene 'E' controls the formation of monoterpene acetates from all monoterpene alcohols (Hefendehl & Murray, 1976).

Reitsema (1958) and Lawrence (1978) reported carvone or dihydrocarvone as the main compounds in *M. rotundifolia* oils. *M. rotundifolia* is a natural sterile hybrid between *M. longifolia* and *M. suaveolens* (Harley & Brighton, 1977). Hendriks *et al.* (1976) reported that the essential oils of the artificial hybrids between *M. suaveolens* and *M. longifolia* were characterized by the main compound piperitone oxide or dihydrocarvone.

The metabolism of monoterpenes is strongly influenced by environmental factors. It has been shown that the diurnal changes in temperature is an important factor of influence regarding the oil composition (Burbott & Loomis, 1967). It is generally accepted that the definition of interspecific chemical races may concern the presence or absence of a particular compound in the secondary metabolism (Tetenyi, 1973; Harbone & Turner, 1984).

Limonene is a key component of *Mentha* essential oils as it was shown to be the precursor of two groups of cyclic p-menthane monoterpenes (Kjonaas & Croteau, 1983; Croteau *et al.*, 1991). A blocking gene L_m causing accumulation of limonene was reported from mints (Hefendehl & Murray, 1973). Isopentenyl pyrophosphate (IPP) is the immediate precursor leading to the formation of over 22000 known terpenoids (Connolly & Hill, 1992).

Chemical investigation on the essential oils of *M. rotundifolia* parent plant and its somaclonal variant revealed that the analyzed plant material may probably belong to a carvone chemotype. In both the samples, the major component remained unchanged even if there is marked variation in the other components. This indicates that the genetic changes due to culture stresses or hormones used did not affect the biosynthetic pathway of major components. The absence of some constituents in the somaclonal variant may be due to some hindrance to the biosynthesis of these components. The appearance of certain new components in the essential oil of somaclonal variant may be due to the triggering of certain diverged biosynthetic pathways. Almost all the secondary metabolites - the monoterpenes, sesquiterpenes and phenyl propenes arise from one of the three biosynthetic pathways or from a combination of two or more of these pathways. These are known as the acetate, mevalonate and shikhimate pathways (Waterman, 1993). Changes in these pathways lead to variation in the chemical composition of the essential oils. Volatile oils are complex mixtures often containing more than 100 individual components. In the present investigation, there is a marked change in the minor components of the essential oil of the somaclonal variant. Most oils have one to several major components, which impart characteristic odour and taste, but many minor constituents also play their part in the final product.

Since there is a close connection between differentiation and developmental process and secondary metabolism in plants, the growth regulators may influence the formation of secondary metabolites (Petri *et al.*, 1989). It is clearly evident that the biosynthesis of secondary plant products is controlled by genetic factors (Franz, 1989). The genetic bases of biosynthesis of monoterpenoids and sesquiterpenoids have already been reported (Lincon *et al.*, 1986). The marked deviation in the essential oil composition of the somaclonal variant from the parent plant may be due to the genetic changes revealed by chromosome studies and RAPD analysis.

Today the modification of oil composition in aromatic plants by 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 an approach to understand control mechanisms regulating the flux of monoterpenic compounds will be of great use to the essential oil industry. In such a study, mint could be considered as a model plant because monoterpene pathways are particularly well known in this plant. Such manipulation experiments were still hampered in *M. rotundifolia* by the lack of suitable and reproducible *in vitro* shoot regeneration system. 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.

SUMMARY

Deena Meria Jose “Chromosome imaging, rapd and gc-ms analyses on a somaclonal variant of mentha rotundifolia (l.) huds. ” Thesis. Department of Botany, University of Calicut, 2002

SUMMARY

Lamiaceae is a family of medicinal and aromatic herbs. The genus *Mentha* is one of the most important taxa of this family. Mint is the most important commercial essential oil bearing plant from the standpoint of worldwide production. The economic importance of mints is due to the production of mint oil as a raw material for confectionary, pharmaceutical and cosmetic industries as well as for flavouring.

M. rotundifolia or apple mint is grown mainly for culinary purposes. It is very important medicinally also. It is used in the preparation of toothpastes, mouthwash, soaps and perfumes. It has been found that our mint products are rated poor in quality and fetch lesser price in the world market.

M. rotundifolia is a sterile diploid natural hybrid. This plant does not flower due to genetic reasons. It is vegetatively propagated through rhizomes. An alternative method for improving this plant is the production of somaclones through tissue culture. Plant tissue culture has the potential to induce genetic variability. In this work, efforts have been concentrated in revealing the cytogenetical aspects and the related phytochemical after-effects of somaclonal variation in *M. rotundifolia*.

1. Micropropagation

MS media with different hormonal concentrations of auxins and cytokinins and coconut water were used for multiple shoot induction. Best results were obtained with BAP 2mg/l & IAA 2.5mg/l from nodal explants. The frequency of shoot induction was very high. The field-transplanted plants having higher oil content were used for further analysis.

