

Studies on Somaclonal variations in Zingiberaceous Crops

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

By
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Certificate

I hereby certify that the thesis entitled 'Studies on Somaclonal variations in Zingiberaceous crops' submitted by Sumathi V for the award of the degree of Doctor of Philosophy in Botany of the University of Calicut, contains the results of bonafide research work done by her during 2001-2007 at Indian Institute of Spices Research, Calicut, Kerala under my supervision and guidance. No part of this work has been submitted to any other university for the award of any other degree or diploma. All sources of help received by her during the course of this investigation have been duly acknowledged. Certified that she has also passed the required qualifying examination.

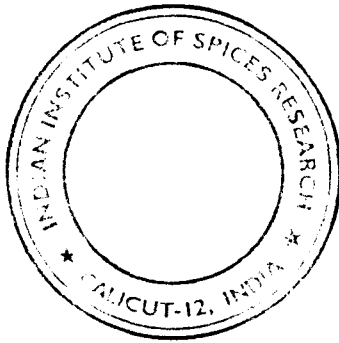
Place: Calicut

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Declaration

I hereby declare that this thesis entitled 'Studies on somaclonal variations in Zingiberaceous crops' submitted by me for the award of the degree of Doctor of philosophy in Botany of the University of Calicut, contains the results of bonafide research work done by me at Indian Institute of Spices Research, Calicut, under the guidance of Dr. K. Nirmal Babu (Senior Scientist, Indian Institute of Spices Research). This thesis or part of it has not been submitted to any other university for the award of any other degree or diploma. All sources of help received by me during the course of this work have been duly acknowledged.

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(Sumathi, V)

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List of Abbreviations Used

Abbreviation	Full form
ANOVA	Analysis of Variance
ASTA	American Spice Trade Association
BA	Benzyl adenine
CR	Callus regenerated plants
CTAB	Cetyl Trimethyl Ammonium Bromide
DNTP's	Dinucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FAA	Formaldehyde: Acetic acid: Alcohol
g / l	Gram per litre
IAA	Indole 3 acetic acid
Kb	Kilobase
LSD	Least significant difference
M	Molar
mg/ l	Milligram per litre
ml	milliliter
mm	Millimetre
mM	Millimole
min	Minutes
MP	Micropropagated plants
Mrh	Microrhizome derived plants
NAA	Naphthalene acetic acid
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
RAPD	Random Amplified Polymorphic DNA
SD	Standard deviation
SE	Standard Error
TAE	Tris Acetic acid EDTA
Taq	Thermophile aquaticus
TE	Tris EDTA
UV	Ultra violet
V	Volume
W/v	Weight/ volume
2,4-D	2,4 - dichlorophenoxy acetic acid
μ M	Micromole
μ l	Microlitre
μ g	Microgram

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Introduction



Zingiberaceae is one of the largest families of the plant kingdom. The earliest published record of Zingiberaceous taxa in India is in *Hortus Indicus Malabaricus* by Hendrik Andriean Van Rheedee (1692), in which 7 genera and 15 species of Zingiberaceae are recorded. Zingiberaceae is considered as a subfamily under the major family Scitaminae which comprises of highly evolved monocotyledons.

Zingiberaceae, is the largest family in the order Zingiberales which includes 53 genera and more than 1200 species (Kress *et al.*, 2002). Members of the family are perennial rhizomatous herbs, distributed mainly in the tropics and subtropics of the world and with the centre of distribution in the Indomalayan region. The greatest concentration of the genera is in the region of Indonesia, Malaysia, Singapore, Brunei, Papua New Guinea and Philippines (Sirirugsa 1999). They are grown in shady habitat and having characteristic creeping tuberous rhizome with an aerial shoot often covered with sheathing leaf bases. The members of Zingiberaceae is an important natural resource that provide many useful products for food, spices, medicines, dyes, perfumes and aesthetics to man. The important genera are *Zingiber*, *Curcuma*, *Elettaria*, *Kaempferia*, *Aframomum* and *Amomum*. Of these ginger, turmeric and cardamom are economically important crops.

Ginger the underground (*Zingiber officinale* Rosc) is one of the oldest and most important spices and has been cultivated in tropical Asia for over 3000 years (Purseglove 1975). It originated in South East Asia and has been used in this continent for very long time. Ginger is very useful spice, it can be used in cooking and in medicine. It is used as carminative and aromatic stimulant to the gastro intestinal tract and externally as a rubifacient and counter irritant. It is also famous as an aphrodisiac. In the West, ginger is used for making ginger bread, biscuits, cakes, puddings, soups, pickles, ginger beer and ginger wine. Which is mainly distributed over India, China, Japan, Taiwan, Philippines, Fiji, Jamaica, Dominican Republic, South Korea, Australia, Nigeria, Sirra Leone, Bagladesh, Srilanka, Thailand, Indonesia, Bhutan, and Malaysia. Of total world's ginger production, India ranks first with respect to ginger production contributing about 303.50

tonnes. India's production during the year 2004 -2005 was 397.99 thousand tonnes from an area of 100.27 thousand hectare with average yield 3969 kg/ha. World's average ginger production in 2004 is 1,072,838 tonnes (Arecanut and Spices Database, 2007).

Turmeric (*curcuma longa* L) is cultivated for the underground rhizomes, which are used as condiment, dyes, drug and cosmetic. It is essentially a tropical crop extending from India to Indochina, East Indies, and parts of China. The centre of origin is believed to be South East Asia and some species are naturalized in North Eastern regions of India and Java. In tropics its cultivation is confined to South East Asian countries. India is the World's largest producer grown in an area of 161.23 thousand hectares with the average production of 716.84 thousand tonnes and productivity 4446 kg/ha (2004 -2005) (Arecanut and Spices Database, 2007). India is the major exporter of turmeric.

Small cardamom (*Elettaria cardamomum* Maton) is a tall perennial, herbaceous rhizomatous plant. It is a native of evergreen forest of South India. Cardamom of commerce, an important spice, is the dried ripe fruit (capsule) of cardamom plant. This is often referred as the 'Queen of spices' because of its very pleasant aroma and taste, which is valued from ancient times. It is grown in hilly region of South India at an elevation of 800-1300m and as under crop in forestlands. Cardamom is used directly as a flavouring material mainly for domestic and culinary purposes. Cardamom oil and oleoresin finds its main application in flavouring processed food, cordials, bitters and occasionally in perfumeries. Cardamom also has distinctive medicinal properties and is found in most pharmacopias. In India the tincture of cardamom is used in indigenous systems of medicine to strengthen the digestive, respiratory and the urinogenital system. (Pruthi 1976; Warriar 1989). More than 90% cardamom of international commerce originated in India. India's production during the year 2004-2005 10,190 tonnes with an area of 65,780 hectare and productivity of 158kg/ha. (Arecanut and Spices Database, 2007). Guatemala with an average annual production of more than 13000 mt has emerged as the top producer and the exporter of the cardamom in the world and India has been relegated to the second position.

These crops are propagated vegetatively, sexual reproduction is absent in ginger, partial in turmeric and efficient in cardamom. Even though sexual reproduction is efficient in cardamom, the 'monotypic' nature of the species leads to reduced variability within the species. The availability of genetic variation is the basis of breeding new cultivars. Parasexual techniques like induced mutagenesis, tissue culture and molecular genetics can be used to compliment recombination and generate variation. Conventional breeding is always dependent on recombination (Mascarenhas 1991). Hence breeding programme of these crops is severely hampered. Natural 'mutation' as sports plays a major role in inducing variations in these crops. Low productivity, disease susceptibility and higher cost of seed rhizome are major production constraints. Most of the crop improvement programmes of these crops are confined to evaluation and selection of naturally occurring clonal variations. Since spontaneous mutation frequency is very low, possibility of getting variant for high yielding, high quality lines with resistance or tolerance to diseases and pests are fewer. This makes conventional breeding programmes ineffective in these crops. Utilizing biotechnological tools creation of genetic variability in these crops has been attempted.

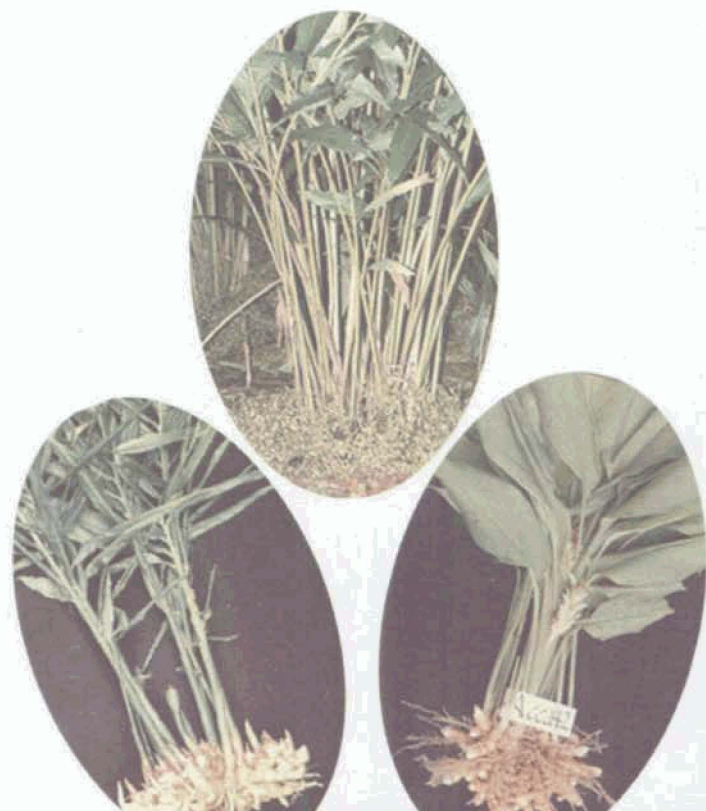
The developments in plant tissue culture have opened up new possibilities in creating genetic variability and supplemented the production of new cultivar by plant breeding. Somaclonal variation has great potential to enhance the advantages of conventional plant breeding and increase the production and productivity of crops to meet the increasing demand for food and food products in future. It could be an important source of variability to evolve high yielding, high quality, and disease resistant lines. Hence the present study in variability of *in vitro* regenerated plants is of primary importance. This would lead to identification of a variety suited to different agro-ecological situation.

In order to exploit the genetic variability generated *in vitro* it is essential to ascertain that the variability is due to genetic changes, which will be inherited further. Several markers have been used to detect the variation derived from the culture. The

extent of variability was detected by means of morphological, cytological, biochemical and molecular markers.

The present investigation attempt to study on emphasizes on the development of new protocols for the generation of somaclones in ginger, turmeric and cardamom and the estimation of extent of variations by different markers.

Review of Literature



The family Zingiberaceae

Zingiberaceae is a monocotyledonous family of perennial rhizomatous herbs consisting of about 50 genera and 1400 species distributed mainly in tropics and subtropics with the centre of diversity in Indo-Malayan region, but extending through Tropical Africa to Central and South America. Most of the members of this family are characterized by the possession of a tuberous, often creeping rhizome, which are used in food and medicine. There are many economically important plants such as ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma longa* L.), cardamom (*Elettaria cardamomum* Maton.), kasturi turmeric (*C. aromatica* Salisb.), mango ginger (*C. amada* Roxb.), large cardamom (*Amomum subulatum* Roxb.), *Aframomum* spp. and *Kaempferia* spp. The most important commercial spices in the family are ginger, turmeric, and cardamom. The other important genera are *Costus*, *Alpinia*, *Hedychium*, etc. Many of these species are used in ayurvedic and other native systems of medicine from time immemorial. This family is present throughout the Tropics but particularly abundant in Southeast Asia.

Characteristics of these members are alternate and distichous leaves, flowers are hermaphroditic, strongly zygomorphic and form in bracts such as racemes and spikes. Flowers typically have labellums, an organ formed by the fusion of two sterile stamens. The perianth is comprised of two whorls. The calyx is herbaceous or membranous, 3-lobed or spathaceous tubular. The plants tissue characteristically yields essential oil.

Ginger, turmeric and cardamom being commercial crops are planted in large areas as monocrops. India is the center of diversity for most of these crops and is the largest producer. The other countries, which cultivate these crops, are China, Nigeria, Australia, Gautemala and Jamaica. All these crops are propagated vegetatively and controlled clonal propagation will help in production of disease free planting materials of elite genotypes. Many high yielding high quality varieties are available for cultivation in these crops.

The occurrence of diseases and pests is the major production constraints in these crops. Diseases of fungal and viral etiology affect most of these crops. In addition, the soil borne nature of most of these diseases contaminates the rhizomes, which are used as vegetative propagules. Under these conditions biotechnological tools play very important role in large scale production of diseases and virus free planting material which can be introduced to newer areas.

Ginger is the underground rhizome of *Zingiber officinale* Rosc. perennial plant and is one of the world's most popular medicinal spices. It is grown throughout the tropical areas of the world. The rhizome (underground stem) is used as both spice and as herbal medicine. In early Indian systems of medicine, ginger was recommended for liver complaints, flatulence, anemia, rheumatism, piles and jaundice (Westland, 1987). The most expensive and highest quality varieties generally come from Australia, South India, and Jamaica. In India there are at least 400 different cultivars, each with varying properties such as the level of potent ginger oil.

Turmeric is the one of the most important and ancient spices of India, and a traditional item of export. Turmeric of commerce is the cleaned, boiled, dried and polished rhizomes (the underground swollen stem) of the plant *Curcuma longa* L. (syn *C. domestica* Val.) It is used in culinary preparations, medicine and cosmetic industries. Turmeric powder and the fresh juice extracted from the rhizomes have been used to treat common cold, diarrhoea, skin infections, leprosy and a number of other ailments. Some of the medicinal properties of turmeric such as anti-inflammatory (Chandra and Gupta, 1972) anti-bacterial and anti-protozoal activities have been demonstrated in experimental animals. Recently, the cholesterol lowering and anti-oxidant properties of turmeric have been demonstrated in human subjects (Despande *et al.*, 1997). The turmeric powder has been used to control seed born diseases in rice (Gangopadhyay, 1998).

Cardamom, popularly known as 'Queen of Spices' is the dried fruit of a tall perennial herbaceous plant *Elettaria cardamomum* Maton. The major use of cardamom is culinary purpose for flavouring food. It is also used in medicine as an aromatic stimulant,

carminative and flavouring agent. Cardamom was used in perfumes by ancient Greeks and Romans and also recommended by the Apicius, a famous Roman empire to counteract over indulgence (Morris and Mackley, 1997).

Ginger (*Zingiber officinale* Rosc.)

History

Ginger was first described by Van Rheedee in 1692 in his '*Hortus Indicus Malabaricus*' and in 1807, Roscoe described *Zingiber officinale* from a plant in the botanical garden at Liverpool (Ravindran *et al.*, 1994). *Zingiber* is included in the tribe Hedychieae of the family Zingiberaceae (Holttum, 1950). It is believed to be native to Asia (Grieve, 1979). However, there is no unanimity of opinion regarding the centre of origin of ginger. Various authors have suggested India (Bailey, 1949), China or Pacific Ocean Islands (Farrell, 1985), and South East Asia (Bailey, 1949; Parry, 1969) as the possible centre of origin. Wild forms of *Z. officinale* have not been found in nature, though the maximum variability of ginger cultivars exist in North Eastern India (Nayar and Ravindran, 1995).

Ginger is a slender rhizomatous perennial herb, which requires a warm and humid climate. It thrives very well, from almost sea level to an altitude of about 1500m. The average optimum elevation is between 1000-1400 m. Ginger is always propagated by portions of rhizomes about 2.5 cm long having 20-30 g weight and at least one or two buds. About 1000-1500 kg of ginger seeds are required to plant one hectare (Purseglove, 1972).

The Latin term '*Zingiber*' is derived from the ancient Tamil root 'ingiver', meaning ginger rhizome. Ginger grows well in warm, humid climatic condition up to an altitude of 1500 m in wide range of soil with good drainage. Ginger is used in medicine as a carminative and aromatic stimulant to the gastrointestinal tract and externally as a rubifacient and counter irritant. It had a reputation as an aphrodisiac. It is widely used in medicine in India and Far East (Purseglove *et al.*, 1981).

The largest ginger producing country is India, which produces about 50 percent of the world's total and is its largest exporter. Other important producers are Jamaica, Nigeria, Sierra Leone, Brazil, China, Japan, Indonesia, Taiwan and Australia. The countries importing the biggest amounts are the United Kingdom, United States and Saudi Arabia. (Purseglove *et al.*, 1981).

Origin and distribution

Ginger has a long respected history as a spice. It is believed to be native to Asia (Grieve, 1979). However, there is no unanimity of opinion regarding the centre of origin of ginger. Rosengarten (1969) recorded that ginger was mentioned by the Chinese philosopher Confucius (551-479 BC). Its medicinal properties are mentioned by Dioscorides (1 century A.D) in his *D. Materia Medica*. It was introduced to Germany and France in the ninth century and to England in the tenth century. Ginger is known in England before the Norman Conquest, as it is commonly found in the 11th century Anglo-Saxon leech books. By the 13th and 14th centuries it was familiar to English palates, and next to pepper, was the most popular spice. Arabs took it from India to East Africa (13th century). The Portuguese Mendoza, introduced ginger into Mexico soon after the discovery of that country. They in turn introduced in Jamaica. Since the ginger rhizomes can be easily transported in a living state, the plant has been introduced to several tropical and sub-tropical countries. Ginger is now commercially cultivated in nearly every tropical and subtropical countries in the world with arable land for export crops. India and Jamaica produce the best quality ginger. In India, about 70% of the total ginger production is confined to Kerala. Other states, which grow ginger, are Assam, Andhra Pradesh, Himachal Pradesh, West Bengal and Sikkim.

Botany

Ginger is a tall (30-100cm) slender perennial herb, usually grown as an annual. The under ground rhizome are robust, branched and bearing leafy shoot which are formed of sheathing leaf bases. The aerial pseudostem usually bearing 8- 12 distichous leaves. Ginger flowers rarely but in India it flowers profusely (Holtum, 1950). The inflorescence

is spike and it arises directly from the rootstock. The spike is cylindrical cone like and possesses ovate or elliptical bracts. The axils of each bract bear single fragile short lived flower. The flower having thin, tubular, spathaceous 1-1.2 cm long calyx and is three toothed. The corolla tube is 2-2.5 cm long with three yellowish lobes. The dorsal lobe of the corolla is curved over the anther and narrowed to the tip. The filament of the stamen is short and broad, the anther is about 9 mm long and cream coloured. The stigma has a circular apical aperture surrounded by stiff hairs. Ovary is inferior, trilocular with several ovules per loculus. The fruit is very rarely produced, is thin walled, three valved capsule, with small, black arillate seeds (Purseglove *et al.*, 1981). An image of *Zingiber officinale* Rosc is presented in Fig.1.



Fig. 1 *Zingiber officinale* Rosc. plant with underground rhizome

Systematic Position

Kingdom	Plantae (Plants)
Subkingdom	Tracheobionta (vascular plants)
Superdivision	Spermatophyta (seed Plants)
Division	Magnoliophyta (flowering plants)
Class	Liliopsida (Monocotyledones)
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae (Ginger family)
Genus	<i>Zingiber</i>
Species	<i>Zingiber officinale</i> Roscoe

Roscoe (1807) described *Z. officinale* from a plant in the Botanical Garden at Liverpool as “*Bracteis ovato-lanceolatis, laciniis corolla revolutis, nectario trilobato*” and referred to *Amomum Zingiber* Willd.sp.Pl 1:p6. Linnaeus’s (1753) *Amomum Zingiber* is the basionym for the species. The genus *Amomum* of Linnaeus is a nomenclature synonym of the conserved generic name *Zingiber* Boehm (Burt and Smith, 1986). The figure given by Rheede (Vol., 11, plate 12, 1692) is the designated lectotype of the species *Z.officinale* Rosc. (Jansen, 1981).

Cytology

The chromosome number of ginger was reported as $2n= 22$ by Moringa *et al.* (1929) and Suguira (1936). Darlington and Janaki Ammal (1945) cited a report from Takahashi who claimed $2n=24$ for *Zingiber officinale*. A more detailed study was carried out by Raghavan and Venketasubhan (1943) on the cytology of three species, *Z. officinale*, *Z. cassumnar* and *Z. zerumbet*, and all three had the somatic chromosome number of $2n=22$. Based on the difference in the ideogram morphology, the above mentioned workers concluded that the chromosome morphology of *Z. officinale* was different from other two species. Darlington and Janaki Ammal (1945) reported two B chromosomes in certain types of ginger in addition to normal complement of $2n=22$. Chakravorti (1948) also found $2n=22$ in ginger. He concluded that in view of normal

pairing of 11 bivalents in species like *Z. cassumnar* and *Z. zerumbet*, *Z. mioga* having a somatic chromosome of $2n=55$ is to be considered as a pentaploid.

Centers of cultivation

Ginger is undeniably the most extensively cultivated spice (Lawrence, 1984). It is not known to occur in the truly wild state. It is believed to have originated in South East Asia and it has spread throughout the tropical and subtropical regions in both hemispheres. The main ginger growing countries are India, China, Jamaica, Taiwan, Sierra Leone, Nigeria, Fiji, Mauritius, Indonesia, Brazil, Costa Rica, Ghana, Japan, Malaysia, Bangladesh, Philippines, Sri Lanka, Solomon Islands, Thailand, Trinidad and Tobago, Uganda, Hawaii, Guatemala and many Pacific Ocean Islands

Cultivation

Though a perennial plant, ginger is usually grown as an annual for harvesting as a spice. Ginger requires a warm and humid climate. It is cultivated from almost sea level to an altitude of 1500 m above MSL either under heavy rainfall conditions (150-300 cm/year) or under irrigation. The crops thrive well in sandy or clayey loam, red loam or laterite loam soils having good drainage and humus content. Ginger does the best in partial to complete shade. It can be grown as a pure crop as well as an inter-crop in coconut, coffee and orange plantations.

Ginger is propagated vegetatively through rhizomes. The size of the planting material varies from place to place and variety to variety. Seed rhizomes are treated with fungicides and insecticides to avoid any seed-borne diseases and scales. Planting is done during April - May with the receipt of pre-monsoon showers. When the crop is raised under rain fed conditions, seed rhizomes are planted in raised beds. In irrigated crop, it is done on ridges. After planting, the beds are mulched with green leaves thrice at intervals of one month.

Ginger is harvested by digging out rhizomes when the tops have died down. The harvesting and processing of dried ginger varies in different countries. The rhizomes are lifted from the earth, cleared of all adhering matter by washing, blanched and then sun-

dried. A few of farmers scald the rhizomes in hot water before drying in the sun. Others scald and then peel or scrape them to remove before drying. In Jamaica, the rhizomes are peeled, washed and sun dried. It requires about 6-8 days to dry the rhizomes. If the ginger is for vegetable purpose or for preparation of candied ginger, salted ginger or for soft drinks, pickles, etc. the harvesting should be done at 4-5 months of planting, when these rhizomes are still immature, tender, succulent and mild in pungency. The ginger of commerce consists of the thick scaly rhizomes (underground stems) of the plant. They branch with thick thumb-like protrusions, thus individual divisions of the rhizome are known as "hands

Cultivars and Varieties

Ginger cultivars are generally recognized by their locality of cultivation or collection. Most of these cultivars are restricted to particular tracts only and are seldom known out side that area. Indian Institute of Spices Research, Calicut has collected 475 accessions of ginger germplasm including exotic land races (10), Indigenous land races (391), improved cultivars (54), mutants (18), and tetraploids (2) (Ravindran *et al.*, 1994). Ginger germplasm is also maintained at Himachal Pradesh Agriculture University (163accessions), NBPGR, Regional station, Trichur (149 accessions) and High Altitude Research Station, Pottangi, Orissa (Ravindran *et al.*, 1994, Mohanty and Panda 1994). Each center of production tends to produce a distinctive type but this may due to soil, climatic and cultural conditions as well as methods of preparation. Some of the prominent indigenous cultivars are Maran, Kuruppampadi, Ernad, Wynad, Himachal and Nadia. Exotic cultivars such as Rio-de-Janeiro have also become very popular among cultivars. Several clones are recognized in India, which differ in the size, fiber and moisture content and yield of rhizomes. The improved varieties of ginger and their salient features are given in Table 1.

Table 1. Popular Cultivars in major ginger growing states in India

State	Culture Name	Specific trails
Kerala	Rio de Janeiro, Burdwan, Jamaica	High yield
Kerala	Nadia, Maran, Bajpai, Narasapattam	High yield
Kerala	Kuruppampadi	Ratoon crop
Kerala	Thingpuri, Assam, China	High yield
Kerala	Ernadan, Mananthavady	High yield
Himachal Pradesh	Himachal, Rio de Janeiro	High yield
Himachal Pradesh	Kerala local	Fresh ginger
Assam	Nadia, Chekerella	High fresh rhizome yield
Nagaland	Thinladium, Nadia, Khasi local	High fresh rhizome yield
Orissa	Rio de Janeiro, China, Vingra selection, Ernad manjeri, Kuruppampadi, Wynad	High yield
Karnataka	Himachal, Jorhat, Wyanad local	High yield rhizome yield
Meghalaya	Tura, Poona, Basar local	High yield
West Bengal	Gurubathani	High yield
Madhya Pradesh	V3S1-8	High yield

Source: Ginger- The Genus *Zingiber* P. N. Ravindran, K. Nirmal Babu (Eds.) (2005).

Table 2. Improved varieties of ginger

Cultivar name	Pedigree	Mean yield t/has	Dry recovery (%)	Oil content (%)	Oleoresin (%)	Crude fibre (%)	Mean days maturity
Suprabha	Selection from Kunduli local	16.6	20.5	1.9	8.9	4.4	230
Suruchi	Induced mutant of Rudrapur local	11.6	23.5	2.0	10.0	3.8	220
Suravi	Selection from germplasm	17.5	23.0	2.1	10.2	4.0	225
IISR Varada	do	22.6	19.5	1.7	6.7	3.3	200
IISR Mahima	do	23.2	23.0	1.7	4.5	3.3	200
IISR Rejatha	do	22.4	19.0	2.4	6.2	4.0	200

Source: Ginger- The Genus *Zingiber*. P. N. Ravindran, K. Nirmal Babu (Eds) (2005).

Table 3. Local cultivars/land races of ginger

Cultivar	Mean yield (fresh) (t/ha)	Maturity (days)	Dry recovery (%)	Crude fibre (%)	Oleoresin (%)	Essential Oil (%)
China	9.50	200	21.0	3.4	7.0	1.9
Assam	11.78	210	18.0	5.8	7.9	2.2
Maran	25.21	200	20.0	6.1	10.0	1.9
Himachal	7.27	200	22.1	3.8	5.3	0.5
Nadia	28.55	200	22.6	3.9	5.4	1.4
Rio-de-Janeiro	17.65	190	20.0	5.6	10.5	2.3

Production and productivity

India is the largest producer of ginger; the annual production is about 263,170 tons from an area of about 77,610 hectares, contributing approximately 30 to 40% of the World production. The productivity is low at about 3,428 kg/ha. Out of the total production, 10 to 15% is exported to about 50 countries around the World. The crop occupies the largest area in the state of Kerala (19%), followed by Orissa (17%), Meghalaya (12%), West Bengal (12%) and Arunachal Pradesh (6%). Kerala and Meghalaya together account for nearly 40% of the country's production. In terms of productivity, Arunachal Pradesh stands first with 7,164 kg/ha, followed by Meghalaya (5,139 kg/ha), Mizoram (5000 kg/ha) and Kerala (3,428Kg/ha). During 1999-2000 India exported 8,773 tons of ginger valued at Rs. 306 million, out of which dry ginger contributed Rs. 199.2 million. During 2000-2001, export decreased to 6,580 tonnes giving a value Rs. 2295.4 lakhs. The major importers of Indian ginger are Pakistan (32.58%), Bangladesh (32.50%), Saudi Arabia (11.15%), UAE (4.30%), Morocco (3.87%) and USA (2.83%). India has capabilities to provide to the requirements of the consuming countries in terms of quantity and quality matching to International standards (Tamil Selvan and Manoj, 2002; Peter and Nybe, 2002). The crop exhibited an annual growth rate of 4.6 per cent in area, 7.4 per cent in production and 2.7 per cent in productivity (Manjunath, 2004). Of total world's ginger production, India ranks first with respect to ginger production contributing about 303.50 tonnes. India's production during the year 2004 -2005

was 397.99 thousand tonnes from an area of 100.27 thousand hectare with average yield 3969 kg/ha. World's average ginger production in 2004 is 1,072,838 tonnes (Arecanut and Spices Database, 2007).

Properties and Uses

Ginger is valued the world over, as a culinary herb, condiment, spice, home remedy, and medicinal agent. The aroma of ginger is pleasant and spicy and flavours penetrating, pungent and slightly biting. It is a common ingredient in Asian cooking and it flavours several products like confectionary, curry powders, pickles, several soft drinks and alcoholic beverages. It is also essential in Western baking like in traditional gingerbreads, cakes, biscuits etc. It is available fresh and preserved in brine or syrup.

Ginger oils and oleoresins also have a variety of uses. The essential oil is used in commercial flavorings. Ginger has several medicinal properties. It is carminative, diaphoretic and spasmolytic. Ginger is truly a world domestic remedy. Asian cultures have used it for centuries. Ginger also claims for use as an anti-vomiting or anti-motion sickness agent. Like the ancient Chinese, in India the fresh and dried roots were considered distinct medicinal products. Fresh ginger has been used for cold-induced disease, nausea, asthma, cough, colic, heart palpitation, swellings, dyspepsia, loss of appetite, and rheumatism. Ginger containing proteolytic enzymes that promote the digestive process and also enhance the action of the gall bladder and protects the liver against toxins (Yamahara *et al.*, 1990). Further studies show that ginger can lower cholesterol levels by reducing cholesterol absorption in the blood and liver. Suekawa *et al.* (1984) reported that gingerol and shogaol present in ginger juice reduce both blood pressure and heart rate.

Ginger extracts have been extensively studied for a broad range of biological activities including antibacterial, anticonvulsant, analgesic, antiulcer, gastric antisecretory, antitumor, antifungal, antispasmodic, antiallergenic, anti-inflammatory and other activities. The juice also acts as a strong diuretic (Kirtikar and Basu, 1935). Gingerols have been shown to be inhibitors of prostaglandin biosynthesis. Other scientific

studies show that gingerol, one of the primary pungent principles of ginger, helps counter liver toxicity by increasing bile secretion. Ginger has potent anti-microbial and anti-oxidant (food preservative) qualities as well.

Diseases and Pests

Soft rot is the most serious disease of ginger in India and in some other countries. It is caused *Pythium* spp. of which Randhawa and Nandpuri (1970) reported *P.aphanidermatum* as the principal species in India. Bacterial wilt of ginger caused by *Ralstonia solanacearum* was recorded from Queensland (Hayward *et al.*, 1967). Rhizome rots also caused by *Fusarium* spp. and *Pythium* spp. (Gupta and Verma, 1966). Leaf rots also caused by *Colletotricchum zingiberis*. Other diseases are also reported for example thread blight by *Pellicularia filamentosa* (Chattopadhyay, 1967), root rot by *Nectriella zingiberi*. A shoot borer *Dichocrosis punctiferlis* attacks the crop and can cause them wilt and die (Yegna Narayan Aiyer, 1944). *Aspidiella bartii* can spoil the appearance of the sample (Fennah, 1947). Scale infects the genus *Aspidiotus* have been reported attacking ginger plants in several countries. *Sitodrepa* and *japanica* and *M. incognia* attacks the crop in a number of countries. The causagents of major ginger diseases and its distribution in India are given in the Table 4.

Table 4. Diseases of ginger and the causal organisms

Disease	Pathogen	Distribution	
Soft rot	<i>Pythium</i> species		
	<i>P. aphanidermatum</i> (Edson)	Andhra Pradesh, Assam,	
	Fitz (syn: <i>P. butleri</i> Subram I,	Bihar, Gujarat, Kerala,	
	<i>P. gracile</i> (deBary) Schrent	Karnataka, Maharashtra,	
		Madhya Pradesh, West Bengal	
	<i>P. deliense</i>	Madhya Pradesh	
Bacterial wilt	<i>Ralstonia solanacearum</i>	All over the country	
	Yellows	<i>Fusarium</i> spp	
		<i>F. oxysporum</i> f.sp.zingiberis	Himachal Pradesh,
		<i>F. solani</i> , <i>F. moniliforme</i>	Rajasthan
		<i>F. graminearum</i> , <i>F. equiseti</i>	
		Leaf spots	<i>Phyllosticta zingiberis</i>
<i>Helminthosporium maydis</i>	Himachal Pradesh		
<i>Colletotrichum zingiberis</i>	Bihar		
<i>Pyricularia zingiberis</i>	Andhra Pradesh		
<i>Leptosphaeria zingiberis</i>	Assam		
<i>Coniothyrium zingiberis</i>	Meghalaya		
<i>Curvularia lunata</i>	Assam		
<i>Vermicularia zingiberis</i>	Bihar		
<i>Septoria zingiberis</i>	Andhra Pradesh		
	Andhra Pradesh		
Leaf blight/Dry rot	<i>Rhizoctonia solani</i>	Himachal Pradesh	
	<i>R. bataticola</i>	Haryana, Kerala	
Thread blight	<i>Pellicularia filamentosa</i>	Kerala	
Basal rot	<i>Sclerotium rolfsii</i>	Maharashtra	
	(<i>Corticium rolfsii</i>)		
Sheath rot	<i>Fusarium</i> sp.	Maharashtra	
Virus diseases	<i>Cucumber mosaic virus</i> (CMV)	Kerala	
	<i>Chlorotic Fleck virus</i> (GCFV)	Assam	
	Chirke virus		

Source: Major spices – Production and processing Eds: V.A.Parthasarathy, P. Rajeev 2006.

Turmeric (*Curcuma longa* L.)

History

The genus *Curcuma* belonging to the family Zingiberaceae which is composed of about 70-80 species of rhizomatous annual or perennial herbs (Purseglove 1974; Sirirugsa 1999). One of the most important species is *Curcuma longa* within the genus which yields turmeric, is the source of an important coloring and aromatic ingredient of curry powders that is enormously used in Asian cuisines (Apavatjirut *et al.*, 1999; Purseglove, 1974) and pharmaceutical industries since it has been considered as an extremely important medicinal plant (Majeed *et al.*, 1995). Turmeric (*Curcuma longa* L.) is essentially a tropical crop extending from India to Indo- china, East Indies and parts of china. The center of origin of this crop is believed to be South East Asia and some species are naturalized in North Eastern regions of India and Java. Turmeric was domesticated in Southern or South East Asia. Raghavan and Venkatasubhan (1943) believe that *C. domestica* is likely to have arisen by a process of triploidy, either from a *C. aromatica* Salisb. or a form similar to it with 42 chromosomes. *C. aromatica* is a native of India spreading from the Eastern Himalayas to Sri Lanka, but it may be that some other diploid form, closely related morphologically to *C. domestica*, may be the true ancestral form. *C. domestica* is not now known in a wild state with certainty, but it seems to have arisen by continued selection and cultivation by vegetative propagation.

Origin and distribution

The genus *Curcuma* is mainly distributed in the Indo-Malayan region. Baker (1898) described 27 species in the *Flora of British India*. He subdivided the genus in to three sections- *Exantha*, *Mesantha*, and *Hitcheniopsis*. The section *Exantha* consists of 14 species including turmeric and other economically important species such as *C. angustifolia* Roxb. (Indian arrow root), *C. aromatica* Salis., and *C. zedoaria* Rosc. From south India 16 species of *Curcuma* were reported (Sabu 1991), out of which 9 are endemic to India.

The species belonging to the genus *Curcuma* can be grown in the diverse tropical conditions from sea level to a height of 1500 m on the hilly slopes, in the temperature range of 20 to 30 ° C. A rainfall of 150 cm or more or an equivalent amount of irrigation is essential for optimum growth and development of *Curcuma* species. Ideal soil requirements for *Curcuma* growing are loose, friable loamy or alluvial suitable for irrigation that should have efficient drainage capacity. The species are naturally found in mixed deciduous tropical forests and tropical broad-leaved evergreen forests of the tropical and subtropical regions. The geographical distribution of the genus reaches from India to Thailand, Indonesia, Malaysia, Indonesia and finally to northern Australia (Apavatjirut *et al.*, 1999). Along with ginger, *C. longa* was probably taken from India, South East Asia, China and Northern Australia to the West Indies and South America by Spaniards. Subsequently its cultivation spread over other countries. There is no available documented literature about the origin and distribution of African and South American *Curcuma* species. The members of the genus in these regions are important resources and have great potentials in terms of commercial values as source of spices, medicines and horticultural products (Apavatjirut *et al.*, 1999; Cao *et al.*, 2001; Cao and Komatsu, 2003; Joe *et al.*, 2004; Maciel and Criley, 2003; Majeed *et al.*, 1995; Paisookasantivatana *et al.*, 2001a and 2001b; Purseglove 1974; Sasaki *et al.*, 2002; Sasaki *et al.*, 2004; Yusuf *et al.*, 2001).

Botany

Turmeric is an erect perennial herb usually grown as an annual. The leafy shoots rarely exceed 1m in height and bearing 6-10 leaves with the leaf sheaths forming a pseudostem. The lamina is lanceolate, acuminate and thin; dark green usually up to 30 cm long and 7-8 cm wide. The inflorescence is a cylindrical terminal spike. The flowers are borne in cincinni of two in the axils of the bracts and are thin textured and fugacious, opening one at a time. The calyx is short and unequally toothed. The upper half of the corolla is cup shaped with three unequal lobes, which is whitish, thin and translucent. There are two lateral staminode, which are creamy white in colour. The stamen is short

filamented and broad and the anther is versatile. The cylindrical styloids are present. The ovary is inferior and trilobular with a slender style passing between anther lobes. Fruits are seldom produced (Purseglove *et al.*, 1981). An image of *Curcuma longa* L is presented in Fig.2.



Fig. 2 *Curcuma longa* L. plant with underground rhizome

Systematic position

The genus *Curcuma* is taxonomically a complicated genus and new species are yet to be described. The first classification of the genus was by Baker (1898). Holtum (1950) simply followed this classification and Valetton (1918) revised the previous classification and pinpointed the anomalies in those. He also made a classification based on Javan species by dividing it into two sub genera namely *Eucurcuma* and *Paracurcuma*. This division is still under question and scientists are till comfortable without this subdivision, hence the taxonomy of genus is still problematic (Maknoi and Sirirugsa, 2002). The name *Curcuma* was coined by Linnaeus in his *Species plantarum* in 1753. *Curcuma longa* of Linnaeus was based on a plant observed by Hermann in Ceylon and is

not this species. *Curcuma longa* Koenig is probably the true turmeric, but as this name belongs to another species. Valetton (1918) proposed the new name of *C. domestica*. Willis (1966) states that there are 5 species of *Curcuma*, which is obviously a mistake. Bailey (1949) says that there are 40-50 species, while Sastri (1950) says there are about 70. Shankaracharya and Natarajan (1973) state that, in India, nearly 30 species have been recognized by botanists. The center of distribution is Indo Malaysia region and they extend to Australia.

Cytology

Curcuma aromatica and *C.amada* each have $2n = 42$ chromosomes, *C. longa* has $2n = 62$ chromosomes according to Raghavan and Venkatasubhan (1943) where as Sugiura (1931) has recorded $2n= 64$. The chromosomes of all the above three species are very small compared to those of the *Zingiber* species. These are rod shaped with submedian or subterminal centromeres. The general appearances of the chromosomes are also similar. *C. zedoaria* has $2n= 64$. The chromosomes are also quite small and rod like. According to Raghavan and Venkatasubhan, *C. longa* is likely to have arisen by a process of triploidy either from *C. aromatica* or a form closely similar to it with 42 chromosomes. This suggestion was made since morphologically *C. longa* and *C. aromatica* are alike except for the aromatic smell of the rhizome of *C. aromatica*. The configuration of the chromosomes in the complements of the three species would appear to be almost identical. This would indicate that the 42 chromosomed species must have got differentiated by structural changes of the chromosomes rather than changes in morphology. *C.amada* and *C. aromatica* may be amphidiploids, which have originated from a cross between some 12 chromosomed and 9 chromosomed ancestors. Aneuploidy with 31, 32 have also been recorded in *Curcuma*. Darligton and Wylie (1955) give the basic chromosome number for the genus *Curcuma* as $x= 16$ and 21. However the most acceptable view is that the chromosome number of *Curcuma longa* (*Curcuma domestica*) is $2n=63$. Sopher (1964) states that *Curcuma domestica* (*Curcuma longa*) is a sterile

triploid. But Aiyadurai (1966) reported the presence of seeds, the germination of which is speeded up by treatment with concentrated H₂SO₄.

Centers of cultivation

Turmeric is grown in most of the states in India and is rich in cultivar diversity. The cultivation is concentrated in the states of Andhra Pradesh, Tamil Nadu, Maharashtra, Madhya Pradesh, Utter Pradesh and Bihar. The total turmeric production in India is around 6, 53,600 metric tones from an area of about 1, 61,300 hectare. Some Kerala cultivars such as Alleppey grown in central Kerala area are regarded as high yielding and of high quality.

Cultivation

Turmeric is vegetatively propagated by rhizomes. According to Aiyadurai (1966) mother rhizomes are better planting materials than the fingers or the secondary mother rhizomes. In Kerala, the field is well prepared during the pre-monsoon showers and the planting is done on raised beds for proper drainage. Planting is done either at the onset of monsoon or before it. Usually, 20x15cm distance is kept between plants. This helps in preventing mixing of tubers of different collections. For commercial cultivation farmyard manure at the rate 25T/ha is mixed with soil and the planted material is well covered with decomposed leaves and crop residues to maintain sufficient moisture for regeneration of the rhizome. Earthing up is usually done for better yield.

The crop is harvested at complete senescence of the aerial portion. The harvested material is cleaned and the roots removed and the fingers and rounds are separated before curing, drying and polishing them to obtain turmeric of commerce. The cleaned material is half cooked with some very dilute alkali solution and then dried in open sun for about 6-7 days. This helps to impart good orange colour to the tubers. Polishing the dried turmeric is done before it is marketed.

Cultivars and Varieties

Turmeric types are known by trade names, based on the appearance, rhizome thickness, colour intensity, aroma and hardness of the core and duration (Rao and Rao

1994). Turmeric cultivars/ types of Andhra Pradesh are classified into 3 groups, namely short duration ‘Kasturi type’, medium duration ‘Kesari’ types and long duration types belonging to *C. longa*. A number of cultivars are available in the country and are known mostly by the name of locality where they are cultivated. Some of the popular cultivars are Duggirala, Mydukur, Armoor, Tekkurpet, Salem, Sugandham, Mannuthy local Amalapuram, Erode local, Alleppey, Moovattupuzha, and Lakdong (Muthuswamy and Ahamad Shah 1982; Srirama Rao 1982; Rao and Rao, 1994).

Efforts have been made to identify and select turmeric types with high yield potential, greater curcumin content and curing percentage. Some of the important released varieties are given in Table 5.

Table 5. Improved varieties and its salient features

Variety	Fresh yield (t/h)	Crop duration (days)	Dry recovery	Curcumin %	Oleoresin %	Essential oil (%)
Suvarna	17.40	200	20.00	4.3	13.5	7.0
Suguna	29.30	190	12.00	7.3	13.5	6.0
Sudarshana	28.80	190	12.00	5.3	15.0	7.0
IISR Prabha	37.5	195	19.50	6.5	15.0	6.5
IISR Prathibha	39.10	188	18.50	6.2	16.2	6.2
IISR Alleppey supreme	35.4	220	19.0	5.6	16.0	-
IISR Kedaram	34.5	215	18.9	5.7	14.0	-
Co-1	30.0	285	19.50	3.2	6.7	3.2
BSR-1	30.70	285	20.50	4.2	4.0	3.7
Krishna	09.20	240	16.40	2.8	3.8	2.0
Sugandham	15.00	210	23.30	3.1	11.0	2.7
Roma	20.70	250	31.00	9.3	13.2	4.2
Suroma	20.00	255	26.00	9.3	13.1	4.4
Ranga	29.00	250	24.80	6.3	13.5	4.4
Rasmi	31.30	240	23.00	6.4	13.4	4.4
Rajendra Sonia	04.80	225	18.00	8.4	-	5.0
BSR -2	32.70	245	20.00	3.15	-	-
RCT-1	25.80	300	16.37	6.8	-	-
Kanthi	37.65	255	18.67	6.8	-	-
Sobha	35.88	255	17.92	7.39	15.95	4.2
TCP-2	29.20	235	21.20	5.7	10.90	4.1
Pant Peetabh	20.00	210	18.50	7.5	-	1.0

(Pujari *et al.*, 1986; Edison *et al.*, 1991; Sasikumar *et al.*, 1996).

Production and Productivity

Turmeric is being cultivated in most areas of the tropics and subtropics. India, Indonesia, Parts of China and Srilanka are the important countries growing turmeric. India is the greatest producer and exporter of turmeric in the world and among spices grown in India, it ranks fifth position in area (1,61,300 hectares), second in production (6,53,600 metric tonnes) and third in export (both in quantity (35,556 t) and value (Rs.121.7 crores) during 1999-2000. (Spices Board, 2000). India is the World's largest producer grown in an area of 161.23 thousand hectares with the average production of 716.84 thousand tonnes and productivity 4446 kg/ha (2004 -2005) (Areca nut and Spices Database, 2007). Important states, which grow turmeric, are Andhra Pradesh (61,400ha), Orissa (23,900ha), Tamil Nadu (21,600ha), Assam (11,700ha), Maharashtra (9,000ha), and to a little extent in Kerala (2,800ha) 2004-2005 (Areca nut and Spices Database, 2007). In production also, Andhra Pradesh leads with a production of 419,000 tonnes followed by Tamil Nadu (118,500t) and Orissa (56,800t) (Areca nut and Spices Database, 2007). India is the major exporter of turmeric. In India, turmeric is produced from 230 districts in 22 states. The states like Andhra Pradesh, Tamil Nadu, Orissa, Karnataka and West Bengal are the major producers of turmeric and these states contribute 90% of the total production. India exports turmeric as turmeric whole, turmeric powder, turmeric oil and oleoresins to Middle East countries, UK, USA, Japan, Sri Lanka and South Africa (Tamil Selvan and Thomas, 2002). The global trade of turmeric is expected to increase at an annual growth rate of two percent (Anonymous, 2005).

Properties and uses

Turmeric is used as primitive medicine, both externally and internally in Southern Asia since time immemorial. It is used as a colouring matter in pharmaceuticals, confectionary and food industries. Turmeric is also used as a natural yellow dye and its powder is used in cosmetics. Turmeric (*C. longa* L.) is an important medicinal plant, which is known for its aromatic stimulant, carminative and antihelminthic properties (Satyavati *et al.*, 1976). It is also known to have cholesterol lowering and antioxidant

properties (Dashpande *et al.*, 1997). In rice, turmeric powder has been used to control seed borne diseases (Gangopadhyay, 1998). Some of the medicinal properties of turmeric such as antiinflammatory (Chandra and Gupta, 1972; Ghatak and Basu, 1972; Sharma *et al.*, 1972; Srimal and Dhawan, 1973). Anti bacterial and anti protozoal activities have been demonstrated in experimental animals.

Several pharmacological effects have been reported. Extracts of turmeric with a high content of curcumin and volatile oil have been used against infectious of the bile duct, gall bladder and liver. In Indian system of medicine, turmeric is used as a stomachic tonic and blood purifier. Mixed with warm milk it is said to good for common cold. The juice of the fresh rhizome is used as an antiparasitic for skin infection. Externally it is applied to indolent ulcers and a paste made from the powdered rhizome along with lime form a remedy for inflamed joints. Oil of turmeric has feeble antiseptic properties. In addition, very recently the wound healing and detoxifying properties of curcumin have also received a considerable attention (Joe *et al.*, 2004).

Turmeric is used for culinary purposes as powder turmeric. It has numerous applications in baking industry and as an essential ingredient in curry powder. Curcumin is the principle colouring constituents, which imparts the characteristic yellow colour to turmeric. It is not only to flavour but also used to colour butters, pickles mustards and other foods. It also used to colour liquors, fruit drinks, cakes and jellies. Pure curcumin is used in colouring hydrogenated vegetable fat (Vanaspati) and other food materials (Sethi and Subha Rao, 1964).

The finest Indian arrowroot derived from *C. angustifolia* Roxb. (Das *et al.*, 1999) has been used as a source of starchy food for centuries. A number of *Curcuma* species have beautiful inflorescence and luxurious foliages that have an immense commercial value in floriculture as a versatile ornamental crop used as cut flowers, pot and landscape plant (Maciel Criley, 2003; Paisooksantivatana *et al.*, 2001 a and 2001 b). Among them *Curcuma alismatifolia* is recognized and popular in international trade as cut flower (Paisooksantivatana *et al.*, 2001 b). Some other species such as *C. aruginosa*, *C. amada*,

C. angustifolia, *C. cesia*, *C. elata*, *C. petiolata*, *C. rubescens*, *C. xanthorrhiza* and *C. zedoaria* have also received considerable attention as cut flowers and tropical glass house ornamentals.

Zedoary oil derived from the rhizomes of *C. zedoaria* is used as a spice, tonic and perfume. The terminal bracts from a sterile cluster called a coma, often brightly coloured and in the case of some species these coma bracts are being rich in volatile oil that are also used to produce perfumes and cosmetics. Recently, leaf essential oils of *C. longa* and *C. aromatica* have been analyzed by Behura *et al.*, (2002). They found several important essential oils such as α - phellandrene, 1,8 cineole, C8- aldehyde and Linalol. These essential oils are valuable for pharmaceutical as well as cosmetic industries.

Diseases and Pest

Turmeric is affected by various diseases of fungal and bacterial origin. Of these the leaf blotch, leaf spot and rhizome rot are the major diseases. Leaf blotch caused by *Taphrina maculans*, leaf spot by *Colletotrichum capsici* and rhizome rot caused by *Pythium graminicolum* result in considerable crop losses (Butler, 1918). Rhizome rot, which accounts for the loss of collections in the farm, is caused by *Pythium graminicolum* L. Subram. (Chattopadhyay, 1967). The long duration types are free from leaf spot disease (Sharma & Krishnamurthy, 1962). Sharma & Nambiar (1974) reported the brown rot disease in the rhizomes of *C. aromatica* type of turmeric. Shoot borer, *Dichocrosis punctiferalis* Gd. is the major pest (Joshi and Sharma 1982). The removal of infected shoot is recommended as a control measure (Anonymous 1950). Other insects of minor importance are *Udasper foleus* Cr., and thrips (*Panchaetothrips indicus*) while *Stegolrum paneceum* attacks stored product. Dubey *et al.*, 1976 noticed the attack of maggots of Dipteran flies (*Mimegralla* species) in fresh rhizomes.

Cardamom (*Elettaria cardamomum* Maton.)

History

Cardamom is the dried fruit or seed of *Elettaria cardamomum* Maton, an herbaceous perennial plant, which can grow to heights of 12 feet. It is a native of moist evergreen forests of Western Ghats of Southern India. It is highly valued from ancient times, because of its very pleasant aroma and taste and it referred as the 'Queen of Spices'. The genus name is derived from the Tamil 'Eletteri' meaning cardamom seeds. The genus consists of about six species (Mabberly, 1987). Only *Elettaria cardamomum* Maton occurs in India, and this is the only economically important species. Rosengarten (1969) states that 'cardamom was an article of Greek trade during the fourth century B.C. Morris and Mackley (1997) said that cardamom grew in the gardens of the king Babylon in 720 B.C. Willis (1966), opined that *Elettaria* has seven species in Indo Malaysia. Based on the nature of panicle Sastri (1952) recognized three varieties of cardamom (Malabar, Mysore, and Vazhukka). Based on the size of the fruit two varieties are recognized as var. major Thwaites and var. cardamomum (syn. var *minor* Watt; Var. *miniscula* Burkill) (Purseglove *et al.*, 1981).

The ancient Indian Ayurvedic texts, *Charaka Samhita* and *Susrutha Samhita*, (1400 – 600 BC) mentioned cardamom on many occasions (Ravindran, 2001). Dioscorides (40-90 AD) mentioned cardamom as a useful medicine in his book *Materia Medica*. Cardamom has distinctive medicinal properties and is found in most pharmacopias. In India, the tincture of cardamom is used in indigenous systems of medicine to strengthen the digestive, respiratory and the urinogenital system (Pruthi, 1976; Warriar, 1989).

Cardamom is ecologically classified as pseophytes meaning shade loving plant and second national spices of India (Miniraj *et al.*, 2000). It's growing regions of South India are located at elevations ranging from 800- 1300 m above mean sea level with annual rainfall of 1500- 7000mm and temperature rang of 10-35 ° C. Cardamom being a

delicate plant is very sensitive to climatic variation. (Miniraj, 2000). Cardamoms require good drainage and cannot tolerate water logging. (Mayne, 1942; Sastry, 1952). The crop thrives best under moderate natural shade (Purseglove *et al*, 1981).

Cardamom is grown on a commercial scale in Guatemala, which incidentally is also now the largest producer of cardamom. If India wants to recapture the lost glory of the 'Queen of Spice', there should be concerted attempts to achieve a quantum jump in productivity not only through high yielding varieties but also high chemical quality and which can be withstand diseases (Ravindran and Madhusoodanan, 2002).

Origin and distribution

The Plant is indigenous to the evergreen rain forest of Western Ghats of Southern India from where it spread to some other tropical countries such as Sri Lanka, Tanzania and a few Central American Countries. Presently, it is being cultivated in not less than twelve countries from three continents lying between 20° latitude north and south. It thrives well in the tropical rain forest of Western Ghats. The tract lies between 8° 30' and 14° 30' N latitude and 75-78° 30' E longitude. The geographical distribution of the crop in India extends northwards from Thirunelveli (Tamil Nadu) for over 1000 kms to Sirsi (Karnataka) through the high ranges of Kerala. Considering the width, it is a narrow belt of land spread over the Western Ghats at an elevation of 800 m and above (Nair *et al.*, 1991).

Botany

Cardamom is a tall herbaceous perennial with branched subterranean rhizomes from which arise several erect leafy shoots and erect or decumbent panicles. The leafy shoots are usually 10-20 in number 2-5.5 m tall and are composed of leaf sheaths and are born in thick clumps. Leaves are distichous with lanceolate acuminate lamina, 25-90 cm long and 5-15 cm wide, dark green and glabrous above and paler beneath. The lower surface may be either glabrous or pubescent depending on the variety and race. The inflorescence is panicle usually 60-120 cm long and arises from the rootstalk at the base of the leafy shoots. The panicle having large alternate bracts with axillary cincinni,

usually two to three flowered. The flowers are hermaphrodite, zygomorphic and about 4 cm long and 1.7 cm cross. The bracteole is tubular, as is the calyx, which is green, shortly three toothed and persistent. The corolla tube is three narrow. Strap shaped spreading, pale green lobes about 1 cm long. The most conspicuous part of the flower is the obovate labellum composed of three modified stamens. There are further two rudimentary staminodes and one functional stamen. The functional stamen has a short, broad filament with a longer anther. The inferior ovary consists of three united carpels with numerous ovules in axile placentation. Style is slender with small capitate stigma. The fruit is a trilocular capsule, fusiform globose and contains 15 –20 seeds with thin mucilaginous aril. (Purseglove *et al.*, 1981). An image of *Elettaria cardamomum* Maton. is presented in Fig.3.



Fig. 3 *Elettaria cardamomum* Maton. plant with fruits

Systematics

The genus *Elettaria* consists of about six or seven species distributed in India, Sri Lanka, Malaysia and Indonesia; only *E. cardamomum* is economically important (Holtum, 1950; Willis, 1967; Mabberley, 1987).

Cytology

The basic chromosome number of *Elettaria cardamomum* as $x = 12$ and the somatic chromosome number as $2n = 48$ was first reported by Gregory (1936). This information indicated the balanced tetraploid nature of the plant. Ramachandran (1969) and Sudharshan (1987, 1989) also substantiate these findings. Chandrasekar and Sampath Kumar (1986) observed that aneuploidy as well as structural alterations in chromosome has contributed to the varietal differentiation. Sudharshan (1989) reported very limited chromosomal aberration and had high pollen fertility and seed set.

Centers of cultivation

Cultivation of cardamom is mostly concentrated in the evergreen forests of Western Ghats in South India. Besides India, cardamom is grown as a commercial crop in Guatemala and on a small scale in Tanzania, Sri Lanka, El Salvador, Vietnam, Laos, Cambodia and Papua New Guinea (PNG). The important areas of cultivation in India are Uttara Kannada, Shimoga, Hassan, Chickmagalur, and Kodagu (Coorg) district in Karnataka State, Northern and Southern foothills of Nilgiris, hill regions of Madurai; Salem and Tirunelveli, Annamalai and Coimbatore districts of Tamil Nadu State; Wynad and Idukki districts as well as in Nelliampathy hills of Palghat district of Kerala.

Cultivation

Cardamom can be propagated vegetatively by the rhizome bits, or seedling usually raised in nurseries. The method will depend upon the region and the area to be planted. The vegetative propagated plants gives an earlier yield than is obtained from the seedling. For large-scale production it is usual to sow selected seeds in specially prepared nursery beds; the seedlings are later transplanted in to further nursery beds and finally in to the field. Sastri (1952) states that the sowing time is usually August to October in

Coorg, Mysore and North Kanara and February and March in Madras. The nursery seedlings are planted in field usually 1.5-1.8 m apart. Maintenance of correct amount of shade is an important feature of cardamom cultivation. It thrives best under moderate shade (Purseglove, 1981). Malabar cardamom is less sensitive to sunlight than Mysore cardamom. Pits are usually 0.6m wide and 0.45m deep. The spacing between the pits varies from 1.5 to 3.0m depending on the type of the cardamom grown, soil fertility. The spacing of Mysore cardamom is usually about 3m each, while for Malabar cardamom the spacing is usually about 1.8m. The after care of cardamoms consists of weeding, removal of old and dying stems, mulching, regulating the shade, manuring and filling up of vacancies. Cardamom comes into bearing three years after planting. In India flowering commences in April and May and continues until July and August. Development and ripening of the fruit take about three months in the case of Malabar and 4 months from Mysore. In Malabar cardamom fruits begin to ripen in August and harvesting is completed in December and in Mysore cardamom ripening starts in September and harvesting may be prolonged until April. Fruits are harvested just before they are fully ripe

Cultivars and varieties

Based on the adaptability, nature of panicle, shape and size of fruit three cultivars of cardamom are recognized (Sastri, 1952). The cv. *Malabar* is characterized by prostrate panicle and cv. *Mysore* possesses erect panicle. The third type cv. *Vazhukka* is considered a natural hybrid between the two and its panicle is semi erect or flexuous. A large number of varieties have been released from various research institutions. Prominent among them are given in Table 6.

Table 6. Improved varieties of cardamom and their characteristics.

<i>Selection/ Variety</i>	<i>Cultivar</i>	<i>Important features</i>
IISR Kodagu Suvasini	Malabar	Suitable for intensive cultivation
IISR Avinash	Malabar	Rhizome rot resistant, Dark green capsules.
IISR Vijetha	Malabar	Katte resistant
ICRI 1	Malabar	Early maturing. Dark green bold capsules.
ICRI 2	Mysore	Tolerant to azhukal disease
ICRI 3	Malabar	Tolerant to rhizome rot
ICRI 4	Malabar	Suitable for low rain fall areas (1500mm).
PV 1	Malabar	Elongated capsules
PV 2	Vazhukka	Green bold capsules
Mudigere 1	Malabar	Tolerant to shoot borer and thrips
Mudigere 2	Malabar	-
Njallani Gold/ Green Gold	Vazhukka	Performs well under intensive cultivation.
Vander cardamom	Vazhukka	Extra bold, deep green capsules

Source: Major spices – Production and processing V.A.Parthasarathy, P. Rajeev (Eds) 2006.

