Micropropagation, diversity study and detection of antioxidants in some medicinal Zingibers

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This work is dedicated to my Mother

DECLARATION

I declare that the thesis entitled "Micropropagation, diversity study and detection of antioxidants in some medicinal Zingibers" has been prepared by me under the supervision of Dr. Arnab Sen, Associate Professor of Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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ABSTRACT

Zingiberaceae is a moderately sized family of relatively advanced monocotyledons. It is the largest family of Zingiberales and is one of the ten largest monocotyledonous families in India. The members of this family occur chiefly in the tropics with the greatest concentration in the Indo-Malayan region of Asia. India is represented by around 22 genera and 178 species.

The study area includes the foothills of Darjeeling and the adjoining plains. The floral elements of the region include various members of the family Zingiberaceae, of which *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* are used in alternative systems of medicine. The whole parts of these plants are aromatic, but it is their underground rhizome, fresh or preserved, that are valuable. *Z. officinale* is used worldwide as a cooking spice, condiment and herbal remedy. The dried rhizomes constitute the spice and are esteemed for its flavour, pungency and aroma. *C. longa* occupies an important position in the life of Indian people as it forms an integral part of the rituals, ceremonies and cuisine. Due to the strong antiseptic properties, turmeric has been used as a remedy for all kinds of poisonous affections, ulcers and wounds. *K. galanga* rhizome extract are used as expectorant, stimulant, diuretic, carminative and stomachic. It is used for the treatment of leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, ulcers, fever, malarial fever, nasal obstruction and for various other ailments.

In the present study of some medicinal Zingibers, three broad objectives (*in vitro* regeneration, study of molecular diversity and detection of antioxidant potential) were considered. Studies in these fields were severely lacking in the perspective of local cultivars of the genera.

Selected places of Jalpaiguri and Darjeeling district of West Bengal were visited for collection of germplasm. The collected samples were planted in separate pots placed at experimental garden of Department of Botany for future use. As, the members of Zingiberaceae are propagated by rhizomes; various pathogens affecting the crop are transmitted through this

process. Moreover, conventional breeding techniques to improve the crops are restricted due to extreme low seed set. *In vitro* culture offers an alternative method for producing variations and disease free planting materials. Unfortunately, this field of research has not been explored for the local cultivars of Zingiberaceous plants.

In vitro regeneration experiment using rhizome sprouts revealed that, Murashige and Skoog basal media supplemented with vitamins, sucrose, agar and plant growth regulators was superior to Gamborg B5 media. The ideal percentage of sucrose required for growth of the sprouts was 30 g/l, when the media was supplemented with 3 or 4 mg/l BAP.

Efficacy of three important cytokinins like benzyl amino purine, kinetin and zeatin were tested in different concentrations and combinations. It has been observed that the growth regulators trigger variable responses. In *Z. officinale* the highest number of shoots per explant with BAP (8.33), kinetin (5.40) and zeatin (7.20) were observed. In *C. longa* the highest number of shoots per explant with BAP (9.08), kinetin (6.85) and zeatin (6.22) were noted, while in *K. galanga* it was BAP (4.33), kinetin (3.44) and zeatin (3.58). In *Z. officinale* the highest average shoot height with BAP (6.90 cm), kinetin (6.06 cm) and zeatin (4.67 cm) were observed. In *C. longa* the highest average shoot height with BAP (6.47 cm), kinetin (5.81 cm) and zeatin (4.43 cm) were observed, while in *K. galanga* it was BAP (4.31 cm), kinetin (2.82 cm) and zeatin (2.48 cm). Among the cytokinins tried, BAP gave better results compared to kinetin and zeatin in regeneration.

The regeneration of plantlets also varied considerably with different combinations of BAP and kinetin. In *Z. officinale* culture, maximum number of plantlets per explant were obtained in the medium supplemented with 4 mg/l BAP + 3 mg/l kinetin (9.60), while the maximum plantlet height was obtained in the medium supplemented with 4 mg/l BAP + 3 mg/l kinetin (8.59 cm). In *C. longa* culture the maximum number of plantlets were obtained in the medium supplemented with 3 mg/l BAP + 4 mg/l kinetin (9.20), while the maximum plantlet height was obtained in the medium supplemented with 4 mg/l BAP + 4 mg/l kinetin (8.36 cm). In *K. galanga* culture the maximum number of plantlets were obtained in the medium supplemented with 3 mg/l

BAP + 4 mg/l kinetin (6.52), while the maximum plantlet height was obtained in the medium supplemented with 2 mg/l BAP + 4 mg/l kinetin (8.23 cm). In general plantlet regeneration was relatively less in the medium supplemented with low BAP and kinetin combination. Good results were obtained when the combined concentrations of BAP and kinetin were more than 5 mg/l. The plant growth regulators worked better in combinations than that when used alone. The plantlets rooted in the same medium and they were moderate to profuse in most of the combinations. The rate of rooting was found to be proportional to the number of plantlets.

Subcultures to obtain maximum number of healthy shoots were experimented by inoculating sets of explants in media having the same composition. The effects of plant growth regulators on regeneration were not the same in primary and secondary cultures. Comparatively less variation were observed in number of shoots per explant in the primary and secondary cultures of *C. longa* than Z. *officinale* and *K. galanga*. In all the cases of *Z. officinale* and *C. longa* regeneration the least number of plantlets were produced in the primary culture and the cultures showed maximum regeneration in the 2nd subculture. In case of *K. galanga* regeneration, the least number of plantlets were produced in the primary culture and it declined after the 1st or 2nd subcultures.

Healthy, *in vitro* grown plantlets with good number of roots were selected for hardening. The regenerated plants *Z. officinale*, *C. longa* and *K. galanga* showed a survival percentage of 94%, 91% and 94% respectively.

Diagnostic tests were performed to detect the presence or absence of the pathogen in the plants. *In vitro*-derived plants raised under field conditions did not show any disease symptoms until maturity. The rhizomes obtained from field planted tissue culture derived plantlets on storage on sand for 6 months did not show any visible root-rot or shoot-rot.

Benzyl Amino Purine showed variable effects on the shooting of twelve different *C. longa* cultivars. The mean number of shoots formed per explant were 3.075 (BAP 1 mg/l), 4.983 (BAP 2 mg/l), 6.891 (BAP 3 mg/l), 6.358 (BAP 4 mg/l) and 5.816 (BAP 5 mg/l). Maximum regeneration potential was observed in the cultivar CLS-2A (8.7 shoots per explant), while the lowest regeneration potential was observed in

the cultivar Allepy (1.8 shoots per explant).

RAPD and ISSR analysis were performed to analyze the genetic relationship among the *C. longa* cultivars. In RAPD analysis, the amplification profiles of the total genomic DNA from the 12 cultivars of *C. longa* using 14 primers resulted in production of 170 bands ranging in between 151 and 1767 bp of which only 10 were monomorphic. The percentage of polymorphism was found to be 94.11%. The similarity matrix obtained using the Dice coefficient of similarity among the 12 cultivars ranged from 0.560 to 0.857. The lowest similarity was observed between *C. longa* cv local-Dhupguri and Roma and *C. longa* cv local-Dhupguri and Sudarshana while the highest value was recorded between *C. longa* cv Suguna and *C. longa* cv TC Assam. Three distinct groups viz. group I (*C. longa* cv Local-Lataguri, Local-Dhupguri, Prova, Suvarna, Suguna, Kasturi and CLS 2A); group II (*C. longa* cv Allepy, PTC13 and Roma) and group III (*C. longa* cv Sudarshana) were noted.

ISSR analysis among 12 cultivars of *C. longa* produced a total of 75 amplified bands by 8 primers of which 69 were polymorphic. The frequency of polymorphism was found to be 92%. The band size ranged between 274bp to 1758bp. Similarity coefficient among the 12 cultivars ranged from 0.523 to 0.904. The lowest similarity was observed between *C. longa* L. cv Local -Dhupguri and *C. longa* L. cv Allepy, while the highest value was recorded between *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam. The dendrogram constructed on the basis of the data obtained from ISSR analysis showed three major groups viz. group I (*C. longa* cv Local-Lataguri and Local-Dhupguri); group II (*C. longa* cv (Prova, Sudarshana and PTC 13) and group III (*C. longa* cv (Suguna, TC Assam, Allepy, Kasturi, CLS 2A, Suvarna and Roma) were noted. Of these the first group with two local cultivars of *C. longa* formed a distinct clade from rest of the cultivars.

Combined RAPD and ISSR based analysis of *C. longa* showed the highest similarity between the cultivars *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam while the lowest was noted between *C. longa* L. cv Local-Dhupguri and *C. longa* L. cv Sudarshana. The highest similarity of the cultivars *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam were also

noted in the results of RAPD and ISSR when calculated separately. The dendrogram showed that, based on the similarity indices, two cultivars of *C. longa* i.e. Suguna and TC Assam formed a cluster sharing a node at 87.2%. Clustering above a similarity of 80% was also formed in between Local -Lataguri and Local-Dhupguri (84.2%) and Kasturi and CLS 2A (82.7%). Three major groups viz. group I (*C. longa* cv Local-Lataguri and Local-Dhupguri); group II (*C. longa* cv Prova, Suguna, TC Assam, Allepy, Kasturi, CLS 2A, Suvarna, Roma and PTC 13) and group III (*C. longa* cv Sudarshana) were noted. Of these the first and the third group with two local cultivars of *C. longa* and Curcuma long a cv Sudarshana respectively formed two distinct clade from rest of the cultivars.

Comparative account of the DNA fingerprinting study showed that both RAPD and ISSR markers are efficient revealing 94.11% and 92% polymorphism respectively among the twelve cultivars of *C. longa* under study. These markers may provide a cheap, rapid and effective means to evaluate the genetic diversity among different *C. longa* cultivars. As flowering is rare in Zingibers so this method can also be useful for cultivar identification.

A total of 10 primers were used to screen somaclonal variations, out of which 6 were RAPD and rest 4 were ISSR primers. All the RAPD and ISSR primers produced distinct and scorable bands which were found to be monomorphic across all the *in vitro* raised plantlets and the parent plant analyzed. This uniformity in the banding pattern confirms the genetic fidelity of the *in vitro* raised *Z. officinale*, *C. longa* and *K. galanga* plantlets.

The TrnL-TrnF region of the sample (*C. longa* cv Local-Lataguri) was sequenced. The sequencing resulted in forward and reverse sequence of 730 bp and 750 bp respectively. The nucleotide BLAST of the forward sequence showed a maximum of 100% identity with *C. longa,* while the reverse sequence showed a maximum of 99% identity with *C. longa.*

Free radical scavenging activity with DPPH showed that the solvent fractions of *Z. officinale*, *C. longa* and *K. galanga* exhibited different levels of antiradical activities. Among *Z. officinale* fractions, Diethyl ether & ethyl acetate (1:1) showed the maximum (80%) antiradical

scavenging activity. In *C. longa*, Diethyl ether & ethyl acetate (3:1) showed the maximum inhibition percent (77.75%), while in *K. galanga* the acetone fraction showed the maximum (66.66%) antiradical scavenging activity.