Genetic variation is a common phenomenon associated with plant tissue culture. Plantlets derived from *in vitro* culture might exhibit somaclonal variation which is often heritable. The morphogenetic response of the explant is mainly based on the type and concentration of the hormone used. In the present study it was observed that a combination of BAP and IAA was most effective in inducing multiple shoots from nodal explants. Stress induced by tissue culture process (hormone effects, nucleotide pool imbalance etc.) can cause alterations in DNA. These alterations could affect the

expression of specific genes. The nature of growth regulators used in the medium may result in the occurrence of somaclonal variation.

2. Cytological Analysis

The cytological preparations revealed that the ploidy level of the parent plant and the cultured plant was invariably the same. Both were diploid (2n=2x=24). Neither chimeral nor aneuploid variations were obtained. Average chromosome length of the parent plant (1.0017 μm) was slightly smaller than the somaclonal variant (1.1561 μm). Average chromosome length of the callus was still smaller (0.9379 μm). The total chromosome length of the somaclonal variant was 27.74 μm and that of the the parent plant was 24.04 μm. The total chromosome length of the callus was 22.52 μm. The disparity index of the parent plant was 35.48, somaclonal variant was 17.76, and that of the callus tissue was 31.37. the variation coefficient was higher in the case of callus (20.20), when compared to the parent plant (17.66) and the somaclonal variant (11.60). The Total Forma percentage of the parent plant was 37.60, the somaclonal variant was 36.50 and the callus was 44.31. A single pair of chromosome with secondary constriction was detected. The karyotype formula of the parent plant was $A_2B_{12}C_8D_2$, of the callus was $A_2B_{20}C_2$ and of the somaclonal variant was $A_2B_{10}C_{12}$.

Plant cells growing in an artificial culture environment show numerous genetic changes. These alterations are manifested as increased frequencies of gene mutations, chromosome breakage, transposable element activation, quantitative trait variation and modifications of normal DNA methylation patterns. The chemical composition of the culture medium has been shown to affect the cytogenetic behavior of plant cells *in vitro*. The concentration and type of hormone in the culture medium also influence the pattern of methylation.

3. Random Amplified Polymorphic DNA Analysis

The total DNA extracted was quantified by comparing the bandwidth of the isolated genomic DNA with that of the weight marker after running in agarose gels. The amount of DNA was found to be greater (~75ng) in the somaclonal variant than in the

parent plant (~50ng). Six different decamer oligonucleotide primers (OPA 02, OPA 08, OPB 07, OPD 19, OPE 14 & OPF 05) were used for the present investigation. Of the six primers used, all successfully amplified the extracted DNA with consistent reproducible bands. The number of bands resolved per primer ranged from a minimum of 3 to a maximum of 11. The size of the amplification products also differed and ranged from approximately below 0.5 Kb to 3 Kb. The RAPD fingerprint of the somaclonal variant differed from the parent plant with 2 primers (OPE 14 and OPF 05) used. A few bands were found to be missing in the somaclonal variant when these two primers were used. No additional bands could be detected in this marker screening. Amplification products with OPE 14 generated no bands above 0.75 Kb length in the somaclonal variant but bands were clearly visible in the parent plant's amplification products in this region. When the primer OPF 05 was used for amplification, a fragment with nearly 1 Kb length was found missing in the somaclonal variant which was clearly present as a prominent band in the parent plant's finger print. In both these cases, no additional bands were present in the variant. The other primers used could not generate any polymorphism, but certain intensity differences in the bands were noticed in the amplification products of all the six primers tested.

The efficacy of RAPD markers in detecting genetic changes after *in vitro* culture is well understood. This method is reported to be an efficient tool in detecting somaclonal variation. The sequence difference between the two genotypes is expressed as the absence of marker and thus as band differences in the RAPD fingerprint.

4. Essential oil Analysis

The oil yield of the parent plant was 1%. The somaclonal variant (TC 1) contained a higher quantity of oil (1.3%). GC-MS analyses revealed 21components each in the parent plant the somaclonal variant. There was a clear difference between the compositions of the two oils tested. The major component was carvone in both the oils, but the percentage was slightly higher in the variant (82 % in parent and 87.4 % in variant). The coefficient of similitude between the parent plant and the somaclonal variant

was 27.27. This low value indicates the dissimilarity of the biosynthetic pathways in both these plants.

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 to produce a new compound. It is presumed that production of new compounds or derivatives might be due to altered gene function in cultured cells compared to the mother plant. Quantitative and qualitative production of mint essential oils is clearly controlled by simple genetic systems. In both the samples, the major component remained unchanged even if there is marked variation in the other components. This indicates that the genetic changes due to culture stresses or hormone used did not affect the biosynthetic pathway of major components. The absence of some constituents in the somaclonal variant may be due to some hindrance to the biosynthesis of these components. The appearance of certain new components in the essential oil of somaclonal variant may be due to the triggering of certain diverged biosynthetic pathways.

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. This study reveals 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.

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