Production and productivity

Cardamom is cultivated mainly in the Western Ghat belts of Kerala, Tamil Nadu and Karnataka states. The altitude, rainfall pattern, temperature and other climatic factors in the Western Ghat belt are suitable for cardamom cultivations. The cardamom production increased from 3170 metric tones in 1970 to 4400 metric tones in 1980 and 4750 metric tones during 1990. India's production during the year 2004-2005 10,190 tonnes with an area of 65,780 hectare and productivity of 158kg/ha. (Arecanut and Spices Database, 2007). The major states which contributing to India's cardamom production are Kerala (8.620t), Karnataka (1.100t) and Tamil Nadu (0.480t) during the year 2004 -2005 (Arecanut and Spices Database, 2007). The changes in climatic factors, decline in the price of cardamom and shifting to other economic crops are the major reason attributed to the decline in cardamom area.

Properties and Uses

Cardamom is in use from ancient times for flavouring and as a component in many indigenous medicines in India. Use of cardamom in 'Guava coffee' among the Arabs is the highest in the World. Indian cardamom is preferred not only for its unique aroma due to high content of volatile that distinguishes the same but also for its pure

green colour and bold size. It is an important ingredient of garam masala, a combination spice for many dishes. It also finds a place in toothpastes, chewing gum, ice cream biscuits, cola and many edible and cosmetic applications.

In Indian systems of medicine- *Ayurveda*, *Sidha* and *Unani*, cardamom is used as a powerful aromatic stimulant, carminative, stomachic and diuretic. It also checks nausea and vomiting and is also reported to be a cardiac stimulant.

Tried and tested home remedies with cardamom are still popular among consumers particularly to stop giddiness, diarrhoea, bad breath and to improve appetite, clear sore throats etc. Gargling with the infusion of cardamom daily protects one from flu infection (Pruthy, 1979). Eating a cardamom capsule daily with a tablespoon of honey improves eyesight; strengthen nervous system and keeps one healthy. Cardamom is a very good cough suppressant. One of the main properties of cardamom is its effect on different dermatological disorders. Medicated cardamom oil and cardamom powder can retard various types of hypo-pigmentation on the face (Nair and Unnikrishnan, 1997).

Cardamom as a preservative and softening agent is popular among the Japanese food industry. In Western cuisine, cardamom adds zip to fruit cups, green peas, soups, rice dishes, Danish pastries, buns, coffee cakes, orange salads, baked apples, jellies, honey and pickles

Diseases and pests

Cardamom is affected by a number of diseases caused by various pathogenic fungi, bacteria and nematodes. The most serious fungal disease of cardamom is capsule rot, popularly known as “Azhukal” disease caused by *Phytophthora* sp. (Menon *et al.*, 1972). Thankamma and Pillai (1973) identified it as *P. nicotianae* Brede de Haan var. *nicotianae* water house and as *P. palmivora* Butler (Radha and Joseph 1974). Nambiar and Sarma (1976) reported the association of *Pythium vexans* and a *Fusarium* sp along with *Phytophthora* sp. Nair (1979) successfully isolated *P. nicotianae* var *nicotianae* from all infected plant parts. Rhizome rot or clump rot is a common disease first

reported by Park (1937). Subba Rao (1938) observed that cardamom rhizome rot caused by *Rhizoctonia solani* Kuhn, and it was associated with a nematode. Ramakrishnan (1949) reported *Pythium vexans* de Barry as the causal organism. Thomas and Vijayan (1994) reported that *Fusarium oxysporum* is also occasionally found to cause rhizome and root rot infections. Another common disease is leaf blight, popularly known as 'chenthal' reported by George *et al.*, (1976), from Idukki district of Kerala State. This was originally reported as a bacterial disease caused by *Corynebacterium* sp. (George and Jayasankar, 1977) and later Govindaraju *et al.*, (1996) reported that it is a fungal disease caused by *Colletotrichum gleosporioides* (Penz.)Penz and Sacc. Suseela Bhai *et al.*, (1988) reported fungus closely resembles *C. gloeosporoides* causing anthracnose disease of capsule. Nematode infestation is a major problem in cardamom plantation cause in heavy crop loss. Ali (1983) reported more than 20 genera of plant parasitic nematode from cardamom soils. The severe damage to the crop is caused only by root knot nematode *Meloidogyne incognita*. Root knot nematode is widely observed in almost all cardamom plantations and nurseries Ramana and Eapen, 1992).

Need for genetic variability

For plant breeders, in their endeavour directed towards increased agricultural production, there is a pressing need for more genetic diversity to work upon, to cater to varied kinds of problems and needs. The wider the range of choice a breeder will have in selecting appropriate kind of diversity the better will be the chances for his particular success for any particular goal. Earlier the breeders were content to go not much further for their material than the old landraces and varieties that were then available in their own countries or from neighboring ones, but in the last 50 years or so, breeders are requiring a much wider range of genetic diversity. The crop possesses a 'narrow genetic base' and uniformity holds a hidden danger for it can offer an open doorway to attacks by pests and pathogens. With a uniform crop variety or a range of rather similar varieties, once a strain of pathogen becomes adapted to attack it, the whole crop can be lost in a very short time.

The narrow genetic base and continuous monoculture practice of the gene pool leads to genetic erosion. Hence steps are taken to broaden the genetic base of these vegetatively propagated crops, as variability is the base for any crop improvement programme. These crops are heavily susceptible to various diseases and pests there by resulting considerable crop loss. Hence the conservation of genetic resources in the field gene bank is very difficult. Therefor production and supply of disease free planting material to the farmers for commercial exploitation is very scanty. There is a need to select tolerant lines for biotic and abiotic stresses and increase the productivity of these crops. This is done with intention of screening a variety, which can overcome the narrow agroclimatic requirements of the crop and to develop resistant clones by screening germplasm against the pathogen *Pythium aphanidermatum*, *Fusarium oxysporum* and *Pseudomonas solanacearum*.

Estimation of genetic diversity of plant populations has been recognized as elementary topic not only to delineate *in situ* and *ex situ* conservation strategies (Holsinger and Gottlieb, 1991; Morden and Loeffler, 1999), but also to establish forms of rational and sustainable exploitation of genetic resources (Chalmers *et al* 1992; Lacerda *et al.*, 2001). The ability of a particular genotype to tolerate drought or inundation, grow in poor or rich soil, resistance against insects, pests or disease, to offer higher protein yields or to produce a better tasting food are traits passed on naturally by its genes. This genetic substance constitutes the raw material that plant breeders and biotechnologists utilize to produce new varieties or cultivars. Without this diversity the ability of better adaptation to ever changing conditions and needs will be lost (Menini, 1998). This mode of reproduction largely determines the transmission of genes in time and space; hence it is widely expected to be a major determinant of population genetic structure (Eckert *et al*, 2003). Most perennial plants combine sexual reproduction through seeds and with clonal reproduction by means of vegetative propagation (Richards, 1986), and the production and recruitment of sexual versus clonal progeny may often vary within a species responding ecological and / or genetic factors that limit

one or other reproductive mode and eventually affect the genetic structure of natural populations (Eckert, 2002). Clonal plants are generally characterized by the capability to generate genetically identical genet that can produce potential independent ramets. For the reason of this life history trait, low genetic diversity and gene flow between populations are expected in clonal plants (Eckert *et al.*, 2003). However such theoretical explanations sometimes do not take place in the natural populations (Auge *et al.*, 2001; Paisookasantivatana *et al.*, 2001a).

Genetic variability in Zingiberaceae

Indo/Malayan region is very rich in Zingiberaceous flora (Holtum, 1950). Considering the present distribution of genetic variability, it is only logical to assume that Indo/Malayan region is probably the major center of genetic diversity for *Zingiber*. It may be inferred that geographical spread accompanied by genetic differentiation in to locally adapted populations caused by mutations could be the main factor responsible for variations encountered in cultivated ginger (Ravindran *et al.*, 1994). Among the ginger growing countries, China has the richest cultivar diversity. In India the cultivar diversity is more in the states of Kerala and in the Northeastern regions of India. In ginger variability was reported in all aspects such as morphological, yield attributes etc. (Mohanty and Sharma, 1979). Variability also reported in essential oil content (Mohanty *et al.*, 1990). There are a few reports on evaluation of somaclones for useful agronomic characters Kulkarni *et al.*, (1987), Samsudeen (1996), Babu (1997) reported variation in somaclones for their reaction to *Pythium* infection. Somaclones have significant exploitable variations with regard to various morphological and biochemical characters like, number of tillers per plant, number of leaves per plant, plant height, rhizome yield per plant, contents of oleoresin, dry recovery and fiber content.

Most of the species of *Curcuma* including cultivated ones are triploids reproducing vegetatively by means of underground rhizomes, however, some diploids and tetraploids have been reported which also do not produce or rarely produce fertile seeds due to incompatibility and high pollen sterility (Joseph *et al.*, 1999). Clonally propagated

plants are thought to be comprised low allelic diversity within the species and are always the risk of extinction. Understanding the genetic structures of the species is a prerequisite to undertake any successful conservation programme, because the species that lack adequate genetic variations are at greater risk of extinction and the existing levels of genetic variations and maintenance of these variations are the major issues for plant genetic diversity conservation.

Many turmeric varieties, mainly identified by the locality of cultivation are grown in different states of India. Even though attempts have been made to study the genetic variability in this clonally propagated crop, most of such studies are done with limited number of accessions (Geetha *et al.*, 1987; Philip *et al.*, 1986; Mohanty, 1979; Mukhopadhyaya *et al.*, 1986; Reddy & Rao, 1988; Sasikumar & Sardana, 1989).

An experiment involving 108 turmeric collection from different states were collected and was laid out in a randomized block design with two replications at the NRCS Farm at Calicut (Nirmal Babu *et al.*, 1993). The various genetic parameters were estimated following standard procedures. The analysis clearly indicates that selection for yield taking in to consideration the most important yield attributes such as weight of mother and finger rhizomes, number and weight of primary fingers, number and weight of secondary fingers would be useful as these traits had high heritability and good genetic gain.

Cell and Tissue culture work in Zingiberaceae

Many tissue culture studies initiated during the 1950's were summarized by Gautheret (1959, 1964). Since then meristem and callus cultures of large number of plant species have been successfully worked out and phenotypic variation in callus regenerated plants have been reported (Bajaj, 1986). Variation frequently occurred from callus regeneration or in strains. Many review articles are available on the subject, some of which attempt to explain the causes and find the origin of this variability (Bajaj, 1978; Betell and Ingram, 1979; Larkin and Scowcroft, 1981; Reisch 1983; Orton 1984; Karp and Bright, 1985; Gould, 1986). This variation was termed differently according to

authors. 'Phenovariants' was first proposed by Sibi (1971) as describing the new generated phenotypes. While 'somaclonal variation' was used to be generally accepted for all types of variations coming from *in vitro* tissue culture.

The first variant regenerated plants from somatic tissue were reported before 1970 in tobacco (Mousseau 1970) and sugar cane (Heinz and Mee 1969), while hereditary transmission after selfing was first observed on Lettuce (Sibi 1976).

Micropropagation

Micropropagation is the true-to-type propagation of selected genotypes using *in vitro* culture techniques (Debergh and Read, 1991). One of the main applications of micropropagation is the mass propagation of superior plants. In many instances, conventional propagation is a slow process during which disease and pest problems can limit production. Micropropagation offers the potential to produce thousands, or even billions of plants per year. Micropropagation offers several advantages not possible with conventional propagation techniques. Once established, actively dividing cultures are a continuous source of microcuttings, which can result in plant production under greenhouse conditions, without seasonal interruption.

Micropropagation allows the production of large numbers of plants from small pieces of a stock plant in relatively short periods of time. Depending on the species, the original tissue explant may be taken from shoot tip, leaf, lateral bud, stem or root tissue. Once the explant is placed on an appropriate culture medium, proliferation of buds and adventitious shoots results in tremendous increases in the number of shoots. Subculture of the buds or shoot is repeated until many plants are produced, all having the genetic characteristics of the original specimen. The mean proliferation rate depends on the species treated. Shoots are generally separated every four weeks and transferred onto a fresh proliferation medium.

Micropropagation of ginger through tissue culture was reported by various authors (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980; Pillai and Kumar, 1982; Sato *et al.*, 1987; Ilahi and Jabeen, 1987; Sakamura 1989; Wang, 1989; Saradha and

Padmanabhan, 1989; Balachandran *et al.*, 1990; Huang, 1995 and Sarma and Singh, 1995). Various explants like young sprouts, dormant buds, rhizome bits, meristem, pseudostem etc. were used as explants. The nutrient medium was used mostly MS (Murashige and Skoog, 1962) but some workers also used Smith's (Kassanis, 1967), Schenk and Hildebrandt's (Schenk and Hildebrandt, 1972). The most commonly used growth regulators were cytokinins (BAP and Kinetin) and Auxins (2, 4 -D, IAA and IBA and in one case Decamba). Sharma and Singh (1995) reported high frequency *in vitro* multiplication of disease free clones by culturing small and active buds of ginger on MS medium supplemented with 2 mg^l⁻¹ kinetin and plant regeneration was obtained in MS with 2.0 mg^l⁻¹, Kinetin and 2.0 mg^l⁻¹ NAA.

Babu (1997) tried explants *viz.*, vegetative bud, immature inflorescence, leaf (pseudostem), ovary and anther were tested for their morphogenetic responses on Murashige and Skoog (1962) basal medium supplemented with cytokinins (BAP and Kinetin) and auxins (NAA and 2,4-D). MS basal medium supplemented with auxin (NAA 0-4 mg^l⁻¹) and cytokinin (BAP 0-4 mg^l⁻¹) gave positive response in inducing multiple shoots and roots. NAA alone at high concentration (2-4 mg^l⁻¹) inhibits shoot growth as well as root induction while BAP alone at higher concentration (4 mg^l⁻¹) induced only multiple shoots and rarely roots. The induction of both roots and shoots in the same medium reduces the time taken for cloning considerably.

Vegetative bud explants containing both shoot tip and axillary buds were used for clonal multiplication. Growth regulators, NAA (1.0-4.0 mg^l⁻¹) and BAP (1.0-4.0 mg^l⁻¹) were tried in various combinations. Only 50 percent of the explants could be established while the rest were lost due to contamination. Both NAA and BAP induced multiple shoots and roots in ginger vegetative bud explants. MS medium supplemented with 4 mg^l⁻¹ NAA and 4 mg^l⁻¹ BAP gave the lowest response (40%) for production of multiple shoots or roots while that supplemented with 1 mg^l⁻¹ NAA and 4 mg^l⁻¹ BAP or NAA alone (1 mg^l⁻¹) gave highest culture response (90%). Considering that both induction of multiple shoots and roots are equally important, development of both in a single medium reduces

the time taken for plant development. In such cases MS medium with 1 mg^l⁻¹ NAA alone or 1 mg^l⁻¹ NAA and 4 mg^l⁻¹ BAP was ideal for multiplication of ginger from vegetative bud explants (Babu, 1997).

In ginger, vegetative shoots were produced in seventy percent of the explants when one week old inflorescences were cultured on MS medium supplemented with 10 mg^l⁻¹ BAP and 0.2 mg^l⁻¹ 2,4-D. One or rarely two plantlets developed per axil of the bract in majority of the cases. In about 26 percent of the cultures multiple shoots ranging from 5-25 were induced. These shoots grew into complete plantlets in 7-8 weeks time. However, in twenty percent of the cultures the older flower bud developed and opened into flowers. These are the meristems which were already 'determined' to develop into flowers (Babu *et al*, 1992).

Successful micropropagation of turmeric has been reported by various workers (Shetty *et al.*, 1982; Keshavachandran and Khader, 1989; Winnar, 1989; Raghu Rajan, 1997; Nadgauda *et al.*, (1978); Balachandran *et al.*, 1990; Yasuda *et al.*, 1988; Nirmal Babu *et al.*, 1993, 1997; Salvi *et al.*, 2001; Meenakshi *et al.*, 2001; Shirgurkar *et al.*, 2001; Nirmal Babu and Minoo 2003; Sunitibala *et al.*, 2001; Prathanturarug *et al.*, 2003; Ali *et al.*, 2004 and Praveen, 2005).

Micropropagation of turmeric was first reported by Nadgauda *et al.* (1978). They cultured young vegetative buds excised from CV Duggirala and Tekkurpeta on MS medium supplemented with coconut milk, kinetin and BAP or on Smith's medium supplemented with coconut milk, kinetin, BAP and inositol. These elongated shoots on transfer to White's liquid medium with 2% sucrose developed a healthy root system and could then be transferred to pots and grown in the field. They again sub cultured explants from the shoot region of these newly formed sterile plantlets on the same media to regenerate multiple shoots. The capacity for shoot formation increased after the first subculture and became constant after 3-4 passages. The number of plantlets obtained was also higher if the cultures were incubated for 4 weeks instead of a shorter period. Plantlet

formation by subculture was observed to take place throughout the year without showing the normal dormancy period of field grown plants.

Balachandran *et al.* (1990) reported that rhizome explants cultured on MS medium supplemented with 3 mg l⁻¹ BA gave the highest multiplication rate in all the three species - *C. domestica* var. 'Koova', *C. aeruginosa* and *C. caesia*. Shetty *et al.* (1982) reported 10-12 multiple shoots per explant (clone 15B) when cultured on modified MS medium (pH 5.6) containing sucrose (40 g l⁻¹) and Kinetin (0.5 mg l⁻¹). Keshvachandran and Khader (1989) reported an average of 2.5 and 2.11 shoots in turmeric cv. Co-1 and BSR-1 respectively on MS medium supplemented with 1 mg l⁻¹ kinetin, 1 mg l⁻¹ BA and 40g sucrose/litre. Nirmal Babu *et al.* (1993, 1997) reported micropropagation of turmeric using young vegetative bud explants in MS medium supplemented with BA 1.0 mg l⁻¹ and NAA 0.5 mg l⁻¹ producing 8-10 shoots in 40 days of culture. Sunitibala *et al.* (2001) reported multiple shoots on 1 mg l⁻¹ NAA + 1 mg l⁻¹ Kin or 1 mg l⁻¹ NAA + 2 mg l⁻¹ BA. Meenakshi *et al.* (2001) reported that the explants of 2.5 and 3.0 mm size on MS + 1.0 mg l⁻¹ BA + 0.1 mg l⁻¹ GA + 0.1 mg l⁻¹ NAA were the best for initiation of cultures of turmeric cv. Cuddapah, which on transfer to 0.3 mg l⁻¹ NAA gave healthy roots. Salvi *et al.* (2002) reported higher shoot production on 10 µM BA and 1 µM NAA with agar at 0.4% and 0.6% in turmeric cv. 'Elite'. Further they reported that carbohydrate sources like xylose, rhamnose, lactose and soluble starch were inhibitory and no variation was observed in RAPD analysis. Rahman *et al.* (2004) developed multiple shoots (14.5) on MS medium supplemented with 2.0 mg l⁻¹ BA and rooting was obtained on half strength of MS with 0.2 mg l⁻¹ IBA, which gave 70% survival rate on hardening. Prathanturarug *et al.* (2003, 2005) were able to induce 11 -18 multiple shoots in 8-12 weeks when the bud explants were supplemented with 18.17 µM -72.64 µM TDZ for one week prior to culture on MS medium. The regenerated plants spontaneously rooted. These results were substantiated by Praveen (2005) who also reported higher rate of multiplication when TDZ was used in culture

medium. Salvi *et al.* (2002) reported that MS medium solidified with agar (0.4 and 0.6%) was superior to liquid media for production of multiple shoots in turmeric. Prathanturarug *et al.* (2005) also preferred solid medium for a better response in turmeric

Several workers have been reported micropropagation of cardamom. Regunath and Bajaj (1992) have given a detailed treatment of micropropagation methods in cardamom. Lukose *et al.* (1993) used MS medium containing 20% coconut water, 0.5 mg^l⁻¹ NAA, 0.2 mg^l⁻¹ IBA, 1.0 mg^l⁻¹ of 6- benzyladenine and 0.2 mg^l⁻¹ kinetin. The plantlets are rooted in white's basal medium containing NAA 0.5 mg^l⁻¹ and hardened in soil vermiculite mixture. Other reports include those Priyadarshan *et al.* (1988), Regunnath (1989), Regunnath and Gopalakrishnan (1991), Nirmal Babu *et al.*, 1997 and Pradipkumar *et al.*, (1997). Micropropagation offers tremendous scope for rapid vegetative propagation of elite clones or varieties, elementary systemic pathogens such as viruses. Replanting of senile, seedling raised plantations with selected high yielding clones multiplied through micropropagation can give 5-6 fold increases in the current average productivity of cardamom (Anonymous 1996).

True to type multiplication provides uniform plants with genetic identity. Morphological and chemical uniformity among plants regenerated by micropropagation technique has been reported in various species of medicinal plants, such as shoot tip and axillary buds cultures *Aconitum carmichaeli* (Hatano *et al.*, 1988), shoot tip cultures of *Atractylodes* spp. (Hatano *et al.*, 1990), node cultures of *Gentiana scabra* (Yamada *et al.*, 1991), and stem tip cultures of *Stevia rebaudiana* (Tamura *et al.*, 1984).

Micropropagation and plant regeneration of other Zingiberaceous taxa

Protocols for micropropagation of many economically and medicinally important Zingiberaceous species like *Amomum subulatum* (large cardamom), *Curcuma aromatica* (kasturi turmeric), *C. amada* (mango ginger), *Kaempferia galanga*, *K. rotunda*, *Alpinia* spp. were developed. (Borthakur and Bordoli, 1992; Thomas *et al.*, 1996; Vincent *et al.*, 1992; Geetha *et al.*, 1997; Sajina *et al.*, 1997; Fatima *et al.* 2001; Jose *et al.*, 2002;

Prakash *et al.*, 2004; Chithra *et al.*, 2005). Most people used MS basal media supplements with BA or kinetin 1-5 mg l⁻¹ for multiplication and around 1 mg l⁻¹ NAA for rooting.

Callus induction and Plant regeneration

Multicellular systems are highly organized. An organized tissue such as root, stem, leaf or any other part of plant can be completely changed to a rapidly proliferating undifferentiated mass of cells, called callus, if cultured on nutrient medium containing growth regulators such as IAA, NAA, IBA, 2, 4-D, kinetin etc. Depending upon the plant species and explants type, growth regulators either individually or in combination will result in callus induction. The callus thus produced can be maintained for an unlimited period by subculture or can be grown in liquid media under agitation as cell suspension culture (Narayanaswamy, 1977). Callus is an undifferentiated mass of rapidly proliferating cells. It can be initiated from any part of a plant and cultured indefinitely by sequential subculture. In many plant species callus can be induced to regenerate plants via the production of adventitious shoots (organogenesis) or somatic embryos commonly known as embryoids (embryogenesis). Plantlets raised from callus (somaclones) may show genetic variations.

The earlier reports on callus induction in ginger were those of Pillai and Kumar (1982), Kulkarni *et al* (1987), Sakamura and Suga (1989), Choi (1991b), Malamug *et al* (1991), Kacker *et al* (1993) Ilahi and Jabeen (1992) and Samsudeen (1996).

Babu (1997) reports that in ginger callus was successfully induced in vegetative bud, young leaf, ovary and anther tissues, on MS medium supplemented with various levels (0.5-5.0 mg l⁻¹) of NAA and 2,4-D for induction and proliferation of callus. Though it is believed auxins in general induce callus formation, he reported that only 2,4-D at concentrations ranging from 0.5-5.0 mg l⁻¹ was effective in inducing callus in all explants tried, the best concentration being 3 mg l⁻¹. NAA induced slight amount of callus at higher concentrations of 3-5 mg l⁻¹ only. The explants differed in their ability to form callus. Callus tissue was not of one kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single experiment. In

ginger, the callus was loose, friable and pale yellow in colour. When fresh calli, immediately after its induction, was cultured on MS medium without growth regulators, it resulted in rhizogenesis. MS medium supplemented with 10 mg l^{-1} BAP and 0.2 mg l^{-1} 2, 4-D gave the best morphogenic response. Once the morphogenic path way of the cultures was determined, they continued to show their morphogenic potential in subsequent subcultures even after 2-3 years.

In vegetative bud derived callus, the number of plantlets developed per culture were higher in BAP than in kinetin. MS medium supplemented with 10 mg l^{-1} BAP and 0.2 mg l^{-1} 2, 4-D was the best for leaf derived callus also. The number of shoots per culture was higher in BAP. Transfer to growth regulator free culture medium after initial morphogenesis resulted in enhancement of plant regeneration to 30-60 shoots.

In callus derived from ovary tissues morphogenesis was observed on MS media media supplemented with 5- 10 mg l^{-1} BAP or kinetin and 0.1- 0.2 mg l^{-1} 2,4-D and also with 5 mg l^{-1} BAP or kinetin and mg l^{-1} 2,4-D after four to five subcultures. In both the media white globular heart shaped embryoid like structures were observed in 67-80 percent of the cultures. The embryogenic calli derived from ovary were canalized and produced increasing number of somatic embryos in the subsequent cultures. These somatic embryos also resulted in repetitive embryogenesis and budding resulting in a large number of somatic embryos in a given culture tube (Babu 1997). Thus the ovary was the best among the explants tried for plant regeneration and recovery. This efficient plant regeneration system is ideally suited for *in vitro* selection, *in vitro* mutagenesis and genetic manipulation experiments. Shoots developed from the callus were placed on the revised. MS medium with 1 mg l^{-1} of NAA for rooting identified as ideal for root induction in earlier experiment. An extensive root system developed within five weeks in this medium. Rooting was better when liquid medium was used instead of solid medium.

Callus formation, development of roots and rhizome like structures were reported from excised ginger anthers cultured on MS medium containing 2,4-D and coconut milk (Ramachandran and Chandrashekhara Nair, 1992b). Samsudeen *et al* (2000) reported

regeneration of plantlets from excised anthers. They cultured excised anthers with uninucleate pollen mother cells and pollens were inoculated on revised MS medium supplemented with 0.2-3.0 mg l⁻¹ 2, 4-D after cold treatment. The anthers developed friable callus in solid as well as liquid medium in about 6 weeks. Occasional development of roots were observed in liquid cultures. Their study has shown that solid medium is better than liquid medium for ginger anther culture. 2,4-D at 3 mg l⁻¹ was good for both callus induction and callus proliferation giving about 3 g of callus in about 30 days of culture when incubated in light. Calli obtained from ginger anthers were cultured on MS basal medium supplemented with 2,4-D (0 - 0.2 mg l⁻¹) and BAP (0 - 10 mg l⁻¹). MS medium with 2, 4-D at 0.2 mg l⁻¹ and BAP at 10 mg l⁻¹ was the best for organogenesis and plant regeneration.

Several authors reported organogenesis and plantlet formation from the callus cultures of turmeric (Shetty *et al.*, 1982., Nirmal Babu *et al.*, 1997, Sunitibala *et al.*, 2001, Salvi *et al.*, 2001, 2002, Praveen, 2005). Shetty *et al.* (1982) reported 10-12 multiple shoots per explant (clone 15B) when cultured on modified M S medium containing sucrose (40 g l⁻¹) and kinetin (0.5 mg l⁻¹). On transfer to a similar medium, callus was produced. When subcultured and exposed to light, the callus produced several buds that turned green and later developed into plantlets. Salvi *et al.* (2000, 2001) also reported plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants showed variation at DNA level. Variants with high curcumin content were isolated from tissue cultured plantlets (Nadgauda *et al.*, 1982). Root rot disease tolerant clones of turmeric cv. Suguna were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum* (Gayatri *et al.*, 2005).

Sunitibala *et al.* (2001) reported callus induction and plant regeneration by organogenesis on MS + 3 mg l⁻¹ 2, 4 -D and 1 mg l⁻¹ kinetin while Salvi *et al.* (2001) induced callusing on MS medium supplemented with 2.0 mg l⁻¹ dicamba or picloram or 5 mg l⁻¹ NAA+ 0.5 mg l⁻¹ BA and plant regeneration on MS with 5 mg l⁻¹ BA. RAPD analysis

of eight regenerated plants showed variation at DNA level indicating somaclonal variation. Praveen (2005) reported efficient micropropagation and plant regeneration from callus using TDZ in two turmeric varieties Prabha and Prathibha with successful induction of both organogenesis and embryogenesis in MS medium supplemented with 0.1 – 0.5 μM TDZ.

The first report of cardamom tissue culture was by Rao *et al.* (1982), who achieved regeneration of plants from callus cultures. Nadgauda *et al.* (1983) achieved a multiplication ratio of 1: 3 when sprouted buds were cultured on MS medium supplemented with BAP (0.5 mg l^{-1}), biotin (0.1 mg l^{-1}) and coconut water (5%). Plantlets were successfully rooted and grown in field. Kumar *et al.* (1985) reported direct shoot formation from inflorescence primordium. When cultured using MS medium containing NAA, Kinetin, and BAP, and they also could get plantlets rooted.

The maximum callus production was reported in MS medium supplemented with 4 mg l^{-1} NAA and 1 mg l^{-1} BAP. This callus on subculturing on an auxin free medium having 3 mg l^{-1} BAP and 0.5 mg l^{-1} kinetin started caulogenesis; each culture producing six-nine meristemoids, and they on culturing on the same medium produced shoots within 28 days. Coconut water (15%) enhanced caulogenesis. Nirmal babu *et al.*, (1997).

An efficient system for callus regeneration is essential to produce large number of somaclones and such a system has been reported earlier by Rao *et al.* (1982) and was also standardized at IISR (Ravindran *et al.*, 1997). High amount of variability was noticed among the somaclones for morphological characters in the culture vessel itself (Ravindran *et al.*, 1997).

Induction of Microrhizomes

Microrhizomes can be produced *in vitro* independent of seasonal fluctuation like induction of tubers and bulbs in culture. (Abbott & Beleher, 1986; Sharma & Singh, 1995; Nayak & Sen, 1995; Haque *et al.*, 1996). These organs serve as planting material, stored and sown like seeds (Bhat *et al.*, 1994). Microrhizomes are very useful for transport, field delivery and help in improved field stand of plants and hence, can be

profitable from commercial point of view as has been seen in the application in germplasm storage, conservation and exchange programme

One of the most important factors is the carbon source in the medium; both its type and concentration have profound effect on tuber growth. Sucrose is considered to be the optimal carbon sources compared to its constituents hexoses – glucose and fructose (Dodds *et al.*, 1992; Khuri and Moorby, 1995). using radiolabel led sugars, Khuri and Moorby (1995) demonstrated that more sugar is translocated to microtubers when sucrose, rather than glucose or fructose, is the carbon source. The reported optimal concentration of sucrose ranges from 60 to 80 g/l (Abbot and Belcher, 1986; Dodds *et al.*, 1992; Garner and Blake, 1989, Hussey and Stacey, 1984). A too high or too low concentration of sucrose leads to slower tuberization and fewer and smaller microtubers.

Sucrose may play a dual role in microtuber development. Apart from being a suitable carbon source that is easily assimilated by the microplants and converted to starch in developing microtubers, sucrose at a concentration of 80g/l, also provides a favourable osmolarity for microtuber development (Khuri and Moorby, 1995). In many other reports it is also stated that a high concentration of sucrose remarkably promotes the *in vitro* formation of storage organs such as bulbs, corms and tubers (Abbot and Belcher, 1986, Alizadeh *et al.*, 1998, Arora *et al.*, 1996; Dantu and Bhojwani, 1987, Garner and Blake, 1989, Gopal *et al.*, 1998, Grewal, 1996, Haque *et al.*, 1996, Kim *et al.*, 2003, Slabbert and Niederwieser, 1999 and Vreugdenhil *et al.*, 1998).

In potato, Garner and Blake (1989) found full strength MS medium supplemented with 8% sucrose better than those supplemented with 4 and 12 % sucrose. The longest storage of microtubers of potato varies from 16-18 months and a large number of potato collections are being maintained in the form of microtubers (Thieme, 1992). More and heavier microtubers were produced when culture period was 70-90 days than 60 d (Yiem *et al.*, 1990).

In vitro rhizome formation and their germination in ginger have reported by various workers (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994;

Sharma and Singh, 1995; Babu, 1997 Tyagi, *et al.*, 2006). Sharma and Singh (1995) reported that 7.5% concentration of sucrose was required for *in vitro* rhizome induction in *Zingiber officinale* Rosc. Tyagi, *et al.*(2006) reported the feasibility of slow growth methods (chemical and physical) by studying the effects of various concentrations of maleic hydrazide and light treatment on *in vitro* rhizome formation and conservation in Rio de Janeiro. *In vitro* rhizomes are conserved for more than 12 months on the same medium and successful recovery of plantlets from these rhizomes after *in vitro* conservation (Tyagi, *et al.*, 2006).

In vitro microrhizomes of turmeric has been reported by Shirgurkar *et al.*, (2001), Sunitibala *et al.*, (2001), Sanghamitra Nayak, (2000), Raghu Rajan (1997), Geetha, (2002), Islam *et al.* (2004). Raghu Rajan (1997) developed a method to induce microrhizomes at the base of the turmeric plantlets. He reported MS medium supplemented with 0.3 mg^l⁻¹ BAP, 0.1 mg^l⁻¹ NAA, 0.5 mg^l⁻¹ ancymidol and 10% sucrose was found to be the best for induction of micro rhizomes. Micro rhizomes harvested at a maturity period of 8 weeks in the induction medium was the best stage for germination under field conditions, without an intervening phase of hardening.

In vitro microrhizome production in four cultivars of turmeric namely, Ranga, Rashmi, Roma, and Suroma was reported by Sanghamitra Nayak (2000). Microrhizomes were produced by transferring them to the MS liquid medium supplemented with 6-benzyl adenine (BA) (1-5 mg^l⁻¹), enhanced concentration of sucrose (50-100g/l) and with reduced photoperiod (0-8 hrs). Microrhizomes were formed at the base of the shoots grown on the medium after 30 days of incubation at 25°C. Concentration of sucrose was found to be most effective in rhizome formation followed by photoperiod and BA in the medium.

Microrhizomes of 1-2.5g fresh weight/explants were induced in turmeric tissue cultures in 8-10 months on MS basal medium supplemented with higher levels of carbon source (12% sucrose) (Geetha, 2002). These microrhizomes could be directly planted in the field and established with 80-100% survival. This eliminated the need of hardening,

saves time and expenses and hence can be directly used by farmers with almost no training. Shirgurkar *et al.* (2001) and Sunitibala *et al.* (2001) reported that 8% sucrose was found to be optimal for *in vitro* microrhizome production. Islam *et al.*, 2004 report that 0.75x strength was most suitable in terms of number and size of microrhizome, however lesser strength of MS medium produced a lower number of smaller rhizome while full strength produced lower number but comparatively larger sized rhizomes.

***In vitro* method to overcome apical dominance**

Apical dominance is a term used to describe the mechanism by which the apex of a shoot inhibits the out growth of secondary or lateral shoots. The apically derived auxin from the dominating shoot moves down to the main stem and then is transported over to the dominated shoot and up to its growing region where growth is repressed. Normally the apical dominance is removed by decapitation of shoot tip which leads to the development of a lateral shoots. Wilson's (2000) recent comprehensive review of apical control in woody plants explores and elucidates various aspects of this phenomenon including dormancy, radial growth, and shoot angle. Sadras and Fitt (1997) found that the stronger the apical dominance, the greater the distance between the plant apex and the nearest vegetative branch.

The role of auxin in apical dominance appears to be indirect and may well involve the action of other hormones (including cytokinins from the roots), signals and /or nutrients (Cline, 1994, 1996; Napoli, Beveridge, and Snowden, 1999, Shimizu-Sato and Mori, 2001). The apical bud produces the growth hormone auxin, which not only promotes cell division, but also diffuses downwards and inhibits the development of lateral bud growth, which would otherwise compete with the apical tip for light and nutrients. Removing the apical tip and its suppressive hormone, allows the lower dormant lateral buds to develop and the buds between the leaf stalk and stem produce new shoots, which compete to become lead growth. Manipulating this natural response to damage (known as the principle of apical dominance) by process such as pruning allows the horticulturist to determine the shape, size and productivity of many fruiting trees and

bushes. Although the involvement of branch orientation, diameter growth, and dormancy/perennial responses in apical control are very significant in many woody species. Hence the scope of this study entails only one aspects of apical control and perhaps the most important branch elongation during one season of growth.

In some species to promote increased seed and/or biomass production (ie, overcompensation) by stimulating lateral branching. The yield increases in herbaceous plants following shoot apex removal either alone or in association with other plant parts, are now common (Clifford, 1979; Tayo, 1980, 1982; Inouye, 1982; Amuti, 1983; Argall & Stewart, 1984; Sheldon 1986; Paige & Whitham, 1987; Aarssen & Turkington, 1987; wein & Minotti, 1988; Benner, 1988; Maschinski& Whitham, 1989; Strauss, 1991; Mopper *et al.*, 1991; Michaud 1991).

Somaclonal Variation

The term ‘somaclonal variation’ has been used by Larkin and Scowcroft (1981) and refers to the phenotypic and genotypic variation observed in regenerated plants from any form of cell culture. When an explant, any plant segment, is subjected to a tissue culture cycle it might be possible to obtain a somaclonal variant (D’Amato, 1978, Rani *et al.*, 1995; Hashmi *et al.*, 1997). This cycle includes establishment of a dedifferentiated cell or tissue under defined conditions and subsequent organogenesis and regeneration of plants (Hammerschlag, 1992). The genetic variation has been shown to originate either from the original explant or from exposure to a tissue culture cycle (Skirvin and Janik, 1976) and is often heritable (Larkin *et al.*, 1984).

Besides somaclonal variation, another source of variations observed in plants regenerated from tissue culture concerns variations from epigenetic origin. Epigenetics refer to modifications in gene expression brought about by heritable, but potentially reversible, changes in chromatin structure and/or DNA methylation (Henikoff and Matzke, 1997).

Factors influencing somaclonal variation

The source of somaclonal variation has not been completely explained and the degree and frequency of genetic changes *in vitro* is uncertain (Phillip *et al.*, 1990). Variation is conditioned by source of material, plant genotype, kind of explant, cell segregation of primary explant, development, organogenesis (D'Amato, 1975; Drew, 1997) and number of subcultures (Skirvin and Janik, 1976). All of these factors are correlated with time of culture and culture media (D'Amato 1964; Evans and Gamborg, 1982).

The importance of culture medium and particularly the exogenous application of plant growth regulators (PGRs) have been discussed (Terzi and LoSchiavo, 1990). The embryogenic potential of explants is closely associated with the content of PGRs, the balance and concentration among naturally produced and exogenous applied PGRs. Due to the complexity and sophistication of the techniques required for PGRs analysis full recognition of these techniques is still in process. Only a few research teams have found links between the embryogenic capacity of plant tissues and a specific endogenous hormonal content (Wenck *et al.*, 1988).

Cytokinins required for shoot induction and multiplication may cause abnormalities such as decreased rooting, stunted or compact plants, increased branching or slender stems and leaves (Karp and Bright, 1985). Albinism is an abnormality that often occurs during *in vitro* propagation. The albino plants might be due to the disorganization of the ribosomal RNAs and plastids *in vitro* and (Torres, 1989). Abnormalities in regenerated plants have been less frequently attributed to auxins as abnormal fertility of the flowers, abnormal petal shapes, decreased vigor, malformed leaves, and increased lateral shoot formation (Bilkey and Cocking, 1981). Other factors that may result in the production of abnormal plants include Gibberellic acid (GA3), temperature, osmotic potential of the medium and agar or gelrite concentration (Torres, 1989).

In general the use of synthetic growth regulators (such as 2,4-D), callus production and long term culture tend to produce genetic as well as epigenetic variations in many species (Larkin and Scowcroft, 1981) Also, the departure from organized growth is a key element in inducing somaclonal variation, suggesting that in disorganized growth the constraints that act to eliminate genetic variations in normal meristems are either suppressed or that mechanisms of genetic instability are induced (Karp, 1995). This may indicate a higher frequency of within clone genetic variation in micropropagated plants than is assumed or generally observed in natural population, particularly with respect to morphological and phenological traits. Chromosome changes are known to occur in high frequency in the early stages of callus or liquid cell cultures, and therefore the occurrence of such abnormalities is not particularly surprising. It is possible, however, to select an appropriate culture medium, which will enhance chromosome stability in the explant, used.

In Poplars (*Populus*), the occurrence of somaclonal variation among tissue culture regenerated plants has been reported for various morphological, physiological, biochemical, disease resistance and herbicide tolerance traits (Fry *et al.*, 1997). Prolonged culture in the presence of synthetic growth regulators is known to induce somaclonal variation (Larkin and Scowcroft, 1981). The frequency of somaclonal variation may be influenced by the regeneration methods employed and tissue source (Ostry *et al.*, 1994

The tissue culture environment may cause a general disruption of cellular controls, leading to numerous genomic changes in the tissue culture derived progeny (Phillips *et al.*, 1994). Stable somaclonal variation may be advantageous for the improvement of certain traits (Karp 1995; Jain *et al.*, 1998).

Somaclonal variation may arise as a result of minor point mutations and rearrangements in nuclear or organellar DNA, the activation of transposable elements, polyploidy, aneuploidy and epigenetic changes (Fry *et al.*, 1997). Poplar somaclonal variation has generally been correlated with changes at the chromosome level; however there is also evidence that Lettuce somaclones may also arise without undergoing major

chromosomal changes (Fry *et al.*, 1997). In poplar somaclonal variation that presumably arose from the addition of a single extra chromosome (aneuploidy) or duplication of chromosome segments arising from breakage and reunion. (Rehman and Rajora, 2001).

Phenotypic changes

Phenotypic variation is expressed principally by changes in morphological characteristics, which can be affected for instance by growth habit, plant height, shoot length, number of primary branches, flower quality, fruit colour, production, uniformity, disease resistance and tolerance to environmental conditions (Skirvin, 1978; Reisch and Bingham, 1981; Evans *et al.*, 1984; Hammerschlag, 1992). This variation in plant regenerates has been reviewed in most of the cases for some herbaceous plants. Some phenotypic changes observed in *in vitro* derived plants include the presence or absence of pubescence, changes in leaf morphology, dwarfs, loss of pigmentation, and alteration in flower morphology. Reduction or enhancement in plant vigour may also occur in plants propagated through tissue culture. Many of these abnormalities appear to have an epigenetic or physiological basis and are therefore reversible. Variegation in leaves may appear in certain species and disappear in others when plants are propagated *in vitro* (Torres, 1989).

Genotypic changes

Somaclonal variation due to karyotypic changes is a widespread phenomenon in plant cell culture, and it might affect plant breeding (Larking and Scowcroft, 1981; Wenzel, 1985). Somaclonal variants in some cases seem to be similar in magnitude to regenerates from cells exposed to mutagenic agents. Although a number of different types of genetic mutations has been described as the basis for somaclonal variation (Larkin and Scowcroft, 1981), the recognition of genetic and epigenetic changes that occurs during *in vitro* culture and on plants derived from these cultures actually remains to be discussed (Reish, 1983). New genome arrangements, aneuploids not separable in culture, mitotic break off that conduces to polyploid lines, genome reorganisation by transposons, amplification or reduction of genes, effects of inversion, trans-location, changes in

chromosome number, chromosomal rearrangements, karyotype variation, prevalence of specific plant karyotype in polysomatic plants or tissues, alterations of the interaction nucleus-cytoplasm have been demonstrated as basis of somaclonal variation (Larkin and Scowcroft, 1981; Karp *et al.*, 1982; McCoy *et al.*, 1982; Orton, 1983). In *P. abies*, an abnormal chromosome number was found in one of two plants presenting dwarf morphology (Fourré *et al.*, 1997). For conifers, these studies suggested that phenotypic variations in regenerated plants from somatic origin could be caused by genetic instability.

Characterization of somaclones

Somaclonal variation can occur for various morphological, physiological, disease resistance and other traits as well as for biochemical and molecular genetic markers (Larkin and Scowcroft, 1981; Karp, 1995; Rani and Raina, 2000). As gross morphological variations are expected to occur at a much lower frequency than cryptic (eg. DNA level) variations (Evans *et al.*, 1984), the absence of visible variation does not preclude the absence of all variations among the micropropagated progeny.

DNA markers are more attractive means for examining somaclonal variation since they are more informative and are not developmentally regulated, but techniques such as random amplified polymorphic DNA (RAPD) suffer from a lack of reproducibility (Riedy *et al.*, 1992; Ellsworth *et al.*, 1993). More over RAPDs are dominant diallelic markers; thus individual parental alleles cannot usually be differentiated by these markers in diploid organisms. There fore, dominant markers, including amplified fragment length polymorphisms (AFLPs), are not quite informative enough for examining somaclonal variation. Co dominant, simply inherited highly polymorphic and reproducible DNA markers are the most suitable markers for detecting somaclonal variation. The sensitivity, reproducibility, Co dominance and strong discriminatory power of microsatellite DNA / SSR (simple sequence repeat) markers (Rajora *et al.*, 2001) make them particularly suitable for detecting somaclonal variation,

but their application in the study of somaclonal variation has been rather quite limited (Wang *et al.*, 1996; Barrett *et al.*, 1997, Chowdari *et al.*, 1998).

The genetic stability or fidelity advisers of *in vitro* produced crop plants are gaining importance (Larkin and Scrowfort, 1981; Goto *et al.*, 1998) with special emphasis on chromosome arrangements (D'Amato, 1978; Whitters, 1989). The characterizations of clone regenerants coming from *in vitro* culture by traditional methods such as morphological descriptions, physiological supervisions and cytological studies have been made. Moreover, biochemical molecular analysis by isoenzymes and electrophoresis techniques to separate molecules has been developed in order to detect genome modifications (Shenoy and Vasil, 1992). However, these analytical approaches have serious limitations because are describing only partially the genetic changes and there are difficulties in assess those changes if any, at the DNA sequence level. In addition the evaluation and interpretation of the results cover many years of research and probes are time dependent. (Rani *et al.*, 1995; Rani and Raina, 1998). The DNA molecule maintains its characteristics permanently because it is not conditioned by the time, irrespectively of its source. The molecular DNA technology by the genetic markers is comparatively less complex, quick to perform and requires only a small amount of probe material. Furthermore DNA extraction protocols are simple, suitable and cover a wide spectrum (Rafalski *et al.*, 1993) Recently several DNA molecular techniques such as Restriction Fragment Length Polymorphic DNA (RFLP) (Shirzadegan *et al.*, 1991), Randomly Amplified Polymorphic DNA (RAPD) (Rani *et al.*, 1995), and DNA Amplification Fingerprinting (DAF) (Vos, 1995) have been introduced and offer several advantages compared with the cytological, biochemical and physiological methods, where the fundamental and practical questions of plant tissue culture remain unsolved (Williams *et al.*, 1990).

Morphological Characterization

It is essential to determine the existence of any somaclonal variation in the regenerants. Morphological variations resulting from somaclonal variation have been

extensively studied for several crop and fruit tree species. For instance, plants showed considerable variation for morphological traits such as flower color and shape, leaf morphology and color, plant height, resistance to disease, and maturity date (Bajaj, 1990; Hammerschlag, 1992). For conifers, phenotypic evaluation might also represent a valuable means for assessing somaclonal variation. In our laboratory, evaluation of ~7000 black spruce (*P. mariana*) and ~4000 white spruce (*P. glauca*) plants regenerated from somatic embryogenesis has been carried out at the phenotypic level within a 5-yr period. For the first time, this work describes in detail several variant phenotypes found in acclimatized spruce plants derived from somatic embryogenesis. Chromosome counts were performed on these phenotypes to see whether, at least at the chromosomal level, genetic instability could be confirmed. Moreover, even if their frequency was low, we were able to correlate statistically the appearance of variant phenotypes with two factors, clone and time in maintenance.

Micropropagated plants from the cultures of preformed structures of such as shoot tips and axillary buds, and from the tissues of hardwood shoot cuttings have been reported to maintain clonal fidelity (Ahuja 1987; Wang and Charles 1991; Ostry *et al.*, 1994). Rani and Raina (2000) reported that the existence of somaclonal variation among micropropagated plants derived through the culture of organized meristem has been shown for various morphological, cytological, biochemical and molecular traits.

Kulkarni *et al.* (1987), Samsuddin (1996) Babu (1997) reported somaclonal variation in ginger. Morphological variations in turmeric somaclones was reported by Praveen (2005). In micropropagated plants of turmeric no variation reported with respect to morphology and RAPD (Praveen 2005). Salvi *et al.* (2003) reported that micropropagated plants from shoot tip showed significant increase in plant height, number of tillers, number and length of leaves, number of fingers and total rhizome weight per plant when compared with conventionally propagated (control) plants. In cardamom somaclones high amount of variation reported by Ravindran *et al.* 1997. Chandrappa *et al* 1997 also reported somaclonal variation in cardamom.

Several authors reported variation in conventional propagated plants of Zingiberaceous crops. Mohanty and Sarma (1979) reported that expected genetic advance and heritability estimates were high for the number of secondary rhizome and total root weight. Genetic coefficient of variation was high for weight of root tubers. Rhizome yield was positively and significantly correlated with number of pseudostems (tillers) leaves, secondary rhizome fingers, tertiary rhizome fingers and total rhizome, plant height, leaf breadth, girth of secondary rhizome fingers and number and weight of adventitious roots. Studies indicated that straight selection was useful to improve almost all characters except the number of tertiaries and straw yield. Rattan *et al.* (1988) reported that plant height was positively and significantly correlated with number of leaves, leaf length, rhizome length, rhizome breadth, and yield per plot. The number of leaves per plant was positively and significantly related with rhizome length, rhizome breadth and yield. The rhizome length was also related with rhizome breadth and yield. Positive correlation of rhizome weight with plant height, number of tillers and leaf number was reported by Sreekumar *et al.* (1980). Mohanty *et al.* (1981) observed significant varietal differences for all the characters except for the number of tillers per plant and number of leaves per plant. Pandey and Dobbel (1993) observed wide range of variability for most of the characters studied by them. Rhizome yield per plant was positively associated with plant height, number of fingers per plant, weight of fingers and primary rhizome.

Sasikumar *et al.* (1992) studied one hundred accessions of ginger germplasm for variability, correlation and path analysis. They found that rhizome yield was positively correlated with plant height, tiller and leaf number, leaf length as well as leaf width. Plant height also had a significant and positive association with leaf and tiller number as well as length and width of leaf. The association of leaf number with tiller number, leaf length and leaf width was also positive and significant. Tiller number had significant negative association with dry recovery. Leaf width had positive significant association with dry recovery.

Yadav (1999) reported high genotypic coefficient of variation for length and weight of secondary rhizomes, weight of primary rhizomes, number of secondary and primary rhizome and rhizome yield /plant. High heritability coupled with high genetic advance as percentage of mean was observed for plant height, leaf length, and suckers per plant, number of mother and secondary rhizomes, weight of primary rhizome and rhizome yield per plant indicating that desirable improvement in these traits can be brought about through straight selection. Plant height followed by number of tillers per plant and leaf length had maximum direct effect on rhizome yield (Singh, 2001).

The partitioning of phenotypic correlation between yield and morphological characters into direct and indirect effects by the method of path coefficient analysis revealed that plant height exhibited a high direct effect as well as high indirect effect in the establishment of correlation between yield and other morphological characters. (Ratnambal 1979; Nair *et al.*, 1980). Rattan *et al.* (1988) indicated that number of leaves per plant had maximum direct contribution to yield per plant, followed by rhizome breadth.

Das *et al.* (1999) reported very high positive direct effects of stomatal number, leaf area, leaf number, and plant height on rhizome yield; leaf temperature, relative humidity of leaves; stomatal resistance and rate of transpiration showed negligible effects. The direct effect of leaf number on rhizome yield was very high (0.631), and this trait is recommended for use as a selection criterion for improving rhizome yield. The study of Pandey and Dobbal (1993) revealed that the strongest forces influencing yield are weight of fingers, width of fingers, and leaf width. Singh *et al.* (2000) grouped 18 cultivars into three clusters under Nagaland conditions based on D^2 analysis. The major forces influencing divergence of cultivars were rhizome yield per plant, oleoresin and fibre contents.

Sasikumar *et al.* (1992) carried out path analysis using 100 accessions of ginger. They reported that plant height followed by leaf length exhibited highest direct effect on rhizome yield. Dry recovery had a negative direct effect on yield. All other direct effects

were negligible. Highest indirect effect was for leaf number through plant height followed by leaf length, again through plant height. In turn, plant height exerted moderately good indirect effect on rhizome yield. Moderate indirect effects were also noticed in the case of leaf width (through plant height, and leaf length and leaf number (through leaf length). However, these authors noticed a residual effect of 0.8217, thereby indicating that the variability accounted for in the study was only 18%. They concluded that plant height should be given prime importance in selection programme as this character had positive and significant correlation as well as good direct effect with rhizome yield.

Biochemical characterization

The recent research on secondary metabolites of ginger such as essential oils, oleoresin, gingerol, shogol and its pharmacological effects described by Schubam and Franz (2000). Singh *et al.* (1999) studied on yield and quality characters in 15 local ginger cultivars. Highest recovery of dry ginger was obtained from cv. RGS3 (22%). Ginger oleoresin was extracted from rhizome with ethanol; isopropanol or liquid CO₂ was analyzed by (Noberga *et al.*, 1997). Ground sun dried Jamaican ginger rhizome was treated with supercritical CO₂ (Kandiah and Spiro, 1990) and the concentrated extract was analyzed by HPLC for [6] gingerol- the major pungent principle of oleoresin.

Babu *et al.* (1997) observed variations among tissue cultured and callus regenerated plants in their oleoresin content, fibre content and dry recovery. The oleoresin content ranged from 1.6 - 7.6%, crude fibre percentage ranged from 3.6 - 6.7 and dry recovery ranges from 21.2 to 31.8% among the tissue cultured plants while the control has 5.2% oleoresin, 5.9 % fibre and 28% dry recovery.

18 ginger cultivars were evaluated for growth, yield and quality in Nagaland during 1991- 92 (Singh *et al.*, 1999) and reported Thinglaidum, Nadia and Rio-de-Janeiro had the best fibre and oil contents. Studies were carried out at 3 sites in each of 7 districts of Nagaland, India Kanjilal *et al.*, (1977) found that the significant differences were observed in growth characteristics and yields components at the various locations. The yield was very low (4.8 to 9.3t/ha) and fibre content high 6.7 to 9.6% at all locations. Oti

et al., (1990) described that the levels of total lipid, total ash, acid insoluble ash, water-soluble ash, cold water soluble extract and alcohol soluble extract decreased with maturity. The crude protein, starch and crude fibre contents of the rhizomes increased with maturity. Essential oils of ginger rhizomes from India and Australia differed markedly in their terpenoid compositions (Erler *et al.*, 1998). A new extraction process for isolation of essential oil from natural substance using liquid and dense carbon dioxide described by Maheswari *et al.*, 1998.

Oleoresin of ginger is the total extract of ginger containing all the flavouring principles as well as the pungent constituents. The oleoresin contains two important compounds – gingerol and shogol - that contribute to the ginger pungency. On long term storage gingerol gets converted to shogol. The quality of ginger thus depends on the relative content of gingerol and shogol. Zachariah *et al.* (1993) classified 86 ginger accession into high, medium and low quality types based on the relative contents of the quality components

There are many ginger cultivars with high oleoresin, a few them like cvs. Rio-de-Janeiro, Ernad Chernad, Wynad, Kunnamangalam, and Meppayyur had high gingerol also. The inter character association showed a positive correlation with oleoresin, gingerol and shogol.

Jogi *et al.* (1972) evaluated 14 cultivars and reported that the fibre content ranged from 4.62% (cv Poona) to 6.98% (cv. Narasapattam). cv. Karakkal was lowest in dry recovery followed by cvs. Wynad local and Rio-De-Janeiro. Cv. Rio had the highest oleoresin, while cv. Karakkal had the highest oil. Crude fibre was least in cv. Nadia and China.

Nybe *et al.* (1980) evaluated 28 cultivars and reported that cvs. Rio-de-Janeiro and Moran had the highest oleoresin content, 10.53 and 10.05% respectively. Essential oil was highest in Karakkal (2.4%) and crude fibre was highest in Kuruppumpadi (6.47%). Sreekumar *et al.* (1980) found that the dry ginger recovery ranged from 17.7% in cv. China to 28.0% in cv. Tura. Cultivars having more than 22% dry recovery (cvs. Moran,

Jugijan, Ernad Manjeri, Nadia, Poona, Himachal Pradesh, Tura and Aripa) are suitable for dry ginger production.

Oil composition of fresh and dried ginger rhizomes of Nigeria was investigated by means of a combination of column chromatography, high resolution GC and GC-MS (Ekundayo *et al.*, 1998). The essential oils contained mainly mono and sesquiterpenoids of which geranial, neral, 1,8-cineole, Zingiberene, β -sesquiphellandrene were the major components. Rhizomes of *Z. officinale* were sliced dried at 4 temperatures, viz 41°C, 46 °C, 54°C and 64.5°C. The drying temperature had no effect on essential oil yield which ranged from 0.64 to 0.89% on dry weight basis (Maia *et al.*, 1991). Volatile essential oils of fresh ginger rhizomes were gamma- irradiated (60 Gy) for sprout inhibition and non-irradiated control rhizomes were isolated by a simultaneous distillation extraction technique described by (Variyar *et al.*, 1997). Major compounds present in these oils are Zingiberene, β -sesquiphellandrene and curcumene which are analyzed by GLC and GC/MS. Chromatographic analysis of pungent compounds of essential oil of ginger extracted by cold press processing was described by Zhang *et al.*, 1993). The major component of essential oil extracted by cold press processing were mono and sesquiterpenes, but other pungent compounds detected were shogaols, methylated shogaols and Zingerone. Miyazawa and Kameoka (1988) analyzed 72 components were α zingiberene, Geranial, Geraniol, β -Bisabolene, Nerol, 1,8-Cineole, α -Terpineol, Boreanol, β -Phellandrene, Linalool, Methyl nonyl ketone and camphene. 3 ginger oils obtained by different extraction methods were analysed (Yu Zhen *et al.*, 1998) quantitatively and qualitatively by GC/MS. In the 3 oils 46, 50 and 61 compounds were identified. The main components of steam- distilled oil were monoterpenes, and sesquiterpenes, the pungent components were not found. Besides sesquiterpenes, the other oil contained mainly the pungent components; the total content was 18.61 % in the cold pressed ginger oil and 23.09% in the supercritical CO₂ extracted ginger oil.

Essential oil, extracted from the rhizomes of ginger, was analyzed (He-Wenshan *et al.*, 2001) by GC-MS 35,36 and 44 constituents were identified respectively from the

methanol, ethyl acetate and hexane extracts. Principal constituents detected in 3 extracts were terpenes, but the composition and contents of the terpenes were different. Chairgulprasert *et al.* (2005) reported the chemical constituents of essential oil and antibacterial activity of *Zingiber wrayi* var. *halabala*. Essential oils of 9 *Zingiber officinale* cultivars were evaluated using GC by (Gopalam *et al.*, 1989). The essential oil yield ranged from 1.5 to 2.2% and the gingerol content of oleoresin varied from 17.7 to 26.67%. Kinetic extractions of the [6] gingerol and hexa hydrocurcumene from ground ginger rhizomes were studied at 30° C (Spiro *et al.*, 1990). Rates were proportional to 0.6, showing the order aqueous aceton > dichloromethane > ethanol > isopropanol. The extraction kinetics of [6] gingerol, 1-(4'-OH-3'methoxy phenyl)-5-OH decan -3-one was investigated by (Spiro *et al.*, 1989). The results are discussed in relation to the industrial production of ginger oleoresin. Low contents of monocyclic terpenes and bicyclic monoterpenes were observed in spring and summer shoot essential oil; higher content of these constituents were observed in oils obtained in the autumn and winter. The oleoresin content of the rhizomes ranged from 4 to 8 % during the year, reaching a maximum in June (Gengaini *et al.*, 1995).