Assay of hydroxyl radical scavenging activity reveals that in all the cases the scavenging activities increased with the increase in concentrations of the extracts. In *Z. officinale*, the chloroform fraction (43.75%) showed maximum hydroxyl radical scavenging activity. In *C. longa*, the diethyl ether fraction (40.00%) showed maximum hydroxyl radical scavenging activity, while in *K. galanga*, the acetone fraction (40.00%) showed maximum hydroxyl radical scavenging activity.

Results indicate that, all the bioactive *Z. officinale*, *C. longa* and *K. galanga* fractions have NO scavenging activity. The scavenging activity increased with decrease in concentrations in all the bioactive fractions other than the hexane fractions *Z. officinale*. In *Z. officinale* the hexane fraction (47.72%) was most active in NO scavenging activity. In *C. longa* the benzene: chloroform (1:1) fraction (42.22%) was most active in NO scavenging activity, while in *K. galanga* the acetone fraction (41.30%).

Determination of lipid peroxidation inhibition activity revealed that the bioactive fractions of *Z. officinale*, *C. longa* and *K. galanga* fractions protected hepatocytes from damage due to lipid peroxidation induced in goat liver homogenate by ferric-ADP and ascorbate in a dose dependent manner. In *Z. officinale* the maximum protective function *was* recorded in chloroform fraction (30.76%). In *C longa* the maximum protective function was recorded in Diethyl ether: Ethyl acetate (3:1) fraction (46.42%), while in *K. galanga* the maximum protective function was recorded in Acetone: Ethanol (3:1) fraction (33.33%).

In different fractions of Ginger, it was observed that phenolic compounds were distributed from chloroform to aqueous fraction whereas gingeroles and arbutin related compounds were mainly restricted in diethyl ether: ethyl acetate fractions.

The antiradical activities in the methanol and water extracts of different *C. longa* cultivars were variable. In all the cultivars the methanol soluble fraction showed more antiradical activity than the water soluble fraction. The maximum

antiradical observed in methanol solvent fraction of Roma (64.61%), while the minimum activity was observed in the cultivar PTC13 (26.92%). The maximum antiradical activity observed in water solvent fractions, was in cultivar Roma (39.75%), while the minimum activity was observed in Kasturi (19.27%) and PTC 13 (19.27%).

Preface

This Ph.D. thesis is a culmination of the research undertaken by me at Molecular Genetics Laboratory, Department of Botany, University of North Bengal over a period of almost eight years (2005-2013). These years have been a challenging trip, with both ups and downs. It would not have been possible for me to write this thesis without the help and support of the kind people around me. I am grateful to numerous personalities who have contributed towards shaping this thesis.

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Finally, I thank each and everyone who were directly and indirectly important in successfully completion of my thesis, as well as I express my apology to all whose name could not be mentioned personally one by one.

For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely of my own.

[Malay Bhattacharya]

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INTRODUCTION

Zingiber officinale Rosc., *Curcuma longa* L. Syn *Curcuma domestica* Val. and *Kaempferia galanga* L. of the family Zingiberaceae are rhizomatous herbs used worldwide in alternative systems of medicine. The members of Zingiberaceae have a very old and glorious history with numerous references in Sanskrit literature and in Chinese medical treatise.

Zingiberaceae is a moderately sized family of relatively advanced monocotyledons. It is the largest family of Zingiberales and is one of the ten largest monocotyledonous families in India (Kress *et al.*, 2002). Several authors have quoted different figures for the total number of genera and species viz. 49 genera with 1300 species (Datta *et al.*, 1988), 52 genera with 1500 species (Sirirugsa, 1999), 53 genera with over 1200 species (Kress *et al.,* 2002) and 45 genera with 1275 species (Singh, 2004).

The systematic position of the family has been studied by several taxonomists. However, the general trend of assigning the systematic position to the family Zingiberaceae is depicted in table 1.1.

The members of the family Zingiberaceae originated in South east Asia. They are distributed mostly in tropical and subtropical areas centering South East Asia. The members occur chiefly in the tropics with about 52

Table 1.1: Systematic position to the family Zingiberaceae (Joy *et al.*, 1998)

Kingdom: Plantae	
Sub-kingdom: Phanerogamae	
Division: Spermatophyta	
Subdivision: Angiospermae	
Class: Monocotyledonae	
Series: Epigynae.	
Order: Scitaminales	
Family:Zingiberaceae	

genera and 1400 species with the greatest concentration in the Indo-Malayan region of Asia. India is represented by 22 genera and 178 species (Jain and Prakash, 1995). Sabu (2006) reported 9 genera and 70 species in South India. Out of the 52 genera and 1500 species found worldwide, 25 genera are found in Malaysia, 21 in China, 20 in Thailand, 18 in India, and 11 in Nepal.

Zingiber officinale is believed to have originated in South Asia. It is widely grown in India, China, Sumatra, Africa, Mexico, Jamaica, Hong Kong, Australia, Nigeria, and Japan. The largest producer and exporter of ginger is India where it is chiefly produced in the states of Kerala and Assam.

Purseglove (1968) and Harlan (1975) believed that the genus *Curcuma* originated in the Indo-Malayan region. Considering the great diversity of the genus represented by over 80 species in Indo-Malayan region, it is legitimate to consider that the genus originated in this region. But the fact that over 40 species indigenous to this country, is supportive to its Indian origin. Further, many species belonging to two subgenera of *Curcuma* (Valeton, 1918) and a newly reported unique species such as *C. vamana* having stoloniferous sessile fingers adds to the cause of its Indian origin. The genus *Curcuma* has a widespread occurrence in the tropical Asia and Australia. At present the crop is distributed in India, Pakistan, Malaysia, Indonesia, Myanmar, Vietnam, Thailand, Philippines, Japan, China, Korea, Sri Lanka, Nepal, South Pacific Islands, East and West African nations, Caribbean Islands and Central America. In India, it is widespread (Purseglove, 1968 and 1981) and it is cultivated in innumerable agro-ecological situations right from the coastal areas to elevations as high as 1880m in the tropics and the sub-tropics of the country. It is also reported to occur widely in Eastern and Western Ghats. India is the largest producer of turmeric in the world accounting for 93.7% of the world production. About 90% is used internally and the rest exported, earning foreign exchange (Kumar *et al*., 1997).

Kaempferia is supposed to have been originated in East Asia, most probably in Burma. The genus is chiefly native to the tropics and subtropics of Asia and Africa (Shirin *et al.*, 2000). Wood (1991) in his study of biogeography and evolution of *Kaempferia galanga*

reported its distribution in Asia, Africa and Australia. It is grown in India, Burma, China, Nigeria, Mexico and other neighbouring countries. In India, it is cultivated mainly in Kerala, Karnataka, Tamil Nadu and West Bengal.

The northern part of the state West Bengal i.e. the study area includes the foothills of Darjeeling and the adjoining plains. This area experiences both cold winter and warm humid summer. Heavy rainfall occurs in this part of Bengal amounting to about 3000 mm at the foothill regions. The soil profile of the region is predominantly shallow to moderately shallow but also deep at places, well drained, coarse-loamy to gravelly loamy in texture which is rich in nutrients, organically rich and acidic in reaction. Most of the rivers originate here. This region is also well known for its diverse range of vegetation and so is one of the richest in India. The hill region of the state forms an important part of Eastern Himalaya, which is recognized as a Biodiversity hotspot in recent times. Many ancient elements have survived in this flora while some have differentiated to different races through the ages (Hara, 1966). A wide range of vegetation structures with extremely rich floral and faunal diversity have developed due to the extreme climatic, edaphic, and physiographic variations. The floral elements of the region include various members of the family Zingiberaceae like *Alpinia galanga*, *Alpinia nigra*, *Alpinia malaccensis*, *Alpinia zerumbet*, *Amomum dealbatum*, *Amomum subulatum*, *Curcuma longa*, *Curcuma amada*, *Cucurma aromatica*, *Cucurma caesia*, *Cucurma zedoaria*, *Hedychium coccineum*, *Hedychium coronarium*, *Hedychium thysiforme*, *Kaempferia galanga*, *Kaempferia rotunda*, *Zingiber officinale*, *Zingiber cassumunar*, *Zingiber zerumbet* etc. both cultivated and/or in the wild condition. But still the list of the zingiberaceous plants remain incomplete due to lack of accessibility to certain regions. Human tribes of diverse origin uses the local flora for their traditional medication. It is certain that the traditional medicinal regime includes several members of Zingiberaceae.

The whole parts of Zingiberaceous plants are aromatic, but it is their underground rhizome, fresh or preserved, that are valuable. *Zingiber officinale* is used worldwide as a cooking spice, condiment and herbal

remedy (Duke and Ayensu, 1985).The dried rhizomes constitute the spice and are esteemed for its flavour, pungency and aroma. Fresh or green ginger is consumed as a vegetable, immature ginger preserved in sugar syrup is mainly used as a desert while crystallized ginger is used as a sweet meat. It is also used in the production of ginger beer, ginger oil and ginger wine. The essential oil of rhizome is used in the manufacture of flavouring essences and in perfumery while oleoresin extracted from the rhizome is used for flavouring and in medicines. Ginger is used extensively in the traditional medicine of India, to block excessive clotting, reduce cholesterol and fight arthritis. In different parts of the world ginger is considered as a pungent, warming herb to be used for ailments triggered by cold and damp weather. It is considered an aphrodisiac (Qureshi *et al*., 1989), digestive, induce sweating, improve the appetite and curb nausea. Ginger root is used for treatment of dyspepsia, prophylactic against motion sickness, anti-inflammatory, for rheumatic diseases (Ippoushi *et al*., 2003), carminative (Kumar *et al.*, 1997), stimulant of the gastro-intestinal tract (Kumar *et al.*, 1997) gastrointestinal disorders, piles, bronchitis, dispels cardiac disorders, cures vomiting, cough, anorexia, fever, anaemia, flatulence, colic, constipation, swelling, elephantiasis and dysuria.

Curcuma longa occupies an important position in the life of Indian people as it forms an integral part of the rituals, ceremonies and cuisine. Due to the strong antiseptic properties, turmeric has been used as a remedy for all kinds of poisonous affections, ulcers and wounds. It gives good complexion to the skin and so it is applied to face as a depilatory and facial tonic. The drug cures diseases due to diabetes, eye diseases, ulcers, anorexia, leprosy, purifies blood by destroying the pathogenic organisms, smallpox and chickenpox. The rhizome juice is also useful in cold, cough, bronchitis, conjunctivitis and liver affections (Nadkarni, 1954; Kurup *et al.*, 1979; Kolammal, 1979). Turmeric paste mixed with a little limejuice and saltpetre and applied hot, mixing with lime is a popular application to sprains and bruises. In Ayurveda, turmeric has been used for various medicinal conditions including rhinitis, wound healing, common cold, skin infections, liver diseases, as a 'blood purifier' (Chainani *et al.,* 2003) an anthelmintic, in asthma, laxative,

diruretic, expectorant, febrifuge (Warrier *et al.*, 1994) and urinary tract diseases. The essential oil of the plant is used as antacid, carminative, stomachic and tonic (Ghani, 1998 and Kirtikar, 1996).

Kaempferia galanga rhizome contains a volatile oil (Wong *et al*., 1992) and several alkaloids, starch, proteins, amino acids, minerals and fatty matters. Leaves and flowers contain flavonoids (Ghani, 1998). The rhizome extract of black thorn are used as expectorant, stimulant, diuretic, carminative, stomachic (Rahman *et al*., 2004), leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, ulcers, helminthiasis, fever, malarial fever, splenopathy, inflammatory tumour, nasal obstruction, and haemorrhoids. The extract is also used to cure nasal block, asthma and hypertension. The leaf extracts are applied to sore eyes, sore throat, rheumatism, swellings, stimulant, expectorant, diuretic and carminative. The *Kaempferia* oil is utilized in the manufacture of perfumes and in curry flavouring. It is also employed in cosmetics, mouth- washes, hair tonics and toiletries (Kumar *et al.*, 1997).

These three plants of Zingiberaceae are rich in various secondary metabolites. Ginger and turmeric contain curcumin as well as less oxygenated curcumin derivatives. Katiyar *et al.*, (1996) showed that water or organic solvent extract of ginger possesses antioxidative property, which inhibits tumour promotion in mouse skin. Thus *Zingiber* extract is postulated to probably contain anti-inflammatory agents with antioxidant activity. *Curcuma* is rich in antioxidants in both *in vitro* and *in vivo* systems (Halim, 2002) *Kaempferia galanga* is also a potential sources of antioxidants and/or cytotoxic agents against tumour cells (Zaeoung *et al.*, 2005).

Zingiber officinale, Curcuma longa and *Kaempferia galanga* are tropical plant adapted for cultivation even in regions of subtropical climate. Being an exhausting crop, they are not cultivated continuously in the same field but shifting cultivation is practiced. Even more as the pathogens of these plants are transmitted by soil and seed rhizomes, so cultivating the crops in the same field are not recommended.

Plants of Zingiberaceae are propagated through rhizomes. So, various pathogens of bacterial and fungal origin get transmitted through the seed materials and affect the cultivation of

these crops (Sit *et al.*, 2005). The potential pests related to the Zingibers are bacterial wilt (*Pseudomonas solanacearum*), soft rot (*Pythium aphanidermatum*), rhizome and root rot (*Pythium graminicolum*), rhizome rot and yellows (*Fusarium oxysporum* f. sp. *Zingiberi*), leafblotch (*Taphrina maculans*) and nematodes (*Meloidogyne* spp. and *Radopholus similis*) (Hosoki and Sagawa, 1977; de Lange *et al*., 1987; Kumar *et al*., 1997).

Problem areas in Zingibers

In traditional method of cultivation the members of Zingiberaceae are propagated by rhizomes; providing the chance to various pathogens like bacteria, fungi and mycoplasma affecting the crop to be transmitted through the seed materials which cause immense loss in yield. Conventional breeding programmes to improve the crops are restricted to selection due to extreme low seed set. So, most crop improvement programs are thus confined to the conventional methods like evaluation and selection of naturally occurring variations (Samsudeen *et al.*, 2000 and Bhattacharya and Sen, 2006). *In vitro* culture offers a method for producing variations and exploring the resultant variations for crop improvement. So, tissue culture methods can be adopted in this field as an alternative means of plant propagation. Biotechnological tools like production of disease free planting materials through tissue culture can improve the yield of rhizome in the field (Sit and Bhattacharya, 2007). But unfortunately this field of research has not been explored for the local cultivars of Zingiberaceous plants.

Study of molecular diversity among the regenerated plantlets can provide suitable tool for crop improvement. Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones, but most have limitations. Polymerase chain reaction (PCR) in conjugation with short primers of arbitrary sequences is useful in analyzing the genomic status of plants (Williams *et al*., 1990). Randomly amplified polymorphic DNA (RAPD) markers were recently shown to be sensitive for detecting variations among individuals between and within species (Carlson, *et al.*, 1991 and Roy *et al*., 1992). There are neither any reports on the gene sequences of the local cultivars nor on the relationships of the local cultivars of turmeric with the established cultivar varieties.

Nutritional and medicinal values of the

genera under study have been worked by several workers. Most of them have concentrated on the extraction of the rhizome by one or a few organic solvents. Separation of active components by column chromatography has not been worked out in these genera. More over there are no reports on the antiradical activities of the local cultivars of the plant materials.

Therefore the research needs

Zingiber officinale, *Curcuma longa* and *Kaempferia galanga* despite being well know spice and medicinal plants of this region have not been extensively studied. Huge possibilities are there to explore the local cultivars in terms of selecting high yielding, disease resistant and antioxidant rich plants. Molecular documentation of the local cultivars and finding its relationship with productivity, disease resistance and active principles may open up new dimensions in exploring the local cultivars.

As the members of Zingiberaceae are vegetative propagated so efforts to improve them by conventional breeding methods are limited due to the problems of heterozygosity and lack of sexual cycle. In this respect little or no work has been done on the locally available cultivars of the genera under study. Hence, extensive study is required in the field to explore the idea of using *in vitro* techniques for isolating useful variants. *In vitro* regeneration by using rhizome buds of *Z. officinale, C. longa* and *K. galanga* as an explant has the potentiality to produce huge quantity of plantlets within a very short period. The plants after hardening can be well maintained in field condition and the line, if superior can be further multiplied by the conventional technique. Moreover, micropropagated plants are free from diseases or pests and show superiority in different aspects. These useful variations can prove to be helpful in providing economic benefit to the farmers and the large farming communities would adopt this technology to ensure superior planting materials for their fields.

Study of floral morphology has been the most important criteria for identification of flowering plants since time immemorial. But, slight variations within the cultivars cannot be made out by this process. So employing molecular techniques is important. Moreover in rhizomatous plants where flowering and seed set is low, study of molecular diversity becomes more

important for taxonomic identification. DNA fingerprinting using RAPD and ISSR primers may prove to be very useful in this process of identification, however, no reports are there on the molecular documentation of the local cultivars of Zingiberaceae. So, the knowledge of the gene sequences of the local cultivars and their relationships with other established cultivars of turmeric will certainly help in judging its origin and relatedness with other members.

Medicinal plants form an important part of indigenous medical systems of India. The system is growing importance in modern system as they can treat disease with very little or no side effects. Numerous herbs with their curative properties are available in literature but unfortunately, little is known about their antiradical activity. Status of the active principles of the Zingiberaceous members of this region have neither been isolated and nor being explored. Study of antiradical activities of plants and separation of the active principle by chromatographic techniques can open new field in the pharmaceutical industry and pave the way to treat diseases like arteriosclerosis, ischemic injury, cancer etc. Fractionation of the extracts with polar and non polar solvents will be of significance in understanding the *in vitro* mechanism of action of these formulations. Keeping all the above facts in mind the present study was designed with the following objectives:

Study of regeneration techniques

- Collection of germplasm of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga.*
- Maintenance of the germplasm in the experimental garden of the Department of Botany.
- Standardization of *in vitro* regeneration protocol of the genera with suitable media and plant growth regulators.
- Hardening of *in vitro* raised plantlets after adequate rooting.
- Comparative study of *in vitro* regeneration of *Curcuma longa* cultivars.

Study of molecular diversity

- Isolation of genomic DNA from fresh and tender leaf samples of *Curcuma longa* cultivars.
- Detection of genetic variability and phylogenetic relationship among the cultivars of *C. longa* by different PCR based DNA

fingerprinting methods.

- Detection of somaclonal variations among the *in vitro* regeneration plantlets of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* by different PCR based DNA fingerprinting methods.
- Sequencing of the taberlet region of *Curcuma longa* genome collected from the locality.

Study of antioxidant activity

- Soxhalation of the rhizomes to prepare extracts.
- Fractionalization of the extracts using silica gel column chromatography with different polar and non polar solvents.
- Isolation of the solutes from the solvents by mild heating.
- Detection of free radical, hydroxyl radical and nitric oxide scavenging activity of the extracts.
- Lipid peroxidation test of the extracts.
- Comparative study of the free radical scavenging activities of the potential solvent fractions of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga.*
- Study of free radical scavenging activity of different *Curcuma longa* cultivars.

Chapter 2
REVIEW OF LITERATURE

2.1 History of Zingiber

The members of the family Zingiberaceae have a very old and glorious history. There are numerous references to ginger and turmeric in Sanskrit literature and in Chinese medical treatise. The Sanskrit name 'Singabera' gave rise to the Greek 'Zingiberi' and to the Latin 'Zingiber'. Zinger has been used in India from Vedic period and is called maha-aushadi implying 'The great medicine'. Ginger is believed to have originated in India and was introduced in China at a very early date. It was known to Europe in the first century AD and was mentioned by Dioscorides and Pliny. Turmeric is native to Southern or South-eastern Asia. It seems to have reached China before the seventeenth century A.D. Turmeric spread early throughout the East Indies and was carried eastwards across the pacific by Polynesians to Hawai and Easter Islands.

2.2 Systematic position of the family

The family Zingiberaceae is the largest family of Zingiberales and is one of the ten largest monocotyledonous families in India **(**Kress *et al.*, 2002). The systematic position of the family has been studied by several taxonomists (table:2.1). However, generally the family Zingiberaceae is placed under the order Scitaminales, Series Epigynae, Class Monocotyledonae, Subdivision Angiospermae, Division Spermatophyta, Sub-kingdom Phanerogamy and Kingdom Plantae (Joy *et al.*, 1998).

2.3 Classification and taxonomic hierarchy of the genera

Zingiber is represented by 90 species, *Curcuma* by 80 and *Kaempheria* by 50 species (Sirirugsa, 1999). Classifications of the family was first proposed in the 19th century and

Table 2.1: Placement of Zingiberaceae (adopted from Singh, G. (2004). *Plant Systematics, 2/E*. Oxford and IBH Publishing).

Hierarchy	Bentham & Hooker	Cronquist	Takhtajan	Dahlgren	Thorne	APGII/APWeb
Division	-	Magnoliophyta	Magnoliophyta	-	-	-
Class	Monocotyledons	Liliopsida	Liliopsida	Liliopsida	Angiospermae	-
Subclass	-	Zingiberidae	Commelinidae	Liliidae	Commelinidae	-
Series*/ superorder	Epigynae*	-	Zingiberanae	Zingiberanae	Commelinanae	Commelinids
Order	-	Zingiberales	Zingiberales	Zingiberales	Cannales	Zingiberanae

refined by others since that time. Four tribes (Globbeae, Hedychieae, Alpinieae, and Zingibereae) were recognized, based on morphological features, such as number of locules and placentation in the ovary, development of staminodia, modifications of the fertile anther, and rhizome-shoot-leaf orientation (Kress *et al.*, 2002). Datta (1988) in his book recognized two subfamilies of Zingiberaceae. The first one Costoideae without any tribe and the second Zingiberoideae, with three tribes named as Globbeae, Hedychieae and Zingibereae. According to him the genera *Curcuma* and *Kaempferia* belongs to Hedychieae and *Zingiber* belongs to Zingibereae. Chase (2004) reported eight families of the order *Zingiberales* (Cannaceae, Costaceae, Heliconiaceae, Lowiaceae, Marantaceae, Musaceae, Strelitziaceae and Zingiberaceae).