Turmeric (*Curcuma longa* L.) rhizome contains volatile oil, pigments, fixed fatty oil, bitter principles resin, protein cellulose, pentosans, starch, mineral elements etc. Turmeric is valued principally for its yellow orange colouring powder, which is determined by the contents of its curcuminoid pigments (Purseglove *et al.*, 1981) However the presence of a significant quantity of volatile oil, which imparts a distinctive aroma and flavour, necessitates classifying turmeric as a spice rather than purely as a colouring agent (Wilhelm, 1958; Hengstenberg, 1958; Jellinek and Cremer, 1958). The pigment and volatile oil contents can differ considerably between cultivars of turmeric, but that a high pigment is not always associated with a high volatile oil content or vice versa (Rama Rao *et al.*, 1975; Krishnamurthy *et al.*, 1975, 1976; Mathai, 1976). Krishnamurthy *et al.*, (1975) have found that the pigment content of turmeric increases to a peak and then declines during the maturation of the rhizome, and that the optimum time for harvesting can differ according to the particular cultivar and the

location of cultivation. They also found that the various methods of curing the rhizome seem to have little effect on the content of the pigment or the volatile oil.

The first report of the chemistry of the pigments of turmeric was published in the early nineteenth century (Vogel and Pelletier, 1815) but the major pigment curcumin was isolated much later (Daube, 1870; Iwanof – Gajewsky, 1870). The molecular formula of $C_{21}H_{20}O_6$ for curcumin was proposed by Ciamician and Silber (1897) and its structure was finally elucidated by Milobedzka *et al.* (1910) as diferuloyl methane. Srinivasan (1953) opined that the pigment extract was comprised of a mixture of analogues in which curcumin is the major component and the structures of the two main pigments accompanying curcumin were desmethoxy curcumin, and that all three compounds exist in the trans- trans keto – enol form.

Curcumin (diferuloylmethane) is a major yellow pigment that has been isolated from the ground rhizome of the *Curcuma* species. Seven major species of *Curcuma* including *Curcuma longa* Linn., *C. xanthor-rhiza* Roxb., *C. wenyujin* (Y.H. Chen et C, Ling); *C. sichuanensis*; *C. kwangsiensis*; *C. aeruginosa* Roxb.; and *C. elata* Roxb. have been cultivated in China and their composition of curcuminoids were analyzed (Chen and Fang, 1997). Three major curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxycurcumin occur naturally in these *Curcuma* species. The contents of curcuminoids of these plants vary with the site and cultivation period. It seems that *C. longa* L. (turmeric) has the highest concentration of curcumin as compared to the other species. Turmeric is widely used as a spice and coloring agent in several foods, such as curry, mustard, bean cake, cassava paste and potato chips, as well as in cosmetics and drugs. Another species *C. wenyujin* (Y.H. Chen et C. Ling) has been used for centuries in tradition Chinese medicine to treat a variety of inflammatory conditions, such as hepatitis and bile duct disorders. Curcumin has been demonstrated to have potent antioxidant (Kunchandy and Rao, 1990; Sreejayan, 1994) and anti-inflammatory activity (Huang *et al.*, 1988, 1991, 1997), and to inhibit the carcinogen-DNA adduct

(Conney *et al.*, 1991) and tumori-genesis in several animal models (Huang *et al.*, 1992, 1994, 1995; Rao *et al.*, 1995).

The various methods have been published for the determination of the colour value of turmeric Salzer (1975) and Malingre (1975). The curcumin is generally determined by spectrophotometric method (American Spice Trade Association, 1968). Jentzsch *et al.*, (1970) have described also a thin layer chromatography/spectrophotometric method to estimate the relative abundance of the individual pigments.

The aroma and flavour of turmeric are determined by the composition of its steam volatile oil. The yield of oil obtained on distillation and its physicochemical properties can also vary between individual samples (Gildemeister and Hoffmann, 1956; Khaliq and Das, 1968; Krishnamurthy *et al.*, 1976). The differences can arise from a number of factors, which include the origin, and the method of curing the spice, its age, and the condition of distillation and the stage of maturity of the rhizome at harvest.

The essential oils obtained by hydrodistillation from the fresh rhizomes of two endemic species of *curcuma* viz. *C. haritha* and *C.raktakanta* were studied by GLC analysis. Eleven components were identified from *C. haritha* of which camphor (21.24%) was the major component and ten components were identified from *C. Raktakanta* of which ethyl p-methoxycinnamate (16.57%) was the major component. A – pinene, camphor, terpinyl acetate, turmerone and ethyl p-methoxycinnamate were common to both the species Mathew *et al.*, 2002. Ibrahim *et al.* (1999) reported essential oil components of four *Curcuma* species, *Curcuma mangga*, *C. xanthorrhiza*, *C. aeruginosa* and *Curcuma longa* (collected from Malaysia), were analysed by GC-MS. Myrcene (81.4%) was the most abundant component in the essential oil of *C. mangga*. The essential oil of *C. xanthorrhiza* was made up mainly of sesquiterpenoids of which xanthorrhizol (44.5%) was the major constituent. 1,8-Cineole [eucalyptol] (23.2%) and curzerenone (28.4%) were the predominant constituents of the essential oil

of *C. aeruginosa*. The essential oil of *C. domestica* contained significant amounts of alpha -tumerone (45.3%), linalool (14.9%) and beta -tumerone (13.5%).

Bordoloi *et al.* (1999) collected essential oils, steam-distilled from leaves and rhizomes of cultivated *Curcuma aromatica* (from India), were investigated by GC and GC-MS. About 50 compounds were identified, accounting for more than 85% of the essential oils. The major constituents of the leaf essential oil were camphor (28.5%), ar-turmerone (13.2%), curzerenone (6.2%), 1,8-cineole [eucalyptol] (6%) and alpha -turmerone (2.5%). The rhizome essential oil consisted mainly of camphor (32.3%), curzerenone (11%), alpha -turmerone (6.7%), ar-turmerone (6.3%) and 1,8-cineole (5.5%). Nguyen-Xuan-Dung *et al.* (1995) steam-distilled essential oil from leaves of *C. domestica* (*C. longa*), was analysed by GC and GC-MS. More than 20 components were identified, of which the monoterpenes alpha -phellandrene (24.5%), 1,8-cineole [eucalyptol] (15.9%), p-cymene (13.2%) and beta -pinene (8.9%) were the major ones.

Dried fruit of cardamom contains steam volatile oil, fixed fatty oil, pigments, proteins, cellulose, pentosans, sugars, starch, silica, calcium oxalate and minerals. The major constituent of the seed is starch (up to 50 %) while in the fruit husk it is crude fibre (up to 30%). Cardamom oil is produced commercially by steam distillation of powdered fruits. Volatile oil content is highest in 20-25 before full maturity. Ratio of the two main components, 1,8 cineole and α terpenyl acetate, determine the critical flavour of the oil. Industrial production of cardamom oleoresin is relatively on small scale. Solvent extraction of oleoresin yields about 10 % and its content is depending upon the raw material and solvent used. Cardamom oleoresin contains about 50 – 58 % volatile oil (Purseglove *et al.*, 1981). Govindarajan *et al.*, (1982) reported that oil from freshly separated seeds or from whole capsules is almost identical. Mathai (1985) evaluated 18 export grades (Agmark) of Indian cardamom for their chemical and physical qualities. Vasanthakumar *et al.*, 1989) reported that cardamom at the black seed stage or “karimkai” is ideal for consumption as well as for essential oil extraction. Gobalakrishnan *et al.*, (1989) reported that thrips affected cardamom capsules contained relatively higher,

1-8 cineole. Nirmala Menon *et al.*, (1999) extracted bound aroma compounds from fresh green cardamom and the free volatile oil were isolated with ether pentane (1:1) and the bound compounds with methanol. Noleau and Toulemonde (1987) reported that 122 compounds from cardamom oil cultivated in Costa Rica.

Anatomical characterization

Systematic anatomy of Zingiberaceae has been described by Tomlinson (1956, 1969). Bell (1980) described the vascular pattern of rhizomatous ginger. Chakraverti (1939) reported the occurrence of fugacious cambium in the rhizomes of *curcuma longa*. Shah and Raju (1975) described the general morphology, growth and branching behavior of the rhizomes of Zingiberaceae. Pillai and Sachadeva (1961) studied root apical organization of members of Zingiberaceae. Much species- specific anatomical variations were noted in the genus *Zingiber*. These variations were brought out in a comparative study of ginger and that of its related taxa (Ravindran *et al.*, 1998).

Leaves are isobilateral and hypostomatic. The stomata are tetracyclic in all the species. The first two subsidiary cells are parallel to the guard cells and the rest two lie at right angles. In *Z. officinale*, *Z. roseum* and *Z. macrostachyam* there is a special thickening in the upper and lower sides of the guard cell, but *Z. zerumbet* showed some extra thickening on the corners of subsidiary cells. Stomatal index was higher in *Z. zerumbet*. The guard cells were largest in *Z. zerumbet* followed by *Z. officinale* and *Z. macrostachyam*. In *Z. roseum* the guard cells were shorter and broader.

Raju and Shah (1975) described the structure and ontogeny of stomata of ginger. Here the differentiation of a guard cell mother cell or a meristemoid occurs by an asymmetrical division of protodermal cells. The meristemoid is distinguished from the adjacent protodermal cells by its small size, dense stainability of cytoplasm and less vacuolation. The anticlinal wall of the meristemoid appears lightly stained with PAS reaction than the lateral walls of the epidermal cell and the meristemoid. The epidermal

cell abutting on either side of the meristemoid divides to form a small subsidiary cell each. This epidermal cell shows dense stainability for nuclear DNA. The young lateral subsidiary cells are smaller than other epidermal cells. Later the meristemoid divides to form a pair of guard cells. In epidermal cell, each lying at the polar region of the guard cell, may divide. It occasionally completely abuts the stomatal complex and appears as a subsidiary cell (Raju and Shah 1975). The upper epidermal cells of leaf are polygonal and predominantly elongated at right angle to the long axis of the leaf. In the lower epidermis the cells are polygonal and irregular, except at the vein region, where they are vertically elongated and thick walled. The epidermal cells in the scale and sheath leaves (the first 2–5 leaves above the ground are without leaf blades) are elongated and parallel to the long axis of the leaf. Oil cells in the upper and lower epidermis are rectangular, thick walled and suberized. Unicellular hairs are present in the lower epidermis of the foliage leaves of ginger. Occasionally a hair is present at the polar side of the stomata.

Ginger leaf is amphistomatic. A distinct substomatal chamber is present. Stomata are either diperigenous or tetraperigenous. Occasionally anisocytic stomata are also observed. The subsidiary cells are completely aligned longitudinally with the guard cell. The lateral subsidiary cells may divide to form anisocytic stomata. Occasionally 3–5 lateral subsidiary cells are formed due to further division (Raju and Shah 1975). The guard cells on the foliage leaves are 40.6 μm long while those on the sheath and scale leaves are 28.9 μm long. The stomata on the scale leaves are rarely on the sheath leaves; show pear shaped guard cells with a large central pore. The nuclei of the guard cells are smaller than those in the subsidiary cells. Raju and Shah (1975) also reported the uncommon wall thickening at the polar ends of the guard cells. This wall thickening may be restricted to the outer wall at the polar regions or may also be extended to the common inner cell wall.

Chromosome indexing

Sharma and Bhattacharya (1959) reported the wide spread occurrence of an inconsistency in chromosome numbers in several species of Zingiberaceae including *Z.*

officinale. Sato (1960) carried out karyotypic studies of 24 species belonging to 13 genera and concluded that basic chromosome of the genus *Zingiber* is $x=11$ and that *Z. mioga* having $2n=55$ is a pentaploid. Ramachandran (1969) reported the cytology of 27 species belonging to 11 genera of Zingiberaceae including five species of *Zingiber* (*Zingiber macrostachyum*, *Z. roseum*, *Z. wightianum*, *Z. zerumbet* and *Z. officinale*.) and found a diploid number of $2n=22$ in all species. He found evidences of structural hybridity involving interchanges and inversion in ginger. Mohanty (1970) studied the cytology of Zingiberales. He reported $2n=22$ for *Z. spectabile* and *Z. Cylindricum* and concluded that the genus *Zingiber* appears to be much more correctly placed in Hydrichieae than in Zingibereae.

Ratnambal (1979) investigated the karyotype of 32 cultivars of ginger (*Z. officinale*) and found that all of them possess a somatic chromosome no. of $2n=22$. The common basic numbers in Zingiberales were presumed to be nine, eleven and twelve chromosome (Sato, 1960). According to Mahanty (1970), most of the species in the tribe Zingibereae have chromosome numbers of $2n = 48$ in their somatic complement with characteristic small to minute chromosomes. On the other hand, the *Zingiber* species mostly have $2n = 22$ chromosomes with comparatively larger chromosomes

Venkatasubban (1943) reported a chromosome survey in 38 species of various families of the order Scitamineae but excluding the genus *Zingiber*. Chakravorti (1948B) also reported a chromosome number of $2n =22$ for *Z. officinale*. He opined that a chromosome number of $2n=22$ in *Z. officinale* reported by Takahashi (1930) was most likely due to the presence of two extra fragments as recorded by Janaki Ammal (Darlington and Janaki Ammal, 1945). Studies on meiosis indicated formation of multivalents and these coupled with self-incompatibility were cited as main reasons for lack of seed set (East, 1940; Ramachandran, 1969; Pillai *et al.*, 1978; Ratnambal, 1979; Nair *et al.*, 1980). Pollen fertility is also as low as 35% (Pillai *et al.*, 1978). Pollen fertility increased to 85% in tetraploid (Ramachandran and Chandrasekharan Nair, 1992 a, b) but did not result in seed set.

Induced polyploidy has been tried in ginger for introducing variability, improving pollen and ovule fertility and for improving growth and yield. Ratnambal *et al.*, (1979) reported induction of polyplidy in the cv. Rio-de-Janeiro through colchicine treatment. The tetraploids showed stunted growth and had reduced length and breadth of leaves. However, in this case a stable polyploid line could not be established and all the plants reverted to diploidy in the succeeding generations.

Ramachandran (1982) and Ramachandran and Nair (1992) reported successful production of stable tetraploid lines in cvs. Maran and Mananthody. The polyploids were more vigorous than the diploids and flowered during the second year of induction. The stable tetraploid lines ($2n=44$) had larger, plumpy rhizomes and high yield. However, the essential oil content was lower (2.3%) than the original diploid cultivar.

The somatic chromosome numbers of *Curcuma* species comprising $2n=20,24,28,32,34,36,42,56,62,63$, and 84 which were previously reported by different workers (Apavatjirut *et al.*, 1996; Beltran and Kiew, 1984; Darlington and Wylie, 1955; Sato, 1960; Das *et al.*, 1999; Eksomtramage *et al.*, 1996 and 2002; Nambiar, 1979; Weerapakdee and Krasaechai, 1997). The variation in chromosome number in *Curcuma* demonstrates that this genus comprises both polyploidy and aneuploidy (Eksomtramage *et al.*, 2002).

The chromosomes of all the species of *Curcuma* between 0.6μ and 1.7μ in length. The basic number of the genus is 21. Two tetraploids of *C. aromatica* studied were aneuploids with 86 ($84+2$) chromosomes. $2n=86$ is the highest number observed in the genus *curcuma* (Ramachandran 1961). Among these wide range of chromosome numbers the basic chromosome number $n=21$ is very frequent in *curcuma* with $2n=2x=42$, $2n=3x=63$, $2n=4x=84$ as described by different authors. It is also mentioned that the genetic improvement of this important crop through conventional breeding is handicapped due to incompatibility and high pollen sterility resulting in no seed set (Joseph *et al.*, 1999). In addition to that, detailed karyomorphological studies on this genus were not tried so far, which may be there as on of very small size of chromosomes.

Cytological studies of cardamom revealed the occurrence of different chromosome numbers such as $2n=48$ (Gregory, 1936; Sharma Bhattacharya, 1959) and $2n=52$ (Chakravarty, 1984). Sharma and Bhattacharya noticed variant nuclei with $2n=26, 31, 44$ and 83 chromosomes in *Elettaria cardamomum* and reported the occurrence of polysomaty. Cardamom is considered to be a balanced tetraploid. Allied genera such as *Globa*, *Balbifera*, *Phoemaria*, *Amomum* sps. and *Alpinia* sps. also possess $2n=48$ and are considered to be evolved from a common basic number, $x=12$. According to Chandrasekar and Sampath Kumar (1986), Mysore and Malabar varieties of cardamom possess $2n=50$ and $2n=48$ chromosome respectively and aneuploidy as well as structural alterations in the chromosomes have contributed to the varietal differentiation.

Molecular Characterization

The Random Amplified Polymorphic DNA markers (RAPD) have recently been applied in woody species (Goto *et al.*, 1998) to assess the reproduction of some segments of the genome, as rapid appraisal of tissue-culture-derived plants (Rani and Raina, 1998). They have been shown to enhance breeding efforts in annual and perennial crops (Rafelski and Tingey, 1993). They are also effective for cultivar identification (Ronning *et al.*, 1995b). The DNA amplification with RAPD not requires previous knowledge of natural target DNA sequence. The amplification of random DNA segments is made with single primers (usually 10-mers) of arbitrary nucleotide sequence (Williams *et al.*, 1990). The assay is not radioactive, requires only nanogram quantities of DNA and is applicable to a broad range of species. To perform a RAPD assay a single oligonucleotide of an arbitrary DNA sequence is mixed with genomic DNA in presence of a thermostable DNA polymerase mixed with a suitable buffer and then is subjected to temperature cycling conditions "polymerase chain reaction". The products of the reaction depend on the sequence and length of the oligonucleotide, as well as the reaction conditions. At an appropriate annealing temperature during the thermal cycle the simple primer binds to sites on opposite strands of the genomic DNA that are within an amplifiable distance of each other and a discrete DNA segment is produced. Allelic variation among individuals

is detected as the presence or absence of the multiplication product visualized as a band after PCR and electrophoresis (Rafalski *et al.*, 1993).

The DNA amplification reaction is repeated on a set of DNA samples with several different primers, under conditions that result in several amplified bands from each primer. The presence or absence of this specific product, although amplified with an arbitrary primer, will be diagnostic for the oligonucleotide-binding sites on the genomic DNA. Polymorphic bands are noted, and the polymorphisms can be mapped in a segregating population. Often a single primer can be used to identify several polymorphisms, each of which maps to a different locus. Analysis of variations at the nuclear genome level using RAPD has advantages over RFLP as a single primer produces several loci, covering a larger portion of the genome. According to Jayasankar *et al.* (1998) RAPD have been used to determine

Suja (2002) and Nirmal Babu *et al.* (2003) used RAPD profiles amplified by 11 Operon primers as an index for estimating genetic fidelity of selected 'variants' among micropropagated and callus regenerated plants . They observed differences in RAPD profiles observed in some of the micropropagated plants indicated that micropropagation even without callus phase induced variations in 9 of the 13 plants tested. Similar differences were noticed among 12 out of 15 Callus regenerated plants. In general this indicates there is high amount of variability among the selected micropropagated and callus regenerated plants of ginger and the majority of the morphological variants selected from earlier studies did show variations in RAPD profiles. Earlier studies by Rout *et al.* (1998) indicated that RAPD profiles did not indicated any polymorphism among the micropropagated plants. The observations of Suja (2002) and Nirmal Babu *et al.* (2003) differ with earlier finding. This may be because bigger population size used by the latter to detect the morphological variants first and confirmation of their genetic nature of variation using RAPD profiles. Other workers also reported somaclonal variation in ginger (Kulkarni *et al.*, 1987; Samsuddin 1996; Babu 1997). This may be because of the genetic nature of ginger which has resulted in many varieties and cultivars

even with out sexual reproduction. However the microrhizome derived plants did show high degree of genetc uniformity as expressed by RAPD profiles. Thus the present study indicated that direct micropropagation of ginger results in somclonal variation hence propagation of ginger through microrhizome pathway significantly reduce this variation.

In turmeric Salvi *et al.* (2002) reported that of the 48 plants, two showed variegated leaves in the tillers. The micropropagated plants showed a significant increase in shoot length, number of tillers, number and length of leaves, number of fingers and total fresh rhizome weight per plant when compared with conventionally propagated plants. RAPD analysis of 11 regenerated plants using sixteen decamer primers did not show any polymorphism.

Salvi *et al.* (2003) reported that micropropagated plants from shoot tip showed significant increase in plant height, number of tillers, number and length of leaves, number of fingers and total rhizome weight per plant in the first generation when compared with conventionally propagated (control) plants. Second and third generation of regenerated plants checked under the same field conditions ascertained the stability of character observed in the first generation. RAPD analysis of 10 turmeric plants, each propagated conventionally, regenerated from shoot tips, leaf base callus and immature inflorescence was carried out using 15 primers. Plants regenerated using shoot tips showed uniform banding pattern, whereas, callus derived and inflorescence derived plants showed polymorphism in banding pattern when compared with conventionally propagated plants. Praveen (2005) also reported variations in turmeric somaclones with respect to both morphological as well as RAPD profiles but could not detect any variations among micropropagatd plants. He reported that *in vitro* mutagenesis increased the spectrum of somaclonal variation. He found variability among somaclones and isolated somaclones with short nodded rhizomes.

An efficient protocol for the isolation of high molecular weight DNA from dry powdered samples of turmeric including market samples is described by Remya *et al.* (2004). This will help in PCR based detection of adulteration in marketed turmeric powders. The method involves a modified CTAB (3 per cent) procedure with 2 M NaCl, 0.3 per cent β -mercaptoethanol coupled with purification of DNA in 30 per cent polyethylene glycol (8000). The yield of the DNA obtained from the samples varied from 2 to 4 μ g/g tissue. The DNA obtained from the five different samples were consistently amplifiable (RAPD primers).

Salvi *et al.* (2001) using RAPD analyzed turmeric plants regenerated from leaf base callus and this study of eight regenerated plants using 14 primers when separated on non-denaturing polyacrylamide gels showed 38 novel bands. About 51 bands present in the control were absent in the regenerants. The result indicates that variation at DNA level has occurred during *in vitro* culture.

Molecular markers like RAPD, PCR - RFLP and ISSR polymorphism were used to characterize 96 collections comprising important cultivars, varieties and related genera of cardamom to develop fingerprints and to study the inter-relationships (Nirmal Babu 2005). The study indicated that there are no duplicates in the 100 lines characterized and the Kerala and Karnataka populations are divergent in that they formed two separate clusters in the Phylograme. Eleven species representing 5 major tribes viz., *Amomum subulatum*, *Amomum aromaticum*, *Amomum ghaticum*, *A. microstephanum*, *Amomum involucreatum*, *Alpinia galanga*, *A. purpurea*, *Alpinia mutica*, *Aframomum melegueta*, *Hedychium coronarium* and *Elettaria cardamomum* were also profiled for polymorphism using RAPD and ISSR primers. The phylogram showed that *Elettaria cardamomum* is clustered with *Amomum subulatum* and *A. microstephanum* indicating that *Amomum* is closest to cultivated cardamom among the genera studied (Jayakumar *et al.*, 2005).

Chen *et al.* (1999) used RAPD polymorphism to differentiate within and among *Curcuma wenyujin*, *C. sichuanensis* and *C. aromatica*. It is hard to differentiate between *C. wenyujin* and *C. sichuanensis* from the DNA level. The relationship between *C.*

wenyujin and *C. aromatica* was also analyzed and based on the morphological and chemical data, it is suggested that these two species should be combined into one and that classification based on peduncle central or peduncle lateral may not be right.

Kress *et al.* (2002) studied the phylogeny of the gingers (Zingiberaceae) based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *matK* regions and suggested a new classification. Their studies suggest that at least some of the morphological traits based on which the gingers are classified are homoplasious and three of the tribes are paraphyletic. The African genus *Siphonochilus* and Bornean genus *Tamijia* are basal clades. The former Alpinieae and Hedychieae for the most part are monophyletic taxa with the Globbeae and Zingibereae included within the latter. They proposed a new classification of the Zingiberaceae that recognizes four subfamilies and four tribes: Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijieae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae).

Cao *et al.* (2001) and Sasaki *et al.* (2002) used sequence analysis of Chinese and Japanese *Curcuma* drugs on the 18S rRNA gene and *trnK* gene and the application of amplification-refractory mutation system analysis for their authentication. The botanical origins of Chinese and Japanese *Curcuma* drugs were determined to be *Curcuma longa*, *C. phaeocaulis*, the Japanese population of *C. zedoaria*, *C. kwangsiensis*, *C. wenyujin*, and *C. aromatica* based on a comparison of their 18S rRNA gene and *trnK* gene sequences with those of six *Curcuma* species reported previously. Moreover, to develop a more convenient identification method, amplification-refractory mutation system (ARMS) analysis of both gene regions was performed on plants. The ARMS method for the 18S rRNA gene was established using two types of forward primers designed based on the nucleotide difference at position 234. When DNAs of four *Curcuma* species were used as templates, PCR amplification with either of the two primers only generated a fragment of 912 base pairs (bp). However, when DNA of the purple-cloud type of *C. kwangsiensis* and *C. wenyujin* were used, PCR amplifications with both primers generated the fragment, suggesting that these two were heterozygotes. The ARMS

method for the *trnK* gene was also established using a mixture of four types of specific reverse primers designed on the basis of base substitutions and indels (insertion deletions) among six species, and common reverse and forward primers. *C. phaeocaulis* or the Chinese population of *C. zedoaria*, the Japanese population of *C. zedoaria* or the purple-cloud type of *C. kwangsiensis*, the pubescent type of *C. kwangsiensis* or *C. wenyujin*, and *C. aromatica* were found to show specific fragments of 730, 185, 527 or 528, and 641 or 642 bp, respectively. All species including *C. longa* showed a common fragment of 897-904 bp. Using both ARMS methods, together with information on producing areas, the *Curcuma* plants were identified. This ARMS method for the *trnK* gene was also useful for authentication of *Curcuma* drugs.

Cao *et al.* (2003) used a molecular approach, *trnK* nucleotide sequencing, for identification of six medicinal *Curcuma* - *C. longa*, *C. phaeocaulis*, *C. sichuanensis*, *C. chuanyujin*, *C. chuanhuangjiang*, and *C. chuanzhu* found in Sichuan, China. The *matK* gene (an intron embodied in *trnK* gene) sequence and the intron spacer region of the *trnK* gene have great diversity within these six medicinal *Curcuma* species. There were six single bases substitutions between *trnK* coding region and *matK* region, the 9-bp deletion and 4-bp or 14-bp insertion repeat at some sites of *matK* region in each taxon. These relatively variable sequences were potentially informative in the identification for these six *Curcuma* species at the DNA level.

A phylogenetic analysis of the tribe *Zingibereae* (*Zingiberaceae*) was performed by Ngamriabsakul *et al.* (2003) using nuclear ribosomal DNA (ITS1, 5.8S and ITS2) and chloroplast DNA (*trnL* (UAA) 5[prime prime or minute] exon to *trnF* (GAA)). The study indicated that tribe *Zingibereae* is monophyletic with two major clades, the *Curcuma* clade and the *Hedychium* clade. The genera *Boesenbergia* and *Curcuma* are apparently not monophyletic. Sasaki *et al.* (2004) applied single-nucleotide polymorphism analysis of the *trnK* gene to the identification of *Curcuma* plants. *Curcuma* plants and drugs derived from *Curcuma longa*, *C. phaeocaulis*, *C. zedoaria*, and *C. aromatica* could be identified by the nucleotide differences at two sites and the existence of a 4-base indel on

trnK gene. Thus Single -nucleotide polymorphism (SNP) analysis was developed to identify four *Curcuma* plants.

Sasikumar *et al.* (2004) was developed a PCR based method for detection of extraneous *Curcuma* species contamination in the powdered market samples of turmeric by. The study revealed the presence of *Curcuma zedoaria* samples mixed with true turmeric (*C. longa*) samples.

Xia *et al.* (2005) under took molecular genetic and chemical assessment of *Rhizoma Curcumae* in China. *Rhizoma Curcumae* (Ezhu) is a traditional Chinese medicine that has been used in removing blood stasis and alleviating pain for over a thousand years. Three species of *Curcuma* rhizomes are being used, which include *Curcuma wenyujin*, *Curcuma phaeocaulis*, and *Curcuma kwangsiensis*. Chemical fingerprints were generated from different species of *Curcuma*, which could serve as identification markers. For molecular identification, the 5S-rRNA spacer domains of 5 *Curcuma* species, including the common adulterants of this herb, were amplified, and their nucleotide sequences were determined. Diversity in DNA sequences among various species was found in their 5S-rRNA spacer domains. Thus, the chemical fingerprint together with the genetic distinction could serve as markers for quality control of *Curcuma* species. Pimchai *et al.* (1999) reported association of a few isozymes markers in the identification of some of the early Flowering *Curcuma* species.

RAPD analysis was used to study genetic variability among somaclones in various crops. (Moon *et al.*, 1996; Godwin *et al.*, 1997; Banerjee *et al.*, 1998; Moukadiri *et al.*, 1999; Yang *et al.*, 1999).

Screening for disease incidence

In India serious efforts are being made for conservation of ginger germplasm The major constraints involved in the conservation of ginger germplasm are the two soil borne diseases: rhizome rot caused by *Pythium spp.*(such as *P. aphanidermatum*, *P. myriotylum* and *P. vexans*) and the bacterial wilt caused by *Pseudomonas solanacearum* (*Ralstonia*

solanacearum). Added to this, a virus disease, the leaf fleck virus, is also posing serious problem for conservation. These diseases are extremely difficult to control or prevent under field conditions.

Kulkarni *et al.* (1984), reported isolation of Pythium-tolerant ginger by using culture filtrate as the selecting agent. *In vitro* selection for resistant types to Pythium and Pseudomonas is in progress at IISR using culture filtrates of the pathogen or pathotoxin as the selecting agent (Nirmal Babu *et al.*, 1996a, Dake *et al.*, 1997).

Babu (1997) and Nirmal Babu *et al.* (1996a) observed variations in both micropropagated and callus regenerated plants with respect to their tolerance to *Pythium aphanidermatum* and *Ralstonia solanacearum* (*Pseudomonas solanacearum*) when they were inoculated with 2 rounds of the organism. Eight somaclones showed low percentage of disease incidence (*P. aphanidermatum* infection). Isolation of Pythium tolerant lines were earlier reported in ginger (Kulkarni *et al.*, 1987). Thus somaclonal variation is an important source of variability and can be exploited for crop improvement programmes in ginger.

There are earlier reports of obtaining somaclones resistance to rhizome rot of turmeric through *in vitro* selection. Root rot disease tolerant clones of turmeric cv. Suguna were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum* (Gayatri *et al.*, 2005).

Several attempts have been made to select disease resistance expressed at the whole plant level by using tissue culture systems. Experimental approaches for such selections have been described by Daub (1984), Grout and weathered (1980) and Ingram (1980).

Brettell *et al.* (1980) and Gengenbach and Green (1975) have selected maize callus resistant to the T-toxin produced by race T of *Bipolaris* (*Helminthosporium maydis*), the causative agent of southern corn leaf blight (SCLB)

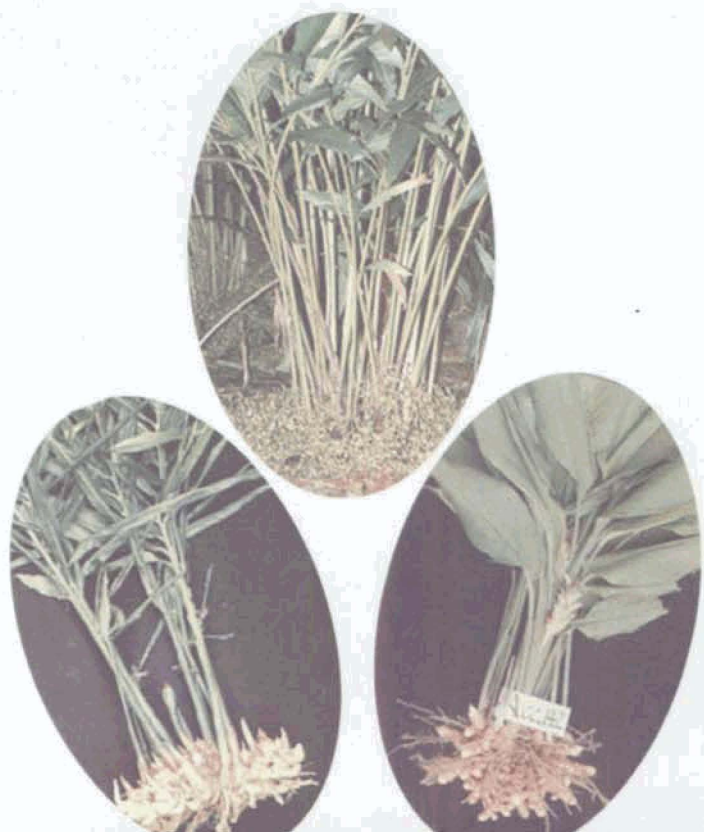
Brettell *et al.* (1980) found that resistance to southern corn leaf blight with a concomitant loss of male sterility was produced in plants derived from callus that had

been in culture for several transfers and that had not been subjected to T – toxin selection pressure (somaclonal variants). A similar event was noted by Larkin and Scowcroft (1983) in the selection of sugarcane plants resistant to eyespot caused by (*Helminthosporium sacchari*). Larkin and Scowcroft (1983) also attempted to select for resistance by using the toxin produced by *H. sacchari*. In this case the toxin was added to the medium used for the last callus maintenance transfer and also to the plant regeneration medium. Regenerated plants showed tolerance to eyespot that was passed through several vegetative propagation cycles. In both the experiments of Brettell *et al.* (1980) and of Larkin and Scowcroft (1983), the recovery of resistant material was greater when toxin was used than when it was dependent only on somaclonal variation.

Behnke (1980) was able to select for potato callus resistant to the crude culture filtrate of *Phytophthora infestans*, the causative agent of late blight of potato. Leaflets from plants regenerated from the resistant callus showed greater tolerance to the culture filtrates and also showed a reduced growth rate of the pathogen when the leaflets were inoculated.

Hartman *et al.* (1984) selected resistance to fusarium wilt of alfalfa in cultures of alfalfa (*Medicago sativa*) using culture filtrates of *Fusarium oxysporum* f. sp. *Medicaginis*. The resistance produced was expressed in cultures placed on inhibitory medium after having been subcultured repeatedly on non inhibitory medium for 5 months. The resistance was also expressed in plants regenerated from the cultures and in callus established for the regenerated plants. Hartman *et al.* (1984), having selected for resistance to a vascular wilt, illustrated that resistance to several types of diseases can be selected in tissue culture. The other reports of development of resistant lines among somaclonal variants and *in vitro* selection are of Behnke (1980), Brettell *et al.* (1980), Carlson (1973), Gengenbach and Green (1975), Gengenbach *et al.* (1977), and Larkin and Scowcroft (1983).

Materials & Methods



Source of explants

Ginger, *Zingiber officinale* Rosc. Cv., Maran, one of the most popular cultivar with high dry recovery (>22%), bold rhizome, high yield and less susceptible to leaf spot was selected as a source material for the present study.

Turmeric, *Curcuma longa* L., variety 'IISR Prabha' (an open pollinated progeny) having high curcumin content (7%) was used as an explant source in the study.

Cardamom, *Elettaria cardamomum* Maton, cultivar Malabar type (CL 37) was used for the study.

Vegetative buds (1.0-2.0 cm) especially apical and axillary buds of fingers were used in the studies.

Culture medium

MS (Murashige and Skoog, 1962) basal medium, the most extensively used medium for plant tissue culture was used in the present study (Table-7).

The chemicals used for micro and macronutrients were obtained from 'Hi-Media', Bombay, while the vitamins and growth regulators were from 'SIGMA' USA.

Carbon source

Sucrose, (Qualigens, Bombay) was used as the carbon source at the concentration of 30g/l. in the experiments.

Growth Regulators

The major auxins namely, α naphthalene acetic acid (NAA), and 2, 4 dichlorophenoxy acetic acid (2,4-D) and the cytokinins; Kinetin, 6 Benzyl adenine (BA) were used in different concentrations to study various aspects such as micropropagation, callus induction and regeneration in cardamom, ginger and turmeric.

Table 7. Composition of Murashige and Skoog* basal medium

Composition	Molecular formula	Concentration (mg l ⁻¹)
Macronutrients		
Ammonium nitrate	NH ₄ NO ₃	1650
Potassium nitrate	KNO ₃	1900
Calcium chloride	CaCl ₂ . 2H ₂ O	440
Potassium orthophosphate	KH ₂ PO ₄	170
Magnesium Sulphate	MgSO ₄ .7H ₂ O	370
Micronutrients		
Sodium EDTA	Na ₂ EDTA	37.3
Ferrous Sulphate	FeSO ₄ . 7H ₂ O	27.8
Boric acid	H ₃ BO ₃	6.2
Manganese Sulphate	MnSO ₄ .4H ₂ O	22.3
Potassium iodide	KI	0.83
Zinc Sulphate	ZnSO ₄ . 7H ₂ O	8.6
Sodium molybdate	Na ₂ MoO ₄ . 2H ₂ O	0.25
Copper Sulphate	CuSO ₄ . 5H ₂ O	0.025
Cobalt chloride	CoCl ₂ . 6H ₂ O	0.025
Vitamins		
Myo-inositol	C ₆ H ₁₂ O ₆	100
Thiamine HCl	C ₁₂ H ₁₇ CIN ₄ OS. HCl	0.1
Nicotinic acid	C ₆ H ₅ NO ₂	0.5
Pyridoxine HCl	C ₈ H ₁₁ NO ₃ . HCl	0.5
Amino acid		
Glycine	C ₂ H ₅ NO ₂	2

*Murashige and Skoog, 1962

Gelling agent

For solidifying the culture medium, Qualigens bacteriological grade agar agar was used at the concentration of 8g/L

Glassware

Borosil culture tubes were used for culture initiation and callus induction. For multiplication culture bottles and 500 ml conical flask were used.

Sterilization of culture medium

The medium and glasswares were sterilized by autoclaving at 121⁰ C for 20 minutes at 16 psi

Plugging the culture vessels

The culture vessels, tubes, bottles and flasks were closed with cotton plugs made of non-absorbent cotton covered with cheesecloth, aluminum foil and polypropylene caps.

Incubation condition

The cultures were incubated at 22⁰ C and were given a photoperiod of 12 hours with a light intensity of 3000 lux, provided by 'Philips' cool white fluorescent tubes.

Distilled water

Double distilled water was used for washing, preparation of stocks, media, buffers etc.

Instruments

Inoculations under aseptic conditions were done in 'Klenzaid's' horizontal laminar flow. Nat Steel horizontal autoclave was used for sterilizing glassware, culture media, stock solutions and other instruments like blades, forceps, and needles.

Nutrient medium

MS (Murashige and Skoog, 1962) medium was used as nutrient medium. Separate stocks were prepared for macronutrients, micronutrients, vitamins and amino acids. Stocks of calcium chloride, ferrous sulphate, Na₂EDTA and glycine were prepared separately. Separate stocks prepared for each of the growth regulators used (Table 8). Sucrose and agar agar, at concentration of 30g/L and 8 g/L respectively were added directly to the culture medium. pH was adjusted to 5.8 before adding agar. The agar was melted to ensure uniform distribution in the medium. The media was autoclaved at 121⁰ C at 16 psi for 20 minutes.

Table 8. Details of various stock solutions for MS medium

Stock	Composition	Stock strength	Quantity / L medium
A	Macronutrients NH ₄ NO ₃ KNO ₃ CaCl ₂ . 2H ₂ O* KH ₂ PO ₄ MgSO ₄ . 7H ₂ O	x 20	50 ml
B	Micronutrients H ₃ BO ₃ MnSO ₄ . 4H ₂ O KI ZnSO ₄ . 7H ₂ O Na ₂ MoO ₄ . 2H ₂ O CuSO ₄ . 5H ₂ O* CoCl ₂ . 6H ₂ O*	x 100	10 ml
C	Micronutrients Na ₂ EDTA* FeSO ₄ . 7H ₂ O*	x 100	10 ml
D	Vitamins Thiamine HCl Nicotinic acid Pyridoxine HCl	x 100	10 ml
E	Amino acid Glycine	x 100	10 ml
F	Myo-inositol Growth regulators NAA IBA 2,4-D BA Kin TDZ	x 100 100 mg/100ml 100 mg/100ml 100 mg/100ml 100 mg/100ml 100 mg/100ml 10 ⁻³ μM	10 ml

* Dissolve separately before mixing in the final stock.

Preparation and surface sterilization of explant

The sprouting cardamom, ginger and turmeric buds were washed in running water and the outer scale leaves were removed. They were then treated with a mixture of wetting agent Tween 20 and copper oxychloride (0.3 %) for 20 minutes in a 250 ml conical flask. This was followed by washing in three changes of sterile distilled water for 3-4 minutes to remove the remnants of the fungicide. Before inoculation, the laminar flow was treated with ethyl alcohol (70 %) for 30-60 seconds, washed again in sterile double distilled water. This was followed by treating the buds with 0.1 % mercuric chloride (HgCl_2) for 15-20 minutes and washed three to four times in sterile distilled water. One more layer of scale was removed and were trimmed to about 1 cm size before inoculating in to culture medium.

Production of Somaclones

Micropropagation

The sterilized buds were cultured on MS medium supplemented with 1mg l^{-1} NAA+ 4mg l^{-1} BA in the case of ginger and turmeric. In cardamom MS + 1mg l^{-1} BA + 0.5mg l^{-1} NAA was used for the establishment of culture from the rhizome bud. These are the most efficient protocol used for micropropagation developed earlier at IISR. The cultures were incubated at 22°C and kept at dark for easy bud breaking.

Callus induction and Plant regeneration

The sterilized ginger rhizome buds cultured for callus induction on MS medium with 2mg l^{-1} 2,4-D and for regeneration the callus were cultured on MS with BA + 2,4-D ($10 + 0.2\text{mg l}^{-1}$). This was the efficient protocol developed at IISR earlier.

Since the existing protocols in turmeric regeneration system did not show consistency in plant regeneration a new protocol was produced. The rhizome buds were inoculated on MS fortified with 2,4-D alone ($0.5 - 1.5\text{mg l}^{-1}$) or in combination with BA ($0.5, 1.0, 2.0, 3.0\text{mg l}^{-1}$) were tried for callus induction and in callus regeneration BA + NAA ($1.0, 2.0, 3.0$ and $4.0 + 0.5, 1.0, 1.5$ and 2.0mg l^{-1}) were used. Three replications were maintained in each treatment

In cardamom MS supplemented 1mg l^{-1} 2,4-D for callus induction and for regeneration MS supplemented with 4 mg l^{-1} BA + 0.5 mg l^{-1} NAA. The cultures were incubated at 22°C and kept in complete darkness.

Induction of Microrhizomes

In ginger the proliferated shoots were separated and cultured on MS basal medium, supplemented with sucrose 6, 8, 10 %. Observations were recorded at 30, 60, 90 and 120 days interval. MS salt concentrations tried for 0.25, 0.5, 0.75 and 1X. Microrhizome induction in turmeric was tried with MS medium supplemented with 5, 7, 9 and 11% sucrose and growth regulators BA ($0.5, 1.0, 1.5\text{mg l}^{-1}$) and NAA (0.5mg l^{-1}). The observation period and MS salt concentrations were same as in ginger. 3 replications were maintained for each treatment.

***In vitro* method to overcome apical dominance**

This experiment was conducted in different culture vessel, 500ml Borosil conical flask, Culture bottle and Tarson's petri plates, containing solid MS medium, 0.8% agar, 30-g l^{-1} sucrose, without the addition of growth hormone. Inoculated the single plantlets in to the same medium were incubated at 25°C , with 12 h of illumination per day. The cultured plantlets with apical dome produce large number of multiple shoots. Rooted plantlets were washed in tap-water and transferred to plastic chambers containing a sterilized mixture of sand and soil (1:1), and covered with a plastic cap that was gradually opened during the acclimatization period of 15 days. Acclimated plants were transferred to the greenhouse and then to outdoor conditions

Subculturing

Subculturing was done at regular intervals to fresh media with same composition. The growth progress was daily observed and data recorded.

Planting out and Hardening

Transfer of plantlets from culture vessels to soil requires a systematic approach. *In vitro* raised plantlets (4-5 weeks old) obtained from subculturing were 5-8 cm tall with 3-5 leaves and 2-6 vigorous primary roots. Hardening cup with drainage holes filled with

potting mixture consist of 2 part river sand, 1 part coir dust. The sticking medium and callus if any were washed off from the *in vitro* plantlets, and planted in the cups. This has covered with a water sprayed polythene bag (12x 24 cm) At three-week stage the plantlets were exposed by removing the polythene cover.

Field planting

Micropropagated, callus regenerated and microrhizome derived plants were planted in pots as well as in filed beds. Preparation of land for planting ginger was started in the month of March with the receipt of early summer showers. All the weeds were collected and removed and clods were broken. The land was then thrown in to raised beds of 3x1 m wide and 15 cm high. Each bed was separated by interspaces of 30 to 45 cm. Each bed containing 40 planting units. Planting was done at the month of June in shallow pits (5 cm deep) dug on the raised beds at a spacing of 22 cm X 22 cm between the pits, which was found to be the optimum for securing the high yield. Bits of seed rhizomes of approximately 20 gm each having at least 2 buds were planted in each pits and covered. Micropropagated and callus regenerated plants of ginger, turmeric and cardamom were used separately for field planting. Microrhizome derived plants of ginger, turmeric were also used for field trials. 10 replicates were maintained for each treatment with the conventionally propagated original cultivar as controls.

Harvesting

The crop was harvested at the month of February. The rhizomes were lifted with *mammatties* and were cleaned of roots and adhering soil particles.

Characterization of somaclones

Morphological characterization

Pot cultured micropropagated, callus regenerated and microrhizome derived plants of ginger; turmeric and cardamom were evaluated for their morphological variations. Conventionally propagated Maran, Prabha and Malabar type (CL 37) were used as control plants. Data of aerial parts were taken four month after planting. Data on number of tillers per plant, number of leaves per tiller, length of the third leaf from the

top, breadth of the third leaf from the top, and inter nodal length were taken as the shoot characteristics. The rhizome characters studied include yield per clump, breadth of finger per rhizome(primary, secondary and tertiary), number of finger per rhizome (primary, secondary and tertiary), number of nodes per finger (primary, secondary and tertiary), inter nodal distance (primary, secondary and tertiary) were recorded at the time of harvesting.

Biochemical characterization

Estimation of Starch (Sadasivam & Manickam, 1992)

Materials used

1. Anthrone reagent
2. 80% ethanol,
3. 52% perchloric acid
4. Standard glucose stock solution: 100mg of glucose 100 ml of distilled water, working standard: 10 ml of stock was diluted to 100 ml with distilled water were used for protein estimation.

Methods used

a. Extraction of starch from sample.

100 mg of sample was weighed and then treated with 80 % ethanol to remove sugars. The sample was centrifuged and retained the residue. The residue was dried over a water bath. Added 0.5 ml of water and 6.5 ml of 52% perchloric acid and centrifuged at 5000 rpm for 20 minutes and save the supernatants. The extraction was repeated using fresh perchloric acid and again centrifuged. Finally the supernatant was made up to 100ml.

b. Estimation of starch

Pipetted out 0.2ml of sample into a series of test tubes and made up the volume of 1ml with distilled water. Added 4 ml of anthrone reagent to each tube. Appropriate standard and blank were also done similarly. Heat all the tubes in boiling water bath for 8 minutes and measured the absorbance at 630 nm.

Extraction of essential oil by hydro distillation (AOAC, 1975)

Materials used

1. Flask (short neck, round bottom type, 1 or 2 liters with S.T 24/40 ground joint)
2. Electric heating mantle,
3. Volatile oil traps (Clevenger type with S.T 24/40 ground joints),
4. West type condenser (400mm in length with water-cooled drip tip and S.T 24/40 ground joint).

Methods used

The samples were weighed and transferred to the flask. Added about 500 ml of water and assembled the apparatus using Clevenger trap. Distilled for 2 ½ to 3 hrs. Volatile oil was collected over water. The distillation rate was maintained of 1- 1½ drops/second. The sample was cooled to room temperature and allowed standing until oil layers was clear and finally the volume of the oil was collected.

Oleoresin by solvent extraction (AOAC, 1975)

Materials used

1. Chromatographic 18mm x 450 mm with stopcock,
2. Beaker
3. Acetone

Methods used

10 g of dry turmeric powder was taken in the glass column. About 70 ml acetone was allowed to percolate into the powder. The apparatus was kept setup overnight. The slurry containing oleoresin and acetone was collected in a pre weighed beaker. The slurry was evaporated over a water bath at 80 °C and finally in an oven at 110 °C for 10 minutes. The dry viscous mass obtained was oleoresin. Percentage oleoresin was computed based on the variation of difference in weight of beaker and initial weight of sample.

Estimation of Curcumin (Sadasivam & Manickam, 1992)

Materials used

1. Flask (flat bottom, 100 ml with TS 24/40 ground joint).
2. Condenser (West type, with water cooled drip tip and TS 24/40 ground joint, 300-400mm in length)
3. Volumetric flask 100ml and 25 ml with TS stopper,
5. Spectrophotometer (capable of accurately measuring absorbance at 425 nm)
6. Ethyl alcohol (95% (or suitable denatured alcohol),
7. Standard curcumin solution

Methods used

Weigh 25 mg of standard curcumin (obtained from East man Kodak company, Rochester) in to a 100 ml volumetric flask. Dissolve and dilute the mark with the alcohol. This standard solution contains 0.0025g/ l.

Weigh accurately 0.1 g of turmeric sample prepared in the extraction flask and add 30 ml of alcohol and boil at 70 °c for 2½ hours. Cool the extract and filter quantitatively in to 100 ml volumetric flask. Transfer the extracted residue to the filter, wash thoroughly and dilute to mark with alcohol. Pipette 20 ml of filtered extract in to 250 ml volumetric flask and dilute to the volume with alcohol. Measure the absorbance of the extract and the standard solution at 425 nm in 1 cm cell against an alcohol blank.

Estimation of total carbohydrate (Sadasivam & Manickam, 1992)

Materials used

1. Phenol 5% (Redistilled (reagent grade) phenol 50 g dissolved in water and diluted to one litre)
2. Sulphuric acid: 96 % reagent grade
3. Standard glucose: Stock 100 mg in 100 ml of water and working standard: 10 ml of stock diluted to 100 ml with distilled water

Methods used

Follow the steps as given in the anthrone method for sample preparation and pipetted out 0.2, 0.4, 0.6, 0.8 and 1 ml of working standard in to series of test tubes. Pipetted out 0.1 and 0.2 ml of the sample solution in two separate test tubes and make up the volume in each tube to 1 ml with water. Set a blank with 1 ml of water and add 1 ml of phenol solution to each tube. Then add 5 ml of 96% sulphuric acid to each tube and shake well. After 10 minutes shake the contents in the tubes and place in water bath 25-30 ° C for 20 minutes and read the colour at 490 nm. Calculate the amount of total carbohydrate present in the sample solution using standard graph.

Determination of reducing sugars (Sadasivam & Manickam, 1992)

Materials used

1. Alkaline copper tartrate (A. Dissolve 2.5 g anhydrous sodium carbonate, 2 g sodium bicarbonate, 2.5 g potassium sodium tartrate and 20 g anhydrous sodium sulphate in 80 ml water and make up to 100 ml. B. Dissolve 15 g copper sulphate in a small volume of distilled water. Add one drop of sulphuric acid and make up to 100 ml. Mix 4 ml of B and 96 ml of solution A before use)
2. Arsenomolybdate Reagent (Dissolve 2.5 g of ammonium molybdate in 45 ml water. Add 2.5 ml sulphuric acid and mix well. Then add 0.3 g disodium hydrogen arsenate dissolved in 25 ml water. Mix well and incubate at 37 ° C for 24 to 48 hours).
3. Standard glucose solution: Stock 100 mg in 100 ml distilled water; working standard: 10 ml of stock diluted to 100 ml with distilled water (100 µg/ ml)

Methods used

Weigh 100 mg of the sample and extract the sugars with hot 80 % ethanol twice (5 ml each time) Collect the supernatant and evaporate it by keeping it on a water bath at 80 ° C. Add 10 ml water and dissolve the sugars. Pipette out aliquots of 0.1 or 0.2 ml to separate test tubes. Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution in to a series of test tubes. Make up the volume in both sample and standard tubes to 2 ml

with distilled water. Pipette out 2 ml distilled water in a separate tube to set a blank. Add 1 ml of alkaline copper tartrate reagent to each tube. Place the tube in boiling water for 10 minutes. Cool the tubes and add 1 ml of arsenomolybdic acid reagent to all the tubes. Make up the volume in each tube to 10 ml with water. Read the absorbance of blue colour at 620 nm after 10 minutes. From the standard graph, calculate the amount of reducing sugars present in the sample.

Estimation of protein (Sadasivam & Manickam, 1992)

Materials used

1. 2% sodium carbonate in 0.1 N sodium hydroxide (Reagent A),
2. 0.5 % Copper sulphate ($\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$) in 1 % potassium sodium tartrate (Reagent B),
3. Alkaline Copper solution: Mix 50 ml of A and 1 ml of B prior to use (Reagent C)
4. Folin – Ciocalteu reagent (Reagent D), Reflux gently for 10 hrs a mixture consisting of 100g sodium tungstate ($\text{Na}_2 \text{WO}_4 \cdot 2 \text{H}_2\text{O}$), 25 g sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot \text{H}_2\text{O}$), 700 ml water, 50 ml of 85% phosphoric acid, 100 ml of concentrated hydrochloric acid in a 1.5 l flask. Add 150 g lithium sulphate, 50 ml water and a few drops of bromine water. Boil the mixture for 15 minutes without condenser to remove the excess bromine. Cool, dilute to 1 l and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1 N NaOH to a Phenolphthalein end point.)
5. Protein solution (Stock standard): Weigh accurately 50 mg of bovine serum albumin (Fraction) and dissolve in distilled water and make up to 50 ml in a standard flask.
6. Working standard: Dilute 10 ml of the stock solution to 50 ml with distilled water in a standard flask. One ml of this solution containing 200 μg protein.

Methods used

- a. Extraction of protein from sample

Extraction is usually carried out with buffers used for the enzyme assay. Weigh 500 mg of sample and grind well with a mortar and pestle in 5-10 ml of the buffer. Centrifuge and used the supernatant for protein estimation.

b. Estimation of protein

Pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard in to a series of test tubes. Pipette out 0.1 ml and 0.2 ml of the sample extract in two other test tubes. Made up the volume to 1 ml in all the test tubes. A tube with 1 ml of water serves as the blank. Add 5 ml of reagent C to each tube including the blank and allowed standing for 10 Minutes. Then added 0.5 ml of reagent D, mixed well and incubated in the dark for 30 minutes. Blue color was developed. The reading was recorded at 660 nm. Drawn a standard graph and calculated the amount of protein in the sample.

Estimation of phenols (Sadasivam & Manickam, 1992)

Materials used

1. 80 % ethanol
2. Folin- Ciocalteau reagent (no dilution)
3. Sodium carbonate
4. 20 % Standard (100 mg catechol in 100 ml) Dilute 10 times for a working standard.

Methods used

The sample was weighed 0.5 to 1.0 g and grinded it with a pestle and mortar in 10 times volume of 80 % ethanol. Centrifuged the homogenate at 10,000 rpm for 20 minutes. Save the supernatant. Re extract the residue with 5 times the volume of 80% ethanol, Centrifuged and pool the supernatants. Evaporate the supernatant to dryness. Dissolved the residue in a known volume of distilled water (5ml). Pipette out different aliquots (0.2 to 2 ml) in to test tubes. Made up the volume in each tube to 3 ml with water and added 0.5 ml of Folin –Ciocalteau reagent. After 3 minutes, 2 ml of 20 % of Na₂ Co₃ solution added to each tube. Mixed thoroughly, Place tubes in boiling water for exactly one minute, cool and measured the absorbance at 650 nm against a reagent blank. Prepared the standard curve using different concentrations of catechol.

Composition of essential oil of *in vitro* microrhizome and *in vivo* rhizome by using GC- MS (Leela *et al.*, 2002)

Oil isolation: *in vitro* microrhizome and *in vivo* rhizome and its leaves were hydrodistilled in a Clevenger type apparatus for 4 hours. The oils thus obtained were dried over anhydrous sodium sulfate and kept in refrigerator at 4°-5°C prior to analysis.

GC/MS analysis: GC/MS data were obtained on Shimadzu QP2010 instrument using RTX wax column (30mx0.25mm, film thickness 0.25µm. Temperature programming: 5 min at 60°C, then rising at 5°C/min to 110°C, then 3°C from 110°-200°C, then 5°C/min to 220°C and maintained at 220°C for 5 minutes. Helium was used as the carrier gas and sample was injected in split mode.

Identification of Compounds: Compounds was identified by comparing the retention indices of the peaks on a RTX wax column with literature values (Bin *et al.*, 1999, Sharma *et al.*, 1997, Mc- Carron *et al.*, 1995, Adams 1989, Jennings and Shibamoto 1980, Swigar and Silverstein 1987, Ramaswamy *et al.*, 1988), computer matching against the library spectra built up using pure substances and components of known essential oils and finally confirmed by comparison of mass spectra of peaks and retention indices with published data (Mc- Carron *et al.*, 1995, Adams, 1989, Jennings *et al.*, 1980, Swigar and Silverstein 1987, Ramaswamy *et al.*, 1988). The relative proportion of each individual component of the oil was expressed as a percentage relative to the total peak area.

Anatomical study of Polyploids

Rhizome anatomy

Rhizomes of normal diploid ginger and polyploid ginger CR 1222 were taken at the time of harvest and fix it in FAA. The hand sections were taken and stained with safranin and mount in glycerin. The photographs were taken by using the camera attached with the Olympus microscope.

Leaf anatomy

Fresh leaf sections of ginger and cardamom somaclones were taken and stained in safranin and mounted on glycerin and observed under the microscope.

Stomatal indexing (Cutler, 1978)

An area of about 1cm square was removed from a central / standard position, always midway between the base and apex, of the mature and fresh leaves of the somaclones. Stomatal preparation was made by boiling these collected specimens of somaclones in different test tubes containing 70% ethanol for 10 minutes. These were allowed to cool and later bleached in 8% sodium hypochlorite solution for 5 minutes. Epidermal peels were stained with 1% ethanol safranin and temporarily mounted in aqueous glycerol solution (Cutler, 1978). Photomicrographs of the epidermal features were taken from the slides using an Olympus microscope fitted with an automatic camera. Ten randomly selected microscopic fields of stomata used for recording the data. The stomatal parameters used are the stomatal size and stomatal density

Histochemical study in turmeric microrhizome

Curcumin cells

Harvested microrhizome from *in vitro* culture at the stage of 120 days were used for studying the development of curcumin in microrhizome compared with the normal rhizome. Fresh sections of *In vitro* derived microrhizome and normal rhizomes of turmeric were taken and treated with diluted alkali to determine the presence of curcumin cells. The sections were mounted in glycerin and observed under the microscope.

Oleoresin and oil

To compare the presences of oleoresin and oil cells in microrhizome with those of normal rhizome. The fresh sections were stained with safranin and temporarily mounted with glycerin and observed under Olympus B x 50 microscope.