Molecular-phylogenetic analyses of Zingiberaceae based on DNA sequences of the nuclear internal transcribed spacer (ITS) and plastid *matK* regions suggest a new classification of the Zingiberaceae that recognizes four subfamilies and four tribes: Siphonochiloideae (Siphonochileae), Tamijioideae (Tamijieae), Alpinioideae (Alpinieae, Riedelieae), and Zingiberoideae (Zingibereae, Globbeae) (Kress *et al*., 2002).

2.4 Relationship of Zingiberales with other monocot taxa

The eight families of the order *Zingiberales* have nearly always been recognized at some level of the taxonomic hierarchy. They are easily recognized by their inferior ovaries, inaperturate pollen (except in Costaceae), reduced numbers of functional stamens (except in *Ensete* and *Ravenala*), often highly modified staminodes (often petaloid), and

essential oils. They formed a clade in the morphological analysis of Stevenson and Loconte (1995) and Chase *et al*. (1995b).

DNA studies have always demonstrated Zingiberales to be monophyletic with high support (Chase *et al*., 1995a, b; Givnish *et al*., 1999). The order has a number of small families that have been kept distinct from their larger sister families as a matter of tradition, but it seems clear that *Canna* (Cannaceae) could easily be included in Marantaceae (Andersson and Chase, 2001), Costaceae in Zingiberaceae (Kress *et al*., 2002), and *Orchidantha* (Lowiaceae) in Heliconiaceae (Kress *et al*., 2001). Combining Musaceae with either Strelitziaceae or Heliconiaceae, as suggested by Dahlgren *et al*. (1985), has not been justified on the basis of molecular studies (Kress *et al*., 2001). Studies of floral development (Kirchoff, 1992, Kirchoff and Kunze, 1995) have provided important insights into homologies of the various floral parts of these often highly modified taxa (Chase, 2004).

2.5 Habitat of Zingiberaceae

Members of the Zingiberaceae are the ground plants of the tropical and sub tropical forests. The plants are cultivated or are grown in wild. They mostly grow in damp and humid shady places. They are also found infrequently in secondary forest. Some species can fully expose to the sun, and is capable of growing on high elevations.

2.6 Botanical description of Zingiberaceae

Plants of the family Zingiberaceae are generally herbs, often large, with a pseudostem of convolute leaf sheaths. The leaves are radical or cauline and usually membranous. Sheaths are generally large, clasping on stem; lamina with a strong central nerve and pinnately closed secondary nerves. Petioles are short or absent. Flowers are hermaphrodite, irregular, solitary or spicate, bracts membranous, bracteoles membranous or toothed or spathaceous tube, inner segments are petaloid, connate in a long or short corolla tube, free or adnate to petaloid staminodes, or 5 perfect with six imperfect. Anthers are linear and 2 celled. Ovary is 3 celled, inferior with many ovules, anatropous and axile. Style is usually slender with 2 short stylodes, crowning the ovary. Stigma is usually entire or sub entire. Fruit is usually loculicidal- a three valved capsule, or indehiscent and

membranous or fleshy, usually crowned by the remains of the perianth. Seeds are often arilate, albumen floury and embryo small (Hooker, 1875; Prain, 1903; Datta, 1988; Joy *et al*., 1998).

2.7 Morphology of the genera

Zingiber officinale Rosc. is herbaceous with elongated, leafy stems and horizontal, tuberous rootstocks; leaves oblong or lanceolate. Flowers in spikes, usually radical, less often terminal, very rarely lateral on the leafy stems; peduncle short or long; bracts persistent, usually 1-flowered. Sepals 3, connate in a cylindrical, shortly three lobed calyx. Petals 3, connate in a corolla with cylindrical tube; lobes lanceolate, the upper concave. Stamens 1 perfect; filaments short; anther 2-celled; cells contagious, with a narrow crest as long as themselves; lateral staminodes absent, or adnate to the ovate-cuneate lip. Carpels 3, connate in a 3 celled ovary; ovules many, superposed; placentas axial; style filiform; stigma small, subglobose. Fruit a long capsule, dehiscent. Seeds large, globose, arillate (Hooker, 1875; Prain, 1903; Datta, 1988; Joy *et al*., 1998).

Curcuma longa L. is a stemless herb with tuberous rootstocks, bearing sessile and long-stipulate tubers; tubers deep orange yellow within; leaves usually oblong, often very large. Flowers in dense, compound spikes, crowned by small, coloured bracts; lower bracts ovate, membranous, enclosing several bracteoles fugitive flowers, which open in succession. Sepals 8, connate in a short, cylindric, minutely toothed calyx. Petals 3, connate in a corolla with funnel shaped tube; lobes usually ovate or oblong, the upper longer and somewhat concave. Stamens 1 perfect; filaments short; anthers uncrested, with contigious cells spurred at the base; lateral staminodes oblong, petaloid, connate with the filaments; lip orbicular, with a deflexed tip. Carpels 3, connate in a 3 celled ovary; ovules numerous, on axial placentas; style filiform; stigma 2 -lipped, the lobes ciliate. Fruits are tardily dehiscent, globose, membranous, 8 valved capsule. Seeds are ovoid or oblong, usually arillate (Hooker, 1875; Prain, 1903; Datta, 1988; Joy *et al*., 1998).

Kaempferia galanga L. is a stemless herb; rootstock often tuberous; leaves few, 3-6 inches long, spreading flat on the ground. Flowers spiked, or radial scapes or at the apex of the leafy stem. Sepals 3, conate in a short, cylindrical

calyx, which splits spathaceously. Petals 3, conate in a corolla with a long, slender tube; lobes equal, usually spreading. Stamens 1 perfect; filaments short, arcuate; anthers 3 celled; cells discrete, on a wide connective, produced above as a petaloid crest, not spurred below; lateral staminodes broad, petaloid; lip broad, usually 2-fid. Carpels 3, connate in a three celled ovary; ovules many, on three axial placentas; style long, filiform; stigma turbinate. Fruit an oblong capsule with thin pericarp. Seeds are subglobose, with a small and lacerate arillus (Hooker, 1875; Prain**,** 1903; Datta, 1988; Joy *et al*., 1998).

2.8 Chemical constituents and their activities

The chemistry of *Zingiber officinale* has been the subject of sporadic study since the early nineteenth century. Fresh ginger contains approximately water 80%, protein 2.3%, fat 1%, carbohydrate 12.3%, fibre 2.4% and ash 1-2%. Dried ginger contains about 10% moisture and 1-3% of volatile oil of which the chief constituent is a sesquiterpene, called zingiberene ($C_{15}H_{24}$). The pungent principle of ginger is zingerone ($C_{11}H_{14}O_3$) which is present in the oleoresin. (Krishnamurthy *et al.*, 1970; Kami *et al.*, 1972; Akhila and Tewari, 1984; Joy *et al*., 1998). It is also rich in secondary metabolites such as oleoresin (Bhagyalakshmi and Singh, 1988).

Ginger owes its characteristic organoleptic properties to gingerols. The odour and much of the flavour of ginger is determined by the constituents of its steam volatile oil, while the pungency is comprised mainly of mono and sesquiterpene hydrocarbons and oxygenated compounds. The monoterpene constituents, though present in trace amounts, contribute most of the aroma of a ginger (Bertram and Walbaum, 1894; Soden and Rojahn, 1900; Schimmel and Co., 1905; Dodge, 1912; Brooks, 1916; Jain *et al.*, 1962; Nigam *et al.*, 1964; Kami *et al.*, 1972; Bednarczyk and Kramer, 1975; Denniff and Whitting, 1976; Thresh, 1879; Joy *et al*., 1998). The sesquiterpene hydrocarbons constitute the major fraction of the essential oil, out of which β- Zingiberene and ar-curcumene are found in high percentages, about 35-40% and 15-20% respectively. Cis-sesquithujene hydrate and Zingiberenol are the two hydrated sesquiterpenes reported (Terhune *et al.*, 1975 and Pagutte and

Kinney, 1982). Several investigations have been carried out on the non-volatile pungent principles of ginger. The major pungent compounds reported are gingerols, shagaols, dihydrogingerols, hexahydrocurcumin, gingerdiols, desmethylhexahydrocurcumin, (n)-paradol, zingerone and ginger diones (Lapworth *et al.*, 1917; Nelson, 1917; Nomura, 1918; Nomura and Tsurani, 1927; Connell and Sutherland, 1969; Connell, 1970; Lapworth and Wykes, 1970; Connell and Mclachlan, 1972; Yamagishi *et al.*, 1972; Masada *et al.*, 1973; Denniff and Whitting, 1976; Macleod and Whiting, 1979; Denniff *et al.*, 1980). Main constituent of essential oil is d-camphene (Husain *et al.*, 1992).

Curcuma longa contains curcumin, alkaloid and an essential oil. *Curcuma longa* rhizome has 1.8-5.4% curcumin, the pigment and 2.5-7.2% of essential oil (Joy *et al*., 1998). A ketone and an alcohol are obtained from the volatile distillate. Fresh rhizomes yield 0.24% oil containing Zingiberine (Chopra, 1980). Essential oil of *Curcuma* contains ar-turmerone, and ar-curcumene as major constituents. Curcumin and related compounds have also been reported as major constituents of the rhizomes. A number of sesquiterpenes have been reported from *C. longa*, viz., the sesquiterpenoids of germacrane, bisabolane and guainane skeletons (Husain *et al.*, 1992). The study of sesquiterpenes has revealed a new compound curlone (Kisoy and Hikini, 1983). The crystalline colouring matter curcumin (0. 6%) is diferuloyl methane (Mathews *et al.*, 1980). Stigmasterol, cholestrol, β-sitosterol and fatty acids, mainly straight chain dienoic acids are reported (Moon *et al.*, 1976). The essential oil (5.8%) obtained by steam distillation of dry rhizomes have been reported to contain β-phellandrene, d-sabinene, cineole, borneol, Zingiberene and sesquiterpene ketones (50%). In the analysis of the essential oil, turmerone (29.3%), ar-turmerine (23.6%) and sabinone (0.6%) have been identified (Lawrence, 1982).

The tuberous rhizome of *Kaempferia galanga* contains an alkaloid, starch, gum, fatty matter with a fragrant liquid essential oil and a solid white crystalline substance and mineral matter. The rhizome possesses a camphoraceous odour with somewhat bitter aromatic taste (Joy *et al*., 1998). The essential oil is reported to contain over 54 components of which the

major constituents are ethyl-trans-p-methoxy, cinnamate 16.5%, pentadecane 9%, 1,8-cineole 5.7%, and borneol 2.7%. Terpenoid constituents amounted to 16.4% (Nerle and Torne, 1984; Wong *et al.*, 1992). Isolation of diterpenes from *Kaempferia* species was done by Parwat *et al.* (1993). The chemistry of *Kaempferia* was studied in detail by Tuntiwachwuttikul (1991). Rhizome yields essential oil, which has antifungal activity. Ethyl-p-Methyl O-trans-cinnamate is the main compound in root (Asolkar, 1992). p-Methoxycinnamic acid and its methyl and ethyl esters have been isolated from the essential oil (Rastogi and Mehrotra, 1991) Essential oil from rhizomes contain n-pentadecane, ethyl-p-methoxy cinnamate, ethyl cinnamate, carene, camphene, borneol, p-methoxystyrene, p-methoxy cinnamate, p-methoxy-trans-cinnamic acid and cinnamaldehyde. Tuber is stimulant, expectorant, diuretic and carminative (Husain *et al.*, 1992).

2.9 Economic importance and health benefits

Zingiber officinale is used worldwide as a cooking spice, condiment and herbal remedy (Duke and Ayensu, 1985). There are 3 primary products of the ginger rhizomes, namely fresh, preserved and dried ginger. Fresh or green ginger is consumed as a vegetable. Immature ginger preserved in sugar syrup is mainly used as a desert. Crystallized ginger is used as a sweet meat. The dried rhizomes constitute the spice and are esteemed for its flavour, pungency and aroma. It is a constituent of curry powder. It is also used in the production of ginger beer, ginger oil and ginger wine. Pressed ginger is prepared by boiling tender fleshy peeled rhizomes after which they are boiled and sold in sugar syrup. Crystallized ginger is produced in the same way, but it is dried and dusted with sugar. The rhizome yields an essential oil, but this lacks the pungent principle. It is used in the manufacture of flavouring essences and in perfumery. An oleoresin is also extracted in which the full pungency of the spice is preserved. It is used for flavouring purposes and in medicines. It is estimated that in India, the average daily consumption is 8 -10 grams of fresh ginger root (Joy *et al*., 1998)

Ginger is used extensively in 'Ayurveda', the traditional medicine of India, to block excessive clotting, reduce cholesterol and fight arthritis. The Chinese have used ginger for at least 2500 years as a digestive aid and

antinausea remedy and to treat bleeding disorders and rheumatism; it was also used to treat baldness, toothache, snakebite, and respiratory conditions (Duke and Ayensu, 1985). In Traditional Chinese Medicine, ginger is considered a pungent, dry, warming, yang herb to be used for ailments triggered by cold, damp weather. In Malaysia and Indonesia, ginger soup is given to new mothers for 30 days after their delivery to help warm them and to help them sweat out impurities. In Arabian medicine, ginger is considered an aphrodisiac (Qureshi *et al*., 1989). Some Africans believe that eating ginger regularly will help repel mosquitoes (Duke and Ayensu, 1985). The Greeks wrapped ginger in bread and ate it after meals as a digestive aid. Subsequently, ginger was incorporated directly into bread and confections such as gingerbread. Ginger was so valued by the Spanish that they established ginger plantations in Jamaica in the 1600's. The physicians of the 19th century relied on ginger to induce sweating, improve the appetite and curb nausea, and as a topical counterirritant. Nowadays, ginger is extensively cultivated from Asia to Africa and the Caribbean and is used worldwide as a nausea remedy, as an anti-spasmodic and to promote warming in case of chills (Kapil *et al*., 1990). Ginger is valued in medicine as a carminative (Kumar *et al.*, 1997) and stimulant of the gastro-intestinal tract (Kumar *et al.*, 1997) gastrointestinal disorders, piles, bronchitis, antipyrexia and treatment of waist pain and rheumatism. It promotes digestive power, cleanses the throat and tongue, dispels cardiac disorders and cures vomiting, ascites, cough, dyspnoea, anorexia, fever, anaemia, flatulence, colic, constipation, swelling, elephantiasis and dysuria. It is also used in diarrhoea, cholera, dyspepsia, neurological diseases, diabetes, eye diseases and tympanitis. In traditional medicine ardraka is extensively used for its specific action in rheumatism and inflammation of liver (Kurup *et al.*, 1979).

It is also used in several domestic preparations (Warrier *et al.*, 1996) It is used as a household remedy for indigestion, flatulence, dypepsia, sore throat, etc. by adding it to tea. Ginger with kernal of castor seed is used in paralysis and with asafoetida in indigestion. In chronic rheumatism, infusion of ginger taken warm just before going to bed, the body being covered with blankets so as to produce

copious perspiration is often attended with the best results. The same treatment has also been found beneficial in colds or catarrhal attacks and during the cold stage of intermittent fever. In headache, ginger paste applied to the forehead affords relief. Tooth-ache and face-ache are relieved by the same application. In the collapse stage of cholera powdered ginger rubbed to the extremities is found to check the cold perspiration, improve the local circulation and so tends to relieve the agonising cramps of that terrible disease. Ginger with salt taken before meals is praised as a carminative, said to clean the tongue and throat, increase the appetite and produce an agreeable sensation (Nadkarni, 1998). Ginger juice produces antimotion sickness action by central and peripheral anticholinergic antihistaminic effects (Qian and Liu, 1992).

Clinical studies made by Singhal and Joshi (1983) and Girij *et al.*, (1984) prove that *Zingiber officinale* reduces the serum cholesterol level considerably in hypercholesterolemic rats. The raw ginger is useful in anorexia, vitiated conditions dypepsia, pharyngopathy and inflammations. The dry ginger is useful in dropsy, otalgia, cephalalgia, asthma, cough, colic, diarrhoea, flatulence, anorexia, dyspepsia, cardiopathy, pharyngopathy, cholera, nausea, vomiting, elephantiasis and inflammations. It has protective activity on gastric ulcerogenesis. Organic solvent extract of ginger rhizomes has also been shown to cause significant inhibition of skin tumour (Katiyar *et al.*, 1996).

The essential oil exhibited remarkable repellent activity against both the kitchen insect *Periplaneta americana* and the agricultural pest *Bruchas pisorum* (Garg and Jain, 1991).

Curcuma longa occupies an important position in the life of Indian people as it forms an integral part of the rituals, ceremonies and cuisine. Due to the strong antiseptic properties, turmeric has been used as a remedy for all kinds of poisonous affections, ulcers and wounds. It gives good complexion to the skin and so it is applied to face as a depilatory and facial tonic. The drug cures diseases due to diabetes, eye diseases, ulcers, oedema, anaemia, anorexia, leprosy and scrofula. It purifies blood by destroying the pathogenic organisms. A paste of turmeric alone, or combined with a paste of neem (*Azadirachta indica*)

leaves, is used to cure ringworm, obstinate itching, eczema and other parasitic skin diseases, and in chicken pox and small pox. The drug is also useful in cold, cough, bronchitis, conjunctivitis and liver affections (Nadkarni, 1954; Kurup *et al.*, 1979; Kolammal, 1979). Turmeric paste mixed with a little limejuice and saltpetre and applied hot is a popular application to sprains and bruises. In smallpox and chickenpox, a coating of turmeric is applied to facilitate the process of scabbing. The smoke produced by sprinkling powdered turmeric over burnt charcoal will relieve scorpion sting when the part affected is exposed to the smoke for a few minutes. Turmeric and alum powder in the proportion of 1:20 is blown into the ear in chronic otorrhoea (Nadkarni, 1998). "*Haridra Khand*", a compound containing powdered turmeric, sugar and many other ingredients is a well-known preparation for cold, cough and flu, and for skin diseases (Joy *et al*., 1998). In Ayurveda, turmeric has been used for various medicinal conditions including rhinitis, wound healing, common cold, skin infections, liver and urinary tract diseases and as a 'blood purifier' (Chainani *et al.,* 2003). The fresh rhizome and dried powder are popular remedies for disorders of blood and various skin diseases. It is also externally used for pains and for beautification by fairness. The rhizome juice is used as an anthelmintic as well as in asthma and urinary diseases. The essential oil of the plant is used as antacid, carminative, stomachic and tonic (Ghani, 1998; Kirtikar, 1996). Curcumin is antiinflammatory. Rhizome is antiprotozoal, spasmolytic, CNS active, antiparasitic, antispasmodic, antibacterial, antiarthritic (Husain *et al.*, 1992). The rhizomes are also anthelmintic, carminative, antiperiodic, emollient, anodyne, laxative, diruretic, expectorant, alterative, alexertive, febrifuge, opthalmic and tonic (Warrier *et al.*, 1994). An extract of the crude drug '*akon'* containing the rhizomes exhibited intensive preventive activity against carbon tetrachloride induced liver injury *in vivo* and *in vitro*. The liver protecting effects of some analogs of ferulic acid and p-coumaric acid, probable metabolites of the curcuminoids have been also evaluated (Kiso *et al.*, 1983).

Essential oil from rhizomes is antiseptic, antacid and carminative. Effect of the oil on cardiovascular and

respiratory systems is not marked, therefore, not of much importance from therapeutic point of view. Chloretic action of the essential oil is attributed to p-tolymethyl carbinol. Dye-stuff acts as a cholagogue causing contraction of the gall bladder. An antioxidant property of curcuma powder is due to phenolic character of curcumin (Dey, 1980).

Protective effect of curcuminoids from *C. longa* on epidermal skin cells under free oxygen stress was analysed by Bonte *et al*. (1997). Anti-inflammatory activity volatile oil of *C. longa* leaves was studied by Iyengar *et al*. (1994). Essential oil (0.1 mg/kg) in rats showed significantly more marked antiinflammatory effect than cortisone acetate (10mg/kg). The uptake, distribution and excretion of curcumin were also studied. Clinical trials showed that plant definitely reduced cough and dyspnoea (Rastogi and Mehrotra, 1991).

Rhizomes are externally effective as insect repellent against houseflies. It is found to inhibit *Clostridium botulinum*. Essential oil from rhizome showed fungitoxicity (Asolkar *et al*., 1992) and nematicidal activity (Kinchi *et al*., 1993).

Kaempferia galanga L. rhizome contains a volatile oil (Wong *et al*., 1992) and several alkaloids, starch, proteins, amino acids, minerals and fatty matters. Leaves and flowers contain flavonoids (Ghani, 1998). The rhizome extract of *Kaempferia galanga* are used as expectorant, stimulant, diuretic, carminative, and stomachic (Rahman *et al*., 2004 and Kumar *et al.*, 1997). The extract is also used to cure nasal block, asthma and hypertension. The leaf extracts are applied to sore eyes, sore throat, rheumatism, swellings and fevers.

The aroma and flavour of the plant is used in flavouring foodstuffs, beverages, perfumes and cosmetics industries (Rahman *et al.*, 2004). The *Kaempferia* oil is utilized in the manufacture of perfumes and in curry flavouring. It is also employed in cosmetics, mouth- washes, hair tonics and toiletries. The pungent, hot, sharp, bitter and aromatic rhizomes find an important place in indigenous medicine as stimulant, expectorant, diuretic and carminative. It promotes digestion and cures skin diseases, piles, phantom tumors, coughs, oedema, fever, epilepsy, splenic disorders, wounds, asthma and rheumatism. The rhizomes are used for protecting clothes against insects and

are eaten along with betel and areca-nut. The rhizomes and leaves are attached to necklaces and added to bath water for perfume. The plant is a reputed remedy for all respiratory ailments like cough, bronchitis and asthma. The drug is reported to be acrid, hot, bitter and aromatic. It cures skin diseases, wounds and splenic disorders. It promotes digestion, removes bad odour of the mouth and destroys pathogenic organisms (Aiyer and Kolammal, 1964). The rhizomes and root-stocks are good for dyspepsia, leprosy, skin diseases, rheumatism, asthma, cough, bronchitis, ulcers, helminthiasis, fever, malarial fever, splenopathy, cephalalgia, inflammatory tumour, nasal obstruction, halitosis, strangury, urolithiasis, and haemorrhoids. The leaves are used for pharyngodynia, ophthalmia, swellings, fever and rheumatism (Warrier *et al.*, 1995). The tubers reduced to powder and mixed with honey are given in case of coughs and pectoral infections. The oil in which they are boiled is useful in applying to open the blockages of the nasal organs (Nadkarni, 1998). Tuber is stimulant, expectorant, diuretic and carminative (Husain *et al.*, 1992).