Starch grain

The fresh sections of *in vitro* microrhizome and normal rhizome were taken and mounted under iodine-potassium iodide solutions. Fully developed starch grains were observed in the microrhizome developed *in vitro* at the stage of 120 days growth period. The sections were observed under the microscope.

Chromosome indexing

For studying mitosis, root tips were collected from healthy and vigorous plants on bright sunny days between 12.00 to 12.30 am. The root tips were pre treated with 0.002M aqueous solution of 8 hydroxy quinoline for 2 hrs at 4 °C. Treated roots were then washed well in tap water and fixed in Carnoy's fluid. The material was given over night fixation. The fixed roots were washed and heated with 1 N HCl. All the trace of HCl was removed by continuous washing with distilled water. The material was stained with Lacto propionic orcein and kept for 2 hours. The root tips were squashed with the help of needle and observe the stages under the microscope.

Molecular Characterization

A 96 well PCR machine used was Peltir thermocycle MJ Research PTC- 200, USA. The primers were obtained from Operon technologies. DNTP's were obtained from Amersham Pharmasica. Taq polymerase, magnesium chloride and PCR assay buffer were obtained from Bangalore Genei. The 1 KB ladder was from SIGMA, USA. For gel electrophoresis, agarose was obtained from Hi-Media. The centrifuges used for DNA isolation and PCR purposes were from Hitachi and Beckman respectively.

PCR technique

The study was to obtain the RAPD profiles and to use them as an index for the extent of somaclonal variation between callus regenerated plants of cardamom, ginger and turmeric. 10 somaclones of cardamom, 20 somaclones of ginger and 20 somaclones of turmeric were used in this study.

Isolation of genomic DNA

Genomic DNA from different ginger, turmeric, Cardamom accessions were isolated using the modified CTAB method of Ausubel *et al.*, (1995). The procedure is as follows and buffers used for this are given in the Table 9.

1. Grind 4 g of cleaned leaf tissue in liquid nitrogen to a fine powder using pre-chilled mortar and pestle.

2. Transfer the powder to 50 ml Oakridge tube containing 16 ml preheated CTAB extraction buffer. Add 2 μ l of RNase and 1 % 40,000 PVP. Use spatula to dispense the material completely.
3. Incubate the sample at 65° C for 30-60 minutes with occasional mixing by gentle swirling.
4. Add equal volume of Chloroform: Isoamyl alcohol (24:1) and mix by gentle swirling. Spin at 10,000 rpm for 10 minutes at 4°C
5. Transfer the aqueous phase to fresh tubes with cut tips.
6. Add 2/3 volume of Isopropanol and mix by gentle inversions and keep at room temperature for 30 min. Then, scoop the DNA strands into eppendorf tubes containing 1000 μ l of 70 % ethanol. Then, spin at 5000 rpm for 2 - 5min, discard the supernatant and vacuum dry for 20 min and dissolve in 500 μ l of TE buffer. If sufficient DNA strands are not formed, the DNA will be obtained by pelleting as described below.
7. Incubate at 4° C for overnight after adding isopropanol
8. Centrifuge at 10,000 rpm for 15 minutes at 4°C
9. Discard supernatant and add 70% ethanol (7ml) and wash the precipitate by gentle swirling for 3-4 minutes.
10. Spin at 10,000 rpm for 15 minutes at 4° C. Pour off supernatant, invert the tubes for 15 minutes to drain off excess alcohol and leave the pellet to air-dry overnight.
11. To the dried pellet add 500 μ l of TE to dissolve the DNA and transfer the solution in to 1ml sterile microfuge tubes.

Table 9. Preparation of buffers used for genomic DNA isolation (Sambrook *et al.*, 1989)

	Buffer	Method of preparation	Comments
1	CTAB Extraction Buffer: 1 litre 100mM Tris HCl (pH 8.0) 20mM EDTA (pH 8.0) 1.4M NaCl 2 % CTAB (w/v) Merck 0.2 %β -Mercapto ethanol. (v/v) - Merck	Measure 100ml Tris (1M), 280ml of NaCl, 40ml of EDTA (0.5M). Mix with about 400ml of hot distilled water, add 20g of CTAB to this. Adjust final volume to 1 litre. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol.	CTAB will take time to dissolve Avoid foaming.
2	TE (0.1mM) buffer 100ml 100mM Tris HCl (pH 8.0) 0.1mM EDTA (pH 8.0)	Take 1ml of Tris HCl (1M), 20ml of EDTA (0.5M) .Mix with 99ml of sterile distilled water taken in a reagent bottle, mix thoroughly, autoclave.	TE (0.1mM) is written since there is TE with 1mM EDTA also
3	TAE buffer 10x: 1 litre	Weigh 48.4g of Tris base; add 20ml of EDTA (0.5M); 11.42ml of Glacial acetic acid and around 150ml d.water. Dissolve the salt and adjust volume to 1 litre. Autoclave	It will dissolve much easily in 500ml solution
4	Gel loading buffer (6x) 100ml 0.25% Bromophenol blue (Sigma) 30% Glycerol (Merck)	Dissolve 0.25g of BPB in 99ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4 °C	Strong dye, handle carefully.

Purification of DNA

1. Add 3 µg /ml RNase that is DNase free and incubate at 37 °C for 1 hour.
2. Add equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24: 1) and mix well.
3. Spin at 10,000 rpm for 10 minutes at 4 °C and transfer aqueous phase to fresh microfuge tubes.
4. Extract with equal volume of Chloroform: Isoamyl alcohol (24:1), spin at 10,000 rpm and transfer aqueous phase to fresh tubes.

5. Add 1/10 volume of 3 M Sodium acetate (pH 5.2) and 0.8 V of isopropanol and mix gently and incubate at 4 °C for overnight.
6. Centrifuge at 5,000 rpm for 5 minutes
7. Decant supernatant carefully. Wash pellet with 70% ethanol
8. Vacuum dry pellet and dissolve in 500µl of TE

Quantification of DNA

DNA quantification was carried out by using UV Scanning Shimadzu Spectrophotometer. OD at 260/280 nm was recorded and the amount of DNA was calculated based on its OD at 260 nm. 0.8% agarose gel was used for DNA visualization for its quality and stored at -20 °C.

The DNA samples were loaded along with the λ DNA standard and DNA quantity was estimated by comparing with that of the standard.

PCR reaction components.

Template DNA concentration

One of the most appealing features of PCR is that the quantity and quality of the DNA sample to be subjected to amplification do not need to be high. When DNA of known concentration is available, amounts of 50-100 ng are typically used for amplifications of single copy loci (Innis *et al.*, 1990).

Buffer

The standard buffer contains 50 mM KCl, 10 mM TrisHCl (pH 8.4), 1.5 mM MgCl₂, and 100µl/ml of gelatin that will be adequate for the majority of genomic PCR (Innis *et al.*, 1990). 10x buffer from Amersham Pharmacia was used from which we directly added 2.5 µl for each reaction

Deoxy Nucleotide Triphosphates

The four dNTPs – dATP, dTTP, dCTP and dGTP are usually used in concentrations between 20-200µM each that result in the optimal balance among yield, specificity and fidelity. All the four dNTPs were used at equivalent concentrations to minimize incorporation errors (Innis *et al.*, 1990).

Enzyme

The optimal concentration of *Taq* DNA polymerase is about 2U/100µl. As a rule, concentrations in excess of 4 U tend to result in the accumulation of non specific amplification products, whereas amounts less than 1U usually reduce the yield of the desired product (Innis *et al.*, 1990).

Taq polymerase from Amersham Pharmacia Biotech having a concentration of 2 U/µl was used in the study.

Primers

Primer concentrations between 0.1 – 0.5 µM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of non-specific product and may increase the probability of generating a template, independent artifact, termed primer - dimer. Non specific products and primer dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers, resulting in a low yield of the desired product (Innis *et al.*, 1990).

10 arbitrary primers (Operon Technologies Inc. Alameda, California) were used for PCR reaction following the protocol of Williams *et al.*, (1990) with minor modifications. Each of the primer contains at least 60 -70% GC content.

Magnesium ion (Mg²⁺) concentration

The Mg²⁺ concentration may affect primer annealing, strand dissociation temperature of both template and PCR product, product specificity, formation of primer dimer artifacts and enzyme activity and fidelity. *Taq* DNA polymerase requires free Mg²⁺ top of that bound by template DNA, primers and dNTPs. Accordingly, PCRs should contain 0.5 – 2.5 mM Mg²⁺ over the total dNTP concentration. The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent Mg²⁺. Therefore, though the buffer contains magnesium, an additional 0.5 mM solution was also added separately.

Table 10. Stock solutions prepared (Sambrook *et al.*, 1989)

Solutions	Method of preparation	Comments
1M Tris (pH 8.0,) 500ml	Dissolve 60.55gm Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500ml. Dispense to reagent bottles and sterilize by autoclaving.	*pH of Tris solution is temperature dependent. *Tris will completely dissolve only when pH becomes 8. Initial pH will be around 11
0.5M EDTA pH 8.0	Dissolve 93.05g of EDTA-disodium salt (sigma) in 300ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml. Dispense in to reagent bottles and autoclave.	*The salt will take time to dissolve
5M NaCl 500 ml.	Weigh 146.1g NaCl (Merck) add 200ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500ml. Dispense in to reagent bottles and autoclave.	*The salt will take much time to dissolve.
3M Sodium acetate (pH5.2) 250 ml.	Dissolve 61.523g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99- 100%). Dispense to reagent bottles and autoclave.	*The salt will take much time to dissolve.
Ethidium Bromide 10mg/ml, 100ml.	Add 1g. Ethidium Bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. Dispense to amber colored reagent bottle and store at 4°C.	*Ethidium Bromide is a powerful mutagen and is moderately toxic. So handle carefully.
70% ethanol, 500 ml	Take 360 ml. of ethanol; mix with 140 ml of distilled water. Dispense to reagent bottle and store at 4°C.	Stock ethanol is 98% that is why 360 ml is taken instead of 350 ml.
Chloroform: isoamyl alcohol (24:1), 500 ml	Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mix well and store in reagent bottle in room temperature	Chloroform will evaporate, so close the cap tightly.
1M MgCl ₂ , 100ml	Weigh 20.33g of Mg Cl ₂ , dissolve in double distilled water, make up to 100ml, dispense in to reagent bottles.	

Optimization of PCR Programming

584.39

Two temperature profiles are tested for optimizing PCR. They are *sumis*

Reaction I Cycles: 3 Ist cycle: 94 °C for 3 minutes

40 °C for 30 second

72 °C for 1 minute Cycle repeats: 1

IInd cycle: 94 °C for 1 minute

40 °C for 30 seconds

72 °C for 1 minute Cycle repeats: 35

IIIrd cycle: 94 °C for 1 minute

40 °C for 30 seconds

72 °C for 5 minutes Cycle repeats: 1

Reaction II Cycles: 3 Ist cycle 94°C, 2 minutes

37 °C, 1 minute

72 °C, 2 minutes Cycle repeats: 1

IInd cycle: 94 °C for 1 minute

37 °C for 1 minute

72 °C for 2 minutes Cycle repeats: 33

IIIrd cycle: 94 °C for 1 minute

37 °C for 1 minute

72 °C for 15 minutes Cycle repeats: 1

Primer Screening

After optimization of PCR conditions and variables, nineteen, 10 mer primers (Operon Technologies Inc. Alameda, California) i.e., OPA-03, OPA-04, OPA-06, OPA-07, OPA-08, OPA-11, OPA-17, OPA-18, OPB-04, OPB-11, OPB-18, OPC-01, OPC-08, OPC-10, OPC-14, OPC-17, OPC-19, OPD-12 and OPD-20 were screened using control plants of ginger, turmeric and cardamom for amplification and polymorphism. Each of the 10-mer samples were resuspended in 1ml TE (pH 7.0), so that the final concentration was 5 picomoles / μ l. For PCR programming, second reaction was taken.

The reaction mixture for PCR

Components	Volume 1X
Assay buffer (10 X)	2.5 0 μ l
<i>Taq</i> DNA polymerase (2U/ μ l)	0.5 μ l
d NTP's (1mM)	3.75 μ l
MgCl ₂ (10 mM)	1.50 μ l
DNA (10 ng / μ l)	3.00 μ l
Primer (5 pmoles/ μ l)	2.00 μ l
Sterile distilled water	11.75 μ l
Total reaction volume	25.00 μl

Amplification Protocol

The reaction volume of 25 μ l containing, 2.5 μ l PCR buffer (10X), 1.5 μ l MgCl₂ (10mM), 3.75 μ l dNTPs (1mM), 2.0 μ l primer (5 p moles/ μ l), 0.5 μ l *Taq* polymerase (2U/ μ l), 3.0 μ l Template DNA (10 ng/ μ l) and the volume was made up with nuclease free water.

Randomly Amplified Polymorphic DNA*Procedure*

Good quality high molecular weight DNA from the isolated samples was selected for the study. 20 MP, CR and MR plants with 10 primers were used in ginger and turmeric, 10 MP and CR plants with 10 primers were used in cardamom.

The primers were used in ginger OPA 06, OPC 10, OPC 01, OPB 18, OPA 11, OPC 14, OPA 07, OPC 19, OPC 08, OPB 11, (Table 11); in turmeric OPA 03, OPA 04, OPA 06, OPA 07, OPA 08, OPA 18, OPB 11, OPB 18, OPC 10, OPC 19 (Table 13) and in cardamom OPA 04, OPA 17, OPB 04, OPB 18, OPC 08, OPC 10, OPC 17, OPC 19, OPD 12, OPD 20 (Table 15).

NB 5634

Six operon primers were used for comparative study of RAPD profiling of micropropagated, callus regenerated and microrhizome derived plants of ginger (OPA 04,



OPA 06, OPA 10, OPA 17, OPC 01, OPC 08) (Table 12), turmeric (OPA 04, OPA 06, OPA 08, OPC 01, OPC 19, OPA 10) (Table 14) and cardamom (OPB 19, OPC 10, OPC 19, OPD 12, OPA 06, OPA 17) (Table 16).

Reaction II was followed for PCR programming. The PCR reaction mix was prepared as mentioned earlier, containing 10x PCR buffer, dNTPs, Taq DNA polymerase, MgCl₂, and sterile distilled water. This mixture was dispensed into the PCR tubes that are placed in ice. Then, required concentration of the selected primers and template DNA was added.

Table 11. Operon primers used in RAPD analysis and their base sequences (MP, CR and MR ginger separately)

Primers	Sequences
OPC 19	5' GTTGCCAGCC 3'
OPA 06	5' GGTCCCTGAC 3'
OPA 07	5' GAAACGGTG 3'
OPA 11	5' CAATCGCCGT 3'
OPB 11	5' GTAGACCCGT 3'
OPB 18	5' CCACAGCAGT 3'
OPC 01	5'TTCGAGCCAG 3'
OPC 08	5'TGGACCGGTG 3'
OPC 10	5' TGTCTGCGTG 3'
OPC 14	5' TCGTGCTTG 3'

Table 12. Operon primers used for comparative RAPD study (*Micropropagated, callus regenerated and microrrhizome derived plants*) in ginger and their base sequences

Primers	Sequences
OPA 04	5' AATCGGGCTG 3'
OPA 06	5' GGTCCCTGAC 3'
OPA 10	5' GTGATCGCAG 3'
OPA 17	5' GACCGCTTGT 3'
OPC 01	5'TTCGAGCCAG 3'
OPC 08	5'TGGACCGGTG 3'

Table 13. Operon primers used in RAPD analysis and their base sequences (MP,CR and MR turmeric separately)

Primers	Sequences
OPA 03	5' AGTCAGCCAC 3'
OPA 04	5' AATCGGGCTG 3'
OPA 06	5' GGTCCCTGAC 3'
OPA 07	5' GAAACGGGTG 3'
OPA 08	5' GTGACGTAGG 3'
OPA 18	5' AGGTGACCGT 3'
OPB 11	5' GTAGACCCGT 3'
OPB 18	5' CCACAGCAGT 3'
OPC 19	5' GTTGCCAGCC 3'
OPC 10	5' TGTCTGCGTG 3'

Table 14. Operon primers used for comparative RAPD study (*Micropropagated, callus regenerated and microrhizome derived plants*) in turmeric and their base sequences

Primers	Sequences
OPA 04	5' AATCGGGCTG 3'
OPA 06	5' GGTCCCTGAC 3'
OPA 08	5' GTGACGTAGG 3'
OPC 01	5' TTCGAGCCAG 3'
OPC 19	5' GTTGCCAGCC 3'
OPA 10	5' GTGATCGCAG 3'

Table 15. Operon primers used in RAPD analysis and their base sequences (MP and CR cardamom separately)

Primers	Sequences
OPA 04	5' AATCGGGCTG 3'
OPA 17	5' GACCGCTTGT 3'
OPB 04	5' GGA CTGGAGT 3'
OPB 18	5' CCACAGCAGT 3'
OPC 08	5' TGGACCGGTG 3'
OPC 17	5' TTCCCCCAG 3'
OPC 19	5' GTTGCCAGCC 3'
OPD 12	5' CACCGTATCC 3'
OPD 20	5' CACCGTATCC 3'
OPC 10	5' TGTCTGCGTG 3'

Table 16. Operon primers used for comparative RAPD study (*Micropropagated and callus regenerated plants*) in cardamom and their base sequences

Primers	Sequences
OPB 19	5' ACCCCCGAAG 3'
OPC 10	5' TGTCTGCGTG 3'
OPC 19	5' GTTGCCAGCC 3'
OPD 12	5' CACCGTATCC 3'
OPA 06	5' GGTCCCTGAC 3'
OPA 17	5' GACCGCTTGT 3'

Electrophoresis

PCR products were visualized in 2% agarose gel containing 0.5µg/ml ethidium bromide and documented in Alpha imager 2220 gel documentation system.

Disease screening

Medium preparation for Pathogen inoculation

Potato Dextrose Agar- PDA (Johnston & Booth, 1983) composition

Potato – 200g

Dextrose – 20g

Distilled water- 1000ml

Peeled potato were sliced and boiled in 500ml distilled water for half an hour. It was later filtered using muslin cloth. Dextrose was added and extracts made up to 1 liter. And pH of the media was adjusted to 6.0. The media thus obtained was dispensed equally in to 500 ml conical flask at the rate of 200ml/ flask and sterilized.

Inoculum preparation.

Potato dextrose broth (with 20% potato and 2% dextrose) was prepared to raise the pathogen *Pythium aphanidermatum* in the conical flask. The broth was uniformly distributed in the conical flask at the rate of 200ml /flask and sterilized at 15 lb pressure for 20 minutes. Each flask was inoculated with 5 discs of 5mm size taken from 48 hrs old culture of the pathogen grown on PDA. The inoculated culture flasks were incubated for 7 days in the room temperature at 28-30 °C. Later the mycelia removed were washed with

water. The weight of the mycelia (100g) were taken and blended thoroughly. The inoculum was applied at the rate 1g/pot in ginger and turmeric somaclones. After the application the soil was mixed well and added fresh soil, mulched with green leaves to maintain humidity.

Disease scoring

Percentage of disease incidence was recorded by visual scoring and also by statistical analysis.

Scoring of gels

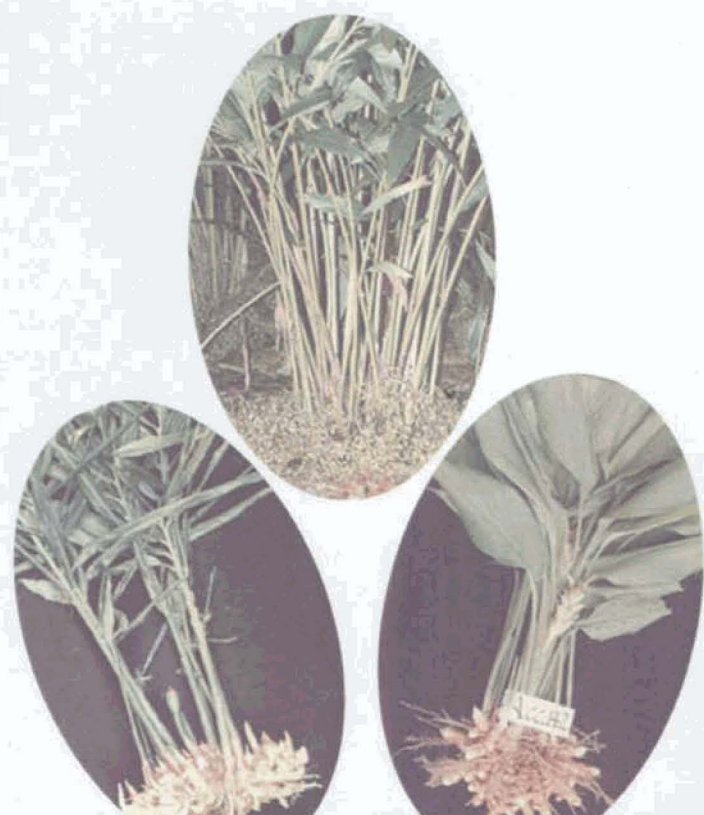
The RAPD profiles were analyzed based on the presence (1) or absence (0) of individual RAPD bands.

Statistical analysis

Analysis of variance (ANOVA)

Analysis of variance (ANOVA) was carried out to test the differences among treatments in the experiments and to test the significance of variation between different types of propagation. The data obtained for each character were analyzed following the randomized complete block design analysis or CRD analysis. MSTATC software was used for the analysis of data. Significant differences were calculated at 5% level according to the least significant difference test (LSD) and Duncan's multiple range test (DMRT).

Results



The objective of the present investigation is to study the utility of *in vitro* technology for creation of somaclonal variation for possible genetic improvement of economically important Zingiberaceous crops like ginger, turmeric and cardamom. All these crops are predominantly vegetative propagated and hence any useful variation can be fixed in the population. The extent of variability is studied using morphological biochemical and molecular markers. Where ever necessary cytological and anatomical data was also obtained.

Ginger

Micropropagation

Vegetative buds of ginger rhizome were inoculated on MS + 1 mg^l⁻¹NAA and 4 mg^l⁻¹BAP for micropropagation as per the earlier protocol developed at IISR (Babu, 1997). On this medium 98.7 % aseptic explants responded and developed multiple shoots. An average of 7.7 shoots per explant with the shoot length of 6.3 cm was obtained within four to eight weeks. The process of micropropagation is given in Fig. 4.

Callus induction and Plant regeneration

Callus was induced in ginger buds on MS medium fortified with 2 mg^l⁻¹2,4-D and regeneration was done on MS medium supplemented with 10 mg^l⁻¹BA and 0.2 mg^l⁻¹2,4-D (Babu, 1997). Activation of the explants was evident from 3 days after inoculation. Culture kept in darkness had better growth and maintenance of calli. 88.7% of the cultures induced callus within two weeks of incubation. The callus was soft and friable and comprised a wide variety of cell shapes and sizes (Fig.5). Differences in growth and differentiation of the callus are examined in ginger in terms of color and fresh weight of the callus tissue. Callus developed rapidly in the presence of 2,4-D. The callus consisted non-embryogenic and embryogenic callus. The embryogenic callus (Fig.5) is carefully collected and cultured separately.

The two-month-old callus derived from vegetative bud was inoculated to the MS + 10 mg^l⁻¹BA and 0.2 mg^l⁻¹ 2,4-D for regeneration. Percentage of shoot production found

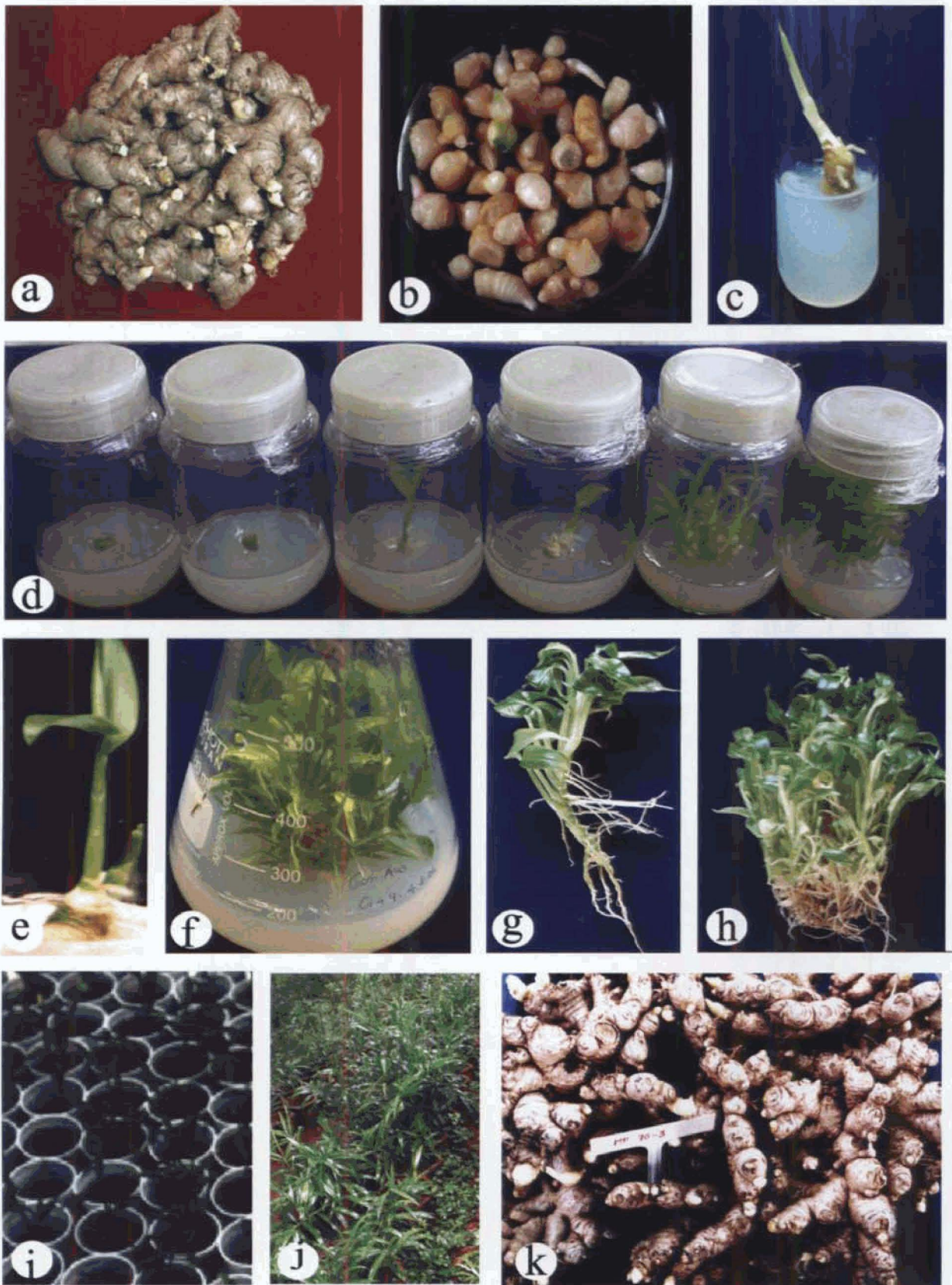


Fig. 4 Various stages in micropropagation of Ginger

a. Sprouting ginger buds b. Explant preparation c. Culture initiation d. Different stages of micropropagation e. Enlarged view of shoot initiation. f. Multiple shoot induction g. Root development pattern h. Multiplication frequency i. Hardening of regenerated plants. j. Field establishment k. Harvested rhizomes of one of the somaclones

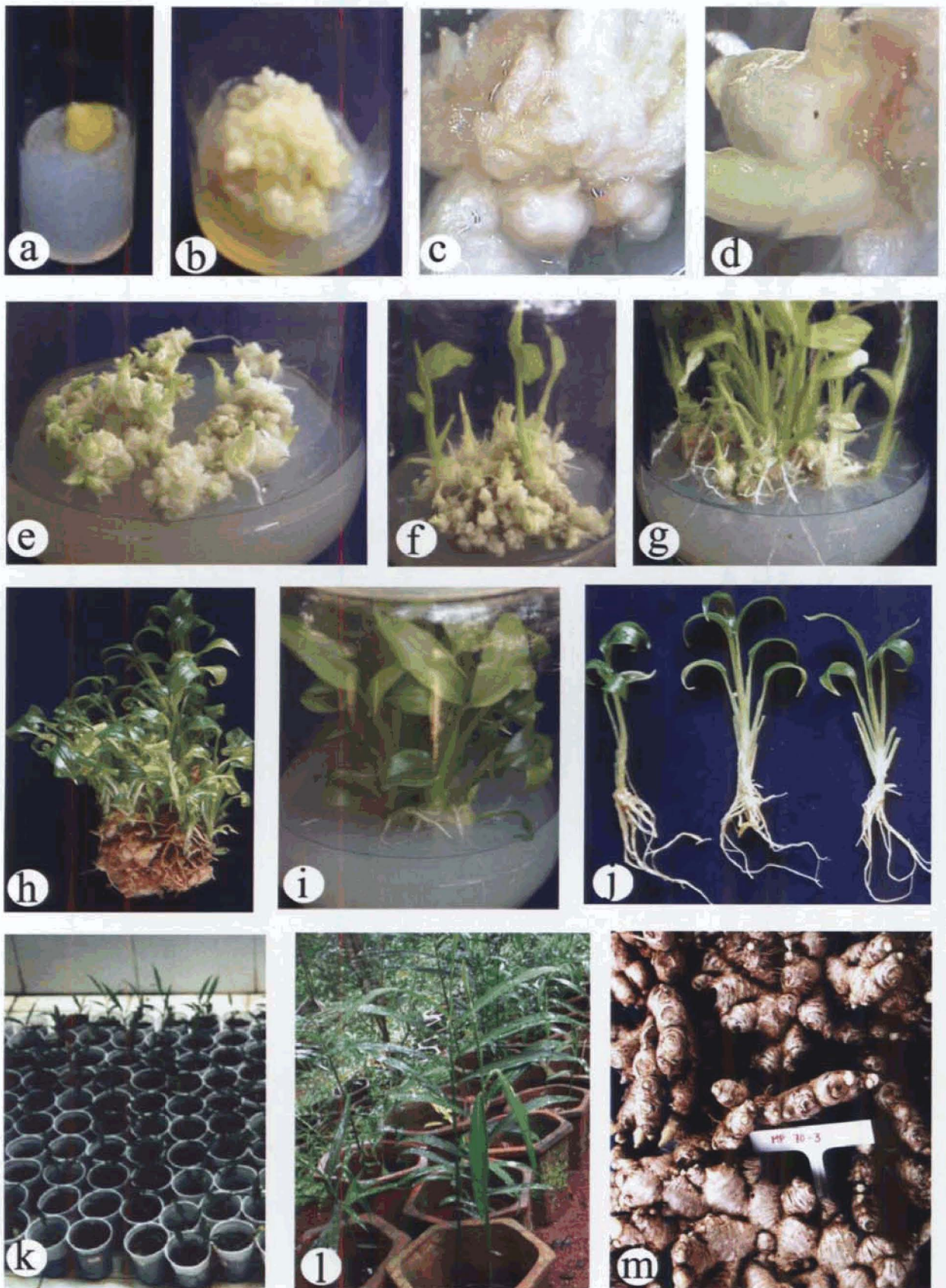


Fig. 5 Plant regeneration from organogenic callus cultures

a. Explant for callus induction b. Non embryogenic friable callus c. Mixed type callus d. Embryogenic callus e. Callus regeneration f&g. Plants regenerated from somatic embryo. h. Callus regeneration frequency (isolated from single culture bottle). i. Regenerated plants multiplied. j. Root development pattern. k. Hardening of regenerated plants. l. Field establishment m. Harvested rhizomes

to be 94.3 in this medium. Where the formation of plantlets also recorded maximum. The root production was also observed on this medium.

Induction of Microrhizomes

The effect of different factors like MS salt, sucrose concentration and duration of the culture period on the production of microrhizome *in vitro* were studied (Table 17). Out of four different concentrations of MS salt tried, 0.75 X is the most favorable salt concentration in which the maximum numbers of microrhizomes were produced (22). The other concentrations of MS salt produced lesser number of microrhizome as well as their fresh weight.

The effect of different concentration of sucrose was also studied. Of the different concentration of sucrose tried 8% sucrose gave the maximum production (21.7) of microrhizome *in vitro*. Where as the lower and higher concentration leads to the reduction in the number and also the fresh weight (Table 17).

Different culture duration were also tried to optimize the production of *in vitro* microrhizome (Table 17). The optimum production of ginger microrhizome obtained at 90 and 120 days of growth period. Where as the optimum fresh weight obtained in 120 days (2.6g) than 90 days. The various stages in *in vitro* microrhizome induction and their field planting are given in Fig.6. The *in vitro* plants were successfully established *in vivo* without hardening.

Table 17. Effect of different media composition on the induction of microrhizome.

Parameters	No. Mrh/culture (Mean± SE)	Fresh Wt/ Mrh (g) (Mean±SE)
MS salt		
	0.25X	6.33±0.46e
	0.5X	10±0.6cd
	0.75X	22±0.6a
	1X	17±0.3b
Sucrose concentration		
	6%	9±0.3d
	8%	21.7±0.46a
	10%	12±0.6c
Duration		
	30 days	10.3±0.46cd
	60days	18.7±0.46b
	90days	23.3±0.34a
	120days	23.7±0.46a
		0.9±0.07ef
		1.5±0.12bcd
		2.3±0.07a
		2.6±0.04a

Each numerical value represents the mean ±standard error from 3 replication after 120 days except the data on culture duration. Mean followed by different letters are significantly different at 5% level according to the least significant difference test (LSD) and Duncan's multiple range test (DMRT).



Fig. 6 Developmental stages of *in vitro* microrrhizome in ginger

a. Plantlets cultured on microrrhizome induction medium. b. Multiple shoot formation frequency. c. Root development frequency in microrrhizome induced culture vs normal culture. d, e, f. Different stages of microrrhizome formation. g. Isolated shoots from single culture bottle after 120 days. h. Individual shoots bearing microrrhizome after 60 days. i. Individual shoots bearing microrrhizome after 120 days. j. Harvested microrrhizome from culture. k & l Normal rhizome and microrrhizome germination. m. Microrrhizome germination on hardening cups. n. Field establishment of microrrhizome. o. Harvested rhizome after 6 months. p. Harvested microrrhizome after 8 months.

Hardening and planting out

The plantlets obtained from various method of propagation were hardened and planting out as described in materials and methods.

Characterization of somaclones

Morphological characterization

Micropropagated, callus regenerated and microrhizome derived plants planted in pots in the subsequent generation after hardening used for the collection of various morphological characters. Uniform planting material was used for micropropagated, callus regenerated and microrhizome derived plants in the second year of planting in pots. Conventionally propagated plant was used as the control. Data on plant height (cm), number of tiller per plant, number of leaves per plant, internodal length (cm), leaf length (cm), leaf breadth (cm) were taken as the shoot character after four months of planting. Rhizome characters like weight of rhizome (g), breadth of rhizome (cm) (M: mother, P: primary, S: secondary, T: tertiary fingers), number of finger per rhizome (M, P, S, T), number of nodes per finger (M, P, S, T) and internodal distance (cm) (M, P, S, T) were recorded at the time of harvest. The observations are presented in the Table 18. The callus regenerated plants show good variation in almost all the characters studied.

The maximum plant height was recorded in callus regenerated plants (56.8 cm) Plant height of conventionally propagated and micropropagated and microrhizome derived plants were almost similar. The callus regenerated plants produced more number of tiller per plant (13.6) followed by microrhizome derived plants (8.7). The number of leaves per plant was more in callus regenerated plants (19.4) and less in the case of microrhizome derived plants (15.3). The conventional and micropropagated plants were similar in the case of number of leaves. Internodal distance was maximum in callus regenerated plants (2.5 cm) and in all others it was similar. The maximum leaf length (16 cm) and leaf breadth (2.2 cm) were recorded in callus regenerated plants compared to others. Minimum leaf length recorded in microrhizome derived plants (12.8 cm).

The highest yield was recorded in callus regenerated plants (573g) and all others the yield was almost similar. The breadth of the mother rhizome was maximum in conventional and micropropagated plants (2.5cm) and minimum in microrhizome derived plants. Breadth of the primary fingers was highest in callus regenerated plants (3.5 cm) followed by micropropagated plants (2.9 cm). Callus regenerated plants showed maximum breadth of secondary fingers (2.4 cm) and minimum in microrhizome derived plants (1.9 cm). Conventional and micropropagated plants were similar in breadth of secondary fingers. Breadth of the tertiary finger was maximum in callus regenerated plants (2.2 cm) and minimum in microrhizome derived plants (1.2cm).

More number of primary and secondary fingers was recorded in callus regenerated plants (6.0 and 9.0) and all others are almost equal. More number of tertiary finger were recorded in microrhizome derived and less in callus regenerated plants (3.7). The conventional and micropropagated plants were almost similar in the case of tertiary fingers (Fig.7)

Number of nodes in mother rhizome was maximum in micropropagated plants (8.8). Number of nodes in primary rhizome was maximum in callus regenerated plants (8.5) and less in microrhizome derived plants. Where as number of nodes in secondary and tertiary fingers were maximum in conventional plants (8) and minimum in callus regenerated plants (7.4). Number of nodes of tertiary fingers in microrhizome derived plants was less (6.2).

Minimum internodal length in mother rhizome was recorded in microrhizome derived plants (0.3 cm). Internodal length of primary and secondary fingers was similar in all the cases. Where as internodal length of tertiary finger was minimum in callus regenerated plants (0.5 cm).

Table 18. Comparative morphology of ginger somaclones (Conventional, Micropropagated, Callus regenerated and microrhizome derived plants).

Plant characters	Conventional propagated plants		Micropropagated plants		Callus regenerated plants		Microrhizome Derived plants		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Aerial characters									
Plant height (cm)	50.7	2.2	50.8	2.6	56.8	4.43	48.5	2.1	
No. tiller / plant	4.3	1.1	4.5	0.8	13.6	1.5	8.7	1.1	
No. leaves / plant	17.2	1.9	16.9	2.5	19.4	3.5	15.3	1.8	
Internodal distance (cm)	2	0.3	2	0.4	2.5	0.3	2.2	0.2	
Leaf length (cm)	15.6	1.4	15.7	0.9	16	1.9	12.8	1.4	
Leaf breadth (cm)	1.8	0.2	1.9	0.1	2.2	0.2	1.7	0.2	
Rhizome characters									
Weight of rhizome (g)	527	79.8	532	83.3	573	85	514	46.4	
Breadth of finger/rhizome (cm)	M	2.5	0.3	2.5	0.3	2.3	0.4	1.8	0.1
	P	2.7	0.3	2.9	0.3	3.5	0.2	2.2	0.2
	S	2.2	0.2	2.1	0.4	2.4	0.4	1.9	0.2
	T	1.9	0.3	1.9	0.3	2.2	0.7	1.2	0.2
No. of finger/rhizome	M	1.0	0.0	1.0	0.0	1.0	0.0	1.0	0.0
	P	4.0	1.0	3.8	1.1	6.0	1.4	4.0	1.0
	S	6.0	0.9	6.2	0.9	9.0	0.7	6.4	0.9
	T	6.6	1.4	6.8	1.7	3.7	1.4	7.5	1.5
No. of nodes/finger	M	7.7	1.0	8.8	1.1	7.9	0.7	8.1	1.0
	P	7.6	0.8	7.6	1.1	8.5	0.8	7.4	1.1
	S	8.0	1.0	7.7	0.6	7.4	0.9	7.5	1.0
	T	7.5	1.2	7.2	0.7	7.0	1.2	6.2	0.9
Internodal distance (cm)	M	0.5	0.1	0.5	0.1	0.4	0.07	0.3	0.1
	P	0.5	0.09	0.5	0.0	0.5	0.0	0.5	0.0
	S	0.6	0.1	0.6	0.1	0.6	0.07	0.6	0.1
	T	0.6	0.1	0.7	0.1	0.5	0.1	0.7	0.1

The quantitative data of aerial characters were taken four months after planting and the rhizome characters recorded at the time of harvest. The data statistically analyzed (ANOVA).

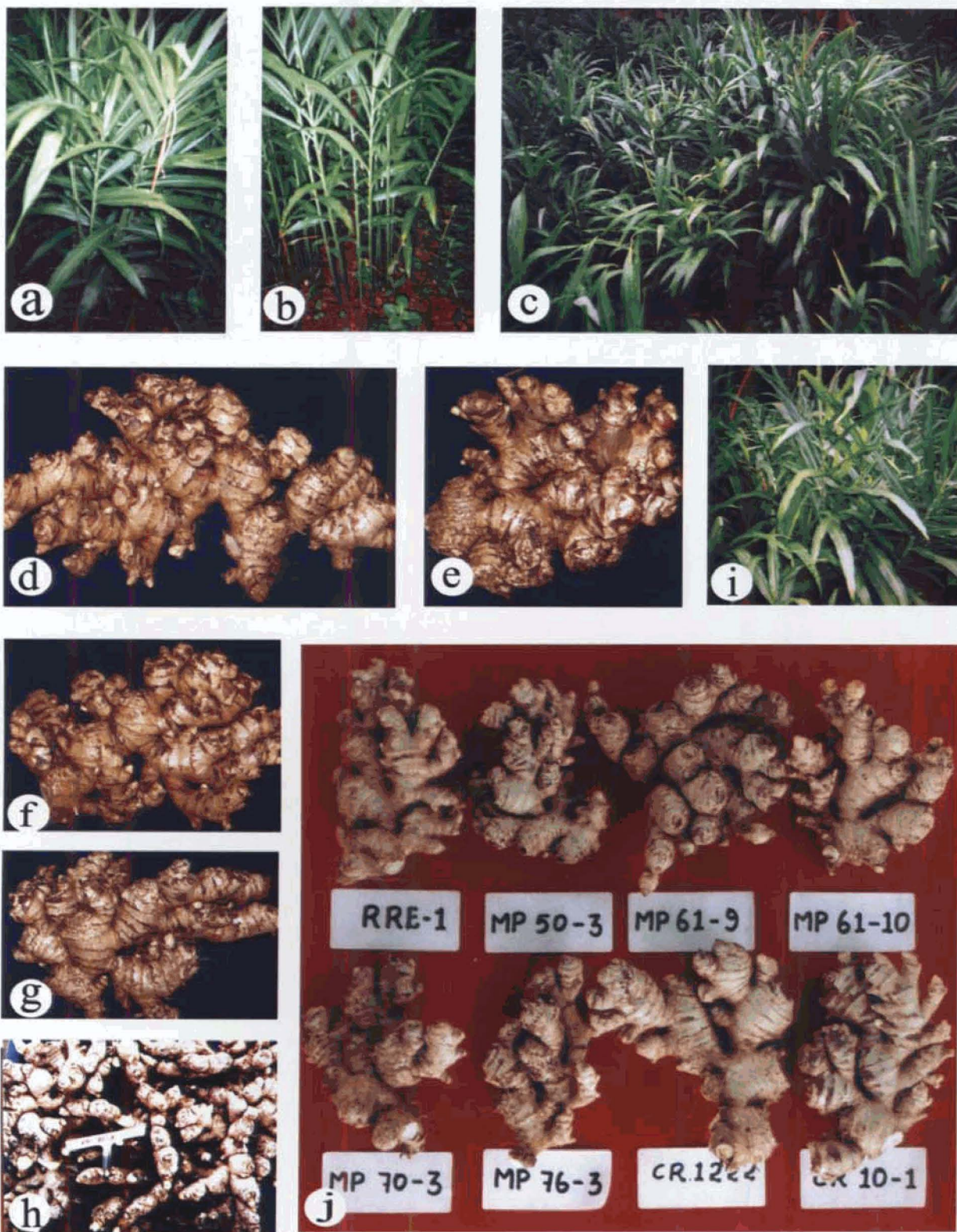


Fig. 7 Morphological variations observed in somaclones

a. Somaclones showing broad leaves. b. Somaclones with maximum number of tiller per plant. c. Field planting of somaclones (3x1m bed). d,e,f,g Harvested rhizome of somaclones showing differences in nature of spreading and presence of persistent scale leaves. h. Harvested rhizome from somaclones showing more number of tertiary fingers. i. Somaclone showing variation in plant height j. Selected somaclones showing variation in rhizome morphology.

Biochemical characterization

The plants showing certain amount of significant variations were selected for further biochemical characterization. The percentage of oil, oleoresin, starch and fibre content were estimated. Significant variations were observed in percentage of oil, oleoresin, starch and fiber content among the somaclones tested. The oil content varied from 1.3 to 2.4%. The highest oil percentage was found in the accession CR 816 (2.4%) and the lowest in MP 61-9 (1.3%). Where as in control (Maran) the percentage of oil was 1.5%. The maximum percentage of oleoresin was observed in CR 1222 (8.1%) followed by CR 816 (7.8%) and CR 10-1 (7%). In the case of starch control (Maran) showed the maximum (47.79%) followed by MP 76-3 (45.7%). CR 816 recorded the maximum percentage of fiber 7.4% followed by MP 50-3 (6%). Microrhizome derived plants showed the minimum fiber content (2%) (Table 19).

Table 19. Data on the percentage of oil, oleoresin, starch and fiber content in dry weight basis

Acc. No.	% of oil	% of oleoresin	% of Starch	% of Fiber
MP 50-3	1.7	5.0	39.75	6
MP 61-9	1.3	3.1	29.41	5.5
MP 61-10	1.4	5.4	43.5	5.5
MP 70-3	1.8	5.3	29.65	5.85
MP 76-3	2	4.0	45.7	3.2
CR10-1	1.5	7.0	29.78	2.6
CR 1222	1.7	8.1	37.01	4.4
CR 816	2.4	7.8	35.4	7.4
Mrh.	2.3	4.2	19.42	2
Maran (Control)	1.5	4.2	47.79	5.4

Each value representing the average of three replications

Composition of essential oil of *in vitro* microrhizome and *in vivo* rhizome by using GC- MS

To study the developmental stages of microrhizome, a comparison were made with microrhizome and conventionally propagated rhizome. The oils were collected by conventional hydrodistillation of the rhizome and leaves in a Clevenger type apparatus. The yield of essential oil obtained from rhizome, leaf of *in vitro* and *in vivo* plants of

ginger are presented in Table 20 on a fresh weight basis. The relative concentrations of the volatile components identified are presented in Table 21 according to their elution order on RTX wax column. The main components of rhizome oil of both *in vitro* and *in vivo* were Zingiberene (14.74% *in vivo* and 10.98% *in vitro*), 1,8 Cineole 11.16 % (*in vivo*) and Camphene 10.38% (*in vivo*) and α -Humulene (7.99 % *in vitro*) (Fig.8). Where as in the case of leaf oil the main components were t- Caryophyllene (22.14% *in vivo* and 24.02 *in vitro*), Epiglobulol (11.05% *in vivo*), α -Humulene (13.97% *in vitro*), z-Citral (5.83% *in vivo*) and β -Muurolene (5.66 % *in vitro*) (Table 21).

Table 20. Essential oil yield from ginger (*Zingiber officinale* Rosc.) mirorhizome

<i>In vitro</i> (% of oil)		<i>In vivo</i> (% of oil)	
Rhizome	Leaf	Rhizome	Leaf
0.16	0.17	0.2	0.12

Table 21. Components of essential oil of ginger (*Zingiber officinale* Rosc) microrrhizome.

Components	Concentration (%)			
	<i>In vivo</i>		<i>In vitro</i>	
	Rhizome oil	Leaf oil	Rhizome oil	Leaf oil
a-pinene	3.05	-	2.56	2.45
Camphene	10.38	-	5.61	1.95
β -pinene	-	-	2.67	3.77
β -Myrcene	1.43	-	-	-
p-Menthadiene/ d-Limonene	2.14	-	1.37	-
1,8 Cineole	11.16	-	4.35	2.59
Aromadendrene	-	1.57	-	-
a-Copaene	-	1.02	1.12	2.38
Linalool	1.68	-	-	-
t- Caryophyllene	-	22.14	5.35	24.02
z- Citral	4.55	5.83		1.5
2-undecanone	-	-	1.02	-
l-a-Terpineol	5.98	-	2.1	-
Alloaromadendrene	-	-	1.58	2.29
d- Cadinene	-	4.69	-	-
Endo-Borneol	2.47	-	1.45	-
a-Humulene	-	-	7.99	13.97
Gamma-Cadinene	1.6	-		-
Zingiberene	14.74	-	10.98	-
β -Bisabolene	2.76	-	2.48	-
β /a-Cubebene	-	-	1	-
?-Muurolene	-	-	3.54	5.66
a-Zingiberene	-	-	-	4.97
Farnesene	3.31	1.94	-	-
t-Geraniol	-	2.99	-	-
?-Selinene	-	-	1.04	-
β -Citronellol	3.53	-	-	1.6
Valencene/ a- Cedrol	-	-	-	2.93
β -Sesquiphellandrene	4.5	-	5.35	1.6
a-Curcumene	2.16	-	1.32	-
Epiglobulol	-	11.05	-	-
Trans- geraniol	1.55	-	-	-
Nerolidol	1.08	4.46	2.09	1.37
Elimol	1.16	-	1.4	-
Cinnamyl acetate	-	4.36	-	-
β -Eudesmol	2.5	1.8	2.66	1.51
Farnisol	-	1.16	-	-
Juniper Camphor	-	-	-	1.45
Phytol	-	1.51	-	-
Trans, trans-farnesal	-	1.06	2.25	-

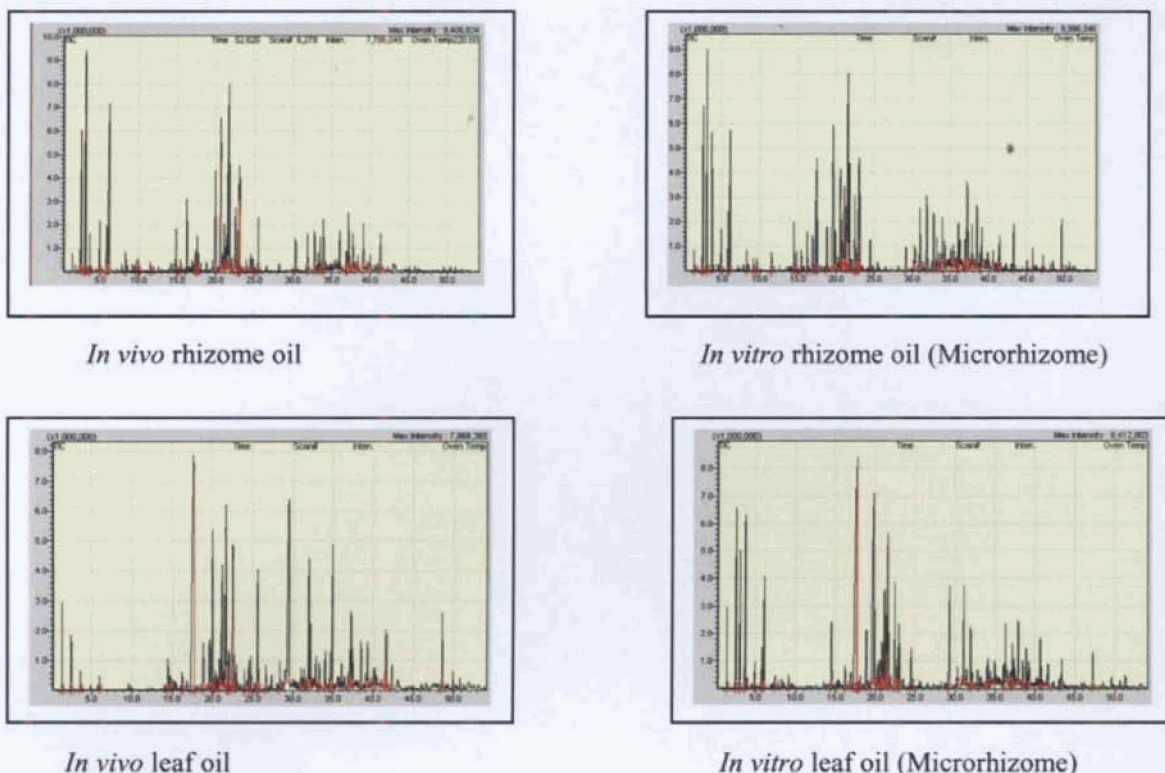


Fig. 8 Showing the differences in essential oil components of ginger microrhizome *in vitro* and *in vivo*

Anatomical characterization

Rhizome anatomy of polyploid and normal plant

Fresh rhizome sections of the identified polyploid (somaclone having bold rhizome and broad leaves - Acc. No. CR 1222) were carried out to study the polyploid nature of the rhizome. The sections were stained with safranin and observed under the light microscope. The single layered epidermis followed by the multi-layered hypodermis was observed in polyploid (Fig.9). Where as in the case of diploid single layered epidermis followed by single layered hypodermis was observed.

Stomatal study and leaf anatomy

Stomatal frequency was high on the lower side of the leaves. Tetracytic stomata were found in diploid and polyploid leaves. In this the first two subsidiary cells are parallel and the remaining two cells lie right angle to the guard cells. In the case of stomatal frequency and size, the polyploid is larger in size and less in stomatal number. Where as in diploid plant the stomatal number was more and size was less. In polyploid

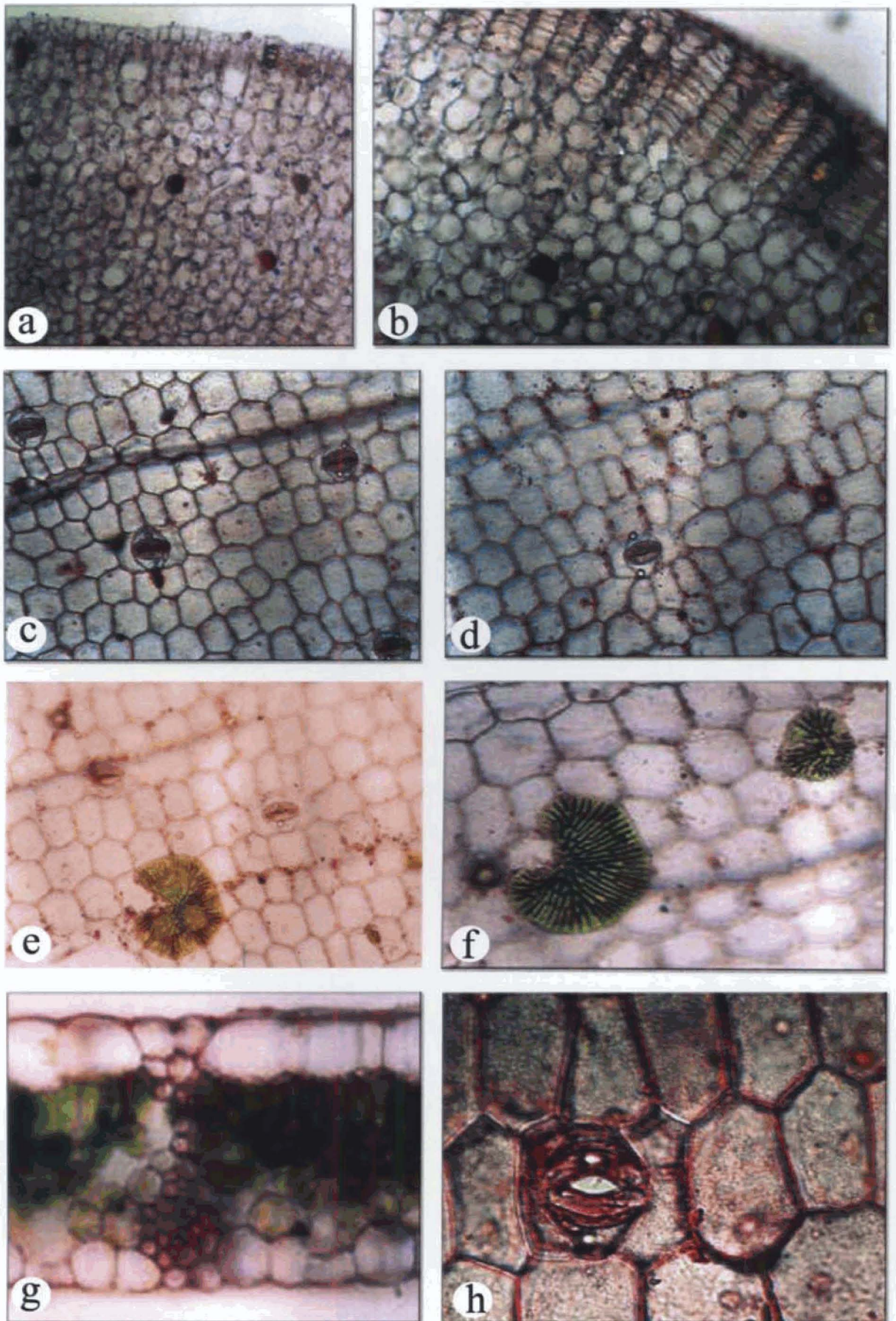


Fig. 9 Anatomical study of ginger polyloid (Acc. No. CR 1222)

a. Normal diploid plant rhizome showing single layered epidermis followed by single layered hypodermis. b. Polyloid showing single layered epidermis followed by multilayered hypodermis. c. Normal diploid showing more number of stomata. d. Polyloid showing less number of stomata. e&f Showing the differences in scleried size and colour in diploid and polyloid respectively. g. Polyloid leaf showing large sized upper and lower epidermal cells. h. Single stomata of polyloid.

leaf also showed large sized and less number of sclereids than the diploid leaf (Fig.9). Large sized upper and lower epidermal layer was observed in polyploid compared to normal diploid plant.

Chromosome indexing

The selected somaclones were cytologically analyzed to find out whether there is any numerical alteration in the chromosome level. The mitotic metaphase chromosome indexing of control plant, micropropagated and microrhizome derived plant showed normal chromosome number as $2n=22$ (Fig.10). The callus derived plants of ginger exhibited deviation from the normal chromosome number (Fig.10). Among the numerical aberrations recorded aneuploids with lower number are most frequent. One of the somaclone CR 1222 showed polyploid number of chromosome (Fig.10).

Molecular characterization

RAPD profile of micropropagated plants

20 micropropagated plants regenerated from vegetative buds were profiled for RAPD polymorphism using 10 primers (OPA 06, OPC 10, OPC 01, OPB 18, OPA 11, OPC 14, OPA 07, OPC 19, OPC 08, OPB 11). In all the primers tried, the micropropagated plants in general showed uniform banding pattern.

RAPD profile of Callus regenerated plants

Twenty plants of callus regenerants were subjected to RAPD analysis using 10 oligonucleotide primers (OPA 06, OPC 10, OPC 01, OPB 18, OPA 11, OPC 14, OPA 07, OPC 19, OPC 08, OPB 11). A total of 113 were produced, of which 27 monomorphic bands and 86 polymorphic bands were present. The maximum percentage of polymorphism (94%) was produced using the primer OPA 06. The frequencies with which the RAPD band changes were observed for ginger somaclones are shown in Table 22.

Using primer OPA 07 a clear-cut extra band was detected in two regenerants (CR 2 and CR 42) at 0.4 kb and 1.4 kb region. While using OPC 10 an extra band was present

in the above regenerants at 0.7 kb region. These regenerants were not identical due to the absence of the bands at 1.6, 1.4 and 0.6 kb region in one of the regenerants.

The marker profiles of nine somaclones ((2) Control, (3) CR-1, (5) CR -3, (6) CR -4, (7) CR -5, (8) CR -7, (9) CR -9, (10) CR 10-1, (11) CR -13) were identical with OPC 14 and OPC 10 (Fig.11,12), and differentiated this group from all other somaclones. However the corresponding amplification product for CR-2 was missing at 0.9 kb region and present at 0.7 kb region in the primer (OPC 10) and an extra band at 0.7 kb region in OPC 14.

The accession number CR-2 showed polymorphism in 6 primers used out of 10 primers (OPA 07, OPC 14, OPB 18, OPC 01, OPC 10, OPA 06). This is clearly indicated that this somaclone is genetically altered. The accession number CR 67 also showed polymorphism in 6 primers (OPA 07, OPA 11, OPB 18, OPB 11, OPC 10, OPA 06). Accession number CR 42, CR 816 showed polymorphism in more number of primers (Accession number CR 42: OPA 07, OPB 18, OPC 10, OPA 06; Accession number CR 816: OPA 07, OPA 11, OPB 18, OPC 10, and OPA 06). Out of 10 primers tried 4 primers showed polymorphism in the case of accession number CR 1222, and 3 primers in the case of CR 13. Out of 20 somaclones were analysed for genetic variability using RAPD techniques, 6 somaclones were identified as genetically variant plants.

Table 22. Different primers used for studying genetic variability of ginger somaclones by means RAPD markers.

Primers	Sequences	Total number of bands	Monomorphic bands (n)	Polymorphic bands (n)	Polymorphic band (%)
OPC 19	5' GTTGCCAGCC 3'	9	4	5	55.5
OPA 06	5' GGTCCCTGAC 3'	17	1	16	94.1
OPA 07	5' GAAACGGTG 3'	15	3	12	80.0
OPA 11	5' CAATCGCCGT 3'	10	4	6	60.0
OPB 11	5' GTAGACCCGT 3'	9	1	8	88.8
OPB 18	5' CCACAGCAGT 3'	12	4	8	66.6
OPC 01	5'TTCGAGCCAG 3'	9	2	7	77.7
OPC 08	5'TGGACCGGTG 3'	11	3	8	72.7
OPC 10	5' TGTCTGCGTG 3'	10	1	9	90.0
OPC 14	5' TGCGTGCTTG 3'	11	4	7	36.3

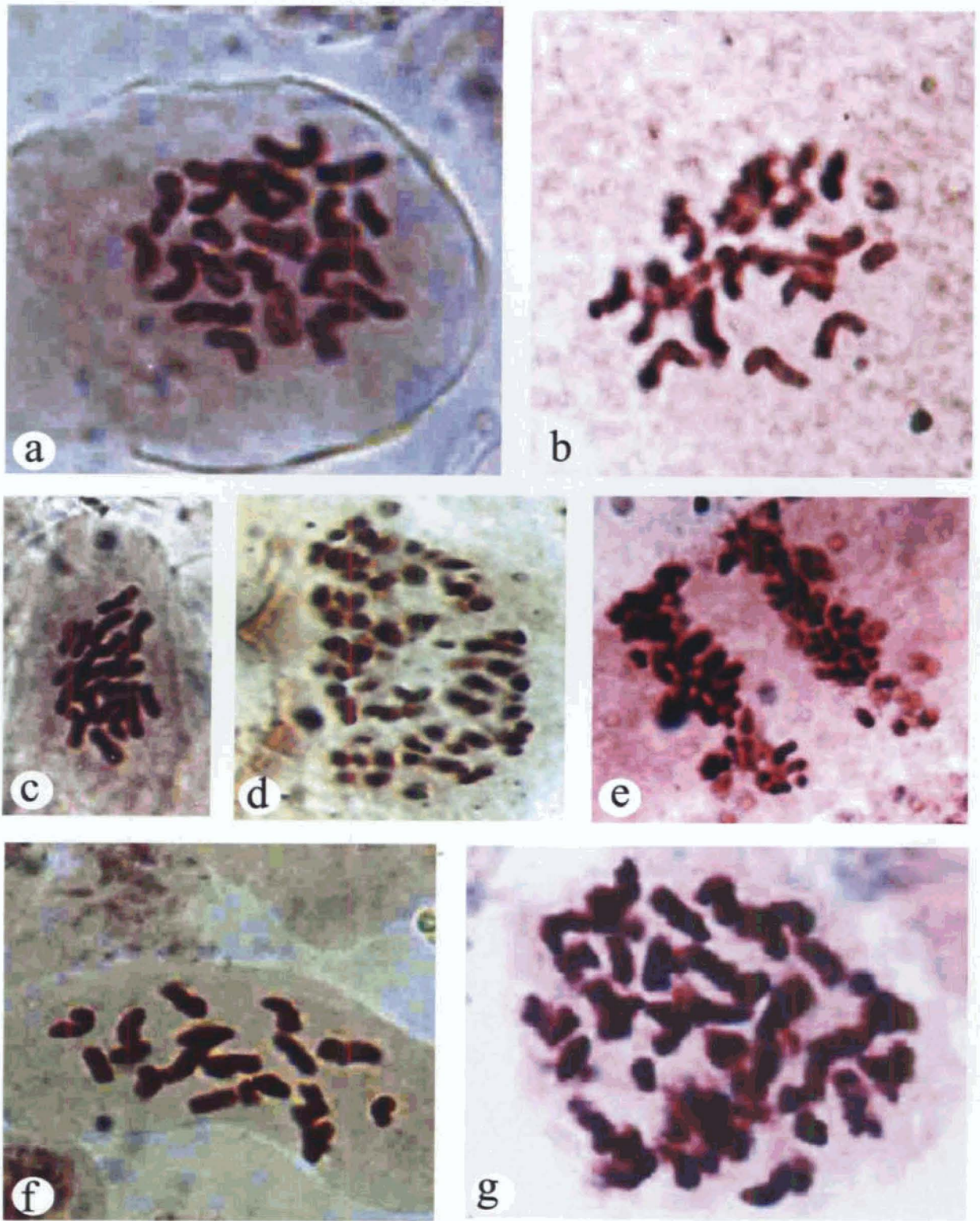


Fig. 10 Cytological indexing of ginger somaclones

a. Conventionally propagated ginger (cultivar maran) showing normal chromosome number $2n= 22$. b. Micropropagated plants of ginger showed no variation in chromosome number. c. Microrhizome derived plants showed similar in chromosome number. d. Callus regenerated plant showed polyploid number of chromosome. e. Anaphase stage of polyploid cell. f. Aneuploid number of chromosome reported in callus regenerated plants of ginger.

RAPD profile of Microrhizome derived plants

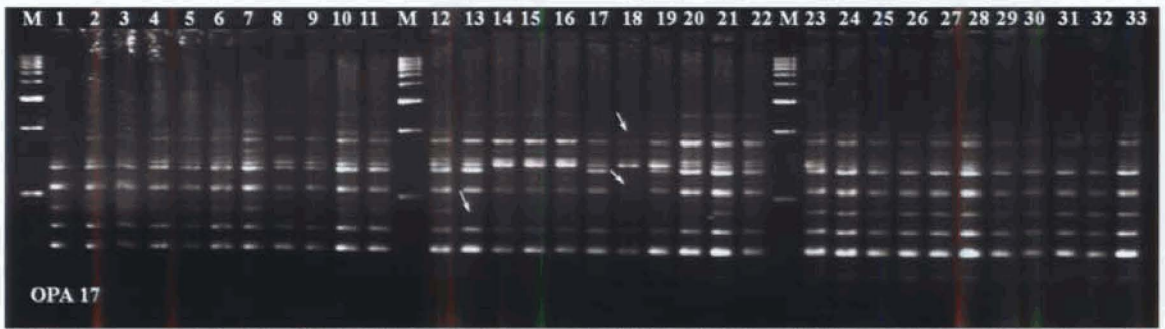
Field grown *in vitro* microrhizome derived plants were profiled for RAPD analysis by using 10 primers (OPA 06, OPC 10, OPC 01, OPB 18, OPA 11, OPC 14, OPA 07, OPC 19, OPC 08, OPB 11) In all these primers, the microrhizome derived plants showed monomorphic banding pattern.

Comparative study of RAPD profiling of micropropagated, callus regenerated and microrhizome derived plants

Comparative RAPD polymorphism of 10 randomly selected micropropagated, callus regenerated and microrhizome derived plants of ginger were produced using 6 operon primers (OPA 17, OPA 10, OPC 08, OPC 01, OPA 06 and OPA 04). The conventionally propagated plant was used as the control plant. The above tried primers showed no variation in micropropagated and microrhizome derived plant but showed variation in callus regenerated plants (Fig.13)

Screening for disease incidence

Nine selected ginger somaclonal 'variants' along with control were inoculated with *Pythium aphanidermatum* for challenging the rhizome rot disease (Fig.14). The somaclones were selected based on its morphological variations. Incidence of disease was observed in aerial portion of susceptible plant within one week and in rhizome it was recorded at the time of harvest. CR 1441 recorded 100 % of disease incidence followed by CR 816 (77.73 %) CR 10-1 (63.3%) and CR 13 (40%) (Table 23). The control showed maximum number of rhizome (9.5) and at the same time there is no disease incidence. The somaclone CR 2, CR 3 and CR 67 observed free from disease symptoms. CR 2 and CR 42 recorded the presence of root tuber.



M-Marker, 1- control, 2-Mp7, 3-Mp 25-6, 4-Mp 47-4, 5-Mp 49-1, 6-Mp 62-3,7 -Mp 63-2, 8-Mp 67-7, 9-Mp 75-1, 10-Mp75-1-3, 11-Mp 80, 12-control, 13-CR 1, 14- CR2, 15-CR3, 16-CR4,17-CR5, 18- CR7, 19-CR9, 20-CR10-1, 21-CR13, 22-CR42, M- marker, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27-Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.



M-Marker, 1- control, 2-Mp7, 3-Mp 25-6, 4-Mp 47-4, 5-Mp 49-1, 6-Mp 62-3,7 -Mp 63-2, 8-Mp 67-7, 9-Mp 75-1, 10-Mp75-1-3, 11-Mp 80, 12-control, 13-CR 1, 14- CR2, 15-CR3, 16-CR4,17-CR5, 18- CR7, 19-CR9, 20-CR10-1, 21-CR13, 22-CR42, M- marker, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27-Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.



M-Marker, 1- control, 2-Mp7, 3-Mp 25-6, 4-Mp 47-4, 5-Mp 49-1, 6-Mp 62-3,7 -Mp 63-2, 8-Mp 67-7, 9-Mp 75-1, 10-Mp75-1-3, 11-Mp 80, 12-CR 1, 13-CR2, 14-CR3, 15-CR4, 16-CR5,17-,CR7 18- CR9, 19-CR10-1, 20-CR13, 21-CR42, M- marker, -, 22-control 23-Mrh1, 24-Mrh2, 25-Mrh3, 26-Mrh4, 27-Mrh5, 28-Mrh6, 29-Mrh7, 30-Mrh8, 31-Mrh9, 32-Mrh10.



M-Marker, 1- control, 2-Mp7, 3-Mp 25-6, 4-Mp 47-4, 5-Mp 49-1, 6-Mp 62-3,7 -Mp 63-2, 8-Mp 67-7, 9-Mp 75-1, 10-Mp75-1-3, 11-Mp 80, 12-control, 13-CR 1, 14- CR2, 15-CR3, 16-CR4,17-CR5, 18- CR7, 19-CR9, 20-CR10-1, 21-CR13, 22-CR42, M- marker, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27-Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.

Fig. 13 Comparative RAPD profiling of micropropagated, callus regenerated and microrhizome derived plants of ginger using operon primers
Mp- micropropagated plants, CR- callus regenerated plants, Mrh- microrhizome derived plants.

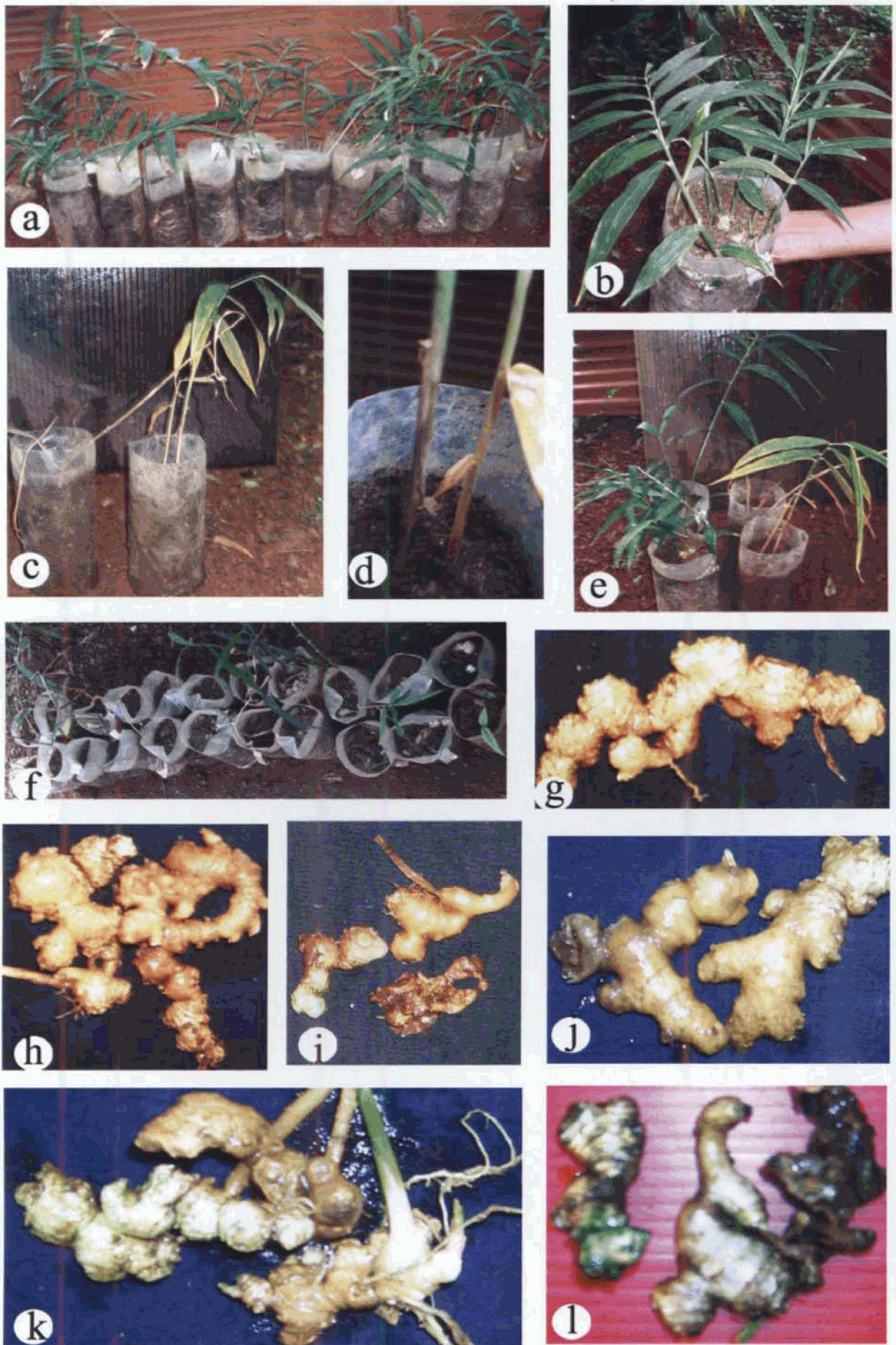


Fig. 14 Rhizome rot disease scoring of ginger somaclones

a. *Pythium aphanidermatum* inoculated ginger somaclones. b. Somaclone showing healthy aerial portion. c&d. Infected somaclones showing symptoms in aerial portion. e. Non infected and infected plants of somaclones. f. Showing the percentage of disease incidence. g&h. Disease free rhizome of somaclone (CR 2, CR3). i,j&k. Somaclones showing infected rhizome with *pythium aphanidermatum*.

Table 23. Incidence of rhizome rot in *Pythium aphanidermatum* treated ginger somaclones.

Acc. No	Total no. Rhizome	Disease incidence (%)	No. of tubers
	Mean	Mean	Mean
Control	9.5 a	0 (0) a	0 a
CR -2	7 abc	0 (0) a	0.5 a
CR-3	7.5 abc	0 (0) a	0 a
CR-10-1	5.5 bcd	63.3 (52.71) de	0 a
CR -13	5 cd	40 (39.23) cd	0 a
CR- 42	4 cd	33.3 (35.25) c	0.3 a
CR- 67	7 abc	0 (0) a	0 a
CR-816	2.7 d	77.73 (61.84) ef	0 a
CR-1222	9.3 ab	4.73 (12.56) ab	0 a
CR-1441	4 cd	100 (90) g	0 a

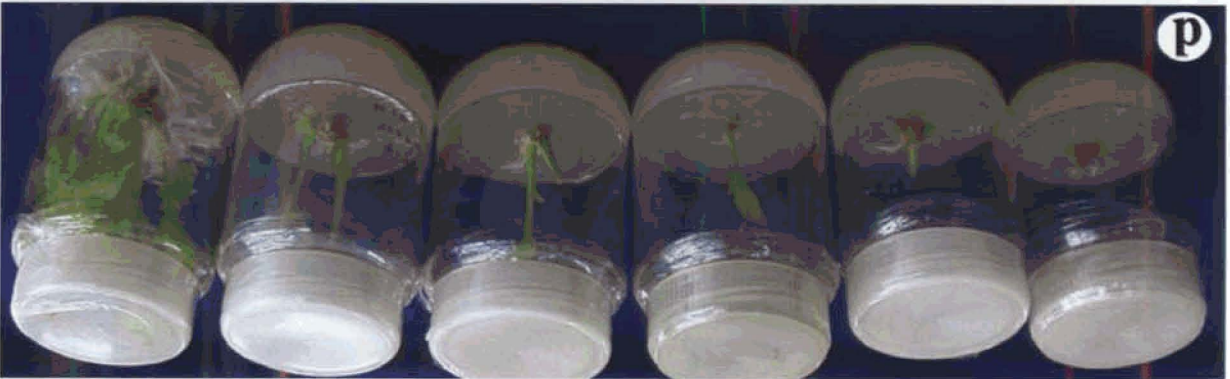
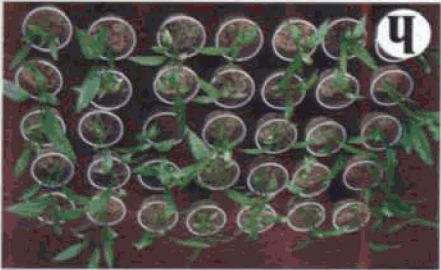
Mean followed by different letters are significantly different at 5% level according to the least significant difference test (LSD) and Duncan's multiple range test (DMRT). Value in parenthesis is transformed value.

Turmeric

Micropropagation

Vegetative buds of turmeric were inoculated on MS supplemented with 1mg l^{-1} NAA and 4mg l^{-1} BA for the clonal propagation as per the earlier protocol developed at IISR (Nirmal Babu *et al.*, 1997). This medium showed consistency in direct regeneration from vegetative bud. On this medium 96.2% aseptic explants responded and developed multiple shoots. The mean frequency of adventitious shoot formation (6.0) was observed in the above concentration. Shoot differentiation and proliferation took 4 -8 weeks. The best results were obtained when the buds (10-20 mm in size) were cultured in solidified medium. The established and *in vitro* derived shoots further multiplied on MS propagation medium containing sucrose (3%), BA 1mg l^{-1} and NAA 0.5mg l^{-1} and agar 0.8%. BA at 1mg l^{-1} was the most beneficial cytokinin for multiplication. By this method it was possible to obtain a 10-fold multiplication in 90 days. The various stages in micropropagation of turmeric are given in fig (Fig. 15).

Fig. 15 Rapid micropropagation of turmeric from rhizome bud
 a. Sterile apical and axillary bud used as explants for micropropagation. b. Shoot developed from the axillary bud c. Shoot developed from apical bud d. Developmental stages of shoots from rhizome. e. Frequency of multiplication. f. Influence of hormone on the development of root. g. Acclimatization of micropropagated plants. h. Field evaluation of micropropagated plants. i. Harvested rhizome. k. Field evaluation of micropropagated plants.



Callus induction

Since the existing protocols in turmeric regeneration system did not show consistency in plant regeneration a new protocol was attempted. Different combinations of 2, 4 -D and BA were used for the efficient induction and proliferation of callus. The maximum amount of callus (5.0g) obtained on the MS medium supplemented with 2,4 -D at 1.0 and BA 0.5 mg^l⁻¹. Proliferation was maximum after 4 weeks of culture. After 5-6 weeks, callus was subcultured for further callus regeneration. The amount of callus ranged from 0.2g to 5.0g. Out of the different types of callus produced (Embryogenic, non embryogenic and mixed type), the percentage of embryogenic callus was found to be highest in 1 mg^l⁻¹ 2, 4-D + 0.5 mg^l⁻¹ BA (100%). Non embryogenic callus percentage was highest in 1 mg^l⁻¹ 2,4-D alone (32%). This was best media used for induction and maintenance of callus. Where as in the case of mixed type of callus, the maximum percentage was found to be highest (23%) in the concentration of 1 mg^l⁻¹ 2, 4-D and 3 mg^l⁻¹ BA (Table 24). The embryogenic callus (Fig.16) was carefully collected and cultured on the media of same composition. The various stages in callus induction and plant regeneration are given in fig. (Fig.16).

Plant regeneration from callus

MS medium with different concentrations of growth regulators were used for plant regeneration in callus cultures obtained earlier (Table 24). Regenerative ability depended on the composition of the medium. Shoot buds and leaf primordia developed from the meristematic protuberances that originated from the surface of the callus. The most effective media contained NAA at 0.5 mg^l⁻¹ and BA at 2.0 mg^l⁻¹ exhibited high efficiency in callus regeneration. Percentage of shoots ranged from 28% to 98.0% and that of root ranged from 20% to 90% (Table 25). Data concerning regeneration efficiency from the callus in the presence of various plant growth regulators, and survival rate for plantlets. Regenerated shoots were multiplied by subculturing shoots on MS medium supplemented with 1 mg^l⁻¹ BA and 0.5 mg^l⁻¹ NAA at 4-week intervals. *In vitro*-produced

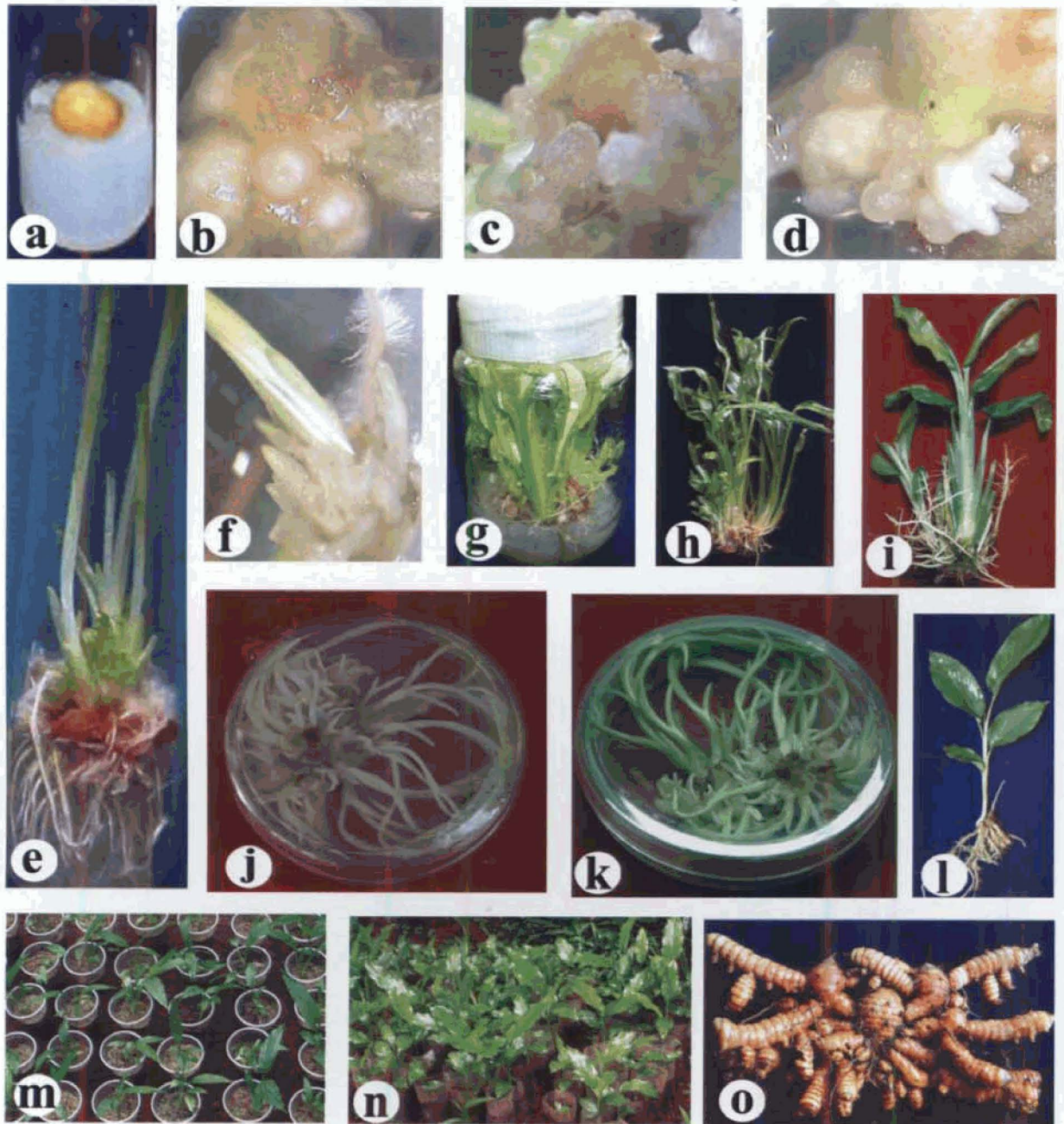


Fig. 16 Plant regeneration from rhizome derived callus cultures

a. Explants used for callus induction. b-d. Callus derived from axillary bud cultured on MS medium supplemented with 2, 4-D + BA for 6 weeks. b. Embryogenic callus c. Non embryogenic callus d. Mixed type of callus. e & f. Different stages of regeneration from callus. g. Fully regenerated plants with callus on its base. h&i. Isolated plants showing difference in shoot and root frequency j & k. Callus regeneration on the same medium with different culture vessel. l. Isolated single plant from the culture vessel showing the vigorous growth. m. Two month old *in vivo* plantlets starting rhizome formation. n. Hardening of callus regenerated plants. o. Hardened plants are planted on polybag. p. Harvested rhizome from callus regenerated plants.

plantlets were fully acclimatized and ready for planting 2 months after transfer to a hardening chamber (Fig 16).

Table 24. Turmeric callus induction

Growth regulators		Amount of callus (g)	% of Non Embryogenic	% of embryogenic	% of Mixed
2,4-D (μ M)	BA (μ M)	Mean \pm SE	Mean \pm SE	Mean \pm SE	Mean \pm SE
0.5	-	0.4 \pm 0.06ef	7 \pm 1.7d	93 \pm 1.7bc	0 \pm 0e
1.0	-	1.2 \pm 0.2cd	32 \pm 1.1a	68 \pm 1.1g	0 \pm 0e
1.5	-	0.8 \pm 0.1def	0 \pm 0f	90 \pm 2.8cd	10 \pm 2.9c
0.5	1.0	0.4 \pm 0.06ef	5.1 \pm 0.6de	79 \pm 2.9ef	15.5 \pm 3.5b
0.5	2.0	0.2 \pm 0.06f	0 \pm 0f	94 \pm 2.3abc	6 \pm 2.3d
1.0	0.5	5 \pm 0.6a	0 \pm 0f	100 \pm 0a	0 \pm 0e
1.0	1.0	1 \pm 0.06cde	0 \pm 0f	93 \pm 2.8bc	7 \pm 0.4d
1.0	2.0	0.4 \pm 0.06ef	15 \pm 2.8c	85 \pm 2.8de	0 \pm 0e
1.0	3.0	0.2 \pm 0.06f	5 \pm 0.6de	72 \pm 1.7g	23 \pm 1.2a
1.5	0.5	2 \pm 0.6b	2.3 \pm 0.9ef	97 \pm 0.9ab	0 \pm 0e
1.5	1.0	1.5 \pm 0.2bc	4.6 \pm 1.5de	95 \pm 1.5abc	0 \pm 0e
1.5	2.0	1 \pm 0.1cde	22 \pm 1.7b	78 \pm 1.7f	0 \pm 0e
1.5	3.0	0.6 \pm 0.06def	7 \pm 1.1d	85 \pm 2.9de	8 \pm 4d

Data recorded after weeks of incubation. The mean within the column \pm standard error followed by the same letters are not significantly different from each other at 5% level by DMRT analysis.

Table 25. Plant regeneration from turmeric callus

Hormone (mg l^{-1})		% of shoot	% of root
BA	NAA	Mean \pm SE	Mean \pm SE
1	0.5	48 \pm 0.5i	40 \pm 0.5h
1	1	65 \pm 0.25de	70 \pm 0.25d
1	1.5	61.3 \pm 0.76ef	68 \pm 0.25d
1	2	50 \pm 0.25hi	58 \pm 0.25e
2	0.5	98 \pm 0.25a	90 \pm 0.25a
2	1	90 \pm 0.5b	82 \pm 0.5b
2	1.5	79 \pm 0.5c	85 \pm 0.25b
2	2	54 \pm 1 gh	75 \pm 0.75c
3	0.5	68 \pm 0.75d	60 \pm 0.25e
3	1	75 \pm 0.75c	78 \pm 0.5c
3	1.5	57 \pm 0.75fg	50 \pm 0.25f
3	2	40 \pm 0.5j	45 \pm 0.75g
4	0.5	35 \pm 0.75k	40 \pm 0.5h
4	1	42 \pm 1 j	34 \pm 0.5i
4	1.5	30 \pm 0.5l	22 \pm 0.5j
4	2	28 \pm 0.75 l	20 \pm 0.5j

The data recorded after 60 days of growth period, the mean values followed by the same letters are not significantly different at 5% level by least significant difference test (LSD) and Duncan's multiple range test (DMRT).

Induction of Microrhizomes

Effect of basal media composition and culture duration on microrhizome induction

Effect of different factors such as MS salt concentration, growth hormones, sucrose and duration of culture were evaluated on the induction of microrhizome. Microrhizomes were formed at the base of the shoots grown on the medium (Fig 17).

Among the various strengths of MS salt tested, 0.75x strength of MS medium was found to be the most suitable in terms of number (16.0) and weight (2.4) both the half and full strength of MS salts decreased the number (Table 26). Lower strength of MS salts produced the lower number of smaller rhizome while full strength produced a lower number but comparatively bigger size of rhizome. Lower strength of the media had light green leaves with the leaves turned yellow on 0.25x strength medium (Fig 17).

The effects of different concentration of sucrose (5, 7, 9, and 11%) were investigated on *in vitro* microrhizome production and its size presented on the table 26. In general sucrose concentration had more significant effect on the number of microrhizome production. It was observed that 9% sucrose produced the highest number (16.3) and the size (2.5) of healthy microrhizome, while the 7% of sucrose reduce the number (6.7) and size (0.7) of microrhizome. However both lower (5%) and higher (11%) concentration of sucrose had significant inhibitory effect on microrhizome production (Table 26).

Different culture durations were tried for the optimum production of microrhizome *in vitro*. Of these, optimum production of microrhizome in culture was at 120 days and rhizome size reached at this stage was optimum for germination. The production is increased with increase in growth period. The maximum number of rhizome produced at 120 days was 17 and size was 2.4 (Table 26).

Table 26. Influence of different parameters on induction of microrhizome in Turmeric plants.

Parameters		No. Mrh. /culture (Mean ±SE)	Fresh wt/Mrh. (g) (Mean ±SE)
MS salt			
concentration	0.25X	3.7±0.33d	1.04±0.18b
	0.5X	7±0.57c	1.6±0.11b
	0.75X	16±0.57a	2.4±0.26a
	1X	10±1.15b	2.7±0.05a
Sucrose			
concentration	5%	2.3±0.9c	0.3±0.03b
	7%	6.7±1.2b	0.7±0.05b
	9%	16.3±0.9a	2.4±0.31a
	11%	3±0.57c	0.4±0.06b
Duration of			
Culture	30 days	3±0.57c	0.38±0.07b
	60days	7.7±0.88b	1.19±0.19b
	90days	15.7±1.2a	2.17±0.32a
	120days	17±0.57a	2.4±0.31a

Table represents mean of three repetitions. Mean ± standard errors followed by same letters are not significant at 5 % level according to Duncan's multiple range tests.

Effect of growth regulators on the production of microrhizome in turmeric

The results indicate that the growth regulators BA and NAA in combination had significant effect on *in vitro* microrhizome induction. Among different combinations of plant growth regulators, 1 mg^l⁻¹ BA together with 0.5 mg^l⁻¹ NAA exhibited a better response than any other treatments in terms of mean number (19.7) and the weight (2.4) of microrhizomes (Table 27). 0.75x concentration of MS salt, 9 % sucrose and pH 5.8 were used for this study.

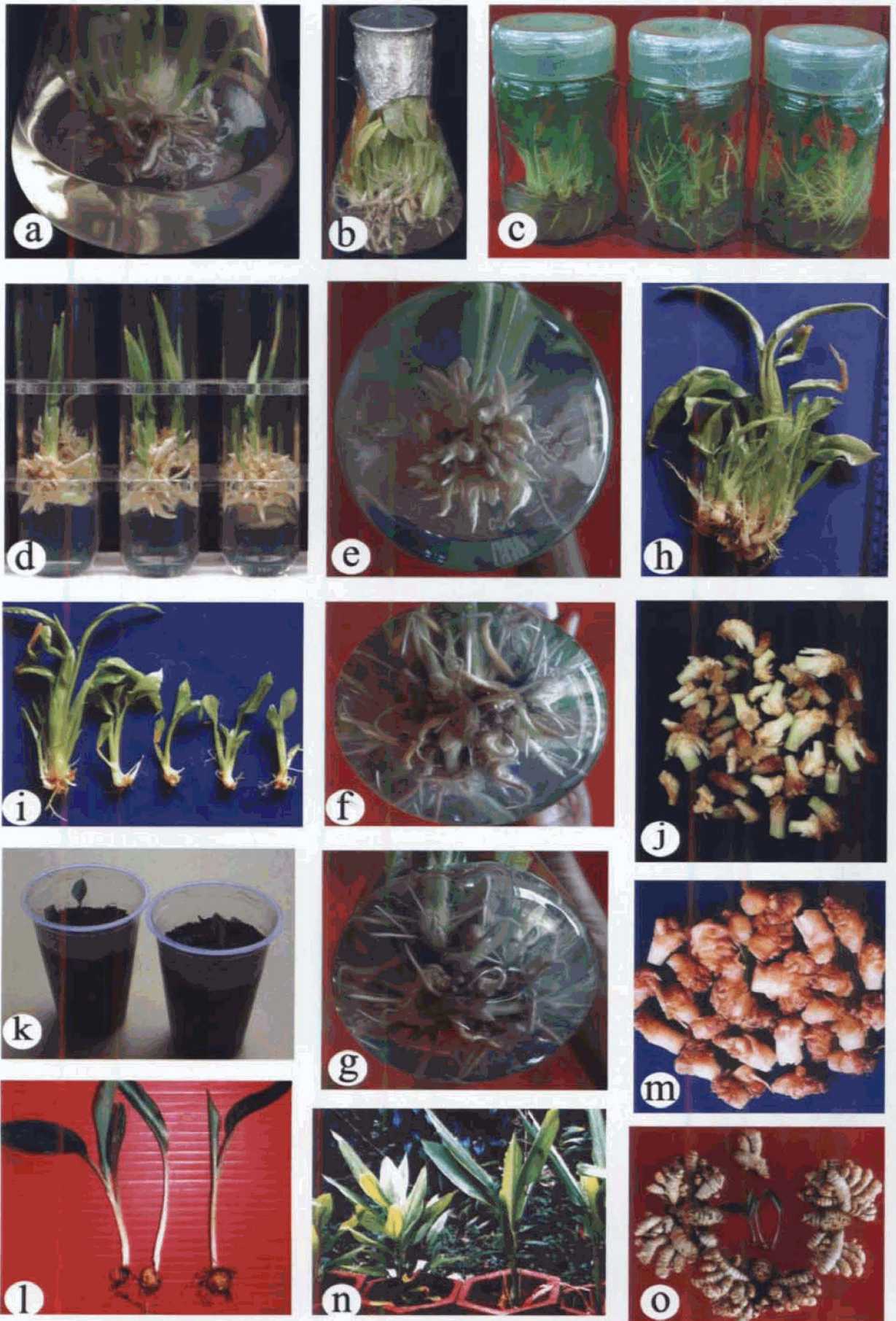


Fig. 17 Developmental stages of *in vitro* microrhizome in turmeric

a.Regenerated shoots cultered on the rhizome induction medium. b.Shoot multiplication frequency on the same medium for 90 days of culture.c. Rooting pattern of microrhizome induced plants.d. Rhizome induction in culture tube after one month. e,f&g. Different stages of *in vitro* rhizome formation. h&i Isolated shoots from culture bottle after 90 days and showing rhizome its base. j. Harvested microrhizome from culutre bottle after 90 days. k&l Germinated microrhizome. m.Harvested rhizome from culutre bottle after 120 days. n. Microrhizome derived plants planted on pots. o. Harvested rhizome from field.

Table 27. Effect of growth regulators on the production of Microrhizome in turmeric.

Treatment	Growth regulators (mg l ⁻¹)			No. of Microrhizome / Culture (Mean± SE)	Fresh weight / Microrhizome (g) (Mean±SE)
	BA	Kinetin	NAA		
Control	0	0	0	2.3±0.3d	0.4±0.02e
1	0.5	0	0	7.3±1.2c	0.5±0.04de
2	1.0	0	0	10.6±1.7b	0.7±0.08d
3	1.5	0	0	5.3±0.3cd	0.5±0.02e
4	0	0.5	0	3±0.6d	0.4±0.02e
5	0	1.0	0	5±0.6cd	0.6±0.05de
6	0	1.5	0	4.7±0.7cd	0.5±0.02e
7	0.5	0	0.5	7.7±0.3c	1.0±0.1c
8	1.0	0	0.5	19.7±0.9a	2.4±0.1a
9	1.5	0	0.5	11.3±1.8b	1.3±0.15b

Table represents mean of three repetitions. Mean ± standard errors followed by same letters are not significant at 5 % level according to Duncan's multiple range tests. This experiment was conducted using 0.75x MS salt + + 9% sucrose+ 0.8% agar. Data was taken after 120 days of growth period.

Hardening and planting out

The plantlets obtained from various method of propagation were hardened and planted out as described in materials and methods.

Morphological characterization

The morphological characters of micropropagated, callus regenerated and microrhizome derived plants were studied and conventionally propagated plants were used as control. Data on plant height (cm), number of tiller per plant, number of leaves/plant, internodal length (cm), leaf length (cm), leaf breadth (cm), weight of rhizome (g), breadth of finger per rhizome (cm), number of finger per rhizome, number of nodes per finger and internodal distance (cm) were recorded. The data are given in the Table 28. The maximum plant height was recorded in callus regenerated plants (49.5 cm) followed by conventionally propagated plants (45.4cm) and minimum recorded in microrhizome derived plants (42.1 cm). The callus regenerated and microrhizome derived plants were deviated from the others in the case of number of tiller per plant. Leaf length were almost similar in both conventionally propagated and micropropagated plants (48.4cm, 48.7cm). The lowest leaf length recorded in microrhizome derived plants (42.2

cm). The maximum leaf breadth (16.7cm) was recorded in callus regenerated plants followed by microrhizome derived plants. The highest yield was recorded in callus regenerated plants (590 g) and in all others almost similar.

Breadth of mother rhizome was maximum in conventionally propagated plants (5.2cm) followed by micropropagated plants (4.92 cm). Breadth of primary finger per rhizome was maximum in conventionally propagated plants (2.6 cm) followed by micropropagated plants (2.45 cm). The breadth of primary finger per rhizome was lowest in microrhizome derived plants (2.25cm). Breadth of secondary fingers were similar in conventionally propagated, micropropagated and callus regenerated plants. Microrhizome derived plants showed minimum breadth of secondary fingers (1.85 cm).

Maximum number of mother rhizomes recorded in microrhizome derived plants (1.4) followed by callus regenerated plants (1.3 cm) (Fig 18) Microrhizome derived plants showed more number of primary finger per rhizome (4.8) and less number recorded in callus regenerated plants (3.4). Secondary finger per rhizome was maximum in microrhizome derived plants (9.0) and minimum in conventionally propagated plants (5.8). Tertiary fingers were maximum in microrhizome derived plants (11.95) followed by callus regenerated plants (10.3). Conventionally propagated plants and micropropagated plants were similar in the case of number of nodes per mother rhizome. Maximum nodes per mother rhizome recorded in microrhizome derived plants (14.65) followed by callus regenerated plants (13.4).

More number of nodes in primary and secondary fingers recorded in callus regenerated plants (11.5, 13.1) and in others it was similar. Maximum number of nodes in tertiary fingers was recorded in conventionally propagated plants (9.6).

Internodal length of the fingers of conventional, micropropagated and callus regenerated plants were almost similar. Microrhizome derived plants recorded minimum internodal length compared to all others.

The noticeable variations in rhizome characters like number of mother rhizome, shape and size of primary, secondary and tertiary fingers and entire rhizome structure observed among the somaclones are given in Fig 18. Clear differences were also observed in internodal distances, shape of the fingertips and elongation (Fig 18).

Table 28. Comparative morphology of turmeric somaclones (conventional propagated, micropropagated plants, callus regenerated and microrhizome derived plants).

Plant characters	Conventional propagated plants		Micropropagated plants		Callus regenerated plants		Microrhizome derived plants		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Aerial characters									
Plant height (cm)	45.4	3.83	42.9	4.48	49.5	2.67	42.1	2.55	
No.tiller / plant	1	0	1	0	1.3	0.48	1.4	0.52	
No.leaves / plant	10.3	1.17	9.1	1.2	9.8	0.95	9.0	1.03	
Internodal distance (cm)	10.1	1.2	10.5	1.45	10.1	1.2	10.4	2.02	
Leaf length (cm)	48.4	2.82	48.7	3.1	45.4	3.92	42.2	3.46	
Leaf breadth (cm)	12.8	1.49	13.8	1.23	16.7	1.34	15.05	1.98	
Rhizome characters									
Weight of rhizome (g)	520	23.5	500	51.42	590	46.38	498	43.08	
Breadth of finger/ rhizome (cm)	M	5.2	0.45	4.92	0.6	4.1	0.48	4.8	1.42
	P	2.6	0.34	2.45	0.39	2.45	0.97	2.25	1.58
	S	2	0.54	1.99	0.54	1.99	1.76	1.85	2.98
	T	1.19	0.23	1.09	0.2	1.51	1.16	1.4	3.02
No. of finger/rhizome	M	1	0	1	0	1.3	0.32	1.4	0.52
	P	3.6	0.88	3.8	1.03	3.4	0.11	4.8	0.29
	S	5.8	1.58	6.4	1.51	20	0.23	9.0	0.21
	T	9.1	1.15	9.7	1.16	10.3	0.29	11.95	0.33
No. of nodes/finger	M	11.1	1.78	11.7	2.16	13.4	1.65	14.65	2.63
	P	10.9	1.2	10.7	1.49	11.5	1.96	9.9	1.2
	S	9.9	0.95	10.1	1.45	13.1	1.91	10.9	1.79
	T	9.6	1.4	8.7	1.49	8.5	1.27	7	1.41
Internodal distance (cm)	M	0.57	0.16	0.62	0.17	0.52	0.09	0.3	0.42
	P	0.51	0.07	0.44	0.05	0.64	0.1	0.4	0.22
	S	0.5	0.09	0.49	0.07	0.49	0.1	0.5	0.25
	T	0.54	0.07	0.47	0.09	0.45	0.08	0.4	0.1

The data on aerial characters recorded four months after planting and rhizome characters recorded at the time of harvest. The data analysed statistically by using the software MSTATC.(ANOVA).

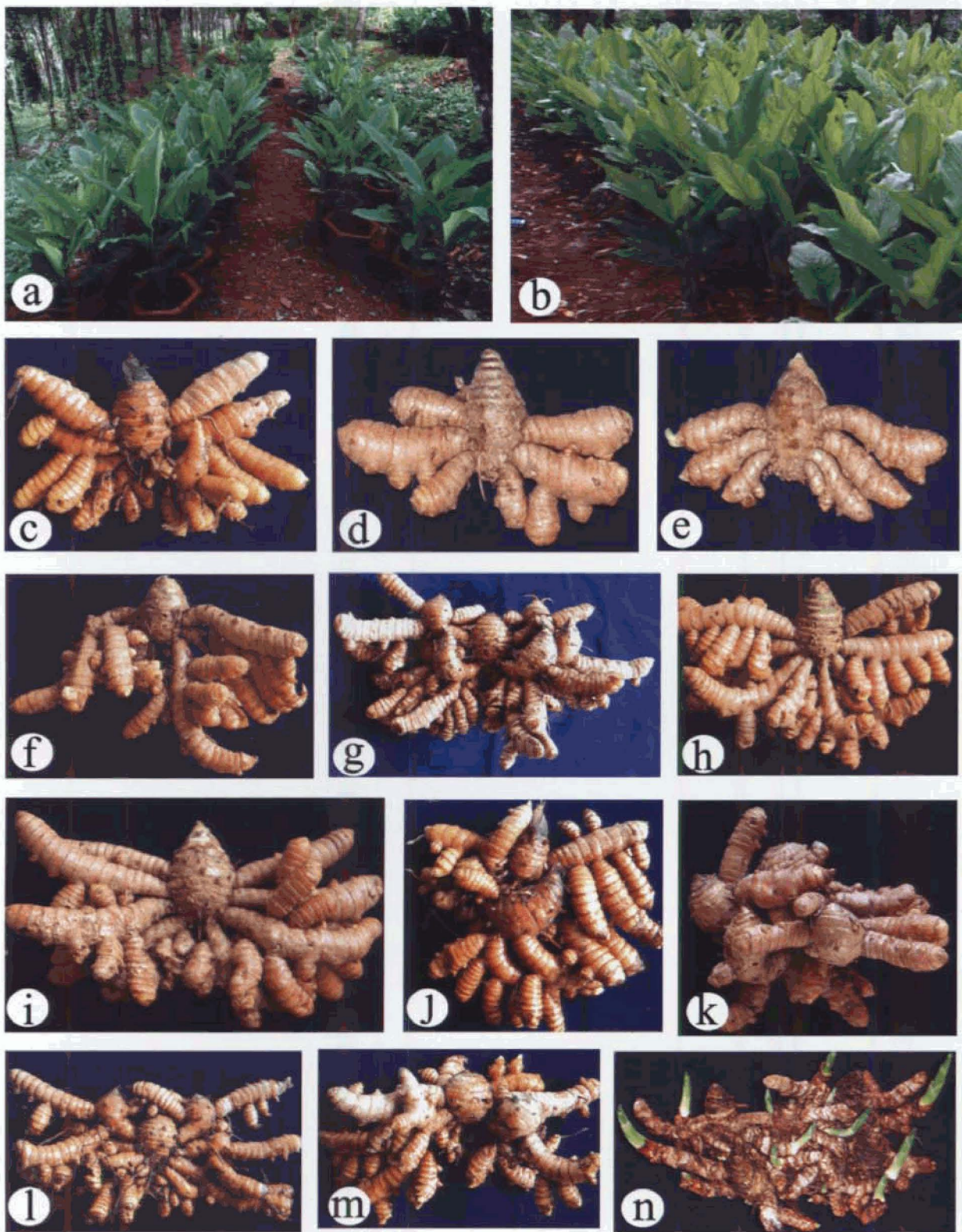


Fig. 18 Morphological evaluation of turmeric somaclones

Va. Somaclones planted in pots. b. Somaclones in field. c-n. Somaclones showing variation in rhizome morphology. c. Bright yellow coloured rhizome with pointed fingers. d. Stout fingers with reduced number. e. Elongated finger with pointed base. f. Downward spreading of fingers. g. Primary fingers turn to mother rhizome. h. More number of secondary fingers. i. Elongated primary fingers. j. Compact nature of mother, primary and secondary fingers. k. Reddish tinted rhizome with more number of mother rhizome. l. Less number of secondary fingers, l. Equal size of primary and secondary fingers. m. Sprouting rhizome showing more number mother rhizome.

Biochemical characterization

The plant showing certain amount of significant variations were selected for further biochemical characterization. Of the sixteen somaclones studied, C4 – 4 recorded the maximum percentage of oil (5%) and minimum in C5-3 (2.0%). Where as in control it was 4.8%. The oleoresin content was low in MP 9 (8.7%) and maximum recorded in MP10 (12.7%) followed by C5-3 (12.5%). The maximum percentage of curcumin was recorded in the accession C4 -2 (5.3%) and minimum in MP 7 and MP 9 (3.9%). MP-8 recorded highest percentage of starch (44.0%) followed by the control (40.2%) and lowest recorded in the somaclone C5 – 3 (30.3%). Maximum percentage of phenol recorded in microrhizome (14.8%) and for reducing sugar maximum recorded in the control (1.85%) followed by the somaclone MP – 10 (1.45%). Where as maximum total carbohydrate recorded in MP – 8 (48.7%). The highest percentage of protein recorded in the accession C 2-4 (1.11%) (Table 29).

Table 29. Quality analysis of turmeric somaclones.

Acc.No	% Oil	% Oleoresin	% Curcumin	% Starch	% Phenol	% Red. Sugar	%Total carbohydrate	% Protein
C1-1	4.0	9.7	4.5	31.4	14.5	1.18	34.9	0.64
C2-1	4.5	10.5	4.0	39.0	12.3	0.82	43.3	0.62
C2-2	3.0	11.2	4.6	36.2	10.8	0.98	40.3	0.55
C2-4	2.9	11.0	4.8	37.0	12.9	1.07	41.2	1.11
C4-2	3.2	12.1	5.3	34.7	14.1	1.19	38.6	0.55
C4-4	5.0	9.8	4.8	25.2	11.3	0.94	39.1	0.48
C5-3	2.0	12.5	4.7	30.3	14.1	1.14	33.7	0.85
MP-1	3.8	11.0	4.3	32.8	12.5	1.02	36.5	0.84
MP-5	4.6	10.9	4.3	33.0	14.0	1.13	36.4	0.79
MP-7	4.0	11.9	3.9	32.7	13.6	0.91	36.3	0.62
MP-8	3.3	11.3	4.1	44.0	11.6	1.17	48.7	0.58
MP-9	3.6	8.7	3.9	37.8	13.4	1.29	42.0	0.43
MP-10	4.6	12.7	4.4	32.7	11.9	1.45	36.3	0.78
MP-12	3.7	10.1	4.0	37.2	14.0	1.38	41.3	0.6
Mrh.	3.9	9.9	4.7	34.2	14.8	0.98	38.0	0.78
Control	4.8	10.8	4.9	40.2	12.7	1.85	42.2	0.98

Each value represents the average of three replications.

Composition of essential oil of *in vitro* microrrhizome and *in vivo* rhizome by using GC- MS

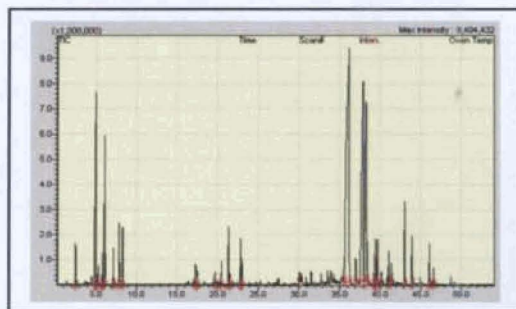
The oils were obtained by conventional hydro distillation of the rhizome and leaves of microrrhizome derived plants of turmeric taken from *in vitro* and *in vivo* in a Clevenger type apparatus. The yield of essential oil obtained from rhizome, leaf of *in vitro* and *in vivo* plants of ginger are given in Table 30 on a fresh weight basis. The relative concentrations of the volatile components identified are presented in Table 31 according to their elution order on column. The main components of rhizome oil of both *in vitro* and *in vivo* were 1 - Phellandrene (10.73 *in vivo* and 15.73% *in vitro*), 1,8 Cineole 5.41 % *in vivo* and 3.15% *in vitro*) and α - Terpinolene 3.24%(*in vitro*). Where as in the case of leaf oil the main components were 1 - Phellandrene (43.87% *in vivo* and 28.15% *in vitro*), α - Terpinolene (15.4% *in vivo* and 10.26% *in vitro*) and 1,8 Cineole (8.45 % *in vivo* and 8.18% *in vitro*) identified. The profile differences are graphically represented in Fig.19.

Table 30. Essential oil yield from turmeric microrrhizome.

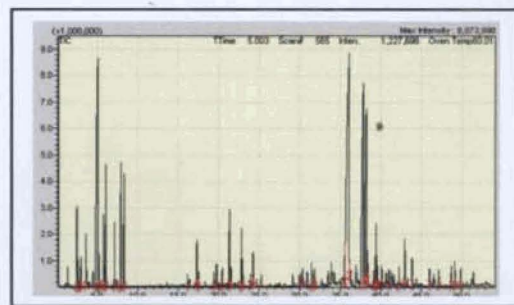
In vitro (% of oil)		In vivo (% of oil)	
Rhizome	Leaf	Rhizome	Leaf
0.22	0.32	0.36	0.32

Table 31. Components of essential oil of turmeric microrrhizome.

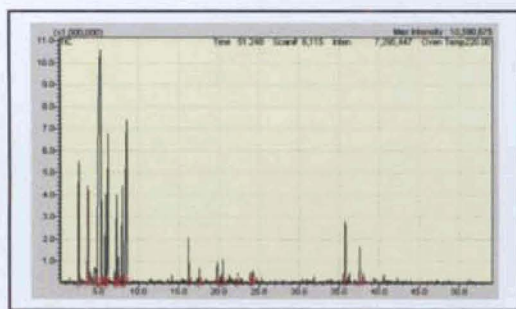
Components	Concentration (%)			
	<i>In vivo</i>		<i>In vitro</i>	
	Rhizome oil	Leaf oil	Rhizome oil	Leaf oil
a - Pinene	-	3.69	1.22	3.15
α - Phellandrene	10.73	43.87	15.73	28.15
β -Pinene	-	4.82	1.01	6.19
1,8 cineole	5.41	8.45	3.15	8.18
d- 3-Carene	-	1.66	1.44	1.44
?-Terpinene	-	3.61	1.28	2.31
p. Cymene	1.17	4.24	3.5	7.68
p- Mentadiene/ d-Limonene		3.85	1.48	3.44
α -Terpinolene	1.12	15.4	3.24	10.26
α -Terpinene	-	1.84	-	1.08
Linalool	-	1.12	-	-
Zingiberene	1.45	-	1.98	-
β -Sesquiphellandrene	1.11	-	1.43	-
t- Caryophyllene	-	-	-	1.09
β -Farnesene	-	-	-	6.44



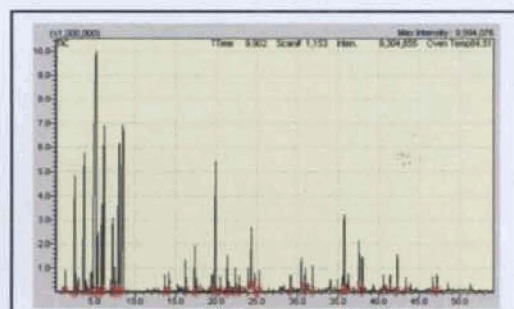
In vivo rhizome oil



In vitro rhizome oil (Microrhizome)



In vivo leaf oil



In vitro leaf oil (Microrhizome)

Fig 19. Showing the differences in essential oil components of turmeric microrrhizome *in vivo* and *in vitro*.

Histology of *in vitro* microrrhizome

Curcumin cells

Fresh sections were taken from *in vitro* microrrhizome and stained with safranin showed a large number of curcumin cells. The cell walls have light yellowish shade and thick walled compared to neighboring cells. The developments of curcumin cells in microrrhizome induced *in vitro* were studied at the stage of 120 days of growth period and compared with the normal rhizome. Well-developed curcumin cells were observed in the microrrhizome derived plants as that of normal rhizome (Fig.20)

Oleoresin and oil

The fresh sections were stained with safranin and observed under Olympus B x 50 microscope. The oleoresins were observed as deep brown colored cells and oil cells appeared as light yellowish transparent. Presences of oleoresin and oil cells were comparable with those of normal rhizome (Fig.20)

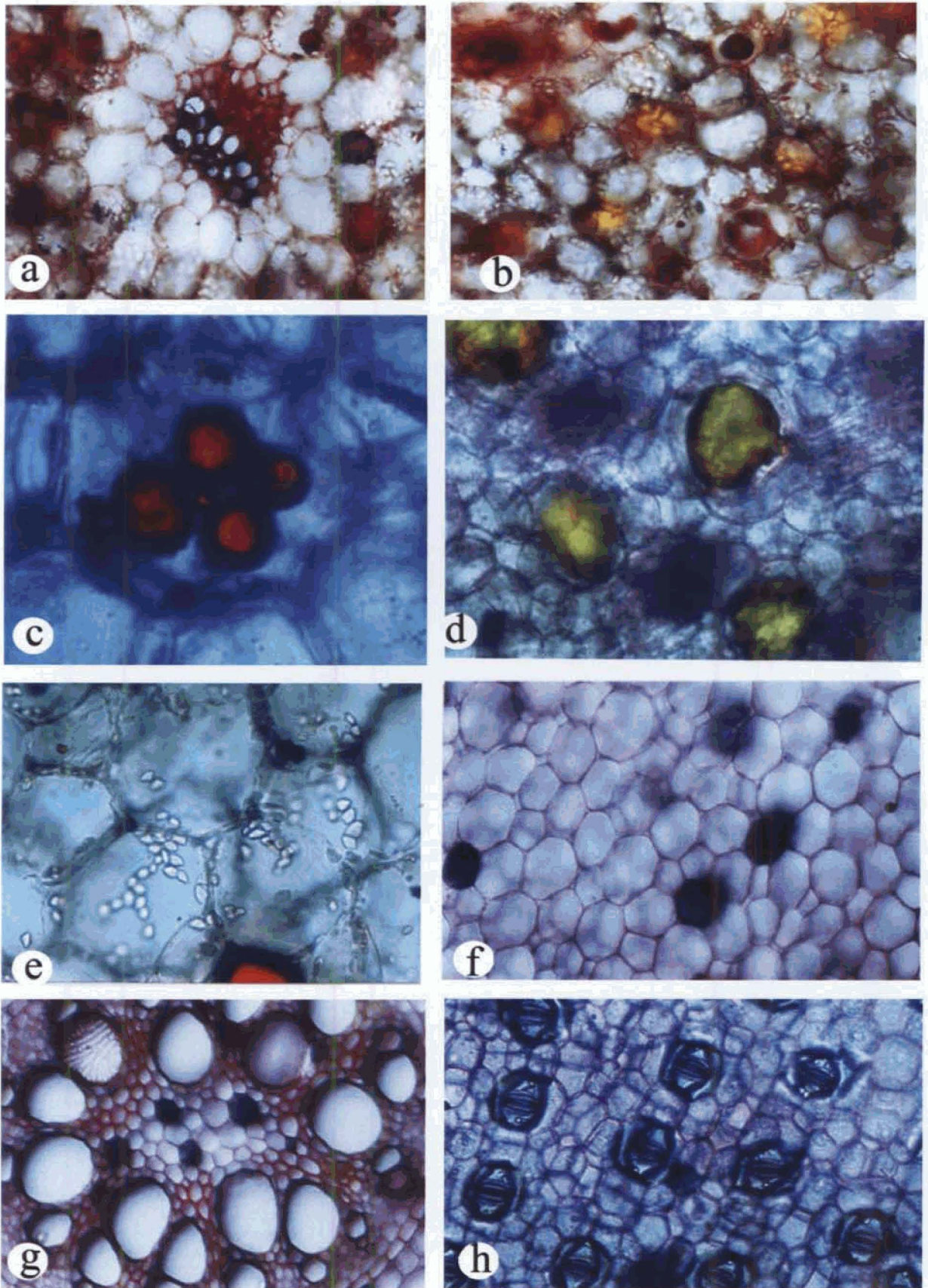


Fig. 20 Secondary metabolites production of *in vitro* microrhizome in turmeric is observed in anatomical sections

a. Developed vascular bundles (Conjoint collateral, exarch and closed) in turmeric microrhizome at the period of 120 days of growth period. b. Presence of curcumin, oil, oleoresin and starch in microrhizome. c. Presence of oleoresin d. Presence of curcumin cells. e. Presence of starch grain. f. Presence of phenol. g. Vascular bundle of roots are polyarch and in radial organization and phloem bundles alternate with xylem bundles; Xylem is exarch; Metaxylem vessels are towards the pith and larger in diameter; barrel shaped endodermis cells completely encircles the stele. h. Stomatal frequency of callus regenerated turmeric leaf.