Kaempferia galanga rhizome extract has been potently active against bacterial infections. Indigenous medical practitioner use these rhizomes for treatment of scariasis, bacterial infections, tumor and it is also applied externally for abdominal pain in women and used topically for treatment of rheumatism (Hirschhornn, 1983). In Thailand, the dried rhizome has been used as cardiotonic and central nervous system stimulant (Mokkhasmit *et al.,* 1971), whereas an acetone extract has an effect on monoamine oxidase inhibition (Noro *et al*., 1983). The 95% ethanol extract of this plant possessed antibacterial activity against *Staphylococcus aureus* and hot water extract against *Escherichia coli* (George and Pandalai, 1949). The rhizome of *K. galanga* has been used for treatment of fungal derived-skin diseases as well as eczema (Tungtrongjit, 1978).

The volatile oil of *Kaempferia galanga* exhibited marked activity against Gram-positive and Gram negative bacteria; and also against *Candida. albicans*, by using agar disc diffusion method The result revealed that the oil of this plant possessed marked antimicrobial activity against Gram-positive bacteria with the inhibition zones from 12.0-16.0 mm., and 8.0-

12.0 mm. against Gram-negative bacteria; whereas it potently inhibited *Candida albicans* with an inhibition zone of 31.0 mm., which was stronger than that of standard antifungal Clotrimazole. It is suggested that the essential oil of this plant may be useful for treatment of the diseases caused by these bacteria and fungi, such as skin diseases and diarrhea. The volatile oil of *Kaempferia galanga* especially ethyl-*p*-methoxycinnamate, could be used as a biomarker for standardization of this plant and the results of bioactivities suggest that the essential oil of *Kaempferia. galanga* could be used for treatment of some microbial infections, which also agrees with the traditional use of this plant in treatment of both fungal and bacterial skin diseases (Tungtrongjit, 1978). Moreover, *Kaempferia. galanga* should also be subjected to more elaborated bioassay for specific pharmacological activities.

Kaempferia galanga even contains insecticidal constituents which were isolated by Pandji *et al.* (1993).

2.10 *In vitro* culture studies

Tissue culture is the foundation on which virtually all plant-related biotechnology experiments rest. In plant tissue culture, micropropagation occupies the key position. Micropropagation is the process of mass propagation of selected clones via *in vitro* techniques. Micropropagation has major supporting role in biotechnology for:-

(i) rapid multiplication of rare, exotic and genetically engineered plants,

(ii) large scale production of superior propagules and,

(iii) conservation of economically important ornamental, horticulture spp., plantation crops and medicinal plants.

Micropropagation is of great importance to overcome the constraints like shortage of seed supply, germination problems, long regeneration time, variation due to cross pollination etc.

The beginning of research in this area can be traced back to the German botanist Gottlieb Haberlandt in 1902, who worked in Berlin. He conducted the first experiments with isolated plant cells. As little was known at that time about the nutritional biochemistry of the hormones essential for growth and differentiation, he could not go very far. Nevertheless, an important concept that a cell is totipotent emerged from his work. Roger

Gautheret (1934) in France laid the foundation of tissue culture studies by raising indefinitely growing callus from explants of cambium taken from the stem of trees. Paul Nobecuort (1937), also from France, contributed significantly to the development of the tissue culture techniques. In the United States, Philip White (1934) established continuing cultures of roots and later also from crown galls (Braun and White, 1943) and played a key role in development of plant tissue culture research (Maheshwari, 1996).

The key to further progress in tissue culture depended on the discovery of hormones. Until about 1930, botanists knew little about hormones. The knowledge of auxins representing the first major class of plant growth substances followed soon after the pioneering work of Frits Went (1926) and Kenneth Thimann (1935) and greatly aided the work of the early investigators. Later, in the 1950s the work of Folke Skoog and co-workers (Miller *et al*., 1955) led to the discovery of cytokinins, another important class of hormones. A reproducible control on differentiation from calli through an adjustment of auxins and cytokinin balance was achieved: a high cytokinin-auxin ratio led to development of shoots and a low ratio to rooting. By the 1960s, a fairly good understanding had been gained of the role of hormones in differentiation, although coconut milk, whose importance had been discovered by Van Overbeek and co-workers (1941), also remained a popular constituent of media in many investigations, and is so even today (Maheshwari, 1996).

With hormones and coconut milk in the arsenal, work for *in vitro* culture of plant tissues and their differentiation was initiated in many laboratories in the 1950s and 1960s. H E Street in the United Kingdom, Georges Morel in France, Jacob Reinert in Germany, and Frederick Steward in the United States led active research groups. Street (1957) made key contributions in successful culture of excised roots *in vitro*. Morel (1963 and 1971) made pioneering contributions to the crown-gall problem as also to the culture of monocots and orchids. However, the most important studies- concluding the first phase of plant biotechnology-were made by Steward and Reinert who demonstrated totipotency of plant cells**.** Using small discs of carrot root as explants for raising cell suspensions, Steward (1958) showed that even somatic cells could form embryos and

then complete plants. In parallel, Reinert (1959) achieved embryogenesis *in vitro*. But totipotency was demonstrated in a most decisive way by Vasil and Hildebrandt (1965). Vasil, while she was working with Hildebrandt in the United States employing tobacco, they showed that an isolated single cell could form an entire plant. By the end of the 1950s, plant tissue culture was already established as a major sub-discipline of plant biology, its importance and ramifications in agriculture, horticulture, and on forestry being far too evident for anyone to ignore (Maheshwari, 1996).

The second phase in 1960s witnessed the development of two special techniques in tissue culture: one relating to production of haploids by anther culture and other concerning isolation and culture of protoplasts. Guha and Maheshwari (1966) decisively demonstrated the haploid nature of embryos from anther and their origin from microspores. The third phase was of studies on genetic engineering and the rise of new biotechnology. The early practitioners of molecular biology were microbiologists or biochemists. Serious plant molecular biology started from 1960s' and that modern plant biotechnology is essentially the outcome of the fusion of tissue culture and molecular biology (Maheshwari, 1996).

In vitro clonal propagation is a complicated process requiring many steps and stages. Murashige (1978a, b) proposed four distinct stages that can be adopted for overall production technology of clones in a commercial way. Stages I to III are followed under *in vitro* conditions, whereas stage IV is accomplished in greenhouse environment. Debergh and Maene, 1981 suggested an additional stage O for various micropropagating systems. The major steps for *in vitro* clonal propagation are as follows:-

Stage O

Selection and maintenance of stock plants for culture initiation

↓

Stage I

Initiation and establishment of aseptic culture (explants isolation, surface sterilization, washing and establishment on appropriate culture medium)

↓

Stage II

Multiplication of shoots or rapid somatic embryo formation using a defined culture medium

↓

Stage III

Germination of somatic embryos and/or rooting of regenerated shoots *in vitro*

↓

Stage IV

Transfer of plantlets to sterilized soil for hardening under green house environment

The adoption of all these stages not only simplifies the daily operation, accounting and product cost but also allows for greater ease in communication with other laboratories (Chu and Kurtz, 1990). Thus a particular plant can be marketed or requested by specifying its stage.

2.10.1 Importance of in vitro regeneration

In zingiberaceae plant breeding is seriously handicapped by poor flowering and seed set. So, most crop improvement programs are confined to the conventional methods like evaluation and selection of naturally occurring variations. *In vitro* culture offers a method for producing variations and exploring the resultant variations for crop improvement. It provides an alternative means of plant propagation and a tool for crop improvement (Rahman *et al.,* 2004). Moreover overexploitation of zingiberous crops are making them endangered. *In vitro* regenerated plants are superior to conventionally propagated plants in respect of productivity and disease resistance. In sterile triploid plants like *Curcuma longa* transmission of pathogens from generation to generation takes place by rhizome, and amplification of particularly useful stocks is a slow process. An *in vitro* propagation method can alleviate these problems (Ma and Gang, 2006). Villamor (2010) indicated that lack of healthy planting materials limit expansion of ginger production which can be rectified by the production of healthy planting stock by tissue culture. In *Curcuma longa* slow multiplication rate, high susceptibility to rhizome rot and leaf spot disease and restricted availability of elite genotype has necessitated application of tissue culture technique to alleviate the problems (Singh *et al.*, 2011).

2.10.2 Establishment of culture

The objective of tissue culture is to successfully place an explant into aseptic culture by avoiding contamination and then to provide an *In vitro* environments that promotes stable shoot production. The important aspects of this are explant disinfection, explant selection and culture medium (Hartmann *et al.,* 1997). The success of establishment of explant in the culture media is dependent on explant selection, sterilization of explant, culture conditions, and composition of the culture media.

The use of tissue culture as a tool for plant propagation could be particularly

relevant for vegetatively propagated crop plants that resist conventional asexual propagation (Hackett, 1966) or when fast methods of mass propagation of single plant is required. The different explants such as axillary bud, shoot tips, meristem tips, root tips are commonly used.

In tissue culture of the members of Zingiberaceae different plant parts like emerging rhizome buds, rhizome pieces, axillary bud, shoot tip, pseudostem, leaves, inflorescence, ovary, anther parts etc are used (table 2.2). In regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempheria galanga* shoot bud/ rhizome buds were the most extensively used explants. The regeneration potential of this part is maximum which has made it popular as explants. *Zingiber* plantlets were regenerated by using shoot bud (Hosoki and Sagawa, 1997, Rout and Das, 1998, Sit *et al*., 2005, Bhattacharya and Sen, 2006 and Behera and Sahoo, 2009), dormant axillary buds of unsprouted rhizomes (Mohanty *et al*., 2008), juvenile shoots (Ilahi and Jabeen, 1987), leaf pseudostem (Ikeda and Tanaba, 1989), shoot tips/meristem (Bhagyalakshmi and Singh, 1988; Inden *et al*., 1988; Choi, 1991; Choi and Kim, 1991; Khatun *et al*., 2003; Lincy *et al*., 2009, Sultana *et al*., 2009), leaf tissue (Nirmal Babu *et al*., 1992, Kackar *et al*., 1993; Sultana *et al*., 2009), immature inflorescence (Nirmal Babu *et al*., 1992)), ovary (Nirmal Babu *et al.*,1996) and anther (Samsudeen *et al.,* 2000). Callus produced from the base or middle of pseudostem showed better responses to differentiation of plantlets than the callus produced from top or bottom of the pseudostem (Choi, 1991).

Curcuma plantlets were regenerated by using shoot bud (Yasuda *et al*., 1988; Kesavachandran and Khader, 1989; Winnar and Winnar, 1989; Balachandran *et al*., 1990; Shirgurkar, 2001; Sunitibala *et al*., 2001; Salvi, *et al*., 2002; Zapata *et al*., 2003; Nayak, 2004; Rahaman *et al*., 2004; Gayatri and Kavyashree, 2005; Prathanturarug *et al*., 2003; Tyagi *et al*., 2007; Naz *et al*., 2009; Behera *et al*., 2010), dormant axillary buds of unsprouted rhizomes (Singh *et al*., 2011), shoot meristem (Sunitibala *et al*., 2001; Nayak and Naik, 2006), leaf tissue (Salvi *et al*., 2001), Ovary (Nirmal Babu *et al*., 1996) and anther (Samsudeen *et al*., 2000).

Kaempheria plantlets were regenerated

by using shoot bud (Geetha *et al*., 1997; Shirin *et al*., 2000; Lakshmi and Mythili, 2003; Swapna *et al*., 2004; Chirangini *et al*., 2005; Rahaman *et al*., 2005; Parida *et al*., 2010; Kochuthressia *et al*., 2012; Bhattacharya and Sen, 2013) and leaf tissue (Swapna *et al*., 2004; Rahaman *et al*., 2004).

In the process of sterilization living materials should not lose their biological activity, but only bacterial or fungal contaminants should be eliminated. The commonly used sterilants are bleach, ethanol, sodium hypochlorite, mercuric chloride etc. The type of sterilant used, concentration and time depends on the nature of explant and species (Razdan, 1993).

Table 2.2: Explants used for *in vitro* regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*

Plant parts	Reference
Zingiber officinale	
Shoot bud/rhizome bud	Hosoki and Sagawa, 1977; PIllai and Kumar, 1982; Malamug *et al.*, 1991; Dogra *et al.*, 1994; Sharma *et al.*, 1997; Rout and Das, 1998; Sit *et al.*, 2005; Bhattacharya and Sen, 2006 and Behera and Sahoo, 2009
Dormant axillary buds of un-sprouted rhizomes	Mohanty *et al.*, 2008
Juvenile shoots	Ilahi and Jabeen, 1987
Leaf pseudostem	Ikeda and Tanaba, 1989
Shoot tips/meristem	Bhagyalakshmi and Singh, 1988; Inden *et al.*, 1988; Choi, 1991; Choi and Kim, 1991; Khatun *et al*., 2003; Lincy *et al.*, 2009 and Sultana *et al.*, 2009
Leaf tissue	Nirmal Babu *et al.*, 1992; Kackar *et al.*, 1993 and Sultana *et al.*, 2009
Immature inflorescence	Nirmal Babu *et al.*,1992
Ovary	Nirmal Babu *et al.*,1996
Anther	Samsudeen *et al.*, 2000
Curcuma longa	
Shoot bud/rhizome bud	Shetty *et al.*, 1982; Yasuda *et al.,* 1988; Keshavachadra and Khader, 1989; Winnar and Winnar, 1989; Balachandran *et al.*, 1990; Sunitibala *et al*., 2001; Salvi *et al.*, 2002; Zapata *et al.*, 2003; Nayak, 2004; Rahaman *et al*., 2004; Gayatri and Kavyashree, 2005; Prathanturarug *et al.*, 2005; Tyagi *et al.*, 2007; Naz *et al.*, 2009 and Behera *et al.*, 2010
Dormant axillary buds of un-sprouted rhizomes	Singh *et al.*, 2011
Shoot tips/meristem	Nayak and Naik, 2006, Sunitibala *et al*., 2001
Leaf tissue	Salvi *et al.*, 2001
Immature inflorescence	Salvi, 2000
Ovary	Nirmal Babu *et al.*,1996
Anther	Samsudeen *et al.*, 2000
Kaempheria galanga	
Shoot bud/rhizome bud	Geetha *et al.*, 1997; Shirin *et al.*, 2000; Lakshmi and Mythili, 2003; Swapna *et al.*, 2004 and Chirangini *et al.*, 2005, Raha-man *et al.*, 2005, Kochuthressia *et al.*, 2012 and Bhattacharya and Sen, 2013
Leaf tissue	Swapna *et al.*, 2004 and Rahaman *et al.*, 2004

Various disinfecting chemicals like commercial bleach, 70% ethyl alcohol and mercuric chloride were used singly or in combinations in the culture of Zingibers. In all the cases the explants were thoroughly washed under running tap water and were trimmed. The buds were soaked in commercial bleach solution for few minutes. Followed by sterilization in Mercuric chloride (0.1% - 0.2%) for 2 - 10 minutes. The sterilant and time required for the regeneration of the members of Zingiberaceae depend on the genera and species.

2.10.3 Media and culture conditions

Successful growth and differentiation of excised plant tissues and organs are possible if they are supplied with nutrients required by it. An artificially prepared nutrient medium is called culture medium which is a is a mixture of several components like inorganic salts, vitamins, aminoacids, sugars, growth regulators and solidifying agents if required. The minerals present in the plant tissue culture medium are used by the plant cell as building blocks for the synthesis of organic molecules or as catalysts. The ions of different salts play an important role in transportation or osmotic regulation ad in maintaining the electrochemical potential of the plant.

The nutrient requirements for the growth of different plants are not the same. Even, it differs for the different organs of a same plant. Therefore, a single media is not suitable for optimum growth of all plant tissues. To overcome this, different nutrient solutions were proposed by different workers from time to time. Consequently the most suitable medium for a particular tissue must be determined by trial and error.

In the regeneration of the members of Zingiberacee, Murashige and Skoog's (1962) medium was used extensively with a few exceptions (table 2.3). In regeneration of *Zingiber ocfficinale* Murashige and Skoog medium (Hosoki and Sagawa, 1977; Ilahi and Jabeen, 1987; Bhagyalakshmi and Singh, 1988; Inden *et al*., 1988; Ikeda and Tanaba,1989; Choi and Kim, 1991; Nirmal Babu *et al.*, 1992; Kackar *et al*., 1993; Dogra *et al*., 1994; Sharma *et al*., 1995; Nirmal Babu *et al*., 1996; Sharma *et al*., 1997; Rout and Das, 1998; Samsudeen *et al*., 2000; Khatun *et al*., 2003; Sit *et al*., 2005; Bhattacharya and Sen, 2006; Mohanty *et al*., 2008; Lincy *et al*., 2009; Sultana *et al*., 2009; Behera and Sahoo, 2009; Villamor, 2010), Schenk

and Hildebrandt (PIllai and Kumar, 1982), Gamborg (Bhattacharya and Sen, 2006) and MS major elements, Ringe and Nitsch minor elements (Choi, 1991 and Malamug *et al.*, 1991). Villamor, 2010**,** studied the effects of MS media strength and sources of nitrogen on shoot and root growth Native variety of ginger. The results indicated that nitrogen in the form of KNO_3 significantly improved proliferation rate of ginger *in vitro*, in both full and half strength media. Leaf growth was better in media devoid of NH_4NO_3. Root formation was significantly better in media without NH_4NO_3.

For regeneration of *Curcuma*, Murashige and Skoog medium (Shetty *et al.*, 1982; Yasuda *et al*., 1988; Keshavachadra and Khader, 1989; Winnar and Winnar, 1989; Balachandran *et al*., 1990; Nirmal Babu *et al*.,1996; Salvi, 2000; Samsudeen *et al*., 2000; Sunitibala *et al.,* Shirgurkar *et al*., 2001; Salvi *et al*., 2001; Salvi, *et al*., 2002; Zapata *et al*., 2003; Islam *et al*., 2004; Nayak, 2004, Rahaman *et al*., 2004; Prathanturarug *et al*., 2005; Nayak and Naik, 2006; Tyagi *et al*., 2007; Naz *et al*., 2009; Behera *et al*., 2010; Singh *et al*., 2011) and LSBM (Gayatri and Kavyashree, 2005) were used.

In regeneration of *Kaempheria*, Murashige and skoog medium (Geetha *et al*., 1997; Shirin *et al*., 2000; Lakshmi and Mythili, 2003; Swapna *et al*., 2004; Rahaman *et al*., 2004; Chirangini *et al*., 2005; Rahaman *et al*., 2005; Kochuthressia *et al*., 2012; Bhattacharya and Sen, 2013) were used. Shirin *et al.* (2000), achieved *in vitro* plantlet production on 0.75 X MS medium.

In the cultures of the members of Zingiberaceae pH of 5.8 were generally maitained in almost all of the cultures. Rout and Das (1998), in their experiment to assess the optimum pH found that shoot bud regeneration of ginger was highest at pH 5.7 - 5.8 and under 24 hour illumination.

Sugar as a carbon source is mainly supplied in the form of sucrose. Sucrose was added at a cocentration of 3% (w/v) in most of the experiments with Zingiberous members (Bhattacharya *et al*., 2014). In regeneration of *Zingiber officinale* 2% sucrose (Choi, 1991), 1% to 4% (Bhattacharya and Sen, 2006) were used. Bhattacharya and Sen (2006) found that 3% sucrose was best for the regeneration of *Zingiber officinale*. In C*urcuma longa* Salvi *et al.* (2002)

tested different carbohydrates like sucrose, fructose, glucose, sugar cubes, maltose, levulose, market sugar, xylose, rhamnose, lactose and soluble starch. They found xylose, rhamnose, lactose and soluble starch were inhibitory in producing shoots. Tyagi *et al.* (2007) replaced laboratory reagent-grade sucrose by locally available commercial sugar (market sugar or sugar cubes) as carbon source and observed no adverse effects on shoot regeneration. In regeneration of related genera of the family, 3% and 10% Sucrose were used in the medium for induction of callus and shoots regeneration of *Mantisia wengeri* respectively (Bhoumick *et al*., 2010). 3% - 7% sucrose and 5% sucrose was used for breaking dormancy of the axilary buds and subculture respectively in the culture of *Costus speciosus* (Punyarani and Sharma, 2010).

The media was solidified with 0.7 to 0.8% (w/v) agar in almost all the cultures of zingiberous plants with a few exceptions. In regeneration of

Table 2.3: Media used for *in vitro* regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*

Media	Reference
Zingiber officinale	
Murashige and skoog (1962)	Hosoki and Sagawa, 1977; Ilahi and Jabeen,1987; Bhagyalakshmi and Singh, 1988; Inden *et al*., 1988; Ikeda and Tanaba, 1989; Choi and Kim, 1991; Nirmal Babu *et al.*, 1992; Nirmal Babu *et al.*, 1992; Kackar *et al.,* 1993; Dogra *et al.*, 1994; Sharma *et al.,* 1995; Nirmal Babu *et al.,* 1996; Sharma *et al.*, 1997; Rout and Das, 1998; Samsudeen *et al.,* 2000; Khatun *et al*., 2003; Sit *et al*,. 2005; Bhattacharya and Sen, 2006, Mohanty *et al.*, 2008; Lincy *et al.*, 2009; Sultana *et al.*, 2009; Behera and Sahoo, 2009 and Villamor, 2010
Schenk and Hildebrandt	PIllai and Kumar, 1982
Gamborg	Bhattacharya and Sen, 2006
MS major elements, Ringe and Nitsch minor elements	Choi, 1991 and Malamug *et al.*, 1991.
Curcuma longa	
Murashige and skoog (1962)	Shetty *et al.*, 1982; Yasuda *et al.,* 1988; Keshavachadra and Khader 1989; Winnar and Winnar, 1989; Balachandran *et al*., 1990; Nirmal Babu *et al.,* 1996; Salvi, 2000; Samsudeen *et al.,* 2000; Sunitibala *et al.*, 2001; Shirgurkar *et al*., 2001; Salvi *et al.,* 2001; Salvi *et al.*, 2002; Zapata *et al.*, 2003; Islam *et al.*, 2004; Nayak, 2004; Rahaman *et al*., 2004; Prathanturarug *et al.,* 2005; Nayak and Naik, 2006; Tyagi *et al.*, 2007; NAZ *et al*., 2009; Behera *et al.*, 2010 and Singh *et al.*, 2011
LSBM	Gayatri and Kavyashree, 2005
Kaempferia longa	
Murashige and skoog (1962)	Geetha *et al.*, 1997; Shirin *et al.*, 2000; Lakshmi and Mythili, 2003; Swapna *et al.*, 2004; Swapna *et al.*, 2004; Rahaman *et al.,* 2004; Chirangini *et al.*, 2005; Rahaman *et al.*, Kochuthressia *et al.*, 2012; Bhattacharya and Sen, 2013

Zingiber officinale, 0.5% (Khatun *et al*., 2003), while in *Curcuma longa* 0.4% and 0.6% agar was successfully used for gelling the media (Salvi *et al.*, 2002). Tyagi *et al.* (2007) replaced agar by isabgol as gelling agent in cultures of *Curcuma longa*. In the cultures of the other members of the family, 0.75 % agar was extensively used (Bhattacharya *et al*., 2014).