Starch grain

Large numbers of simple starch grains are observed in the cells near the intermediate layer and in ground parenchyma of the central zone as well as in the inner cortical layer. Each cells contains more number of starch grains. The fresh sections were stained with safranin and temporarily mounted in glycerol. Fully developed starch grains were observed in the microrhizome developed *in vitro* (Fig. 20).

Stomatal study of somaclone

The stomatal parameters used are the stomatal size and stomatal density. Those, which showed maximum stomatal size, showed least stomatal frequency. Stomatal frequency of the five callus regenerated plants were studied, of these one of the plants showed more number and smaller in size than the other somaclones (on average 15 stomata per sq.mm; where as in the case of control it was 9 stomata per sq. mm (Fig. 20).

Chromosome indexing

The root tips cells of selected somaclones were cytological analyzed for the detection of variation in their chromosomal number. The root tips of conventionally propagated plants were taken as control. The somatic chromosome number of turmeric is $2n=63$. Micropropagated and microrhizome derived plants showed normal chromosome number as that of the control (Fig. 21) Callus regenerated plants showed variation in the chromosome number and also structural aberrations. The numerical variations were mainly aneuploids with lesser number of chromosomes ($2n=53, 55, 57$) (Fig. 21) than the normal number except one plant with higher number of chromosome ($2n=65$). (Fig. 21). This reduction in the chromosome number may be due to the effect of growth hormone in the culture medium.

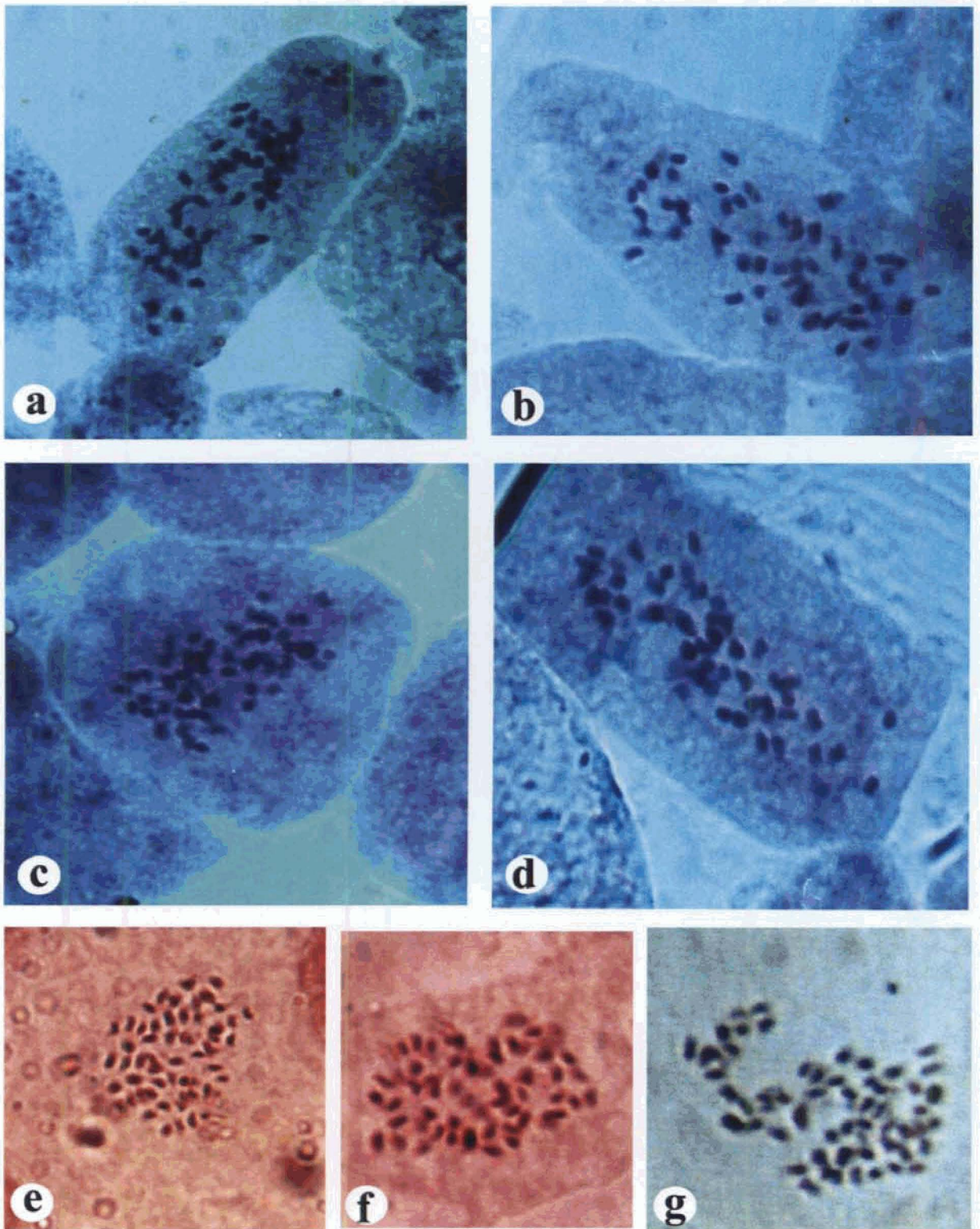


Fig. 21 Mitotic metaphase of root tip cells in turmeric

a. Somatic cells showing normal chromosome number in control plant $2n=63$. b. Micropropagated plant showing same chromosome number as in control plant. 63 . c. Callus regenerated plants showing less chromosome count $2n=53$. d. Microrhizome derived plants showed $2n=63$. e, f, & g. Callus regenerated plant showing aneuploidy. (e. $2n=55$; f. 57 ; g. 56).

Molecular characterization

RAPD profiles of micropropagated plants

RAPD analysis of 20 micropropagated plants and a conventionally propagated plant taken as the control were done using 10 operon primers. The primers used for RAPD analysis were OPA 03, OPA 04, OPA 06, OPA 07, OPA 08, OPA 18, OPB 11, OPB 18, OPC 10 and OPC 19. These plants showed uniform banding pattern in all the above primers tested.

RAPD profiles of Callus regenerated plants

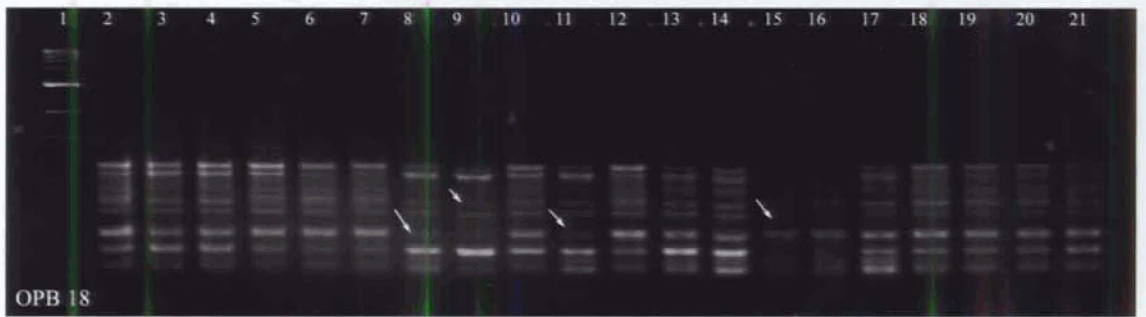
The 19 selected callus-regenerated plants were subjected to RAPD study using 10 oligonucleotide primers. Conventionally propagated plant was taken as the control plant. A total of 89 bands were produced, of which 21 monomorphic bands and 78 polymorphic bands were present. The maximum percentage of polymorphism (85%) was produced using the primer OPA 08. The frequencies with which the RAPD band changes were observed for turmeric somaclones are shown in Table 32.

Out of 10 primers used the accession number C3-2 showed polymorphic bands in 9 primers (OPB 18, OPB 11, OPA 08, OPA 06, OPA 03, OPA 07, OPC 19, OPA 18, OPC 10). The accession number C4-2 showed polymorphism in 7 primers (OPB 18, OPB 11, OPA 08, OPA 07, OPC 19, OPA 04, OPC 10). C9-2 showed polymorphism in 6 primers (OPB 11, OPA 03, OPC 19, OPA 18, OPA 04 and OPC 10). C5-3, C7-1 and C10-4 showed polymorphism in 5 primers (C5-3: OPB 18, OPB 11, OPA 08, OPA 06 and OPA 07. C7-1: OPB 18, OPA 08, OPA 07, OPA 18 and OPC 10. C10-4: OPB 11, OPC 19, OPA 18, OPA 04, OPC 10). C8-2 in 3 primers (OPB 11, OPA 18, OPC 10) and C8-1 in 2 primers (OPB 18, OPA 07) showed polymorphism. Out of 20 somaclones were analysed 8 plants were identified as variant.

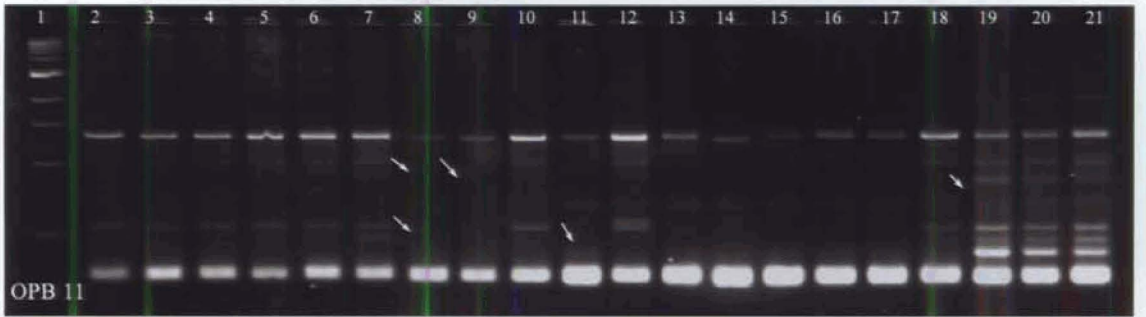
The genotypes C3-2, C7-1 and C8-1 clearly showed distinct polymorphism from the rest of the genotypes at 0.8 kb region and 1.1 kb region and absence of a clear cut band at 1.0 kb region in the genotype C7-3 using the primer OPA 07 (Fig. 22, 23).



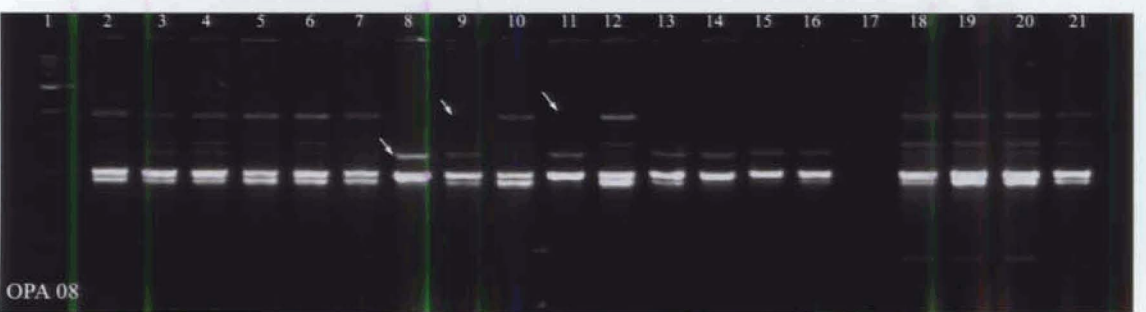
Fig. 22 RAPD profile of callus regenerated plants of turmeric expressed by operon primers



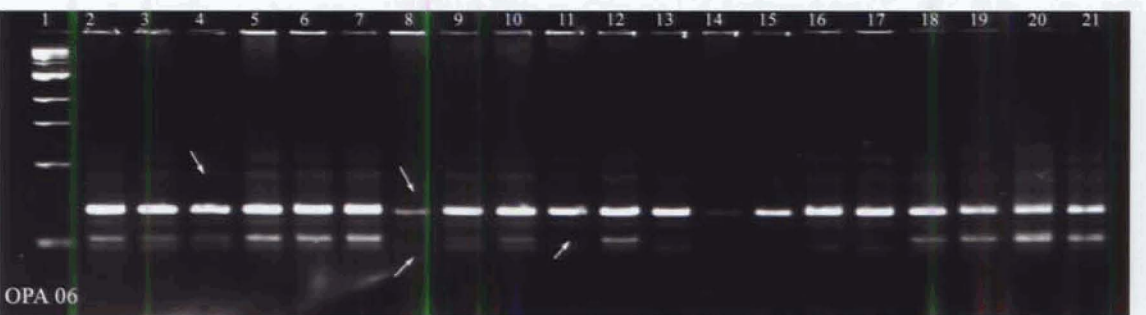
1- Marker, 2- control, 3-C1-1, 4-C1-5, 5-C2-1, 6-C2-2, 7-C2-4, 8 -C3-2, 9-C4-2, 10-C4-4, 11-C5-3,12-C5-4, 13-C5-5, 14-C6-2, 15- C7-1, 16-C7-3, 17-C8-1,18-C8-21, 19- C9-2, 20-C10-2, 21-C10-4.



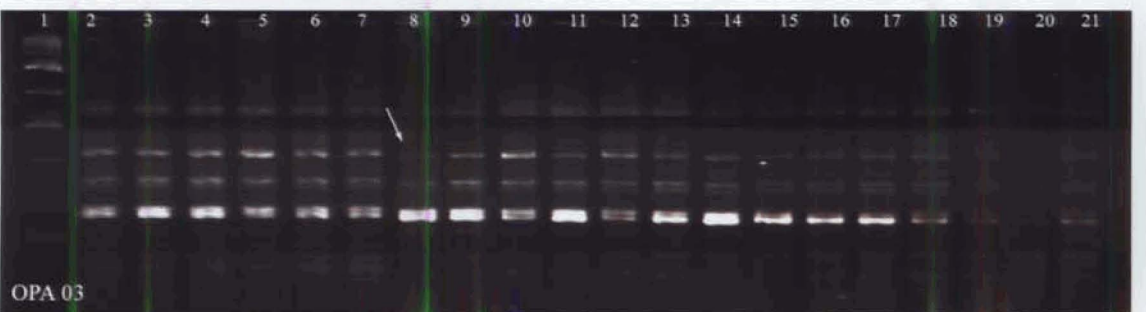
1- Marker, 2- control, 3-C1-1, 4-C1-5, 5-C2-1, 6-C2-2, 7-C2-4, 8 -C3-2, 9-C4-2, 10-C4-4, 11-C5-3,12-C5-4, 13-C5-5, 14- C6-2, 15- C7-1, 16-C7-3, 17-C8-1,18-C8-21, 19- C9-2, 20-C10-2, 21-C10-4.



1- Marker, 2- control, 3-C1-1, 4-C1-5, 5-C2-1, 6-C2-2, 7-C2-4, 8 -C3-2, 9-C4-2, 10-C4-4, 11-C5-3,12-C5-4, 13-C5-5, 14-C6-2, 15- C7-1, 16-C7-3, 17-C8-1,18-C8-21, 19- C9-2, 20-C10-2, 21-C10-4.



1- Marker, 2- control, 3-C1-1, 4-C1-5, 5-C2-1, 6-C2-2, 7-C2-4, 8 -C3-2, 9-C4-2, 10-C4-4, 11-C5-3,12-C5-4, 13-C5-5, 14- C6-2, 15- C7-1, 16-C7-3, 17-C8-1,18-C8-21, 19- C9-2, 20-C10-2, 21-C10-4.



1- Marker, 2- control, 3-C1-1, 4-C1-5, 5-C2-1, 6-C2-2, 7-C2-4, 8 -C3-2, 9-C4-2, 10-C4-4, 11-C5-3,12-C5-4, 13-C5-5, 14- C6-2, 15- C7-1, 16-C7-3, 17-C8-1,18-C8-21, 19- C9-2, 20-C10-2, 21-C10-4.

Fig. 23 RAPD profile of callus regenerated plants of turmeric expressed by operon primers

Table 32. Different primers used for studying genetic variability of turmeric somaclones by means RAPD markers.

Primers	Sequences	Total number of bands	Monomorphic bands (n)	Polymorphic bands (n)	Polymorphic band (%)
OPA 03	5' AGTCAGCCAC 3'	9	2	7	77.7
OPA 04	5' AATCGGGCTG 3'	8	3	5	62.5
OPA 06	5' GGTCCCTGAC 3'	6	1	5	83.3
OPA 07	5' GAAACGGGTG 3'	7	2	5	71.4
OPA 08	5' GTGACGTAGG 3'	7	1	6	85.0
OPA 18	5' AGGTGACCGT 3'	9	2	7	77.7
OPB 11	5' GTAGACCCGT 3'	9	2	7	77.7
OPB 18	5' CCACAGCAGT 3'	9	2	7	77.7
OPC 19	5' GTTGCCAGCC 3'	12	4	8	66.6
OPC 10	5' TGTCTGCGTG 3'	14	3	11	78.5

RAPD profiles of microrhizome derived plants

The microrhizome derived plants were analysed for RAPD profiling by using 10 operon primers (OPA 03, OPA 04, OPA 06, OPA 07, OPA 08, OPA 18, OPB 11, OPB 18, OPC 10 and OPC 19). The microrhizome-derived plants showed similar banding pattern with that of the conventionally propagated control plant in all the primers listed above.

Comparative study of RAPD profiling of micropropagated, callus regenerated and microrhizome derived plants

Comparative RAPD polymorphism of 10 randomly selected micropropagated, callus regenerated and microrhizome derived plants of turmeric were produced using 5 operon primers (OPA 04, OPA 08, OPC 19, OPC 01, OPA 06 and OPC 10). Micropropagated and microrhizome derived plants showed monomorphic banding pattern in all the primers tested. Callus regenerated plants showed polymorphic banding pattern in almost all the above primers (Fig. 24).

Screening for disease incidence

Selected 10 somaclones and a control plant of turmeric variety 'Prabha' were challenged with *Pythium myriotylum* for rhizome rot disease. Plants were planted in pots with four



OPA 08
 M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp3, 5-Mp 4, 6-Mp5,7 -Mp6, 8-Mp7, 9-Mp8, 10-Mp9,11-Mp10, 12-control, 13-C1-1, 14- C1-5, 15-C2-1, 16-C2-2,17-C2-4, 18- C3-2, 19-C4-2, 20-C4-4, 21-C5-3, 22-C5-4, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27- Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.



OPC 19
 M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp3, 5-Mp 4, 6-Mp5,7 -Mp6, 8-Mp7, 9-Mp8, 10-Mp9,11-Mp10, 12-control, 13-C1-1, 14- C1-5, 15-C2-1, 16-C2-2,17-C2-4, 18- C3-2, 19-C4-2, 20-C4-4, 21-C5-3, 22-C5-4, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27-Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.



OPA 04
 M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp3, 5-Mp 4, 6-Mp5,7 -Mp6, 8-Mp7, 9-Mp8, 10-Mp9,11-Mp10, 12-control, 13-C1-1, 14- C1-5, 15-C2-1, 16-C2-2,17-C2-4, 18- C3-2, 19-C4-2, 20-C4-4, 21-C5-3, 22-C5-4, 23- Control, 24- Mrh1, 25-Mrh2, 26-Mrh3, 27- Mrh4, 28-Mrh5, 29-Mrh6, 30-Mrh7, 31-Mrh8, 32-Mrh9, 33-Mrh10.

Fig. 24 Comparative RAPD profiling of micropropagated, callus regenerated and microrhizome derived plants of turemric

MP- micropropagated, C callus regenerated, Mrh- microrhizome derived plants

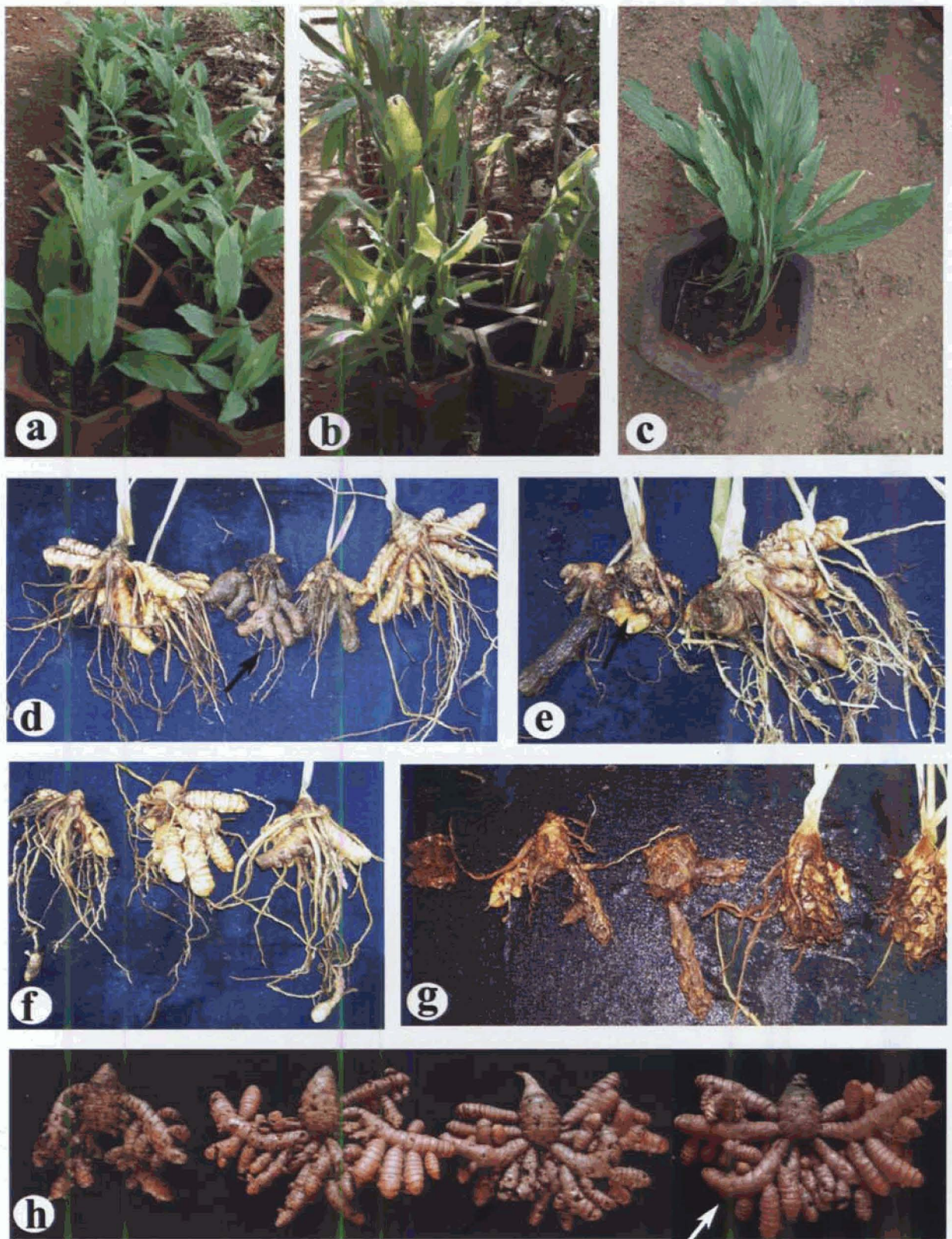


Fig. 25 Disease screening of turmeric somaclones

a. *Pythium myriotylum* inoculated somaclones. b. Inoculated plants at the period of seven months. c. Rhizome infected plant with no aerial symptoms. d-g Somaclone showing different range of disease incidence. h. Somaclones showing the range of symptoms of unidentified pest attack.

replications. The selection of the somaclones was based on its morphological expression. The maximum disease incidence was recorded in the accession no. C4-2 (91.8%) followed by C5-3 (48.15 %) and C6-2 (33.3%). The control plant with pathogen showed 6.25% of disease incidence. The somaclones such as C10-2 (2.5%), C3-2 (2.7%) and C8-2 (4.17%) showed minimum disease incidence (Fig. 25). The maximum number of rhizome was recorded in C3-2 (4.6) and C4-2 (4.4). The somaclone C8-2 also recorded the maximum number of root tuber (3.3%) followed by C3-2 (1.5%) (Table 33).

Table 33. Incidence of rhizome rot in *Pythium myriotylum* treated turmeric somaclones planted in pots.

Acc. No	Total no. Rhizome	Disease	
		incidence (%)	No. of tubers
	Mean	Mean	Mean
Control	4.2 abc	6.25 (14.47) ab	0 b
C3-2	4.6 a	2.7 (9.46) a	1.5 b
C4-2	4.4 a	91.8 (73.36) d	0 b
C5-3	3.2 abc	48.15 (43.94) cd	0.7 b
C6-2	2.7 c	33.3 (35.25) cd	0.5 b
C7-1	4.3 ab	17.5 (24.73) bc	1.1 b
C8-1	4 abc	10.47 (18.88) bc	0 b
C8-2	2.8 bc	4.17 (11.78) ab	3.3 a
C9-2	4.2 abc	23.8 (29.2) c	1.3 b
C10-2	3.2 abc	2.5 (9.1) a	1.3 b
C10-4	3.5 abc	23.6 (29.07) c	0.5 b

Mean followed by different letters are significantly different at 5% level according to the least significant difference test (LSD) and Duncan's multiple range test (DMRT). Value in parenthesis is transformed value.

Cardamom

Micropropagation

Rhizome cuttings with shoot bud primordia and juvenile shoots were cultured on MS medium supplemented with BA at 1mg l^{-1} and NAA at 0.5mg l^{-1} (Nirmal Babu *et al.*, 1997). This

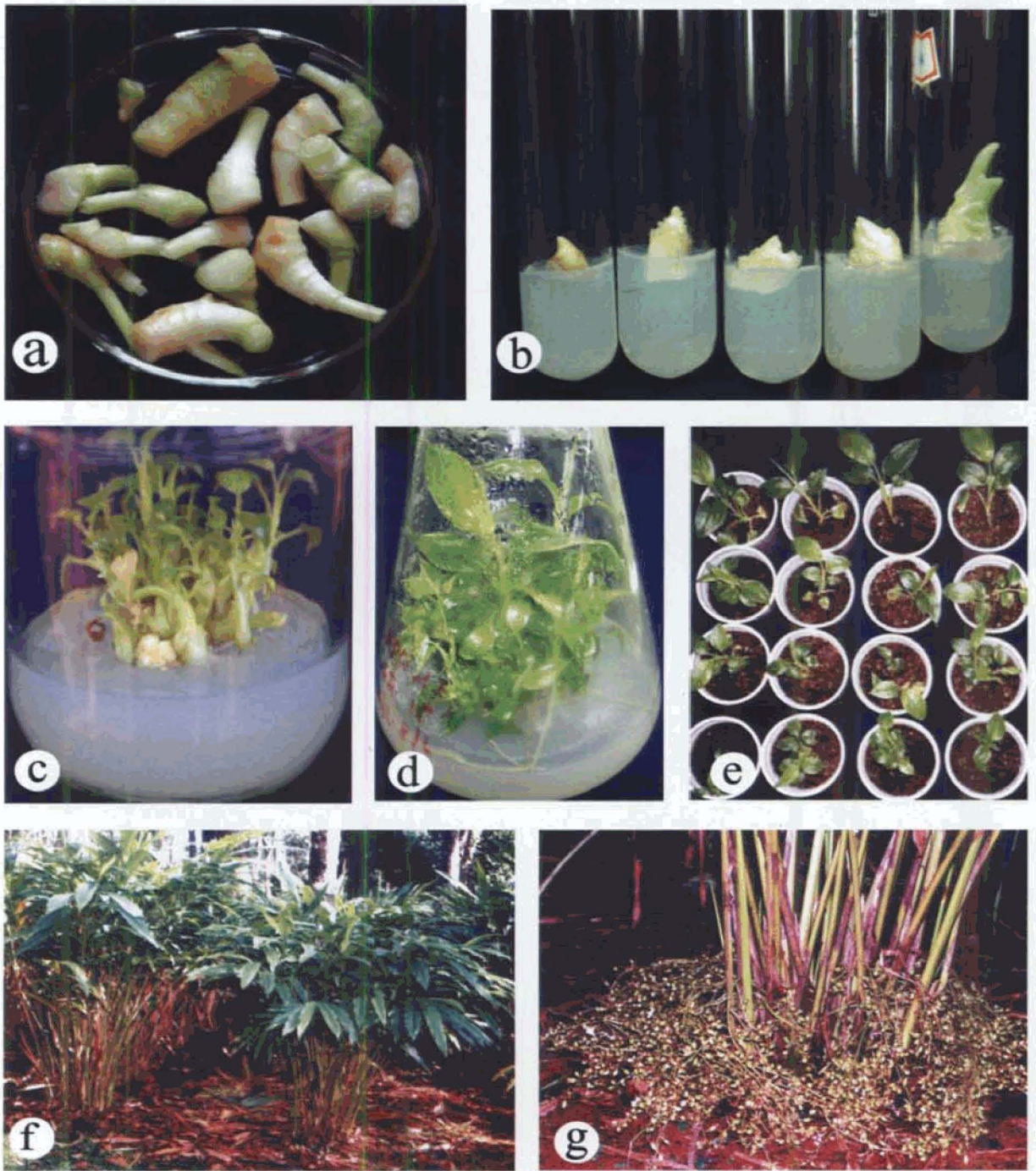


Fig. 26 Rapid micropropagation of cardamom from rhizome bud

a. Sterile axillary bud used as explants for micropropagation. b. Different stages of micropropagation. c&d. Shoot elongation and multiplication on MS medium. e. Regenerated plantlets established in potting soil in green house for 3 months. f. Field planting of cardamom. g. Yield of cardamom somaclones.

was the efficient protocol used for micropropagation of cardamom, developed earlier at IISR. Each rhizome produced a mean of 4 shoots, each approximately 5.4 cm in length. BA was the most effective cytokinin for promoting shoot production. Proliferation could be maintained by subculturing the plants once in every 6 weeks. Cultures were maintained at 3000 lux for 12 h per day at 27°C. Eight to 10 weeks after transfer, rooted plantlets were planted in pots containing a 2:1 mixture of sand and coir dust (Fig. 26).

Callus induction and Plant regeneration

Vegetative bud cultured on MS supplemented with 1mg^l⁻¹ 2,4-D for callus induction and regeneration on MS + 4 mg^l⁻¹ BA+ 0.5 mg^l⁻¹ NAA. This was the high frequency plant regeneration protocol standardised at IISR (Nirmal Babu *et al.*, 1997). The optimum percentage of callus (96%) and fresh weight (3.6g) of callus obtained from this medium (Fig.27). Darkness for 14 days at the beginning of the culture period increased the rate of callus production.

Maximum regeneration response was observed after 2 weeks in the MS medium containing 4 mg^l⁻¹ BA+ 0.5 mg^l⁻¹ NAA (80%). This was most suitable for shoot multiplication and development from differentiating callus (Fig. 27). Once shoots were excised, the calli maintained their morphogenetic competence over several subcultures in the hormone-free MS medium. Regenerated plants showed variation in morphology on *in vitro* culture itself. Regenerated shoots rooted readily and the plants produced are being studied for somaclonal variation (Fig. 27).

***In vitro* method to overcome apical dominance**

A method using petri plates replaces growth regulators with a mechanical means to control apical dominance and thus activating axillary bud development. Shoot-tip explants were induced to produce multiple shoots (18- 25) in a petri plate containing MS basal medium with 0.8% agar, 30g/l sucrose. This method stimulated vigorous root growth also with high rate (80%) of establishment in soil (Fig. 28).

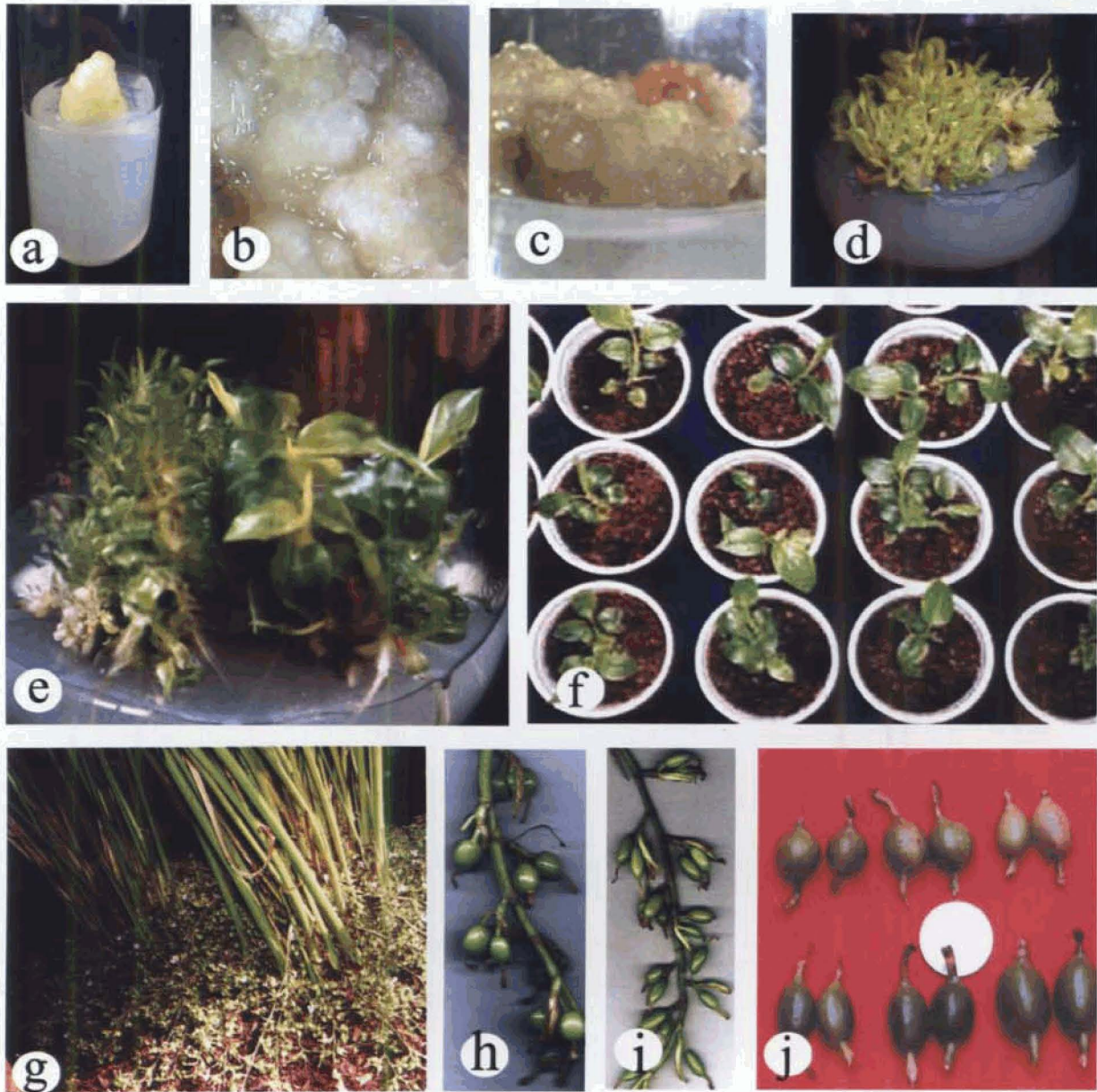


Fig. 27 Regeneration of plantlets from vegetative callus of cadamom

a. Explant inoculated in callus induction medium. b, c, d. Callus formation and regeneration of plantlets from vegetative bud. e. Regenerated plants showing variation in leaf shape. f. Hardened plants. g. Field grown plant showing panicle density. h, i & j. Somaclones showing variation in fruit shape.

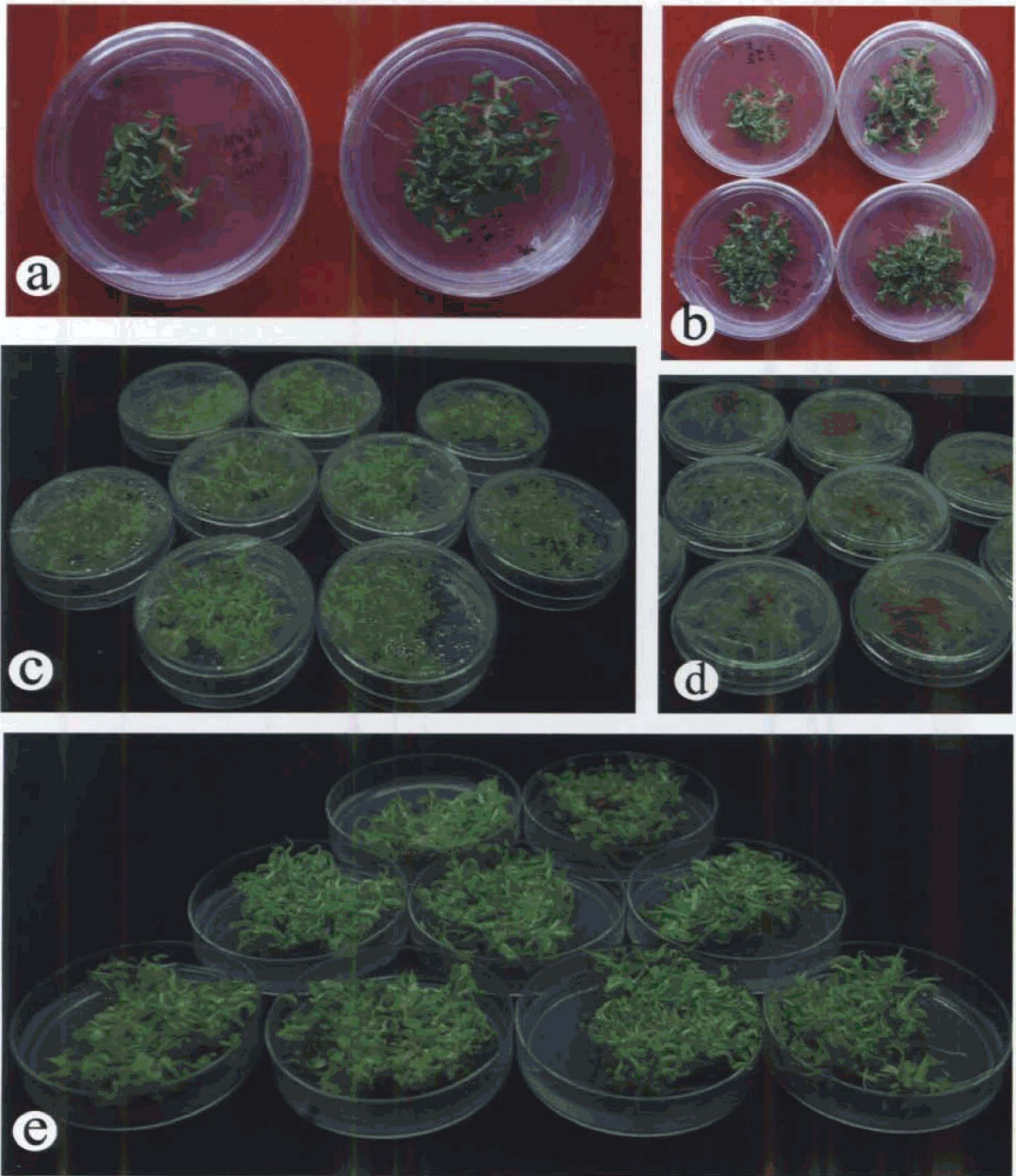


Fig. 28 An *in vitro* method to control apical dominance by replacing growth regulators
 a. Multiple shoot induction in cardamom. b. Different stages of multiplication c. Rate of multiple shoot formation
 d. Root formation frequency e. Opened vessels with multiple shoots

Hardening and planting out

The plantlets derived from the different method of propagation were hardened and planted out as described in material and methods.

Morphological characterization

The growth characters varied significantly in various types of propagation with regard to plant height, number of tiller/plant, number of leaves, primary leaf length, number of panicle per plant, number of nodes per panicle, panicle length and number of flowers per panicle (Table 34). The plant height varied from 175.45 cm to 207.2 cm. Maximum plant height observed in micropropagated plants (207.2 cm). Number of tiller was recorded maximum in conventionally propagated plants 22.45 followed by micropropagated plant (19.5). More number of leaves also recorded in conventionally propagated plants (157.35) followed by micropropagated plants (139.55) and minimum recorded in callus regenerated plant (130.8). Primary leaf length recorded high in conventionally propagated plants (9.69) followed by micropropagated plants 8.72.

Greater number of panicle was recorded in callus regenerated plants (17.1) and in micropropagated plants it was less (12.37). Where as the greatest number of nodes per panicle observed in callus regenerated plants (19.1). Panicle length and number of flowers per panicle was high in micropropagated plants (28.49 cm and 29.6).

The noticeable variations in panicle branching and size of the fruit observed among the somaclones are given in Fig. 27.

Table 34. Morphological characterization of cardamom somaclones

Plant characters	Conventional propagated plants		Micropropagated plants		Callus regenerated plants	
	Mean	SD	Mean	SD	Mean	SD
Plant height (cm)	180.15	35.96	207.2	24.82	175.45	32.88
No.tiller / plant	22.45	7.07	19.5	8.68	18	9.03
No. leaves / plant	157.35	80.34	139.55	77.95	130.8	70.2
Primary leaf length (cm)	9.69	2.97	8.72	3.56	8.09	3.53
No. of panicle/plant	13.19	6.64	12.37	8.05	17.1	8.55
No. of nodes/panicle	18.85	24.29	15.3	3.92	19.1	11.85
Panicle length	23.24	8.49	28.49	7.65	16.08	8.28
No. of flowers/panicle	23.77	9.38	29.6	15.33	22.22	9.11

The data were analysed by ANOVA using MSTATC software

Petiole anatomy

The single layered upper and lower epidermis followed by patches of hypodermal sclerenchyma occurs associated with both upper and lower epidermal region, but they are prominent below the upper epidermis. The median portion of the lamina is thickened on the adaxial side and has a number of conjoint and collateral vascular bundles. In the rest of the lamina (on either side of the mid rib) the vascular bundles of various sizes alternate with one another. The vascular bundles are surrounded by a definite sclerenchymatous bundle sheath. In one of the somaclones, the bundle sheath is connected upper and lower epidermis by a group of compact sclerenchymatous cells called bundle sheath extension.

Stomata are distributed mostly in the lower epidermis only. The stomata are paracytic, having the guard cells flanked by somewhat elongated, conical shaped subsidiary cells. The above somaclone also showed altered size of the subsidiary cells. Some were oval or round in nature (Fig. 29).

Molecular characterization

RAPD profile of micropropagated plants

RAPD analysis of micropropagated plants was conducted using 10 operon primers (OPA 04, OPA 17, OPB 04, OPB 18, OPC 08, OPC 17, OPC 19, OPD 12, OPD 20 and OPC 10). 10 Micropropagated plants and a conventionally propagated control plants were studied by this technique. The entire 10-micropropagated plants showed monomorphic banding pattern in all the above primers tested.

RAPD profile of Callus regenerated plants

The selected 10 callus regenerated plants were used for RAPD analysis with 10 operon primers (OPA 04, OPA 17, OPB 04, OPB 18, OPC 08, OPC 17, OPC 19, OPD 12, OPD 20 and OPC 10). A total of 103 bands were produced, of which 30 monomorphic bands and 73 polymorphic bands were present. The maximum percentage of polymorphism (95%) was

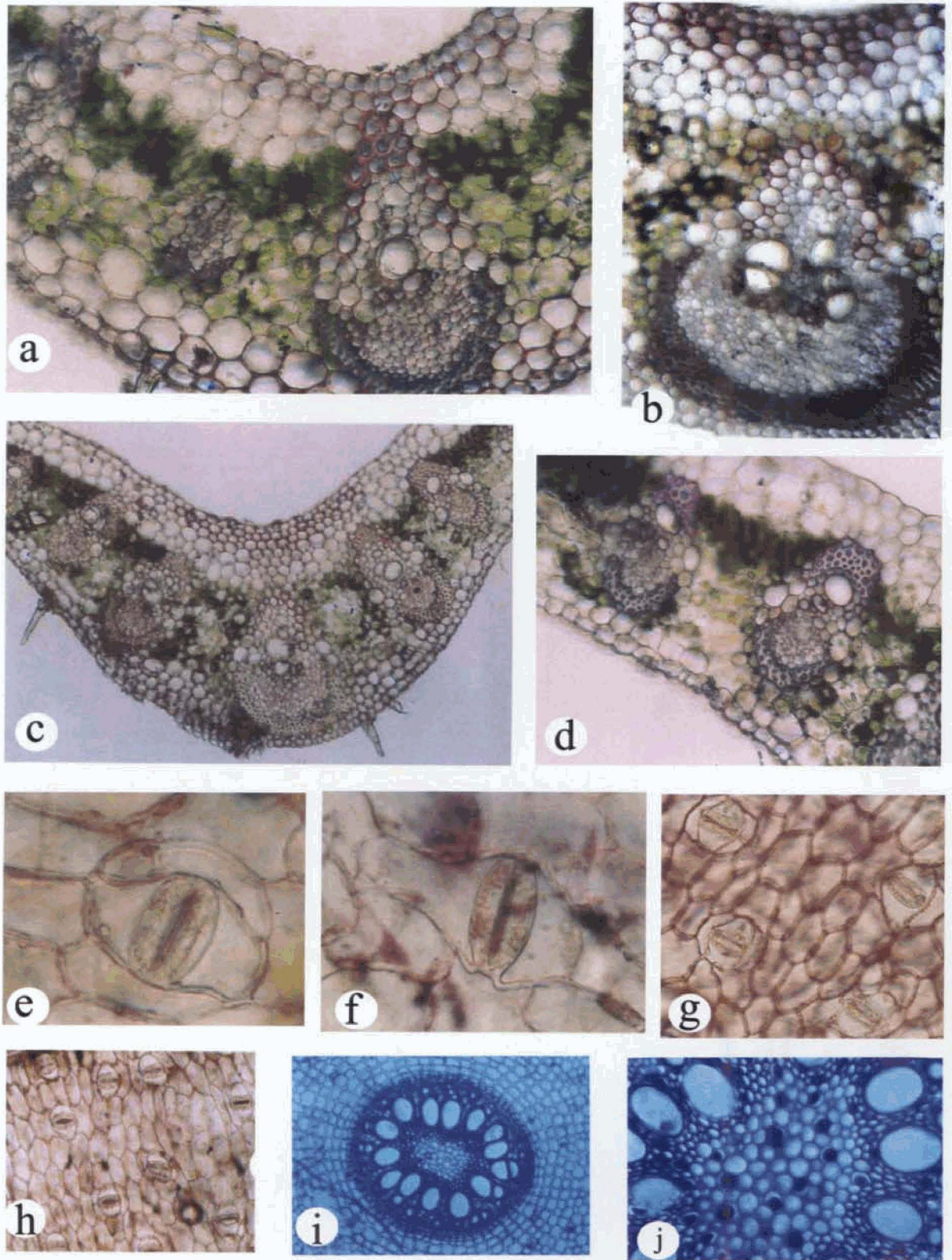


Fig. 29 Anatomy of cardamom somaclones

a. Somaclone showing bundle sheath extension b. Control plant showing no bundle sheath extension. c. Sclerenchymatous patches of hypodermal cells prominent below the upper epidermis. d. Conjoint collateral vascular bundle of the lamina. e&f. Paracytic stomata showing differences in size and shape. g&h. Stomatal difference in number. i&j. More number of vascular bundles present in the root.

produced using the primer OPC 10. The frequencies with which the RAPD band changes were observed for cardamom somaclones are shown in table 35.

The somaclone (SC 08) showed polymorphism in 6 operon primers (OPA 04, OPA 17, OPD 12, OPC 10, OPB 18 and OPB 04). The somaclone (SC 15) also showed polymorphism in 6 primers (OPA 04, OPD 12, OPC 10, OPB 18, OPC 17 and OPB 04) (Fig 30,31). Somaclone 13 (SC 13), somaclone 6 (SC 6) and somaclone 18 (SC 18) showed polymorphism with 5 and 4 primers respectively (SC 13: OPA 17, OPD 12, OPC 10, OPB 18 and OPC 17; SC 6: OPD 12, OPC 19, OPC 10, OPB 04 and SC 18: OPD 12, OPB 18, OPC 17 and OPB 04) (Fig. 30,31).

Table 35. Different primers used for studying genetic variability of cardamom somaclones by means RAPD markers

Primers	Sequences	Total number of bands	Monomorphic bands (n)	Polymorphic bands (n)	Polymorphic band (%)
OPA 04	5' AATCGGGCTG 3'	8	5	3	37.5
OPA 17	5' GACCGCTTGT 3'	4	2	2	50.0
OPB 04	5' GGACTGGAGT 3'	5	2	3	60.0
OPB 18	5' CCACAGCAGT 3'	9	4	5	55.5
OPC 08	5' TGGACCGGTG 3'	11	3	8	72.7
OPC 17	5' TTCCCCCAG 3'	10	2	8	80.0
OPC 19	5' GTTGCCAGCC 3'	12	7	5	41.6
OPD 12	5' CACCGTATCC 3'	16	3	13	81.25
OPD 20	5' CACCGTATCC 3'	8	1	7	87.25
OPC 10	5' TGTCTGCGTG 3'	20	1	19	95.0

Comparative study of RAPD profiling of micropropagated and callus regenerated plants

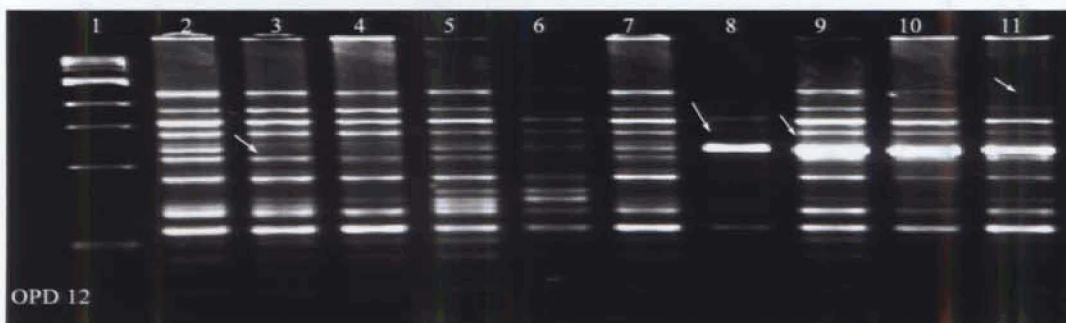
Comparative RAPD profiling of micropropagated and callus regenerated were studied by using 6 operon primers OPB 19, OPC 10, OPC 19 OPD 12. OPA 06 and OPA 17. Conventionally propagated cardamom was used as control plant, 10 micropropagated plants regenerated from vegetative buds and 10 callus regenerated plants from vegetative buds were used for the study. Micropropagated plants showed monomorphic banding pattern in all the primers tested and at the same time callus regenerated plants showed polymorphic banding pattern in all the above primers (Fig. 32).



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



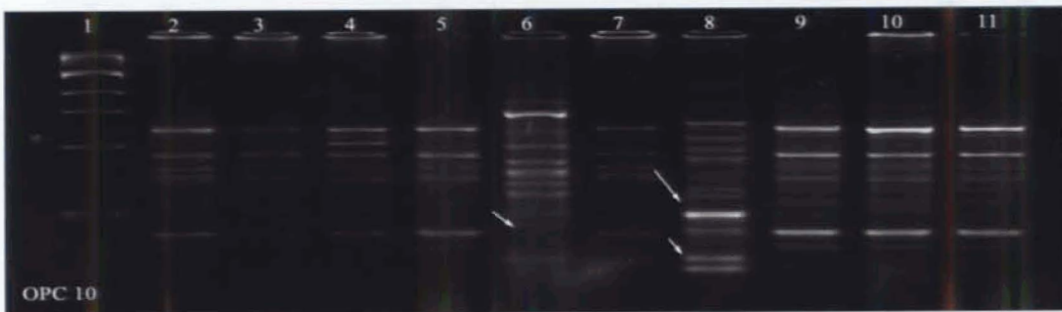
1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



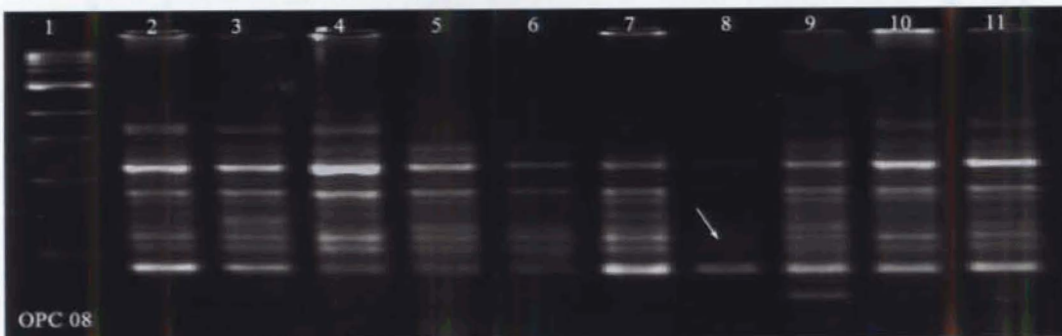
1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.

Fig. 30 RAPD profile of callus regenerated plants of cardamom expressed by operon primers

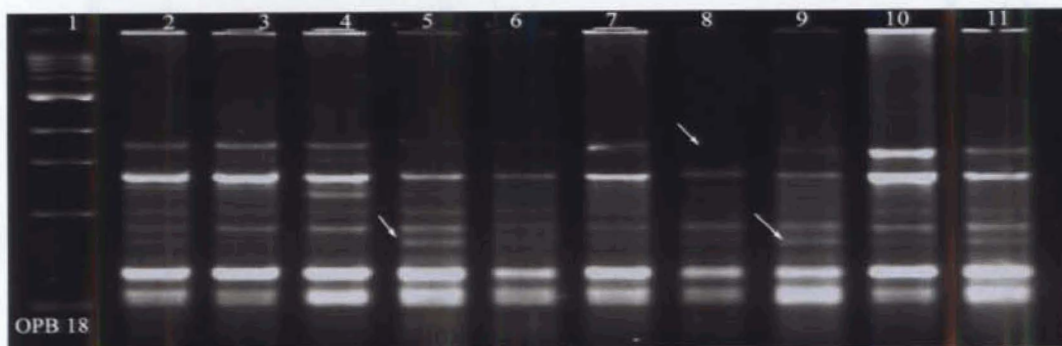
SC- Somaclone (regenerated from callus).



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



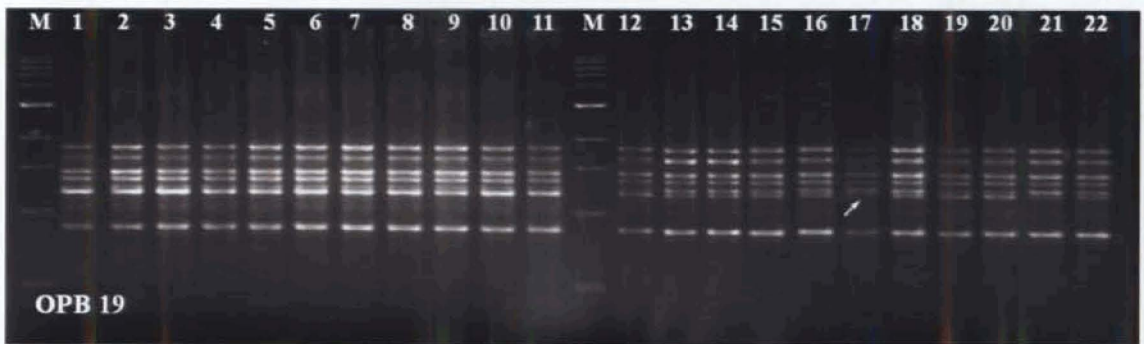
1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.



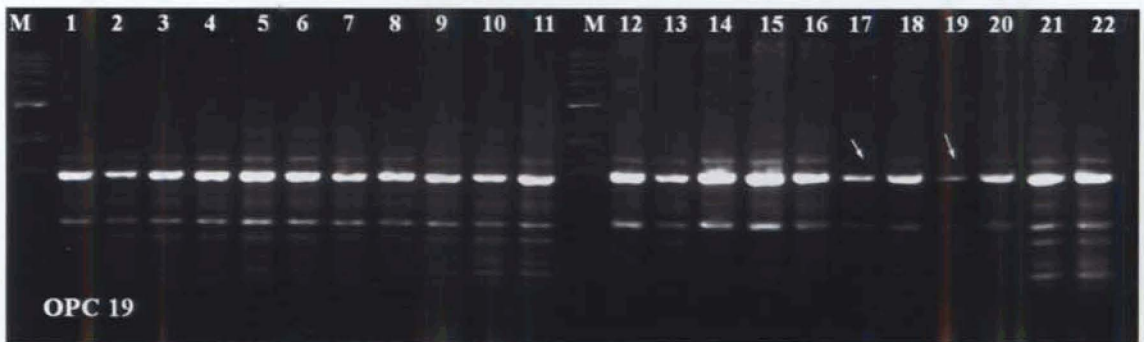
1- Marker, 2-control, 3-SC 5, 4-SC6, 5-SC7, 6-SC8,7-SC13, 8- SC14, 9-SC15, 10-SC16, 11-SC17.

Fig. 31 RAPD profile of callus regenerated plants of cardamom expressed by operon primers

SC - somaclone (regenerated from callus).



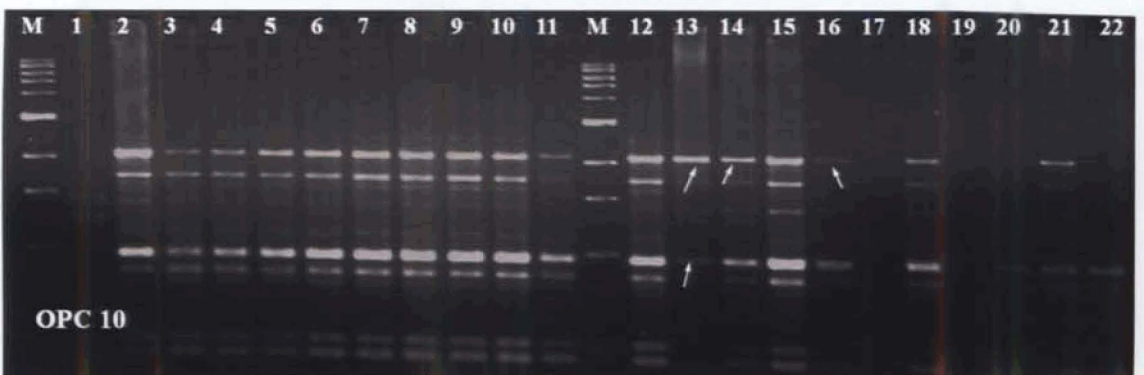
M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp 3, 5-Mp 4, 6-Mp 5,7 -Mp 6, 8-Mp 7, 9-Mp 8, 10-Mp9,11-Mp 10, 12-control, 13-SC 5, 14- SC6, 15-SC7, 16-SC8,17-SC13, 18- SC14, 19-SC15, 20-SC16, 21-SC17, 22-SC18.



M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp 3, 5-Mp 4, 6-Mp 5,7 -Mp 6, 8-Mp 7, 9-Mp 8, 10-Mp9,11-Mp 10, 12-control, 13-SC 5, 14- SC6, 15-SC7, 16-SC8,17-SC13, 18- SC14, 19-SC15, 20-SC16, 21-SC17, 22-SC18.



M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp 3, 5-Mp 4, 6-Mp 5,7 -Mp 6, 8-Mp 7, 9-Mp 8, 10-Mp9,11-Mp 10, 12-control, 13-SC 5, 14- SC6, 15-SC7, 16-SC8,17-SC13, 18- SC14, 19-SC15, 20-SC16, 21-SC17, 22-SC18.



M- Marker, 1- control, 2-Mp1, 3-Mp 2, 4-Mp 3, 5-Mp 4, 6-Mp 5,7 -Mp 6, 8-Mp 7, 9-Mp 8, 10-Mp9,11-Mp 10, 12-control, 13-SC 5, 14- SC6, 15-SC7, 16-SC8,17-SC13, 18- SC14, 19-SC15, 20-SC16, 21-SC17, 22-SC18.

Fig. 32 Comparative RAPD profiling of micropropagated and callus regenerated plants of cardamom

Mp- Micropropagated plants, SC- Somaclones (Callus regenerated plants).

Discussion



The present investigation was carried out with an objective of studying the utility of *in vitro* technology for creation of somaclonal variation for possible genetic improvement of economically important Zingiberaceous crops like ginger, turmeric and cardamom. These crops are predominantly vegetative propagated and hence useful variation can be fixed in the population. The attempts were made to induce somaclonal variation and with limited sexual reproduction especially in ginger and turmeric. The extent of variability is studied using morphological biochemical and molecular markers supplemented with cytological and anatomical evidences.

Ginger

Micropropagation

Vegetative buds of ginger rhizome were multiplied on MS + 1 mg^l⁻¹ NAA and 4 mg^l⁻¹ BAP with 98.7 % success. An average of 7.7 shoots per explant was obtained with the shoot length of 6.3 cm within four to eight weeks.

Micropropagation of ginger was reported earlier by various workers using shoot meristems (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980; Pillai and Kumar, 1982; Ilahi and Jabeen, 1987; Bhagyalakshmi and Singh, 1988; Saradha and Padmanabhan, 1989; Balachandran *et al.*, 1990; Choi, 1991a; Choi and Kim, 1991, Samsudeen, 1996; Babu 1997), base of the pseudostem (Ikeda and Tanabe, 1989; Choi, 1991b, Babu 1997) and roots (Nel, 1985) for clonal multiplication. Most of them used MS basal medium (Murashige and Skoog, 1962) supplemented with IAA, IBA, NAA, 2,4-D, kinetin and BAP in different concentrations and combinations. Only Pillai and Kumar (1982) used SH (Schenk and Hildebrandt, 1972) medium.

Babu (1997) tried explants viz., vegetative bud, immature inflorescence, leaf (pseudostem), ovary and anther were tested for their morphogenetic responses on Murashige and Skoog (1962) basal medium supplemented with cytokinins (BAP and Kinetin) and auxins (NAA and 2,4-D). The presence of NAA at low concentrations (1 mg^l⁻¹) resulted in good growth of culture, root induction and shoot multiplication and addition of BAP at 4 mg^l⁻¹ increased the multiple shoot induction. NAA alone at high

concentration (2-4 mg^l⁻¹) inhibits shoot growth as well as root induction while BAP alone at higher concentration (4 mg^l⁻¹) induced only multiple shoots and rarely roots. Earlier workers also reported that BAP and NAA combinations were best for shoot multiplication in ginger (Sakamura *et al.*, 1986; Charlwood *et al.*, 1988; Sakamura and Suga, 1989).

Considering that both induction of multiple shoots and roots are equally important, development of both in a single medium reduces the time taken for plant development. In such cases MS medium with 1 mg^l⁻¹ NAA alone or 1 mg^l⁻¹ NAA and 4 mg^l⁻¹ BAP was ideal for multiplication of ginger from vegetative bud explants. The plantlets from the vegetative bud cultures are healthy, robust, 8-12 cm tall, with 3-4 roots.

These plantlets were hardened in humid chamber for 20-25 days with 85 percent establishment.

Callus induction and Plant regeneration

Callus was induced in ginger buds on MS medium fortified with 2mg^l⁻¹ 2,4-D and regeneration was done on MS medium supplemented with 10 mg^l⁻¹ BA and 0.2 mg^l⁻¹ 2,4-D. Culture kept in darkness showed better growth and maintenance of calli. 88.7% of the cultures induced callus within two weeks of incubation. Callus developed rapidly in the presence of 2,4-D. The callus consisted non-embryogenic in between embryogenic callus. The embryogenic callus is to be separated and cultured for better embryogenic regeneration. Embryogenesis was observed after 2 weeks of culture.

Plant regeneration was better (94.3%) when embryogenic callus was cultured on MS + 10 mg^l⁻¹ BA and 0.2 mg^l⁻¹ 2, 4-D. The regenerated plants developed root system on the same media.

The earlier reports on callus induction in ginger were those of Pillai and Kumar (1982), Kulkarni *et al.* (1987), Sakamura and Suga (1989), Choi (1991b), Malamug *et al.* (1991), Kacker *et al.* (1993) Ilahi and Jabeen (1992) and Samsudeen (1996)

Babu (1997) reports that in ginger callus was successfully induced in vegetative bud, young leaf, ovary and anther tissues, on MS medium supplemented with various levels (0.5-5.0 mg^l⁻¹) of NAA and 2,4-D for induction and proliferation of callus. Though

it is believed auxins in general induce callus formation, he reported that only 2,4-D at concentrations ranging from 0.5-5.0 mg l⁻¹ was effective in inducing callus in all explants tried, the best concentration being 3 mg l⁻¹. NAA induced slight amount of callus at higher concentrations of 3-5 mg l⁻¹ only. The explants differed in their ability to form callus. The best is vegetative bud followed by anther explant. Leaf explant gave the least amount of callus. The amount of callus produced was 2.7g per tube in leaf and 3.4g per tube in vegetative bud. Callus tissue was not of one kind. Strains of callus differing in appearance, colour, degree of compaction and morphogenetic potential commonly arise from a single experiment. In ginger, the callus was loose, friable and pale yellow in colour. In the subsequent cultures, the callus contained some hard organized embryogenic 'lumps' (meristemoids) within the mass of loose cells (Babu, 1997). The callus could be maintained and multiplied by monthly subcultures on the same media. In vegetative bud derived callus, the number of plantlets developed per culture with same media was higher in BAP (28-60) than in kinetin (8-26). The present study agrees with these findings.

Among the other reports, callus formation, development of roots and rhizome like structures were reported from excised ginger anthers cultured on MS medium containing 2,4-D and coconut milk (Ramachandran and Chandrashekharan Nair, 1992b).

Samsudeen *et al.* (2000) reported regeneration of plantlets from excised anthers. They cultured uninucleate pollen mother cells and pollen on MS medium supplemented with 0.2-3.0 mg l⁻¹ 2,4-D after cold treatment. The cold treatment was given for 1, 2 and 7 days at 0°C. The anthers developed friable callus in solid as well as liquid medium in about 6 weeks. 2,4-D at 3 mg l⁻¹ was good for both callus induction and callus proliferation giving about 3 g of callus in about 30 days of culture when incubated in light. Calli obtained from ginger anthers were cultured on MS basal medium supplemented with 2,4-D (0 - 0.2 mg l⁻¹) and BAP (0 - 10 mg l⁻¹). MS medium with 2,4-D at 0.2 mg l⁻¹ and BAP at 10 mg l⁻¹ was the best for organogenesis and plant regeneration.

Induction of Microrhizomes

The effect of different factors like MS salt, sucrose concentration and duration of the culture period on the production and multiplication of microrhizomes *in vitro* were studied. Out of four different concentrations of MS salt tried, 0.75 X is the optimum basal salt concentration in which the maximum numbers (22) of microrhizomes were produced. While in the other concentrations the number of microrhizome and their weight was less.

Sucrose studied 8% was found to be optimum and gave the maximum production (21.7) of microrhizome *in vitro*. Where as the lower and higher concentration leads to the reduction in the number and also their fresh weight. The optimum production of ginger microrhizome obtained at 90 and 120 days of growth period. Where as the optimum fresh weight obtained in 120 days (2.6g) than 90 days.

In vitro induction of rhizomes and their germination in ginger was reported earlier by various workers (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994, Sharma and Singh, 1995 and Babu, 1997). Bhat *et al.* (1994) reported *in vitro* induction of rhizomes in ginger at higher sucrose concentrations (9–12%). Quality analysis of *in vitro* developed rhizomes indicated that they contain the same constituents as the original rhizome but with quantitative differences. The composition of basal medium seems to affect the composition of oil (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Charlwood *et al.*, 1988). Sharma and Singh (1995) reported microrhizomes with 4-5 buds weighing 73 – 459 mg were induced on MS medium with 75 g /l sucrose. After storage in moist sand at room temperature for 2 months 80 % of the micro rhizomes sprouted in to plants.

Geetha (2002) and Peter *et al.* (2002) tried various combinations of sucrose and mannitol in different concentrations induces micro rhizomes in ginger. Microrhizomes of 0.05–15 g fresh weight per explant were induced in ginger tissue cultures in 1–12 months on MS basal medium supplemented with higher levels of carbon source. In the medium with 1% or 1.5% each of sucrose and mannitol, time taken for microrhizome formation

was reduced to 8 months with 50–60% of the cultures responding. In this combination comparatively smaller microrhizomes, weighed up to 0.05–1.2g, were produced. Microrhizomes were produced in 80–100% of cultures when sucrose concentrations were increased to 9, 10 and 12%. The microrhizomes from these cultures were larger with a fresh weight of 3–15 g in 1-6 months. The other combinations of sucrose and mannitol did not induce microrhizomes. Microrhizomes resembled the normal rhizomes in all respects, except for their small size. The microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds. Presence of well-developed oil cells, fibres, and starch grains was also observed. These micro rhizomes were directly planted in the field with out any hardening with 80 % success. The microrhizomes though gave lesser yield per bed gave very high recovery based on the relative weight of seed material used (Geetha 2002)

Microrhizomes also have the aromatic flavour of ginger and they resembled the normal rhizome in anatomical features in the presence of well-developed oil cells, fibres, and starch grains were observed. These microrhizomes were directly planted in the field without any hardening with 85 % success. These microrhizomes gave a fresh rhizome yield of 100 – 525g per plant with an average per 3 m² bed yield of 10. 5 kg while the control gave per bed yield of 15 kg. 20- 30g of seed rhizome was used for conventionally propagated plants, where as in microrhizome seed rhizome weight was 2-8g. Thus microrhizomes though gave lesser yield per bed gave very high recovery based on the weight of seed material used. This coupled with its disease free nature will make microrhizomes an ideal source of planting material. Microrhizome derived plants although slow in initial field establishment was able to pick up with the control ones within 4-5 months. The present study agrees with the findings of Peter *et al.* (2002). Though there are earlier reports (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994, Sharma and Singh, 1995; Babu, 1997 and Geetha 2002; Nirmal Babu *et al.*, 2003) on induction of microrhizomes this is by far the most efficient result in that maximum number of microrhizomes were induced in shortest time possible that too with

the no use of growth regulators. This helps to maintain the genetic fidelity. This is the first report on field performance of ginger microrhizomes.

Characterization of somaclones

The success of any *in vitro* culture technique depends either on the ability to clone the genotypes for production of uniform planting material, or the ability to bring about variations, which can be exploited in crop improvement programmes. The genetic uniformity of plants multiplied by tissue culture depends on a number of factors, the two most important being the method of multiplication and the genotype.

Plants regenerated from callus or cell suspension cultures may show a varying proportion of structural or physiological abnormalities depending upon the species, origin and the age of culture (Yeoman, 1986). Other factors like growth regulators (Singh and Harvey, 1975; D'Amato, 1978; Zakhlenyuk and Kunakh, 1987), composition of the culture medium (Bayliss, 1977; Feng and Quyang, 1988), culture conditions (Cerutti, 1985; Jackson and Dale, 1988) and culture method (Wilson *et al.*, 1976) influence somaclonal variation. The reasons for variations in micropropagated plants can also be due to the variation that existed in the source plant (pre-existing variation), epigenetic or physiological effects and genetic changes (Swartz, 1991; George, 1996). Extensive studies conducted during the last decade have shown that the cell and callus cultures especially on periodical subculture undergo various morphological and genetic changes i.e., polyploidy, aneuploidy, chromosome breakage, deletions, translocations, gene amplifications, inversions, and mutations (Nagl, 1972, Meins, 1983; D'Amato, 1985). In addition, there are changes at the molecular and biochemical level including changes in the DNA, rearrangement of genes, somatic crossing over, altered nucleotide methylation, perturbation of DNA replication by altered nucleotide pools, slicing or activation of genes by mutations in associated non coding regions and transposons (Scowcroft, 1984) and enzymes (Cullis, 1983; Day and Ellis, 1984; Ball and Seilleur, 1986; Brettel *et al.*, 1986). Thus *In vitro* technology is a powerful tool for the induction of much-needed genetic variability in ginger.

Morphological characterization

The plantlets obtained through micropropagation, callus regeneration and microrhizome pathways were morphologically characterized in pot culture and certain amount of variations were observed. The variations were observed in all the plantlets studied (Table 20). The callus regenerated plants show maximum variation in almost all the characters studied (Fig.33,34)

The characters, which showed maximum in callus regenerated plants compared to others were plant height (56.8cm), number of tiller per plant (13.6), number of leaves per plant (19.4), leaf length (16 cm), yield (573g) and primary and secondary fingers (6.0 and 9.0).

The characters, which showed similarity in conventional, micropropagated, and microrhizome derived plants were plant height, number of leaves, yield, primary and secondary fingers, Number of nodes of mother rhizome, internodal length of primary and secondary fingers (Fig.33,34).

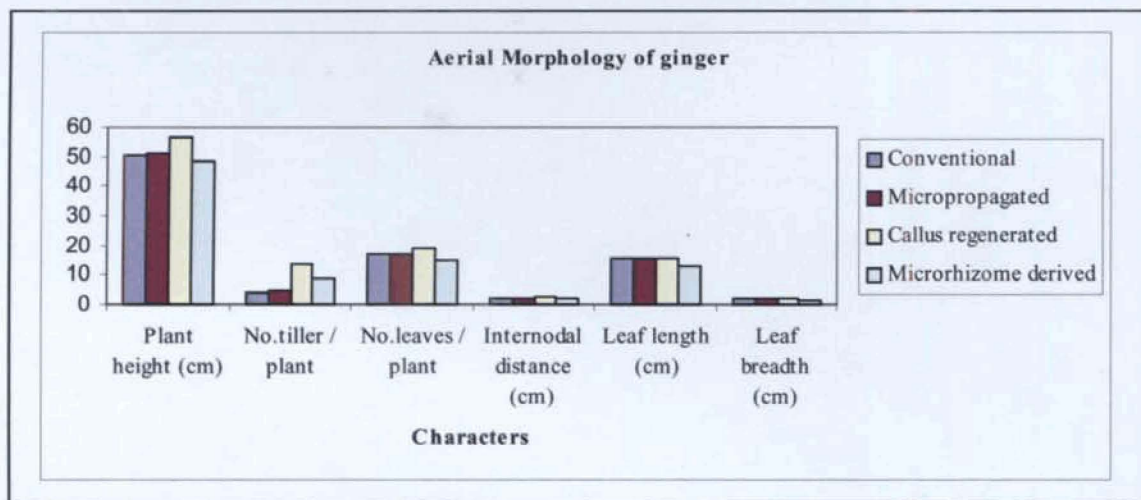


Fig. 33 Comparative aerial morphology of ginger somaclones (conventional, micropropagated, callus regenerated and microrhizome derived plants)

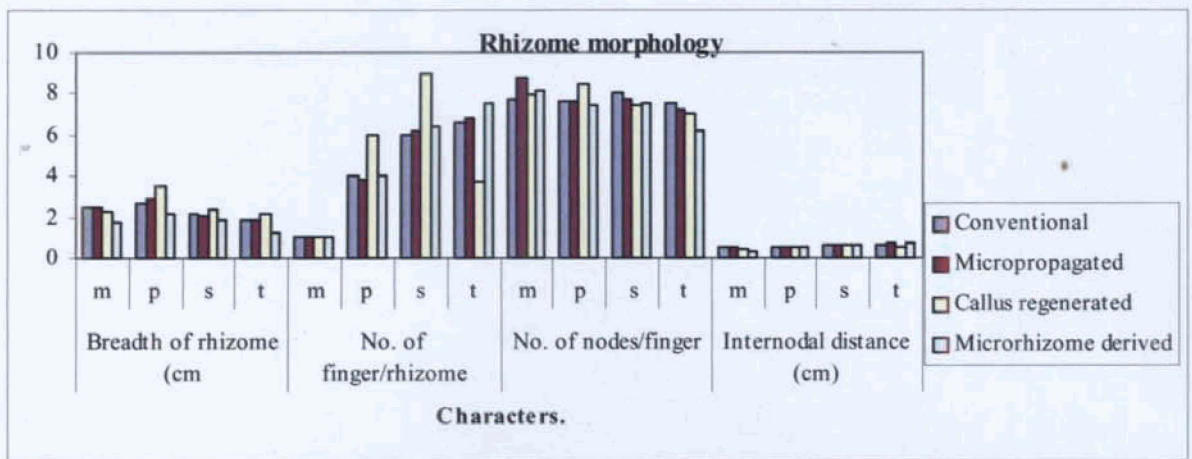


Fig. 34 Comparative rhizome morphology of ginger somaclones (Conventional micropropagated, callus regenerated and microrrhizome derived plants)

Morphological characterization is a traditional method for identifying phenotypic variation in many plants (Pereira *et al.*, 1996). Morphological characterization of micropropagated as well as callus regenerated plants was reported by Babu (1997) in comparison with conventionally propagated plants. Direct regenerated plants and callus regenerated plants as separate groups when compared with conventionally propagated plants revealed good amount of variation with regard to plant height, number of tillers and of leaves per plant, width of rhizome, number of nodes per finger, internodal distance and yield per plant. Micropropagated and callus regenerated plants have higher mean values with regard to plant height, number of tillers, number of nodes per finger, yield per plant compared to controls while they have lesser mean values with regard to number of leaves per plant. With regard to width of rhizomes and inter nodal distance all the three groups are on par. When we consider the range observed within the group, micropropagated plants showed highest range with regard to plant height, number of tillers and leaves per plant, width of rhizome, number of nodes per finger, while callus regenerated plants showed highest range with regard to internodal distance and yield. The present study is also in tune with these findings.

A few promising lines having important yield attributes and other useful characters could be selected from both micropropagated and callus regenerated lines

(Babu 1997). However Smith and Hamil 1996 reported that micropropagated plants had significantly reduced rhizome yield in the first generation of *ex vitro*.

The partitioning of phenotypic correlation between yield and morphological characters into direct and indirect effects by the method of path coefficient analysis revealed that plant height exhibited a high direct effect as well as high indirect effect in the establishment of correlation between yield and other morphological characters. (Ratnambal 1979; Nair *et al.*, 1980). Rattan *et al.* (1988) indicated that number of leaves per plant had maximum direct contribution to yield per plant, followed by rhizome breadth. Das *et al.* (1999) reported very high positive direct effects of stomatal number, leaf area, leaf number, and plant height on rhizome yield; leaf temperature, relative humidity of leaves; stomatal resistance and rate of transpiration showed negligible effects. The direct effect of leaf number on rhizome yield was very high (0.631), and this trait is recommended for use as a selection criterion for improving rhizome yield. The study of Pandey and Dobbal (1993) revealed that the strongest forces influencing yield are weight of fingers, width of fingers, and leaf width. Singh *et al.* (2000) grouped 18 cultivars into three clusters under Nagaland conditions based on D² analysis. The major forces influencing divergence of cultivars were rhizome yield per plant, oleoresin and fibre contents.

Sasikumar *et al.* (1992) carried out path analysis using 100 accessions of ginger. They reported that plant height followed by leaf length exhibited highest direct effect on rhizome yield. Dry recovery had a negative direct effect on yield. All other direct effects were negligible. Highest indirect effect was for leaf number through plant height followed by leaf length, again through plant height. In turn, plant height exerted moderately good indirect effect on rhizome yield. Moderate indirect effects were also noticed in the case of leaf width (through plant height, and leaf length and leaf number (through leaf length). However, these authors noticed a residual effect of 0.8217, thereby indicating that the variability accounted for in the study was only 18%. They concluded that plant height

should be given prime importance in selection programme as this character had positive and significant correlation as well as good direct effect with rhizome yield.

Multiple regression analysis by using morphological characters indicated that the final yield could be predicted fairly accurately by taking into consideration plant height, number of leaves, and breadth of last fully opened leaf at 90th and 120th day after planting (Ratnambal *et al.*, 1980). Rattan *et al.* (1988) found that to improve yield per plant, emphasis should be given to number of leaves per plant and rhizome length by using partial regression analysis.

Nybe and Nair (1979) suggested that morphological characters are not reliable to classify the types, although some of the types can be distinguished to certain extent from rhizome characters. All the morphological characters were found to vary among types except for breadth of leaf, leaf area index, and number of primary fingers. Manmohandas *et al.* (2000) in a study found that all the cultivars differed significantly for tiller number, leaf number, and yield stability analysis revealed the superiority of cvs. Ernad and Kuruppumpadi as they expressed high mean yield, non significant S²di values and a regression nearing unity. Thus the variations observed in the present study with respect to plant height, number of tiller per plant, number of leaves and leaf length characters will contributes morphology enhances to improvement in yield.

Several authors reported morphological variation in somaclonal variants in many crop plants. Nishi *et al.* (1968) and Henke *et al.* (1978) both reported phenotypic variants amongst somaclonal plants regenerated from rice callus. The variations were in traits such as number of tillers per plant, number of fertile tillers per plant, average panicle length, frequency of fertile seed, plant stature and flag leaf length. Cummings *et al.* (1976) reported in Oat (*Avena sativa*) an altered phenotype in plant height, heading date, twin culms, yellow leaf stipes, awn morphology and fertility. Many of these variants were transmissible to later generations (both true breeding and segregating lines occurred). (McCoy *et al.*, (1978) have also indicated a high frequency of cytogenetic abnormality Skirvin and Janick (1976a, 1976b) have observed a remarkable degree of

variability in plants regenerated from the cultures of 5 pelargonium cultivars. Tokumasu & Kato (1979) regenerated homogeneous plants from genetic tissue of diploid anthers. Amongst these plants are two variants for essential oil compositions. A study of the tissue culture literature shows many passing comments on the occurrence of abnormalities amongst regenerated plants. Some examples include carrot (Ibrahim 1969), Chrysanthemum (Ben-Jacov and Langhans 1972) (Stimart *et al.*, 1980), carnation (Hacket and Anderson 1967), and clover (Beach and Smith 1979), Gamborg *et al.* (1977) found variants amongst sorghum culture regenerants. Some had reduced fertility; some had altered leaf morphology and growth habit. All these variant sorghum somaclones were normal in karyotype ($2n=20$)

Wakasa (1979) 448 somaclones of pineapple (*Ananas comosus*). Many variants were observed in spine and leaf colour, wax secretion, foliage density, leaf width, and leaf spine formation. The explant source had a dramatic effect on the occurrence of variation. Novak (1980) indicated that garlic (*Allium sativum* L) somaclones from shoot tip callus and leaf base callus showed great phenotypic variability. Somaclonal variation has also been encountered in lettuce (*Lactuca sativa* L.) for plant morphological traits (Sibi 1976).

Biochemical characterization

The observed 'significant variants' were selected and made more satisfied to biochemical characterization. Significant variations were observed in percentage of oil, oleoresin, starch and fiber content among the somaclones tested. The highest oil percentage was found in the accession CR 816 (2.4%) and the lowest in MP 61-9 (1.3%). Where as in control (Maran) the percentage of oil was 1.5%. The maximum percentage of oleoresin was observed in CR 1222 (8.1%) followed by CR 816 (7.8%) and CR 10-1 (7%). In the case of starch control (Maran) showed the maximum (47.79%) followed by MP 76-3 (45.7%). CR 816 recorded the maximum percentage of fiber 7.4% followed by MP 50-3 (6%). Microrhizome derived plants showed the minimum fiber content (2%). Variation observed between micropropagated, microrhizome and conventional with

regard to biochemical characters is minimal, the plants regenerated through callus pathway gave significant variation.

Babu *et al.* (1997) observed variations among tissue cultured and callus regenerated plants in their oleoresin content, fibre content and dry recovery. The oleoresin content ranged from 1.6 - 7.6%, crude fibre percentage ranged from 3.6 - 6.7 and dry recovery ranges from 21.2 to 31.8% among the tissue cultured plants while the control has 5.2% oleoresin, 5.9 % fibre and 28% dry recovery.

Thus the variation observed between micropropagated, microrhizome and conventional with regard to biochemical characters is minimal, however the plants regenerated through callus pathway gave significant variation. This will in course of time lead to development of chemotypes which can be maintained in population through vegetative reproduction.

Oleoresin of ginger is the total extract of ginger containing all the flavouring principles as well as the pungent constituents. The oleoresin contains two important compounds – gingerol and shogoal - that contribute to the ginger pungency. On long term storage gingerol gets converted to shogoal. The quality of ginger thus depends on the relative content of gingerol and shogoal. Zachariah *et al.* (1993) classified 86 ginger accessions into high, medium and low quality types based on the relative contents of the quality components.

There are many ginger cultivars with high oleoresin, a few them like cvs. Rio-de-Janeiro, Ernad Chernad, Wynad, Kunnamangalam, and Meppayyur had high gingerol also. The inter character association showed a positive correlation with oleoresin, gingerol and shogoal (Jogi *et al.*, 1972, Nybe *et al.*, 1980, Sreekumar *et al.*, 1980).

Somaclonal variation may find its greatest application for plant improvement in concert with selection for desirable mutations at the cellular level. Cellular selection is conceivable for the recovery of variants resistant to antimetabolites such as amino acid analogues, antibiotic drugs, pathotoxins, herbicides and physiological stress (Maliga 1978; Thomas *et al.*, 1979; Brettell and Ingram 1979).

Composition of essential oil of *in vitro* microrrhizome and *in vivo* rhizome by using GC- MS

The developmental status of microrrhizomes, were studied using GCMS profiling (Table 23). The microrrhizomes behaved like storage organs though there are differences in storage contents compared to control. This may due to the differences *in vitro* and *in vivo* condition in which these storage organs are induced.

The *in vitro* Microrrhizome have some essential oil components, which were absent in the control. The components like β - pinene, 2-undecanone, Alloaromadendrene, α - Humulene, γ - muurolene, γ -Selinene, Trans, trans-farnesal are present in the *in vitro* microrrhizome while this components are missing from *in vivo* control. At the same time components like β -myrcene, Linalool, d- cadinene, γ - cadinene, Farnesene, t- geraniol, Trans geraniol were absent *in vitro* microrrhizome and these components were present in *in vivo* control plant.

There is no earlier report available in essential oil components *in vitro* microrrhizome of ginger. The reports are available in chemical constituents of normal rhizome of ginger.

Pino *et al.* (2004) examined the chemical composition of the essential oil obtained from the rhizomes of *Zingiber officinale* Rosc. from Cuba. The oil was characterized by the presence of α -curcumene (22.1%), α -zingiberene (11.7%), beta-bisabolene (11.2%) and β -Sesquiphellandrene (10.5%).

Gurib-Fakim *et al.* (2002) reported chemical composition of the essential oils obtained from the hydrodistillation of the rhizomes of the common ginger (*Zingiber officinale*) and three ginger-lilies (*Hedychium coccineum*, *H. flavescens* and *H. coronarium*) grown in Mauritius was investigated by GC and GC/MS. *Z. officinale* oil was characterized by the presence of geranial (16.3%), neral (10.3%), zingiberene (9.5%), beta -sesquiphellandrene (6.3%) and α -curcumene (5.1%). The oils of the ginger lilies were characterized as follows: *H. coccineum*: (E)-nerolidol (44.4%), trans-sesquisabinene hydrate (24.2%); *H. flavescens*: linalool (35.0%), 1,8-cineole [eucalyptol] (15.3%), beta -

pinene (14.7%), alpha -terpineol (14.5%) and alpha -pinene (5.3%); *H. coronarium*: alpha -muurolol (16.8%), alpha -terpineol (15.9%), 1,8-cineole (11.2%), an unknown sesquiterpene alcohol (7.0%), alpha -fenchyl acetate (5.6%), citronellal (5.5%) and (E)-methyl cinnamate (5.1%).

Rhizome anatomy of polyploid and normal plant

One of the somaclone CR 1222 has exhibited gigantism in morphological characters like plant height, leaf breadth, chlorophyll content and bold rhizome, is also confirmed this polyploid through stomatal study and rhizome anatomy. This line CR 1222 has multi-layered hypodermis, compared to control which has single layered hypodermis.

In the case of stomatal frequency and size, the polyploid CR 1222 has larger stomata with less in stomatal number, typical of polyploids. Where as in diploid plant the stomatal number was more and size was less. In polyploid leaf also showed large sized and less number of sclereids than the diploid leaf. Large sized upper and lower epidermal layer was observed in polyploid compared to normal diploid plant. The polyploid has larger stomata with less in stomatal number, typical of polyploids indicating that CR 1222 may be a polyploidy.

Induced polyploidy is known to enhance the content and yield of secondary metabolites in various plant species like *Papaver somniferum* (Andreev, 1963), *Mentha arvensis* (Janaki and Sobti, 1962), *Catharanthus roseus* (Kulkarni *et al.*, 1984), *Rauvolfia serpentina* (Janaki, 1962) and others. In the present study the somaclone CR 1222 produced maximum percentage of oleoresin (8.1%).

Increased guard cell size was found to be a consistent indicator of a doubling of the chromosome number (Watrous and Wimber, 1988). Stomatal size decreased with increase in stomatal density. High positive correlations were found between area and stomatal size.

Bhattacharjee (1956) reported that the autotetraploids are often shorter than diploids in some of the morphological parameters in pigeon pea. Such an effect often results from fewer cell division accompanied by increased cell size and larger stomata.

These facts also suggest that duplication of genes responsible for quantitative characters does not always enhance but some times cause reduction (Gupta and Sinha, 1978) depending on the optimum level of ploidy.

Jane Fiuza *et al.* (2005) used additional characteristics like stomata length, width, area and frequency to discriminate polyploid and diploid shoot. Tetraploid shoots presented, on average, a lower stomata number per leaf area unit. Several studies evaluating ploidy level based on these traits succeeded in discriminating plants with different ploidy levels (Tan and Dunn, 1973; Griesbach, 1990). Chloroplast number per guard cell is useful in determining ploidy levels (Compton and Veilleux, 1991; Otoni *et al.*, 1995).

The rhizome size and stomatal size and density indicate that CR 1222 is a polyploid.

Chromosome indexing

The selected somaclones especially CR 1222 was cytologically analyzed to find out whether there is any numerical alteration in the chromosome level. One of the callus derived plants of ginger exhibited deviation from the normal chromosome number in acc.no. CR 1222 Among the numerical aberrations recorded aneuploids with lower number are most frequent. One of the somaclone CR 1222 showed polyploid number of chromosome (Fig.9). No reports are available in occurrence of polyploids in ginger somaclones.

Induced polyploidy has been tried in ginger for introducing variability, improving pollen and ovule fertility and for improving growth and yield. Ratnambal *et al.* (1979) reported induction of polyploidy in the cv. Rio-de-Janeiro through colchicine treatment. The tetraploids showed stunted growth and had reduced length and breadth of leaves. However, in this case a stable polyploid line could not be established and all the plants reverted to diploidy in the succeeding generations.

Polyploids produced from chromosome doubling are often a little bigger, luxuriant, with thicker leaves and larger flower, than corresponding diploids. Such

characteristics are called gigas features and are consequence of larger cells. Induced polyploids have many uses including facilitation of genetic transfer in genetic complexes, synthesis of new crop species, broadening of the genetic base, development of commercial cultivars

Ramachandran *et al.* (1982) and Ramachandran and Nair (1992) reported successful production of stable tetraploid lines in cvs. Maran and Mananthody. The polyploids were more vigorous than the diploids and flowered during the second year of induction. The stable tetraploid lines ($2n=44$) had larger, plumpy rhizomes and high yield. (198.7 g/plant). However, the essential oil content was lower (2.3%) than the original diploid cultivar. There was considerable increase in pollen fertility in the tetraploids. These tetraploids are maintained in the gemplasm collection of IISR, Calicut. Induced polyploids should be considered as new and sometimes valuable raw material for further breeding work requiring a long-term commitment. The realization that polyploids are possible through chromosome doubling or through interspecific and intergeneric crosses has allowed plant breeders to fulfill an age old desire to produce new species. Thus in ginger tetraploidy may enhance pollen fertility (Ramachandran and Nair 1992).

Following a great deal of effort with polyploids, the practical out come has been small. Where seed is economic component, auto tetraploids have, in effect, failed because of sterility. Several tetraploid rye cultivars have been released and are marginally acceptable. Success has been enjoyed in ornamental crops and in triploid banana, hops, pyrethrums, and watermelons notably where seed set is undesirable.

The gigas characteristic generally has not proven to be agriculturally useful because larger cells are associated with increased moisture content rather than with increased biomass potential.

The plantlets regenerated from these callus tissues posses a vast array of genetic changes. The changes in structure and number induced in cultured cells may provide new variations for use in breeding programmes aimed at crop improvement. Ratnambal (1984)

reported that the karyotype analysis also showed that the trend of evolution in the genus *Zingiber* has been towards increasing karyotype asymmetry.

Similarly aneuploids are also observed in callus cultures by Ezhova *et al.* (1988) in pea and Sekerka (1988) in *Vicia sativa*. Similarly about 4% aneuploid cells in callus cultures of pea have been reported by Gostimskii *et al.* (1985). Along with the presence of variation in chromosome number in callus cultures, a fair amount of structural changes of chromosome were also observed. These findings are in agreement with reports of Anju and Sarbhoy (1990) and Kiyamova *et al.* (1991) in pea.

Novak (1980) reported variation in a range of phenotypic characters including plant height, leaf number, bulb weight and shape and number of cloves within a bulb; cytological variation was also observed with also most half of the regenerates being tetraploid, aneuploid or mixoploid. In another study, a garlic somaclones was found to possess a consistently higher bulb weight than the parental clone (Vidal *et al.*, 1993).

Genetic variability is valuable only if it is handled skillfully and the useful variants selected and evaluated judiciously. Occurrence of somaclonal variation during culturing is a frequent and consistent event. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg *et al.*, 1977) but also by their biochemical, physiological and genetic characteristics. Several reports in the literature indicate that variation can be detected by identifying chromosome numbers (Bayliss 1973, Ogura 1990)

There is a need to establish the nature and sources of variation in culture cells and to regulated the degree of variation with a view to explore the possibility of regenerating plants with varying chromosome numbers (Madock, 1985; Swedlund and Vasil, 1985).the genotypes at different concentrations and combinations of NAA and BA revealed wide variation in chromosome number and structure.

Though somaclonal variations are observed in morphological characters in many crop plants, many of these traits are either environmentally induced or transient leading to loss of these variations on subsequent generations. To confirm whether these variations

expressed at DNA level and selected the ginger somaclones were characterized using RAPD profile.

Molecular characterization

Micropropagated, callus regenerated and microrhizome derived plants were subjected to RAPD profiling with 6 operon primers (OPA 17, OPA 10, OPC 08, OPC 01, OPA 06 and OPA 04). RAPD polymorphism expressed in callus regenerated plants of ginger (Fig.35).

No variation observed in micropropagated and microrhizome derived plant but showed variation in callus regenerated plants. This indicates that many of the morphological variants in callus regenerated plants may be due to genetic variations.

Suja (2002) and Nirmal Babu *et al.* (2003) used RAPD profiles amplified by 11 Operon primers as an index for estimating genetic fidelity of selected 'variants' among micropropagated and callus regenerated plants. They observed differences in RAPD profiles observed in some of the micropropagated plants indicated that micropropagation even without callus phase induced variations in 9 of the 13 plants tested. Similar differences were noticed among 12 out of 15 Callus regenerated plants. In general this indicates there is high amount of variability among the selected micropropagated and callus regenerated plants of ginger and the majority of the morphological variants selected from earlier studies did show variations in RAPD profiles. Earlier studies by Rout *et al.* (1998) indicated that RAPD profiles did not indicated any polymorphism among the micropropagated plants. This may be because bigger population size used by the latter to detect the morphological variants first and confirmation of their genetic nature of variation using RAPD profiles. Other workers also reported somaclonal variation in ginger (Kulkarni *et al.*, 1987, Samsudeen, 1996, Babu, 1997). This may be because of the genetic nature of ginger, which has resulted in many varieties and cultivars even without sexual reproduction. However the microrhizome derived plants did show high degree of genetic uniformity as expressed by RAPD profiles.

In the present study good amount of variation in RAPD profiles are observed among the callus regenerated plants. A total of 113 were produced by using 10 primers,

of which 27 monomorphic bands and 86 polymorphic bands were present. This indicates that possibility of gave more ratios of ginger lines leading to high variation.

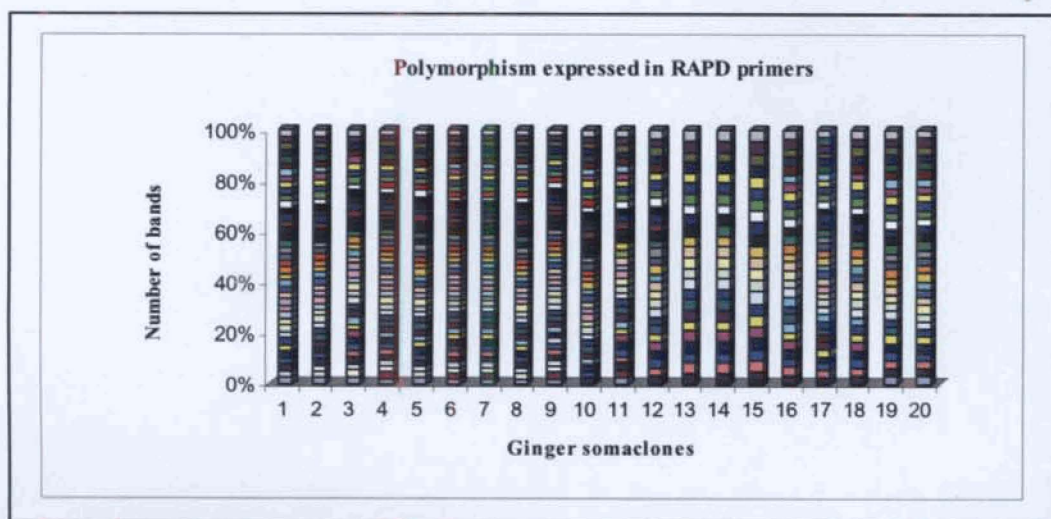


Fig. 35 RAPD profile differences among ginger somaclones (1- Control, 2-20 Callus regenerated plants of ginger)

Screening for disease incidence

Nine selected ginger somaclonal 'variants' along with control were inoculated with *Pythium aphanidermatum* for challenging the rhizome rot disease (Fig.13). The disease incidence ranged for 100% - 4.73%. The somaclones like CR2, CR-3 and CR 67 observed as tolerant to rhizome rot disease indicate these are variants for rhizome rot disease.

Kulkarni *et al.* (1984), reported isolation of *Pythium*-tolerant ginger by using culture filtrate as the selecting agent. *In vitro* selection for resistant types to *Pythium* and *Pseudomonas* is in progress at IISR using culture filtrates of the pathogen or pathotoxin as the selecting agent (Nirmal Babu *et al.*, 1996a, Dake *et al.*, 1997).

Babu (1997) and Nirmal Babu *et al.* (1996a) observed variations in both micropropagated and callus regenerated plants with respect to their tolerance to *Pythium aphanidermatum* and *Ralstonia solanacearum* (*Pseudomonas solanacearum*) when they were inoculated with 2 rounds of the organism. Eight somaclones showed low percentage of disease incidence (*P. aphanidermatum* infection). Isolation of *Pythium* tolerant lines were earlier reported in ginger (Kulkarni *et al.*, 1987). Thus somaclonal variation is an

important source of variability and can be exploited for crop improvement programmes in ginger

Turmeric

Micropropagation

Vegetative buds of turmeric were inoculated on MS supplemented with 1mg l^{-1} NAA and 4 mg l^{-1} BA for the clonal propagation. 96.2% explants responded and developed shoots with an average of 6.0 number of shoot per explant. The best results were obtained when the buds (10-20 mm in size) were cultured on this medium. This established and *in vitro* derived shoots further multiplied on MS with BA 1 mg l^{-1} and NAA 0.5 mg l^{-1} and agar 0.8%.

Various workers have reported successful micropropagation of turmeric. (Nadgauda *et al.*, 1978, Shetty *et al.*, 1982, Yasuda *et al.*, 1988, Keshvachandran and Khader 1989, Nirmal Babu *et al.*, 1993 and 1997, Meenakshi *et al.*, 2001, Sunitibala *et al.*, 2001, Nirmal Babu and Minoo, 2003, Ali *et al.*, 2004, Rahman *et al.*, 2004 and Praveen 2005).

Nadgauda *et al.* (1978) were the first to report micropropagation of turmeric. They cultured young vegetative buds excised from CV Duggirala and Tekkurpeta on MS medium supplemented with coconut milk, kinetin and BAP or on Smith's medium supplemented with coconut milk, kinetin, BAP and inositol. These elongated shoots on transfer to White's liquid medium with 2% sucrose developed a healthy root system and could then be transferred to pots and grown in the field. They again sub cultured explants from the shoot region of these newly formed sterile plantlets on the same media to regenerate multiple shoots. The capacity for shoot formation increased after the first subculture and became consistent after 3-4 passages.

Shetty *et al.* (1982) reported 10-12 multiple shoots per explant (clone 15B) when cultured on modified MS medium (pH 5.6) containing sucrose (40 g/l) and Kinetin (0.5 mg l^{-1}). Keshvachandran and Khader (1989) reported an average of 2.5 and 2.11 shoots in turmeric cv. Co-1 and BSR-1 respectively on MS medium supplemented with 1mg l^{-1}

¹kinetin, 1mg l⁻¹BA and 40g sucrose/litre. Balachandran *et al.* (1990) reported that rhizome explants cultured on MS medium supplemented with 3 mg l⁻¹ BA gave the highest multiplication rate in all the three species *C. domestica* var. 'Koova', *C. aeruginosa* and *C. caesia*. Nirmal Babu *et al.* (1993, 1997) reported micropropagation of turmeric using young vegetative bud explants in MS medium supplemented with 1mg l⁻¹ NAA and these explants responded readily to culture conditions. Meenakshi *et al.* (2001) reported that the explants of 2.5 and 3.0 mm size on MS + 1.0 mg l⁻¹ BA + 0.1 mg l⁻¹ GA + 0.1 mg l⁻¹ NAA were the best for initiation of cultures of turmeric cv. Cuddapah, which on transfer to 0.3 mg l⁻¹ NAA gave healthy roots.

Sunitibala *et al.* (2001) reported multiple shoots on 1 mg l⁻¹ NAA + 1 mg l⁻¹ Kin or 1 mg l⁻¹ NAA + 2 mg l⁻¹ BA. Salvi *et al.* (2002) reported higher shoot production on 10 µM BA and 1 µM NAA with agar at 0.4% and 0.6% in turmeric cv. 'Elite'. Further they reported that carbohydrate sources like xylose, rhamnose, lactose and soluble starch were inhibitory and no variation was observed in RAPD analysis. Rahman *et al.* (2004) developed multiple shoots (14.5) on MS medium supplemented with 2.0 mg l⁻¹ BA and rooting was obtained on half strength of MS with 0.2 mg l⁻¹ IBA, which gave 70% survival rate on hardening. Prathanturarug *et al.* (2003, 2005) were able to induce 11 -18 multiple shoots in 8-12 weeks when the bud explants were supplemented with 18.17 µM -72.64 µM TDZ for one week prior to culture on MS medium. The regenerated plants spontaneously rooted. These results were substantiated by Praveen (2005) who also reported higher rate of multiplication when TDZ was used in culture medium.

Most of the reports used MS basal medium for *in vitro* culture of turmeric and related species. But Mukhri and Yamaguchi (1986) used Ringe and Nitsch media containing 1mg l⁻¹ BA for shoot and root development in turmeric. On increasing the concentration of BA to 10 mg l⁻¹ and 1 mg l⁻¹ 2, 4-D or 10 mg l⁻¹ BA and 10 mg l⁻¹ NAA produced callusing.

Salvi *et al.* (2002) reported that MS medium solidified with agar (0.4 and 0.6%) was superior to liquid media for production of multiple shoots in turmeric. Prathanturarug

et al. (2005) also preferred solid medium for a better response in turmeric.

The present study is in agreement with earlier findings of Nirmal Babu *et al.*, 1997 and can be used for production of disease free planting material of elite genotypes of turmeric.

Callus induction and Plant regeneration

Different combinations of 2, 4 -D and BA were used for the efficient induction and proliferation of callus. MS medium supplemented with 2,4 -D at 1.0 and BA 0.5 mg^l⁻¹ was the best for induction and proliferation of callus after 4 weeks of culture. The percentage of embryogenic callus was found to be highest in 1 mg^l⁻¹ 2, 4-D + 0.5 mg^l⁻¹ BA (100%) and non embryogenic callus percentage was highest in 1 mg^l⁻¹ 2,4-D alone (32%). The embryogenic callus need to carefully separated and cultured on the media contained NAA at 0.5 mg^l⁻¹ and BA at 2.0 mg^l⁻¹ to get high rate of plant regeneration.

Organogenesis and plantlet formation were achieved from the callus cultures of turmeric (Shetty *et al.*, 1982., Nirmal Babu *et al.*, 1997., Sunitibala *et al.*, 2001., Salvi *et al.*, 2001, 2002., Praveen 2005). Shetty *et al.* (1982) reported 10-12 multiple shoots per explant (clone 15B) when cultured on modified M S medium (pH 5.6) containing sucrose (40 g/l) and kinetin (0.5 mg^l⁻¹). On transfer to a similar medium, callus was produced. When subcultured and exposed to light, the callus produced several buds that turned green and later developed into plantlets.

Salvi *et al.* (2000, 2001) also reported plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants showed variation at DNA level.

Sunitibala *et al.* (2001) reported callus induction and plant regeneration by organogenesis on MS + 3 mg^l⁻¹ 2, 4 -D and 1 mg^l⁻¹ kinetin while Salvi *et al.* (2001) induced callusing on MS medium supplemented with 2.0 mg^l⁻¹ dicamba or picloram or 5 mg^l⁻¹ NAA+ 0.5 mg^l⁻¹ BA and plant regeneration on MS with 5 mg^l⁻¹ BA. RAPD analysis of eight regenerated plants showed variation at DNA level indicating somaclonal variation.

Praveen (2005) reported efficient micropropagation and plant regeneration from callus using TDZ in two turmeric varieties Prabha and Prathibha with successful induction of both organogenesis and embryogenesis in MS medium supplemented with 0.1 – 0.5 μ M TDZ. When the concentration of TDZ was raised to 10 μ M direct plant regeneration was observed from leaf tissues without intervening callus. This study revealed occurrence of cytotypes and morphological variants among callus regenerated progenies. RAPD profiling also indicated the occurrence of molecular variants.

In the present study excellent plant regeneration was obtained using 2,4-D and BA. The result was as good if not support to the report of Praveen 2005 by TDZ. The reports of Nirmal Babu *et al.*, 1997, Salvi *et al.*, 2000, 2001 and Sunitibala *et al.*, 2001 also supports this view.

Induction of Microrhizomes

Effect of different factors like MS salt concentration, growth hormones, sucrose and duration of culture period on the production of microrhizome *in vitro* were studied. Out of four different concentrations of MS salt tried, 0.75 X is the optimum basal salt concentration in which the maximum numbers of microrhizomes were produced (16.0). While in the others the number of microrhizome and their weight was less. The result was same as in ginger.

Among the concentration of sucrose studied 9% sucrose gave the maximum production (16.3) of microrhizome *in vitro*. Where as the lower and higher concentration leads to the reduction in the number and also their fresh weight

The optimum production of turmeric microrhizome obtained at 120 days of growth period. The rhizome size reached at this stage was optimum for germination. The maximum number of rhizome produced at 120 days was 17.

Among different combinations of plant growth regulators tried 1 mg l^{-1} BA together with 0.5 mg l^{-1} NAA with 0.75x concentration of MS salt, 9 % sucrose, exhibited a better response than any other treatments in terms of mean number (19.7) and the their fresh weight (2.4).

Earlier Islam *et al.* (2004) also found that best medium at 0.75x strength was most suitable in terms of number and size of microrhizome, however lesser strength of MS medium produced a lower number of smaller rhizome while full strength produced lower number but comparatively larger sized rhizomes. The present study also supports this view. Shirgurkar *et al.* (2001) reported that half strength MS medium is suitable for microrhizome production in turmeric obtaining an average number 5.8 ± 0.7 with biggest size being 0.55 ± 0.06 g, while Nayak (2000) and Sunitibala *et al.*, (2001) used full strength MS basal medium for microrhizome induction in *Curcuma*. Sharma and Singh (1995) also found full strength MS basal medium along with 7.5% sucrose and $35.2 \mu\text{M}$ BA optimal for the production of *in vitro* microrhizome in ginger. In potato, Garner and Blake (1989) found full strength MS medium supplemented with 8% sucrose better than those supplemented with 4 and 12 % sucrose. In the present study three fourth strength of MS is the best for microrhizome induction.

Shirgurkar *et al.* (2001) reported that 8% sucrose was found to be optimal for microrhizome production. Sunitibala *et al.* (2001) reported that the *in vitro* rhizome formation was obtained with MS + NAA (0.1mg l^{-1}) + kin (1.0mg l^{-1}) and 8% sucrose. Sanghamitra Nayak (2000) reported *in vitro* microrhizome production in four turmeric rhizome production in four turmeric varieties (Ranga, Rashmi, Roma and Surama) after 30 days of culturing on MS liquid medium, supplemented with 3mg l^{-1} BA and 60g l^{-1} sucrose and 4 hrs of photoperiod. He also observed that at an obligatory concentration of sucrose (3%), plants could not produced any microrhizomes even by increasing the concentration of BA from $1-7 \text{mg l}^{-1}$ or by increasing the duration of the photoperiod and also stated that a lower concentration of sucrose decreased the size and number of rhizomes or even completely prevented the induction of any microrhizomes. Raghu Rajan (1997) found that MS medium supplemented with 0.3mg l^{-1} BAP [benzyladenine], 0.1mg l^{-1} NAA and 0.5mg l^{-1} ancymidol and 10% sucrose was best for induction of microrhizomes. The enhanced rate of *in vitro* organ formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in the

form of sucrose since storage organs mostly store carbohydrates Nayak (2000). Sharma and Singh (1995) reported that a concentration of 7.5% was required for *in vitro* rhizome induction in *Zingiber officinale* Rosc. In many other reports it is also stated that a high concentration of sucrose remarkably promotes the *in vitro* formation of storage organs such as bulbs, corms and tubers (Abbot and Belcher 1986, Alizadeh *et al.*, 1998, Arora *et al.*, 1996; Dantu and Bhojwani 1987, Garner and Blake 1989, Gopal *et al.*, 1998, Grewal 1996, Haque *et al.*, 1996, Kim *et al.*, 2003, Slabbert and Niederwieser 1999 and Vreugdenhil *et al.*, 1998). In the present study microrhizome production was best with 9% sucrose.

Raghu Rajan (1997) states that microrhizomes harvested after 8 weeks from the induction medium (rather than after 12, 16 or 20 weeks) gave the best results for germination under field conditions, without an intervening phase of hardening. Tyagi *et al.* (2006) reported that in ginger *in vitro* rhizomes are conserved for more than 12 months on the same medium and successful recovery of plantlets from these rhizomes after *in vitro* conservation. The longest storage of microtubers of potato varies from 16-18 months and a large number of potato collections are being maintained in the form of microtubers (Thieme, 1992). More and heavier microtubers were produced when culture period was 70-90 days than 60 d (Yiem-*et al.*, 1990)

Nayak (2000) reported that 5 mg l⁻¹ BA in *C. aromatica* Salisb. enhance the microrhizome production. Sharma and Singh (1995) reported 8 mg l⁻¹ BA enhance the microrhizome in ginger. However Shirgurkar *et al.* (2001) stated that maximum concentration of BA (35.2 µM) had an inhibitory effect on *in vitro* microrhizome production where as in lowest level of BA (4.4 µM) did not have much adverse effect on the production of microrhizome. Islam *et al.* (2004) reported that Kinetin alone or in the presence of NAA did not show any significant result where as 12.0 µM BA and 0.3 µM NAA in combinations exhibited better responses in terms of number and weight of rhizome. Sunitibala *et al.* (2001) reported that Kn (1 mg l⁻¹) and NAA 0.1 mg l⁻¹ is suitable for *in vitro* rhizome induction in *C. longa* L. Peak and Murthy (2002) reported that NAA

is the most effective auxin for the induction of bulblets *in vitro* from scale sections of *Fritillaria thunbergii*. Kim *et al.* (2003) also reported that 0.1 mg l⁻¹ NAA together with 11 % sucrose and 10 µM jasmonic acid was the most effective medium for *in vitro* multiplication of bulblets in garlic. The size of the culture vessel may play an important role to produce the uniform size of rhizomes. In yams bigger sized vessels (Magenta box) resulted in a significant increase in micro tuber weight (Jean Cappadocia, 1991).

Microrhizomes resembled the normal rhizomes in all respects, except for their small size. They resembled the normal rhizome in anatomical features in the presence of well-developed curcumin cells, oil cells, oleoresin, and starch grains were observed. These micro rhizomes were directly planted in the field without any hardening with 85 % success. Microrhizome derived plants were almost similar in morphology with normal plant. But are smaller in size with higher number of mother rhizomes per plant. These micro rhizomes gave a fresh rhizome yield of 100 - 550g per plant compared with controls with 300- 1200g per plant.

The microrhizome is an ideal pathway for production of disease free planting material in rhizomatous crops like ginger and turmeric. These can be directly served as planting material without hardening. This system can be sealed up in similar lines of micro and minituber production in potato for large scale production of quality planting material.

Morphological characterization

The plantlets obtained through micropropagation, callus regeneration and microrhizome pathways were morphologically characterized in pot culture. The variations were observed in all the plantlets studied though not significant in some cases (Table 28). Certain amount of variation was observed in micropropagated, microrhizome and conventional plants also.

The callus regenerated plants show maximum variation in almost all the characters studied. The important yield attributing characters, which showed good

variation in callus regenerated plants, were plant height, leaf breadth, yield and nodes in primary and secondary fingers.

The characters, which showed variation in conventional, micropropagated, and microrhizome derived plants were leaf length, yield, breadth of secondary fingers, nodes per mother rhizome and internodal length of the fingers (Fig.36,37).

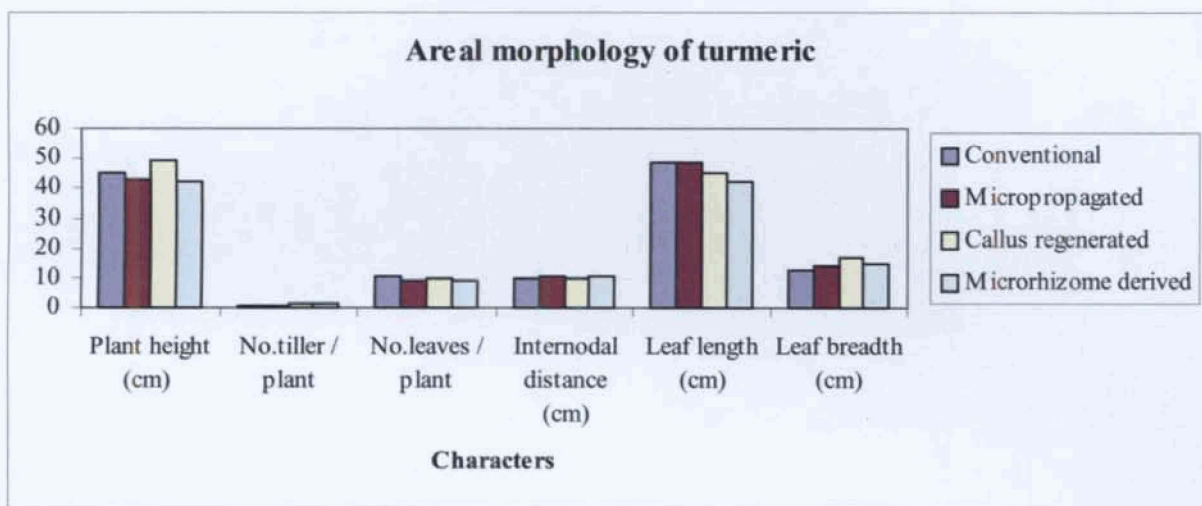


Fig.36 Aerial morphology of turmeric somaclones (Conventional, Micropropagated, Callus regenerated and Microrhizome derived plants)

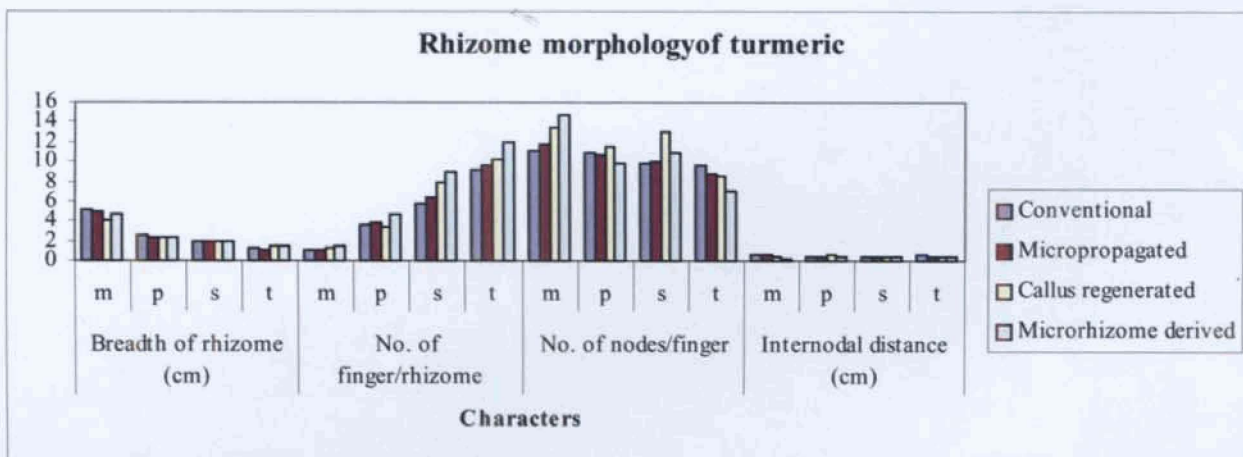


Fig. 37 Rhizome morphology of turmeric somaclones (Conventional, Micropropagated, Callus regenerated and Microrhizome derived plants)

Field evaluation of micropropagated and conventionally propagated plants have conducted by Salvi *et al.* (2002) and reported that micropropagated plants were superior in most of the morphological and rhizome characters studied. Micropropagated plants of

turmeric cv 'elite', had a higher number of tillers and leaves. It was suggested that this might be due to the residual effect of cytokinins used in the culture medium.

Praveen 2005 observed somaclone with distinct variation in patterns of rhizome development, orientation and number of primary, secondary and tertiary rhizome, internodal and nodal distances. Appearance of 'horn' like primaries and longer internodal distances were observed in some of the somaclones. Rhizomes with much shorter internodes and more number of nodes in primaries and secondaries were also observed. Variations in colour of rhizome (dark pink) were observed in a few somaclones.

In the present study also variation with respect to leaf length, yield, breadth of secondary fingers, nodes per mother rhizome and internodal length of the fingers, were observed among micropropagated and microrhizome derived plants. The plants derived from callus showed variation with respect to plant height, leaf breadth, yield and nodes in primary and secondary fingers.

Biochemical characterization

A few of the morphological variants were subjected to further biochemical characterization. Significant variations were observed in percentage of curcumin, oil, oleoresin, starch, reducing sugar, and total carbohydrate among the somaclones tested. The callus regenerated C4 -2 recorded maximum percentage of curcumin (5.3%) where as C4 – 4 recorded the maximum percentage of oil (5%). The highest percentage of protein recorded in the accession C2 – 4 (1.11) and MP10 gave maximum percentage of oleoresin (12.7%). MP-8 recorded highest percentage of starch (44.0%) and total carbohydrate (48.7%). Reducing sugar was high in the control (1.85%) (Table 31).

Though the micropropagated and microrhizome derived plants showed minimal biochemical variation, the plants regenerated through callus pathway gave significant variation in biochemical characters.

Earlier reports in turmeric states that variants with high curcumin content were isolated from tissue cultured plantlets (Nadgauda *et al.*, 1982). Metabolic changes during *in vitro* multiplication of *Curcuma longa* reported by Rout *et al.* (1995). They observed

chlorophyll content was higher during shoot multiplication than during shoot elongation or rooting in both cultivars, while total protein and total carbohydrates were highest at the rooting stage. Catalase and acid phosphatase activities were both highest during shoot multiplication, but peroxidase activity increased through elongation, multiplication and rooting.

Several authors reported increase in the biochemical constituents due to effect of different media components. Hazarika *et al.* (2000) reported in Citrus cultures, Wang and Steffens, (1987) reported in apple seedlings on addition of paclobutrazol to the medium. Increase in starch levels may be due to decreased starch hydrolysis as result of reduced amylase activity of as observed by Steffins *et al.* (1983) and Upadhyaya *et al.* (1986).

Parthasarathy *et al.* (2002) reported decrease in protein levels in the presence of cytokinin. Wang and Steffens (1987) have reported an increase in total phenol in the presence of paclobutrazol in apple seedling.

Nayak *et al.* (2003) reported, among 100 somaclones of Jamrosa, a wide range of variation was recorded for several traits, including plant height, tiller number, herb weight, oil content and total oil yield. Qualitative analysis of essential oil was carried out for 45 somaclones, which performed better or equal to the donor parent. Eleven somaclones selected on the basis of total oil yield and qualities were further evaluated in a replicated trial. Five superior somaclones showing a total oil yield twofold higher than the donor and possessing quality oil containing a high geraniol content > 84% were selected for further analysis in the field for six clonal propagations. Out of the five superior somaclones two improved somaclones which showed relative stability in oil yield and quality were subjected to RAPD analysis. Changes in RAPD banding pattern in the improved somaclone as compared to donor parent revealed occurrence of gross genetic changes.

Composition of essential oil of *in vitro* microrrhizome and *in vivo* rhizome by using GC- MS

The developmental status of *in vitro* and *in vivo* microrhizomes of turmeric were studied using GCMS profiling. The relative concentrations of the volatile components identified are presented in according to their elution order on column. The components, which present in the *in vitro* rhizomes, were α -pinene, β -pinene, γ -Terpene and d-Limonene. These components were absent *in vivo* rhizomes. This may be due to the differences *in vitro* and *in vivo* condition in which the storage organs were induced.

Oil composition of rhizome oil matched to a great extent with that of rhizome oil reported by Sharma *et al.* (1997) with respect to major constituents ar-turmerone (33.34% and 30.1%), turmerone (17.23 % and 14.7%) and α -phellandrene 10.73% *in vivo*. The major constituents of *in vitro* rhizome are similar with *in vivo* even though its percentage of content varied. The *in vitro* rhizome content of ar- turmerone 25.82%and turmerone 14.79% and α -phellandrene 15.73% were reported in our study.

Ibrahim *et al.* (1999) reported essential oil components of four *Curcuma* species, *Curcuma mangga*, *C. xanthorrhiza*, *C. aeruginosa* and *Curcuma longa* (collected from Malaysia), were analysed by GC-MS. Myrcene (81.4%) was the most abundant component in the essential oil of *C. mangga*. The essential oil of *C. xanthorrhiza* was made up mainly of sesquiterpenoids of which xanthorrhizol (44.5%) was the major constituent. 1,8-Cineole [eucalyptol] (23.2%) and curzerenone (28.4%) were the predominant constituents of the essential oil of *C. aeruginosa*. The essential oil of *C. domestica* contained significant amounts of α -tumerone (45.3%), linalool (14.9%) and β -tumerone (13.5%).

Bordoloi *et al.* (1999) collected essential oils, steam-distilled from leaves and rhizomes of cultivated *Curcuma aromatica* (from India), were investigated by GC and GC-MS. About 50 compounds were identified, accounting for more than 85% of the essential oils. The major constituents of the leaf essential oil were camphor (28.5%), ar-turmerone (13.2%), curzerenone (6.2%), 1,8-cineole [eucalyptol] (6%) and α -turmerone (2.5%). The rhizome essential oil consisted mainly of camphor (32.3%),

curzerenone (11%), alpha -turmerone (6.7%), ar-turmerone (6.3%) and 1,8-cineole (5.5%).

Nguyen-Xuan-Dung *et al.* (1995) steam-distilled essential oil from leaves of *C. domestica* (*C. longa*), was analysed by GC and GC-MS. More than 20 components were identified, of which the monoterpenes alpha -phellandrene (24.5%), 1,8-cineole [eucalyptol] (15.9%), p-cymene (13.2%) and beta -pinene (8.9%) were the major ones.

There is no report available on essential oil composition of *in vitro* microrhizome of turmeric.

Histology of *in vitro* microrhizome

The developmental stages of *in vitro* microrhizome at 120 days of growth period were anatomically studied with regard to the production of secondary metabolites. The presence of well-developed curcumin cells, oil, oleoresin and starch cells were observed in the *in vitro* microrhizome at this stage of growth period (Fig).

Cortella and Pochettino (1994) reported starch grains are an important diagnostic feature in the identification of plant material because of their abundance, ubiquity and varied morphological characteristics.

Stomatal study of somaclones

One of the somaclone showed more number of stomata with smaller size compared to other somaclones. An average of 15 stomata per sq.mm recorded where as in the case of control it was 9 stomata per sq. mm (Fig. 24).

Chromosome indexing

The observed 'variants' were cytologically indexed to observe variations in chromosome number if any. Micropropagated and microrhizome derived plants showed normal chromosome number as that of the control. A few of the callus regenerated plants showed variation in the chromosome number and also structural aberrations. The numerical variations were mainly aneuploids with lesser number of chromosomes ($2n=53, 55, 57$) than the normal number except one plant with higher number of

chromosome ($2n=65$). This may due to triploid nature of *C longa* leads to cytological aberrations.

Novak (1980) reported phenotypic as well as cytological variations in callus derived garlic plant. Almost half of the regenerants being tetraploid, aneuploid or mixoploid. Dolezel and Novak 1985; Novak *et al.*, 1986 reported changes in the basic chromosome number within garlic culture. After 30 days of callus culture derived from leaf bases of garlic, Dolezel and Novak (1985) observed that 70% of the cells were polyploidy (mostly tetraploid) with a lower frequency of aneuploids. It is well documented that *in vitro* culture conditions induce a genomic stress that might result in chromosome breakage. Many studies indicated that the break positions do not appear to be random but occur in a heterochromatic region and could lead to chromosomal translocation, inversion or deletion (Benzion and Phillip 1988; Lapitan *et al.*, 1988).

Molecular characterization

Micropropagated, callus regenerated and microrhizome derived plants were subjected to RAPD profiling with 6 operon primers (OPA 04, OPA 06, OPA 08, OPC 01, OPC 19 and OPC10). The monomorphic banding pattern was observed in micropropagated and microrhizome derived plants and callus regenerated plants showed polymorphic banding pattern almost all the above primers. This indicates that most of the variation expressed in the somaclones developed especially callus are reflected in genetic make up (Fig. 38).

Earlier reports also stated that though there are many morphological variations in tune somaclones these variations are reflecting in genetic nature. Salvi *et al.* (2002) reported that of the 48 plants, two showed variegated leaves in the tillers. The micropropagated plants showed a significant increase in shoot length, number of tillers, number and length of leaves, number of fingers and total fresh rhizome weight per plant when compared with conventionally propagated plants. RAPD analysis of 11 regenerated plants using sixteen decamer primers did not show any polymorphism. In some cases

genetic variations were found as seen in RAPD profile differences among somaclones.

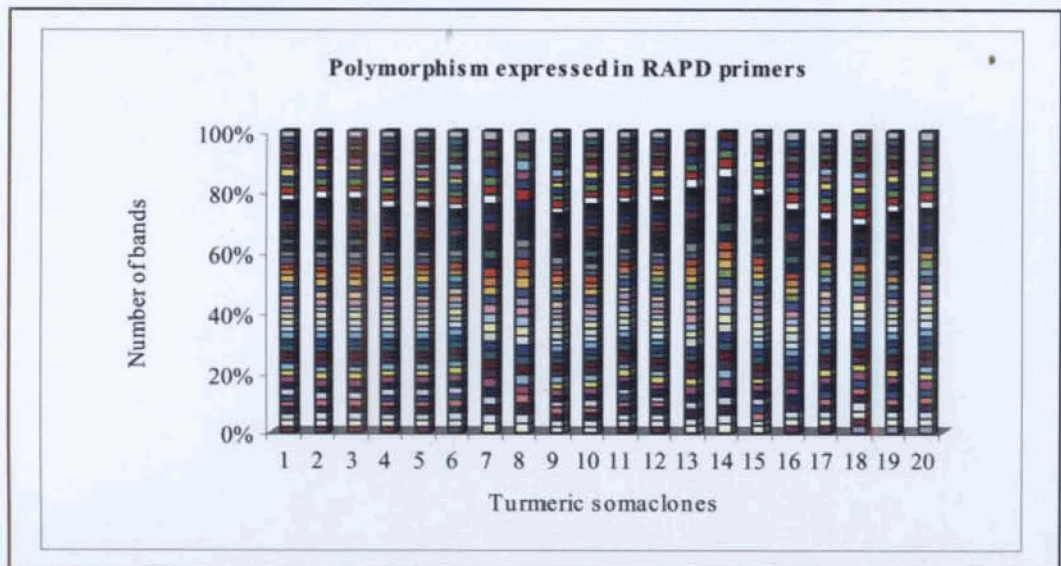


Fig. 38 RAPD profile differences among turmeric somaclones (1-Control, 2-20 Callus regenerated plants of turmeric)

Salvi *et al.* (2003) reported that micropropagated plants from shoot tip showed significant increase in plant height, number of tillers, number and length of leaves, number of fingers and total rhizome weight per plant in the first generation when compared with conventionally propagated (control) plants. Second and third generation of regenerated plants checked under the same field conditions ascertained the stability of character observed in the first generation. RAPD analysis of 10 turmeric plants, each propagated conventionally, regenerated from shoot tips, leaf base callus and immature inflorescence was carried out using 15 primers. Plants regenerated using shoot tips showed uniform banding pattern, whereas, callus derived and inflorescence derived plants showed polymorphism in banding pattern when compared with conventionally propagated plants.

Praveen (2005) also reported variations in turmeric somaclones with respect to both morphological as well as RAPD profiles but could not detect any variations among micropropagated plants. He reported that *in vitro* mutagenesis increased the spectrum of

somaclonal variation. He found variability among somaclones and isolated somaclones with short noded rhizomes. The present study agrees with the findings of Salvi *et al.* (2001, 2003) and Praveen (2005).

Salvi *et al.* (2001) using RAPD analyzed turmeric plants regenerated from leaf base callus and this study of eight regenerated plants was using 14 primers when separated on non-denaturing polyacrylamide gels showed 38 novel bands. About 51 bands present in the control were absent in the regenerants. The result indicates that variation at DNA level has occurred during *in vitro* culture.

Improvement in turmeric is hindered by reduced or rare seed set. No source of resistance either to rhizome rot or leaf spot disease is available in the germplasm (Nair *et al.*, 1980). Improvement in turmeric is also sought for high curcumin content and yield. Somaclonal variation is a tool that can be used by plant breeders for improving crop plants and is true in turmeric as well and is the most useful in crops like turmeric with narrow genetic base, limited scope for hybridization and vegetative propagation (Karp, 1995). Considering these factors, somaclonal variation finds a prime place in the breeding programme of turmeric.

Screening for disease incidence

Ten selected somaclonal ‘variants’ of turmeric along with control plant were challenged with *Pythium myriotylum* for their reaction to rhizome rot disease. The somaclones like C4-2, C5-3 and C6-2 showed susceptible reaction to rhizome rot disease. The control plants also showed 6.25% of disease incidence. The somaclones such as C10-2, C3-2 and C8-2 showed minimum disease incidence, indicating variations among somaclones for disease resistance.

There are earlier reports of obtaining somaclones resistance to rhizome rot of turmeric through *in vitro* selection. Root rot disease tolerant clones of turmeric cv. Suguna were isolated using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum* (Gayatri *et al.*, 2005).

Several attempts have been made to select disease resistance expressed at the whole plant level by using tissue culture systems. Experimental approaches for such

selections have been described by Daub (1984), Grout and weatherhed (1980) and Ingram (1980).

Brettell *et al.* (1980) and Gengenbach and Green (1975) have selected maize callus resistant to the T-toxin produced by race T of *Bipolaris (Helminthosporium maydis)*, the causative agent of southern corn leaf blight (SCLB)

Brettell *et al.* (1980) found that resistance to southern corn leaf blight with a concomitant loss of male sterility was produced in plants derived from callus that had been in culture for several transfers and that had not been subjected to T – toxin selection pressure (somaclonal variants). A similar event was noted by Larkin and Scowcroft (1983) in the selection of sugarcane plants resistant to eyespot 9caused by (*Helmintosporium sacchari*). Larkin and Scowcroft (1983) also attempted to select for resistance by using the toxin produced by *H sacchari*. In this case the toxin was added to the medium used for the last callus maintenance transfer and also to the plant regeneration medium. regenerated plants showed tolerance to eyespot that was passed through several vegetative propagation cycles. In both the experiments of Brettell *et al.* (1980) and of Larkin and Scowcroft (1983), the recovery of resistant material was greater when toxin was used tan when it was dependent only on somaclonal variation.

Benhke (1980) was able to select for potato callus resistant to the crude culture filtrate of *Phytophthora infestans*, the causative agent of late blight of potato. Leaflets from plants regenerated from the resistant callus showed greater tolerance to the culture filtrates and also showed a reduced growth rate of the pathogen a when the leaflets were inoculated.

Hartman *et al.* (1984) selected resistance to *Fusarium* wilt of alfalfa in cultures of alfalfa (*Medicago sativa*) using culture filtrates of *Fusarium oxysporum* f. sp. *Medicaginis*. The resistance produced was expressed in cultures placed on inhibitory medium after having been subcultured repeatedly on non inhibitory medium for 5 months. The resistance was also expressed in plants regenerated from the cultures and in callus established for the regenerated plants. Hartman *et al.* (1984), having selected for

resistance to a vascular wilt, illustrated that resistance to several types of diseases can be selected in tissue culture. The other reports of development of resistant lines among somaclonal variants and *in vitro* selection are of Behnke (1980), Brettel *et al.* (1980), Carlson (1973), Gengenbach and Green (1975), Gengenbach *et al.* (1977), and Larkin and Scowcroft (1983).

Cardamom

Micropropagation

Rhizome buds were cultured on MS medium supplemented with BA at 1mg l^{-1} and NAA at 0.5mg l^{-1} produced a mean of 4 shoots, each approximately 5.4 cm in length.

Efficient *in vitro* methods for rapid clonal propagation of cardamom are available. (Nadgauda *et al.* (1983), Priyadarsan and Zachariah (1986), Vatsya *et al.* (1987), Reghunath and Gopalakrishnan (1991). Kumar *et al.* (1985) reported successful conversion of immature floral buds to vegetative plantlets and inflorescences form an excellent source for reducing culture contamination especially since other sources are prone to high rate of contamination. Many commercial laboratories are using micropropagation techniques for large-scale production of cardamom clonal material. Clonal multiplication of elite cultivars, coupled with their being rendered free of pathogens etc have made the methods very useful

Vatsya *et al.* (1987) reported multiple shoot formation was induced on a modified MS medium. The best results were obtained when the buds (10-20 mm in size) were cultured in liquid medium at 25°C , with an irradiance of $40\ \mu\text{mol m}^{-2}, \text{s}^{-1}$, and a 12 h photoperiod. By this method it was possible to obtain a 10-fold multiplication in 90 days. Shoots were rooted *in vitro* on an MS medium containing IBA and a reduced sucrose concentration, or were separated, dipped in a dilute solution of rooting hormone (Seradix-B1 [IBA]) and transferred to pots containing soil and compost. *In vitro* rooting gave the highest survival rate.

High rate of multiplication coupled with additional advantage of obtaining disease free planting material makes micropropagation an important and viable alternative to conventional propagation.

Callus induction and Plant regeneration

Vegetative bud cultured on MS supplemented with 1mg l^{-1} 2,4-D for callus induction. 96% of the culture induced callus and 3.6g fresh weight of callus obtained from this medium. Darkness for 14 days at the beginning of the culture period increased the rate of callus production.

80% of callus gave positive response for plant regeneration when cultured on MS medium with 4 mg l^{-1} BA+ 0.5 mg l^{-1} NAA This was most suitable for shoot multiplication and development from embryogenic callus. The calli maintained their morphogenetic competence over several subcultures in the hormone-free MS medium.

Successful regeneration of plantlets from callus of seedling explants of cardamom was reported earlier Rao *et al.* (1982), Priyadarshan and Zachariah (1986), Nirmal Babu *et al.* (1993).

Ravindran *et al.* (2002) reported callus induction and proliferation from cardamom anthers initially in MS medium containing 0.1mg l^{-1} TDZ and thereafter the swollen anthers on MS medium containing 0.5 mg l^{-1} 2, 4-D and 0.1mg l^{-1} TDZ. Plant regeneration was obtained from anther derived callus on MS medium with 0.5 mg l^{-1} 2,4-D, 0.1 mg l^{-1} TDZ, 0.2% Trypton along with 25% sucrose and 5% glucose or 15% sucrose and 15% glucose.

High frequency plant regeneration from rhizome and vegetative bud-derived callus cultures was standardised at IISR and this excellent regeneration system was used for development of somaclonal variation and selection of useful genotypes from them. Good morphological variation was observed among the somaclones in the culture vessels itself.

***In vitro* method to overcome apical dominance**

Controlling of apical dominance by mechanical means using petri plates replaces growth regulators and thus activating axillary bud development. 18- 25 multiple shoots were produced by this method and stimulated vigorous root growth also. 80% of the plants established in the soil.

The apically derived auxin from the dominating shoot moves down to the main stem and then is transported over to the dominated shoot and up to its growing region where growth is repressed. Normally the apical dominance is removed by decapitation of shoot tip which leads to the development of a lateral shoots. In some species to promote increased seed and/or biomass production (ie, overcompensation) by stimulating lateral branching. The yield increases in herbaceous plants following shoot apex removal either alone or in association with other plant parts, are now common (Clifford 1979, Tayo 1980, 1982, Inouye 1982, Amuti 1983, Argall & Stewart 1984, Sheldon 1986, Paige & Whitham 1987, Aarssen & Turkington 1987, wein & Minotti 1988, Benner 1988, Maschinski & Whitham 1989, Strauss 1991, Mopper *et al.*,1991, Michaud 1991). This helps in easy multiplication of plant lets without the use of growth regulators which indirectly enhances the genetic fidelity of multiplied plants. This is the first report in cardamom.

Morphological characterization

The plantlets obtained through micropropagation, callus regeneration pathways were morphologically characterized. Maximum plant height, panicle length and number of flowers per panicle were recorded in micropropagated plants. Number of tiller, number of leaves and primary leaf length were recorded maximum in conventionally propagated plants. Where as more number of panicle and number of nodes per panicle recorded in callus regenerated plants (Fig. 39).

Large scale field evaluation of tissue cultured plants of cardamom sponsored by the Department of Biotechnology has been carried out by Spices Board and IISR and the results showed that the micropropagated plants performed on par with suckers (Lukose 1993, Sudharshan *et al.*, 1997, Chandrappa *et al.*, 1997).

Variations observed among the TC plants, suckers and seedlings were non significant for most of the characters. Lukose *et al.*, 1993 reported that the variations observed among the TC plants, suckers and seedlings were non significant for most of the characters. Yielding tillers, panicles per plant, green capsule yield and cumulative yield were significantly more in tissue cultured plants. Sudharshan *et al.* (1997) reported that variability was observed in the clonal population for vegetative characters. The overall variability in tissue cultured plants was 4.5 % as against 3 % in open pollinated seedling progenies for a given set of characters. However inspite of occurrence of variations, tissue cultured plants yield 34% more than the seedlings. The causes of variations were attributed to adventitious bud formation during micropropagation via axillary buds, genetic instability of adventitious meristem and tissue culture induced disorganization of meristems.

Chandrappa *et al.* (1997) tested eight tissue cultured cardamom selections against their suckers and two local checks. They identified three clones TC 5, TC 6, and TC 7 with promising yield and yield attributes.

A few Katta tolerant somaclones were identified (Nirmal Babu *et al.*, 1997, Peter *et al.*, 2001) among somaclones of cardamom.

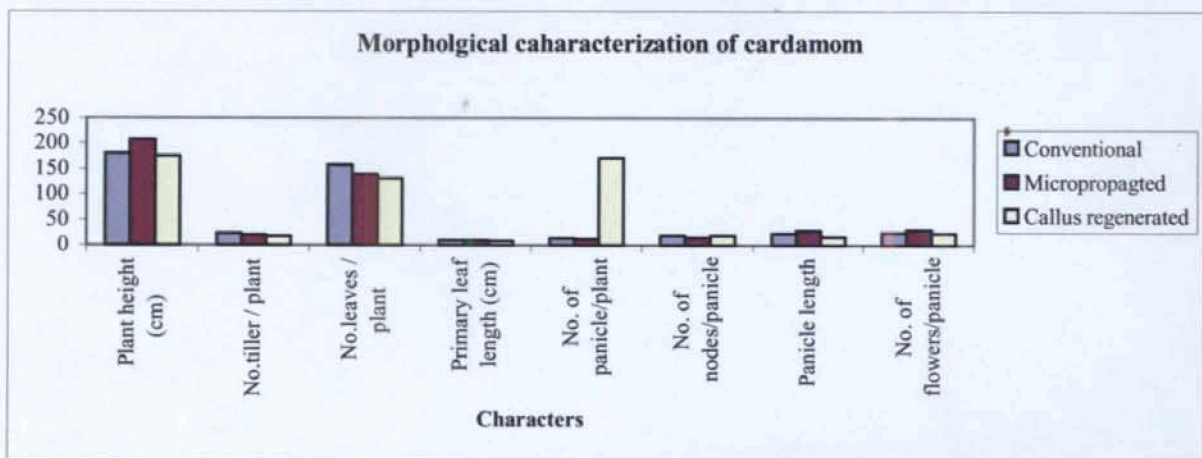


Fig. 39 Comparative morphology of cardamom somaclones (Conventional, micropropagated, callus regenerated plants)

In one of the somaclones, the bundle sheath is connected upper and lower epidermis by a group of compact sclerenchymatous cells called bundle sheath extension. The stomata are paracytic, having the guard cells flanked by somewhat elongated, conical shaped subsidiary cells. The above somaclone also showed oval or round stomatal subsidiary cells.

Molecular characterization

Comparative RAPD study of micropropagated and callus regenerated plants were conducted using 6 operon primers OPB 19, OPC 10, OPC 19 OPD 12. OPA 06 and OPA 17. Micropropagated plants showed monomorphic banding pattern in all the primers tested and at the same time callus regenerated plants showed polymorphic banding pattern in all the above primers (Fig. 40). This indicates that reasonable genetic variation could be obtained from cardamom somaclones.

RAPD analysis was used to study genetic variability among somaclones in various crops. (Moon *et al.*, 1996; Godwin *et al.*, 1997; Banerjee *et al.*, 1998; Moukadiri *et al.*, 1999; Yang *et al.*, 1999)

Molecular markers like RAPD, PCR - RFLP and ISSR polymorphism were used to characterize 96 collections comprising important cultivars, varieties and related genera of cardamom to develop fingerprints and to study the inter-relationships (Nirmal Babu *et*

al 2005)). The study indicated that there are no duplicates in the 100 lines characterized and the Kerala and Karnataka populations are divergent in that they formed two separate clusters in the Phylograme. Eleven species representing 5 major tribes viz., *Amomum subulatum*, *Amomum aromaticum*, *Amomum ghaticum*, *A. microstephanum*, *Amomum involucreatum*, *Alpinia galanga*, *A. purpurea*, *Alpinia mutica*, *Aframomum melegueta*, *Hedychium coronarium* and *Elettaria cardamomum* were also profiled for polymorphism using RAPD and ISSR primers. The phylogram showed that *Elettaria cardamomum* is clustered with *Amomum subulatum* and *A. microstephanum* indicating that *Amomum* is closest to cultivated cardamom among the genera studied (Jayakumar *et al.*, 2005).

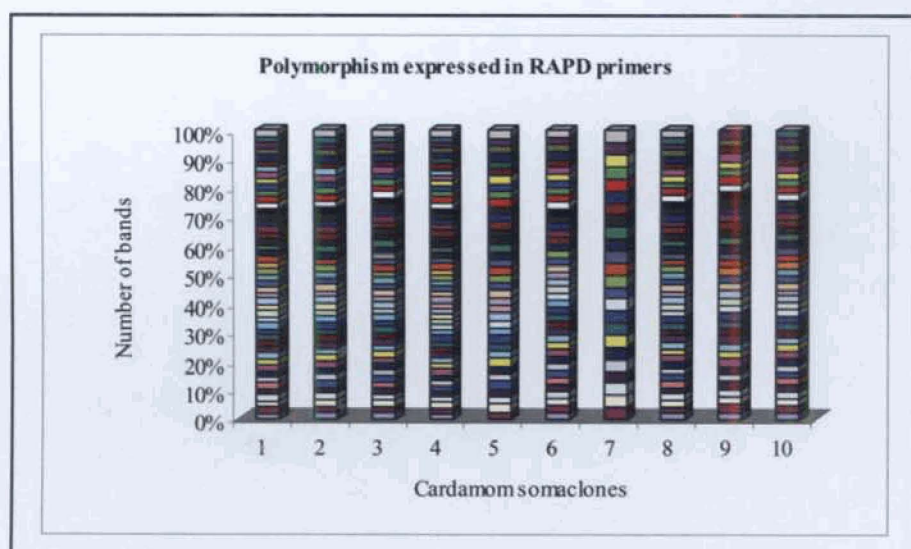


Fig.40 RAPD profile differences among cardamom somaclones (1-Control, 2-10 callus regenerated plants of cardamom)

A study of the tissue culture literature shows many passing comments on the occurrence of abnormalities amongst regenerated plants. Some examples include carrot (Ibrahim 1969), Chrysanthemum (Ben-Jaacov and Langhans 1972), carnation (Hacket and Anderson 1967), and clover (Beach and smith 1979), Gamborg *et al.* (1977) found variants amongst sorghum culture regenerants. Some had reduced fertility; some had altered leaf morphology and growth habit. All these variant sorghum somaclones were normal in karyotype ($2n=20$)

Nishi *et al.* (1968) and Henke *et al.* (1978) both reported phenotypic variants amongst somaclonal plants regenerated from rice callus. The variations were in traits such as number of tillers per plant, number of fertile tillers per plant, average panicle length, frequency of fertile seed, plant stature and flag leaf length.

In Oats (*Avena sativa*), Cummings *et al.* (1976) reported altered phenotype in plant height, heading date, twin culms, yellow leaf stipes, awn morphology and fertility. Many of these variants were transmissible to later generations (both true breeding and segregating lines occurred). McCoy *et al.* (1978) has also indicated a high frequency of cytogenetic abnormality.

Skirvin and Janick (1976a, 1976b) have observed a remarkable degree of variability in plants regenerated from the cultures of 5 pelargonium cultivars. Wakasa (1979) 448 somaclones of pineapple (*Ananas comosus*). Many variants were observed in spine and leaf colour, wax secretion, foliage density, leaf width, and leaf spine formation. The explant source had a dramatic effect on the occurrence of variation.

Many authors (Heinz *et al.*, 1977; Liu and Chen 1976, 1978a; Skirvin 1978, Hoffman 1978; Oono 1978b, Brettell and Ingram 1979; Thomas *et al.*, 1979; shepard *et al.*, 1980; Barbier and Dulieu 1980) have given substantial acknowledgement to the existence of variability among plants regenerated from cell cultures. generally their direct or allusive judgements imply that somaclonal variation is artefactual noise, confined to vegetatively propagated plants, produced by karyotype changes and occurs in chimeric tissues, Oono (1978b) Skirvin (1978), Thomas *et al.* (1979), Shepard *et al.* (1980) and Barbier and Dulieu (198) have suggested that the phenomenon may prove to be of some value as a tool in plant improvement. The spectrum of characters affected can be diverse and the frequency of variant occurrence comparatively high in predominantly vegetative crops.

Somaclonal variation may find its greatest application for plant improvement in concert with selection for desirable mutations at the cellular level.

Cellular selection is conceivable for the recovery of variants resistant to antimetabolites such as amino acid analogues, antibiotic drugs, pathotoxins, herbicides and physiological stress (Maliga 1978; Thomas *et al.*, 1979; Brettell and Ingram 1979).

Thus in the present study in vitro technology could be used for generating and utilizing somaclonal variation as a good source of variability in vegetatively propagated Zingiberaceous crops like ginger, turmeric and cardamom. Useful variants with good rhizome and yield characters, disease resistance, extra bold rhizome were identified in ginger, variant with resistance to *Pythium myriotylum*, good rhizome characters like closer nodes, quality attributes were identified in turmeric and variants with fruit shape and size were identified.

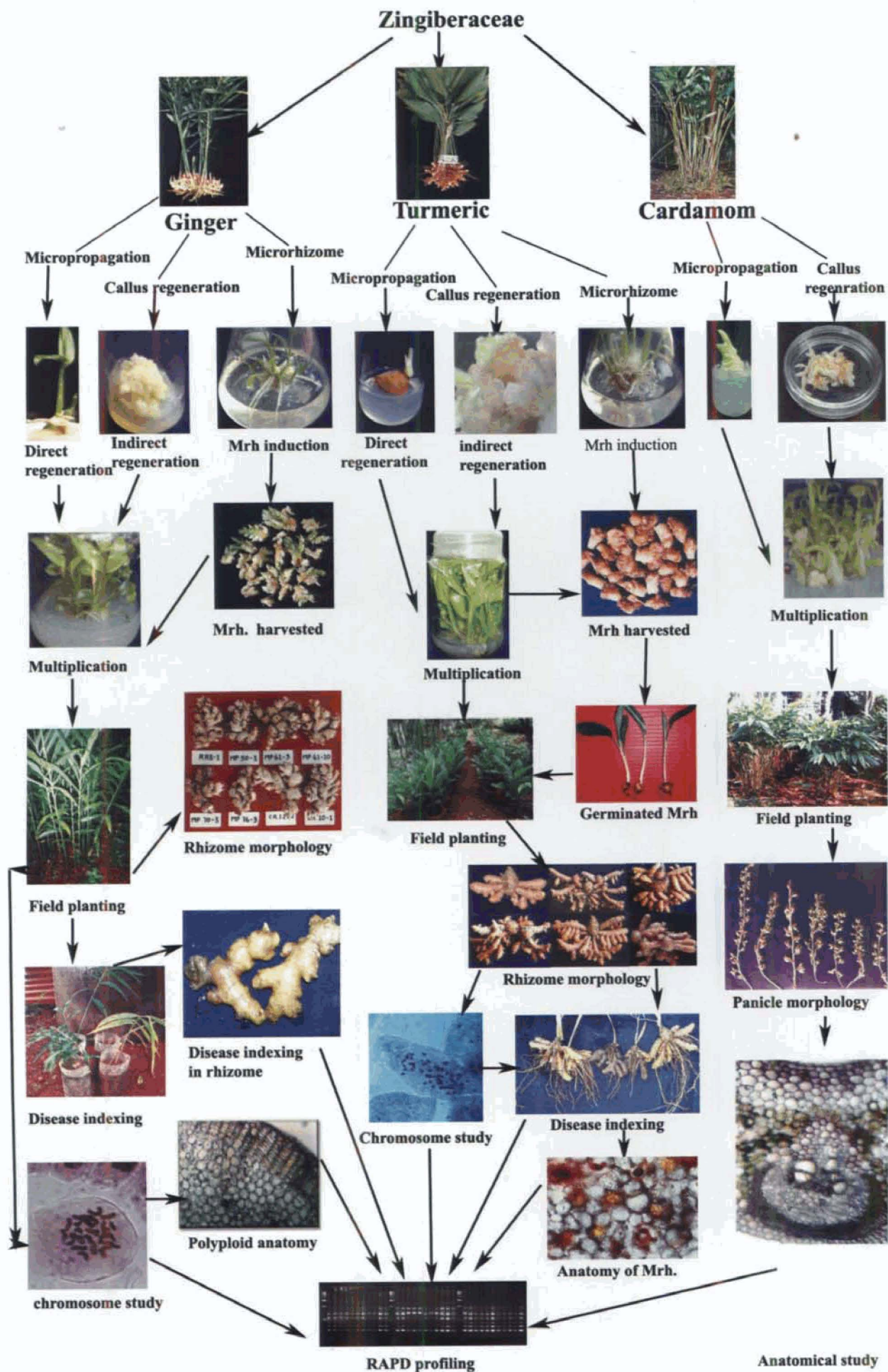
Genetic variability is valuable only if it is handled skillfully and the useful variants selected and evaluated judiciously. Occurrence of somaclonal variation during culturing is a frequent and consistent event. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg *et al.*, 1977) but also by their biochemical, physiological and genetic characteristics

Conclusions

Most of the plant species in the family Zingiberaceae are solely or predominantly vegetative propagated plants. In some plant species like ginger, sexual reproduction is completely absent or rare in plant species like turmeric, this leads to narrow genetic diversity in intra and inter species levels. In plant species like cardamom, though sexual reproduction is very efficient, the monotypic nature of the species also reflects narrowness of the genetic base. Sexual reproduction is essential for intra and interspecies gene flow and in addition to variability that occurs due to recombination. However in these crops with rare or no sexual reproduction there seems to be high degree of variability observed, for e.g., huge number of variable cultivars are available in completely vegetatively propagated plants like ginger and turmeric, it is particularly impossible to have so many variants occurring in populations without sexual reproduction. Obviously factors other than recombination, like natural mutations must be

playing a major role in generating this variability. These natural mutations whose rate of occurrence may be comparatively higher in these crops are fixed in populations by the predominantly vegetative propagation and are unconsciously selected for their preferential performance. This indirectly indicate natural mutation leading to genetic mosaicism among the vegetatively propagated propagules, must have contributed significantly in the development of variations among the vegetatively propagated taxa of Zingiberaceae especially in ginger and turmeric.

These natural variations can be effectively harvested and increased if necessary using micropropagation and *in vitro* culture technology. Thus somaclonal variation can effectively be used to compensate the lack of variability through sexual reproduction and recombination in crops like ginger. Many useful somaclonal variants with increased yield, quality and resistance to biotic and abiotic stresses have been identified in crops like ginger, turmeric and cardamom.



Schematic representation of major activities of the present study

Summary & Conclusion



Zingiberaceae, a subfamily under the major family Scitaminae, is the largest family in the order Zingiberales in which includes 53 genera and more than 1200 species (Kress *et al*, 2002). Members of this family are highly evolved monocotyledons perennial rhizomatous herbs, predominantly vegetatively propagated, distributed mainly in the tropics and subtropics of the world and with the centre of distribution in the Indo-Malayan region. The members of Zingiberaceae are an important natural resources that provide many useful products for food, spices, medicines, dyes, perfumes and aesthetics to man. The important genera are *Zingiber*, *Curcuma*, *Elettaria*, *Kaempferia*, *Aframomum* and *Amomum*. Of these ginger, turmeric and cardamom are the economically important crops. Ginger (*Zingiber officinale* Rosc), native of South East Asia, is one of the oldest and most important spices, has been cultivated in tropical Asia for over 3000 years (Purseglove, 1975). Small cardamom (*Elettaria cardamomum*. Maton), often referred as the 'Queen of spices', is a tall perennial, herbaceous rhizomatous plant. It is native of evergreen forest of South India.

India is the major producer and exporter of ginger, turmeric and cardamom. These crops are propagated vegetatively and sexual reproduction is absent in ginger and partial in turmeric and efficient in cardamom. Even though sexual reproduction is efficient in cardamom, the 'monotypic' nature of the species leading to reduced variability within the species. Irrespective of predominance of vegetative propagation and less efficient or absence of sexual reproduction, there is reasonable variability in germplasm of these crops (Ravindran *et al*, 2002, 2005, 2007). This may indicate a possible role of natural mutations conserved in populations by predominant vegetative propagation as a major factor contributing to the existing variability in these crops.

Conventional breeding is always dependent on recombination (Mascarenhas, 1991). Hence breeding programme of these crops is severely hampered especially in Zingiberaceous crops. Utilization of biotechnological approaches for implementing para sexual techniques like somaclonal variation, induced mutagenesis can be used to complement recombination to generate variation.

The present investigation was carried out with an objective of studying the utility of *in vitro* technology for creation of somaclonal variation for possible genetic improvement of economically important Zingiberaceous crops like ginger, turmeric and cardamom. The extent of variability is studied using morphological, biochemical and molecular markers supplemented with cytological and anatomical evidences.

Ginger

Micropropagation

Vegetative buds of ginger rhizome were multiplied on MS + 1 mg^l⁻¹NAA and 4 mg^l⁻¹ BAP showed 98.7 % success. An average of 7.7 shoots per explant with the shoot length of 6.3 cm were obtained within four to eight weeks.

Though Micropropagation of ginger was reported by various workers using (Hosoki and Sagawa, 1977; Nadgauda *et al*, 1980; Pillai and Kumar, 1982; Ilahi and Jabeen, 1987; Balachandran *et al*, 1990; Samsudeen, 1996; Babu 1997) the present system is simple and efficient and is similar to that of Babu 1997. Induction of both multiple shoots and roots in a single medium reduces the time taken for plant development.

Callus induction and Plant regeneration

Callus was induced in ginger buds on MS medium with 2 mg^l⁻¹ 2,4-D and plant regeneration was obtained on MS medium supplemented with 10 mg^l⁻¹ BA and 0.2 mg^l⁻¹ 2,4-D. Culture kept in darkness showed better growth and maintenance of calli. 88.7% of the cultures induced callus within two weeks of incubation. Callus developed rapidly in the presence of 2,4-D. The callus consisted non-embryogenic in between embryogenic callus. The embryogenic callus is to be separated and cultured for better embryogenic regeneration.

Embryogenesis was observed after 2 weeks of culture. Plant regeneration was optimum (94.3%) when embryogenic callus was cultured on MS + 10 mg^l⁻¹BA and 0.2 mg^l⁻¹ 2,4-D. The regenerated plants developed root system on the same media. The

earlier reports on plant regeneration in ginger are mainly those of Kulkarni *et al*, (1987), Kacker *et al*, (1993) Ilahi and Jabeen (1992) Samsudeen (1996), Babu (1997) and Samsudeen *et al* (2000). This protocol is very efficient and agrees with the findings of Babu (1997).

Induction of Microrhizomes

The effect of different factors like MS salt, sucrose concentration and duration of the culture period on the production of microrhizome *in vitro* were studied. MS basal salts at 0.75 X concentration supplemented with 8% sucrose gave optimum results with an average of 20 microrhizomes with a fresh weight of 2.6g after 90 and 120 days of culture.

Microrhizomes resembled the normal rhizomes in all respects, except for their small size. The microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds. They also have the aromatic flavour of ginger and they resembled the normal rhizome in anatomical features in the presence of well-developed oil cells, fibres, and starch grains were observed. These microrhizomes were directly planted in the field without any hardening with 85 % success. These microrhizomes gave a fresh rhizome yield of 100 – 525g per plant with an average per 3 m² bed yield of 10.5 kg while the control gave per bed yield of 15 kg. 20- 30g of seed rhizome was used for conventionally propagated plants, whereas in microrhizome seed rhizome weight was 2-8g. Thus microrhizomes though gave lesser yield per bed gave very high recovery based on the weight of seed material used. This coupled with its disease free nature will make microrhizomes an ideal source of planting material

Though there are earlier reports (Sakamura *et al*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994, Sharma and Singh, 1995 Babu, 1997 and Geetha 2002 Nirmal Babu *et al*, 2003) on induction of microrhizomes this is by far the most efficient result in that maximum number of micro rhizomes were induced in shortest time possible that too with the no use of growth regulators. This helps to maintain the genetic fidelity. This is the first report on field performance of ginger microrhizomes.

Characterization of somaclones

The success of any *in vitro* culture technique depends either on the ability to clone the genotypes for production of uniform planting material, or the ability to bring about variations, which can be exploited in crop improvement programmes.

Morphological characterization

Morphological characterization is a traditional method for identifying phenotypic variation in many plants (Bailey 1983; Pereira *et al.* 1996). The plantlets obtained through micropropagation, callus regeneration and microrhizome pathways were morphologically characterized and certain amount of variations were observed. The callus regenerated plants show maximum variation in almost all the characters studied. The main characters, which showed maximum variation in callus regenerated plants compared to others, were plant height, number of tiller per plant, number of leaves per plant, leaf length, yield and primary and secondary fingers

The characters, which showed similarity in conventional, micropropagated, and microrhizome derived plants were plant height, number of leaves, yield, primary and secondary fingers, Number of nodes of mother rhizome, internodal length of primary and secondary fingers.

A few promising lines having important yield attributes and other useful characters could be selected from callus regenerated plants.

Biochemical characterization

A few of the morphological 'variants' were subjected to biochemical characterization. Significant variations were observed in percentage of oil, oleoresin, starch and fiber content among the somaclones tested. Variation observed between micropropagated, microrhizome and conventional with regard to biochemical characters is minimal, the plants regenerated through callus pathway gave significant variation.

The developmental status of microrhizomes, were studied using GCMS profiling. The microrhizomes behaved like storage organs though there are differences in storage

contents compared to control. This may be due to the differences *in vitro* and *in vivo* condition in which these storage organs are induced.

No earlier reports are available on biochemical characterization of *in vitro* microrhizome of ginger.

Rhizome anatomy

One of the somaclone CR 1222 has exhibited gigantism in morphological characters like plant height, leaf breadth, chlorophyll content and bold rhizome, is also confirmed this polyploid through stomatal study and rhizome anatomy. This line CR 1222 has multi-layered hypodermis, compared to control which has single layered hypodermis. The polyploid has larger stomata with less stomatal number, typical of polyploids indicating that CR 1222 may be a polyploid.

Chromosome indexing

The selected somaclones were cytologically analyzed to index the deviations in chromosome numbers among somaclones. Micropropagated and microrhizome derived plants showed normal chromosome number as that of the control. Callus regenerated plants showed variation in the chromosome number and also structural aberrations. The numerical variations were mainly aneuploids with lesser number of chromosomes ($2n = 19, 20$) than the normal number except one plant with higher number of chromosome

Molecular characterization.

Micropropagated, callus regenerated and microrhizome derived plants were subjected to RAPD profiling with 6 operon primers (OPA 17, OPA 10, OPC 08, OPC 01, OPA 06 and OPA 04). No variation observed in micropropagated and microrhizome derived plant but showed variation in callus regenerated plants. This indicates that many of the morphological variants in callus regenerated plants may be due to genetic variations.

Nirmal Babu *et al* (2003) observed differences in RAPD profiles in some of the micropropagated as well as Callus regenerated plants, whereas Rout *et al.* (1998) did not

find any polymorphism among the micropropagated plants. This may be because bigger population size used by the latter to detect the morphological variants first and confirmation of their genetic nature of variation using RAPD profiles. These high amounts of variations in ginger supports the view that the genetic mosaic nature of ginger, due to accumulated natural mutations, which has contributed to occurrence of many varieties and cultivars even without sexual reproduction.

Screening for disease incidence

Nine selected ginger somaclones were screened for resistance to *Pythium aphanidermatum* infection which causes the dreaded rhizome rot disease. Variation in resistance reaction was observed among somaclones of callus regenerated plants. CR2, CR-3 and CR 67 gave tolerant reaction to rhizome rot disease. This supports the earlier view of Kulkarni *et al*, (1984) Nirmal Babu *et al*, (1996) that somaclonal variation could be exploited for developing *Pythium* tolerant varieties in ginger.

Turmeric

Micropropagation

Vegetative buds of turmeric were inoculated on MS supplemented with 1 mg^l⁻¹ NAA and 4 mg^l⁻¹ BA for the clonal propagation. 96.2% explants responded and developed shoots with an average of 6.0 number of shoot per explant. The best results were obtained when the buds (10-20 mm in size) were cultured on this medium. This established and *in vitro* derived shoots further multiplied on MS with BA 1 mg^l⁻¹ and NAA 0.5 mg^l⁻¹ and agar 0.8%.

This is in tune with the earlier reports of Nadgauda *et al*, (1978), Balachandran *et al*, (1990), Nirmal Babu *et al*, (1997), Sunitibala *et al*, (2001), Salvi *et al*, (2002) and Rahman *et al*, (2004). These micropropagation techniques could be used for production of disease-free planting material of elite genotypes.

Callus induction and Plant regeneration

Different combinations of 2, 4 -D and BA were used for the efficient induction and proliferation of callus. MS medium supplemented with 2, 4 -D at 1.0 and BA 0.5 mg^l⁻¹

¹ was the best for induction and proliferation of callus after 4 weeks of culture. The percentage of embryogenic callus was found to be highest in 1 mg l⁻¹ 2, 4-D + 0.5 mg l⁻¹ BA (100%) and non embryogenic callus percentage was highest in 1 mg l⁻¹ 2,4-D alone (32%). The embryogenic callus need to carefully separated and cultured on the media contained NAA at 0.5 mg l⁻¹ and BA at 2.0 mg l⁻¹ to get high rate of plant regeneration.

Organogenesis and plantlet formation were reported earlier by Shetty *et al*, (1982)., Nirmal Babu *et al*. (1997)., Sunitibala *et al* (2001)., Salvi *et al*, (2001)., Praveen (2005).

Induction of Microrhizomes

The effect of different factors like MS salt, sucrose concentration and duration of the culture period on the production of microrhizome *in vitro* were studied. MS basal salts at 0.75 X concentration supplemented with 9% sucrose gave optimum results with an average of 16 microrhizomes with a fresh weight of 2.4g after 120 days of culture.

Microrhizomes resembled the normal rhizomes in all respects, except for their small size. The microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds. They resembled the normal rhizome in anatomical features in the presence of well-developed curcumin cells, oil cells, oleoresin, and starch grains were observed. These micro rhizomes were directly planted in the field without any hardening with 85 % success.

Microrhizome derived plants were almost similar in morphology with normal plant. But are smaller in size with higher number of mother rhizomes per plant and gave reasonably big rhizomes. These micro rhizomes gave a fresh rhizome yield of 100 - 550g per plant. The fresh rhizome yield of controls around 300- 1200g per plant. For microrhizomes 2 – 2.5 g was used as seed material where as in control 20- 30g of seed rhizome was used. Thus microrhizomes though gave lesser yield per bed gave very high recovery based on the weight of seed material used. Microrhizomes have the aromatic flavour of turmeric and resembled the normal rhizomes in all respects, except for their small size. The presence of well-developed curcumin cells, oil, oleoresin and starch cells were observed in the *in vitro* microrhizome.

Though there are earlier reports (Sharma and Singh 1995; Rajan *et al*, 1997; Shirgukar *et al*, 2001; Nayak 2000; Sunitibala 2001; Nirmal Babu *et al*, 2003; Geetha 2002) on induction of microrhizomes this is by far the most efficient result in that maximum number of micro rhizomes were induced in shortest time possible that too with the no use of growth regulators. This is the first report on field performance of ginger microrhizomes.

Characterization of somaclones

Morphological characterization

The plantlets obtained through micropropagation, callus regeneration and microrhizome pathways were morphologically characterized. The variations were observed in all the plantlets studied though not significant in some cases. The callus regenerated plants show maximum variation in almost all the characters studied. The main characters, which showed variation in callus regenerated plants compared to others, were plant height, leaf breadth, yield, nodes in primary and secondary fingers.

The characters, which showed variation in conventional, micropropagated, and microrhizome derived plants were leaf length, yield, breadth of secondary fingers, nodes per mother rhizome and internodal length of the fingers.

Salvi *et al*, (2002) reported that micropropagated plants of turmeric cv 'elite', had a higher number of tillers and leaves, which suggested a residual effect of cytokinins used in the culture medium. Praveen (2005) also observed somaclones with distinct variation in patterns of rhizome development, orientation and number of primary, secondary and tertiary rhizome, internodal and nodal distances. The present study agrees with the findings of these authors in that somaclonal variation is an important source of variation for turmeric improvement.

Biochemical characterization

A few of the morphological 'variants' were subjected to biochemical characterization. Significant variations were observed in percentage of curcumin, oil, oleoresin, starch, reducing sugar, and total carbohydrate among the somaclones tested.

Though the micropropagated and microrhizome derived plants showed minimal biochemical variation, the plants regenerated through callus pathway gave significant variation in biochemical characters.

The developmental status of microrhizomes, were studied using GCMS profiling. The microrhizomes behaved like storage organs though there are differences in storage contents compared to control. The components, which present in the *in vitro* rhizome, were α -pinene, β -pinene, γ -Terpene and d-Limonene. These components were absent *in vivo* rhizome. This may be due to the differences *in vitro* and *in vivo* condition in which the storage organs were induced.

Variants with high curcumin content were isolated from tissue cultured plantlets (Nadgauda *et al*, 1982). Nayak *et al*, 2003 reported variations in oil content and total oil yield among turmeric somaclones and qualitative analysis of essential oil revealed that the somaclones performed better or equal to the donor parent. Changes in RAPD banding pattern in the improved somaclone as compared to donor parent revealed occurrence of gross genetic changes. *Chromosome indexing*

The selected somaclones were cytologically analyzed to index the deviations in chromosome numbers among somaclones. Micropropagated and microrhizome derived plants showed normal chromosome number as that of the control. Callus regenerated plants showed variation in the chromosome number and also structural aberrations. The numerical variations were mainly aneuploids with lesser number of chromosomes ($2n = 53, 55, 57$) than the normal number except one plant with higher number of chromosome ($2n = 65$).

Molecular characterization.

Micropropagated, callus regenerated and microrhizome derived plants were subjected to RAPD profiling with 5 operon primers (OPA 04, OPA 08, and OPC 19, OPC 01, OPA 06). No variation observed in micropropagated and microrhizome derived plant but showed variation in callus regenerated plants. This indicates that many of the morphological variants in callus regenerated plants may be due to genetic variations.

Salvi *et al.* (2002, 2003) reported that though the somaclones showed variable morphological characters when compared with conventionally propagated plants RAPD analysis indicated that plants regenerated using shoot tips showed uniform banding pattern, whereas, callus derived and inflorescence derived plants showed polymorphism in banding pattern. Similar results were obtained by Praveen (2005) also. The present study agrees with these findings.

Salvi *et al.* (2001) using RAPD analyzed turmeric plants regenerated from leaf base callus and this study of eight regenerated plants was using 14 primers when separated on non-denaturing polyacrylamide gels showed 38 novel bands. About 51 bands present in the control were absent in the regenerants. The result indicates that variation at DNA level has occurred during *in vitro* culture

Screening for disease incidence

Ten selected somaclones were screened for resistance to *Pythium myriotylum* infection which causing rhizome rot of turmeric. Variation in resistance reaction was observed among somaclones and callus regenerated plants C10-2, C3-2 and C8-2 gave tolerant reaction. This supports the view of Gayatri *et al.*, (2005) that somaclonal variation and *in vitro* selection could be exploited for developing *Pythium* tolerant varieties in ginger.

Cardamom

Micropropagation

Rhizome buds of cardamom were multiplied on MS medium supplemented with BA at 1 mg^l⁻¹ and NAA at 0.5mg^l⁻¹ produced a maximum of 4 shoots, each approximately 5.4 cm in length.

Efficient *in vitro* methods for rapid clonal propagation of cardamom are available. (Nadgauda *et al.*, 1983, Vatsya *et al.*, 1987, Reghunath and Gopalakrishnan 1991). High rate of multiplication coupled with additional advantage of obtaining disease free planting material makes micropropagation an important and viable alternative to conventional propagation.

Callus induction and Plant regeneration

Callus was induced in cardamom buds on MS medium with 1 mg l⁻¹ 2, 4-D in 96% of cultures. Plant regeneration was obtained on MS medium supplemented with 4 mg l⁻¹ BA+ 0.5 mg l⁻¹ NAA with 80% response. This excellent regeneration system was used for development of somaclonal variation and selection of useful genotypes from them. Good morphological variation was observed among the somaclones in the culture vessels itself.

Successful regeneration of plantlets from callus of seedling explants of cardamom was reported (Rao *et al* 1982, Priyadarshan and Zachariah 1986, Nirmal Babu *et al*, 1997).

In vitro method to overcome apical dominance.

Controlling of apical dominance by mechanical means using petri plates replaces growth regulators and thus activating axillary bud development. 18- 25 multiple shoots were produced by this method and stimulated vigorous root growth also. 80% of the plants established in the soil. This helps in easy multiplication of plantlets without the use of growth regulators which indirectly enhances the genetic fidelity of multiplied plants. This is the first report in cardamom.

Characterization of somaclones

Morphological characterization

The plantlets obtained through micropropagation and callus regeneration pathways were morphologically characterized. Maximum plant height, panicle length and number of flowers per panicle were recorded in micropropagated plants. Number of tiller, number of leaves and primary leaf length were recorded maximum in conventionally propagated plants. Where as more number of panicle and number of nodes per panicle recorded in callus regenerated plants.


Earlier reports on large scale field evaluation of tissue cultured plants showed that the micropropagated plants performed on par with suckers (Lukose 1993, Sudharshan *et al*, 1997, Chandrappa *et al*, 1997, Kuruvilla *et al*, 2005). Lukose *et al*, 1993 reported that

the variations observed among the TC plants, suckers and seedlings were non significant for most of the characters. Yielding tillers, panicles per plant, green capsule yield and cumulative yield were significantly more in tissue cultured plants. Sudharshan *et al* (1997) reported that variability was observed in the clonal population for vegetative characters. The overall variability in tissue cultured plants was 4.5 % as against 3 % in open pollinated seedling progenies. A few Katte tolerant somaclones were identified (Peter *et al* 2001).

Molecular characterization.

Comparative RAPD study of micropropagated and callus regenerated plants were conducted using 6 operon primers OPB 19, OPC 10, OPC 19 OPD 12. OPA 06 and OPA 17. No variation observed in micropropagated and microrhizome derived plant but showed variation in callus regenerated plants. This indicates that many of the morphological variants in callus regenerated plants may be due to genetic variations.

CONCLUSIONS

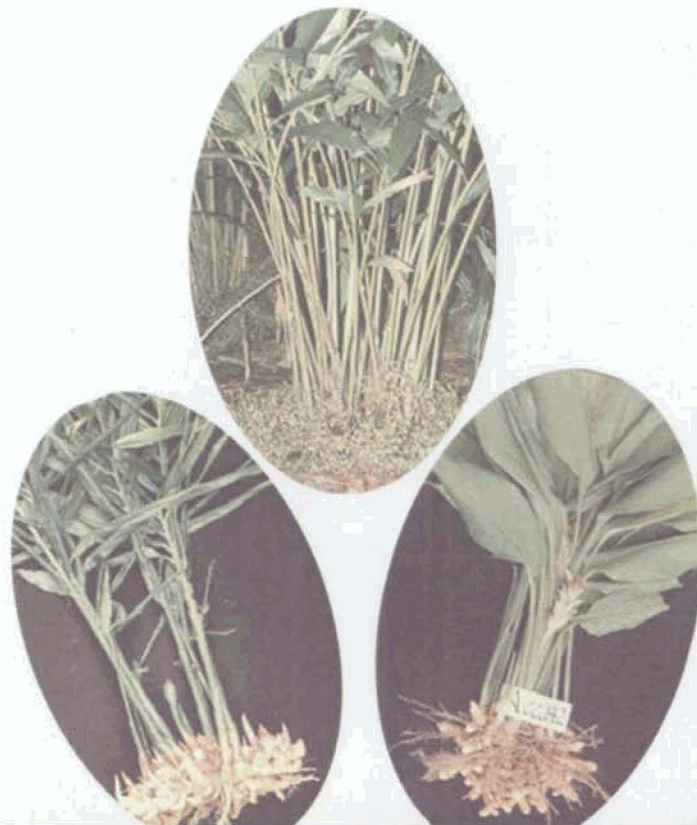
Most of the plant species in the family Zingiberaceae are solely or predominantly vegetative propagated plants and sexual reproduction is completely absent or rare in some species like ginger and turmeric respectively. This leads to narrow genetic diversity in intra and inter species populations.  Some other plant species like cardamom, though sexual reproduction is very efficient, the monotypic nature of the species also reflects narrowness of the genetic base. Sexual reproduction is essential for intra and interspecies gene flow and in addition to variability that occurs due to recombination. However in these crops with rare or no sexual reproduction there seems to be high degree of variability observed. For example huge number of variable cultivars are available in completely vegetatively propagated plants like ginger and turmeric. It is particularly impossible to have so many variants occurring in populations without sexual reproduction. Obviously factors other than recombination, like natural mutations must be playing a major role in generating this variability. These natural mutations, whose rate of occurrence may be comparatively higher in these crops, are fixed in populations by the

predominantly vegetative propagation and are unconsciously selected for their preferential performance. This indirectly indicate natural mutation leading to genetic mosaics among the vegetatively propagated propagules, must have contributed significantly in the development of variations among the vegetatively propagated taxa of Zingiberaceae especially in ginger and turmeric.

These natural variations can be effectively harvested using *in vitro* culture technology. Thus the resultant somaclonal variation can effectively be used to compensate the lack of variability through sexual reproduction and recombination in crops like ginger. Many useful somaclonal variants with increased yield, quality and resistance to biotic and abiotic stresses have been identified in crops like ginger, turmeric and cardamom.

Occurrence of somaclonal variation during culturing is a frequent and consistent event. Somaclonal variations not only can be distinguished by their morphological traits (Gamborg *et al* 1977) but also by their biochemical, physiological and genetic characteristics. Somaclonal variation was found to be an important source of variation in many crop plants though in many cases these variations were found to have progressively lost in subsequent generations. Some success has been realized in selecting for qualitative traits such as resistance to anti metabolites, herbicides, toxins and abiotic stresses. Genetic variability is valuable only if it is handled skillfully and the useful variants selected and evaluated judiciously (Larkin and Scowcroft 1981). This has more relevance in vegetatively propagated Zingeberaceous plants more so in crops like ginger and turmeric where the sexual reproduction is either absent or rare.

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