Many researchers prefer to call plant hormones as plant growth substances or plant growth regulators. Plant hormones added to plant tissue culture media are taken up and increase the level within the tissue. Most of the increase is however, transient because plant hormones are rapidly inactivated after uptake. Usually only very small amounts of the applied hormones remain the free form. It has been seen that, for auxins, equilibrium exists between the free and conjugated form, of which only less than 1% being present in the freeform. The effect of hormones not only depends on the rate of uptake from the medium, or on the stability in the medium and in the tissue, but also on the sensitivity of the target tissue (Razdan, 1993).

The main plant growth regulators used in tissue culture are auxins (indole-3-acetic acid, indole-3-buturic acid, 1-napthaleneacetic acid, 2,4-dichlorophenoxyacetic acid, picloram etc); cytokinins (zeatin, 6-benzylamino purine, kinetin, thidiazuron etc); gibberellins (GA_1, GA3, GA_4, GA_7 etc); abscissic acid; ethylene etc. A list of the plant species and the plant growth regulator used for its regeneration are provided in table 2.4. In the regeneration of Zingibers plant growth regulators like thidiazuron (TDZ), 6-benzylamino purine (BA/ BAP), kinetin (kn), 1-napthaleneacetic acid (NAA), indole-3-acetic acid (IAA), TRIA, 2,4-dichlorophenoxyacetic acid (2,4-D) etc were extensively used. Organic additives like coconut water were used as a supplement in some media for the regeneration of the Zingibers.

Incubation condition is very important for micropropagation. High temperature is likely to lead to dissociation of the culture media and tissue damage while very low temperature tissue growth is slow. Moreover some tissue grows in dark while other prefers light conditions. The amount of light also has substantial effect on the regeneration.

2.10.4 Regeneration of plantlets through callusing

Callus tissue is an unorganized and

undifferentiated proliferated mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium in glass vials under controlled experimental conditions. Formation of callus tissue is the outcome of cell expansion and cell division of the cells of the explants.

Initiation of callus culture is done with juvenile parts like leaf, stem segment, roots etc containing meristematic tissue. Such tissue has a pre-existing growth momentum. On implantation, the merstematic tissue absorbs the exogeneously supplied nutrients and growth regulators; divide asynchronously to form the unorganized mass of tissue. During the initial growth phase the cells enlarge or swell to rupture. This indicates the response of tissue to the medium for callus formation. Some endogeneous growth substances oozes out through the injured tissue through the cut end and stimulates the cell division along with the penetration of the exogeneously supplied hormone and nutrients. The unorganized callus tissue gradually increases in size and ultimately the whole part of the explants starts to divide (Bhattacharya *et al*., 2014).

In tissue culture of *Zingiber officinale*, callusing was achieved by Pillai and Kumar (1982) on Schenk and Hildebrandt liquid medium containing different concentrations of hormone but the calluses were unable to proliferate. Later on Ilahi and Jabeen (1987) succeeded to regenerate plantlets from callus supplementing various concentrations of 2,4-D and BA. They were able to produce callus from juvenile shoots of ginger but failed to produce callus from stem explants on MS medium containing different concentrations of bioregulators. Choi (1991) observed that both shoots and roots formation took place on medium containing NAA (0.1- 1.0 ppm) and BA (1.0 ppm). Shoot tips of ginger cv. Kintoki were used to induce callus on medium containing MS major elements, Ringe and Nitsch minor elements and organic additives, sucrose (2 %), agar (0.8 %) with various concentrations of 2, 4-D (0.5 mg/l) on combination with BA (1 mg/l). They found that shoot regeneration also occurred in the same medium but best shoot regeneration occurred on media containing BA (5 mg/l) and NAA (1mg/l). Leaf tissues of ginger cultivar Maran produced callus on revised MS medium with

Table 2.4: Plant growth regulators (PGR) used for *in vitro* regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*

PGR and other additives	Reference
Zingiber officinale	
BA/BAP	Hosoki and Sagawa, 1977, Rout and Das 1998 and Sit *et al.*, 2005
Kn	Sharma *et al.,* 1997
NAA	Dogra *et al*., 1994
2,4-D	Lincy *et al*., 2009
BAP/BA and Kn.	Khatun *et al.,* 2003
Kn and NAA	Sharma *et al.,* 1997
BA/BAP and NAA	Inden *et al*., 1998; Ikeda and Tanaba, 1989; Choi and Kim, 1991; Dogra *et al*., 1994; Behera and Sahoo, 2009
BA/BAP and 2,4-D	Ilahi and Jabeen, 1987; Choi, 1991; Nirmal Babu *et al*., 1992; Samsudeen *et al.*, 2000
2,4-D, BA/BAP and NAA	Malamug *et al*., 1991; Nirmal Babu *et al*., 1992; Nirmal Babu *et al.*, 1996
BA/BAP, Kn and Zn	Bhattacharya and Sen, 2006
BA/BAP, IAA, and Adenine sulfate	Mohanty *et al*., 2008
2,4-D, IAA, NAA and Dicamda	Kackar *et al.,* 1993.
BA/BAP, Calcium pantothenate, GA_3 and NAA	Sharma *et al., 1995*
Dicamba, 2,4-D, Kn, BA/BAP and IBA	Sultana *et al*., 2009
BA/BAP, Coconut milk, AA, Glutamine & Activated charcoal	Bhagyalakshmi and Singh 1988
Curcuma longa	
BA/BAP	Nadgauda *et al.*, 1978; Winnar and Winnar, 1989; Prathanturarug *et al.*, 2005; Nayak and Naik, 2006; Tyagi *et al*., 2007; Singh *et al*., 2011
2,4-D	Samsudeen *et al.*, 2000
BA/BAP and Kn	Shetty *et al.,* 1982; Nayak, 2004; Keshavachadra and Khader, 1989,
NAA and Kn	Yasuda *et al.,* 1988
NAA and BA/BAP	Behera *et al*., 2010
BA/BAP, Kn and Coconut water	Shirgurkar *et al.*, 2001
2,4-D and BA/BAP	Nirmal Babu *et al.,* 1996; Gayatri and Kavyashree, 2005; Zapata *et al*., 2003
BA/BAP and Kn, and NAA	Islam *et al*., 2004
BA/BAP, NAA and TDZ	Naz *et al*., 2009
BA/BAP, 2,4-D, NAA and Kn	Sunitibala *et. al.,* 2001
BA/BAP, AA, IBA and IAA	Rahaman *et al.,* 2004
BA/BAP, 2,4-D, NAA, TDZ and IAA	Salvi, 2000;
Dicamba, picloram, NAA, BA/BAP, Kn, TIBA and 2-4-D	Salvi *et al*., 2001
BA/BAP, Kn, kn-riboside, Zn, 6-,-dimethylallylaminopurine, Adenine, Adenine sulfate or Metatopolin and NAA	Salvi, 2002
Kaempheria galanga	
BA/BAP and NAA	Shirin *et al.*, 2000
2-4-D and BA/BAP	Lakshmi and Mythili, 2003
BA/BAP and Kn	Kochuthressia *et al*., 2012
Kn, NAA and BA/BAP .	Geetha *et al*., 1997
IAA, Kn and BA/BAP	Chirangini *et al*., 2005
BA/BAP, Kn and Zn	Bhattacharya and Sen, 2013
IAA, BA/BAP, NAA, 2-4-D and Kn	Swapna *et al*., 2004
NAA, IBA, IAA, BA/BAP and Kn.	Rahaman *et al*., 2005
BA/BAP, IAA, IBA, NAA and adenine sulphates	Parida *et al*., 2010

high concentrations of 2, 4-D (9.0–2.6 µM/l) and plantlet formation occurred at considerable low concentrations of 2, 4-D (0.9 µM/l) in combination with BA (44.4 µM/l) (Nirmal Babu *et al.*, 1992). It was interestingly noted that the rate of plant regeneration increased when the bioregulators were completely removed from the medium in the subsequent subcultures. Nirmal Babu *et al.* (1996) observed the same result when they found that the embroyoid formation was more pronounced after the removal of growth regulators from the culture medium after initial embryogenesis. Development of embryos from the ginger callus cv. Eruttupetta on MS medium was done by Kackar *et al.* (1993). They used various concentrations of auxins like 2, 4-D. IAA, NAA and dicamda. They found dicamda at 2.7 µM/l was most effective to produce embryos whereas IAA, AA failed to produce embryos. The embryonic culture produced plantlets on MS medium containing BA (8-9 µM/l) and plantlets were successfully transferred to the soil. Dogra *et al*. (1994), cultured callus derived from different plant parts of ginger for regeneration of plants on different media containing different concentrations of bio-regulators). Samsudeen *et al.* (2000), regenerated ginger from anther derived callus culture of ginger. They collected anthers at the uninucleate microspore stage and the anthers were subjected to a cold treatment (0º) for 7 days. The treated anthers were induced to develop profuse callus on MS medium supplemented with 2-3 mg/l 2,4D. Plantlets were regenerated on MS media supplemented with 5-10 mg/l BAP 0.2 mg/l 2,4D.

In *Curcuma longa* tissue culture Yasuda *et al.* (1988) was able to regenerate shoots from rhizome explants on MS medium containing NAA (1 ppm) and kinetin (0.1 ppm). Sufficient number of shoots was obtained from the callus when media was supplemented with NAA (0.1 PPM) or BA (0.3 PPM). Sunitibala *et al.* (2001) observed multiplication and callus induction starting from the rhizome buds and shoot on MS medium. A concentration of 2.5-3.0 mg/l of 2,4-dichlorophenoxy-acetic acid (2,4-D) was found to be optimum for callus induction. Regeneration of plantlets from a callus was successfully conducted in MS medium supplemented with standard growth hormones. Salvi *et al.* (2001), initiated

callus cultures from leaf bases of turmeric on Murashige and Skoog's basal medium supplemented with dicamba, picloram (2 mg/l) or 1-naphthaleneacetic acid (5 mg/l) in combination with benzyladenine (0.5 mg/l). On transfer of callus cultures to medium supplemented with benzyladenine (5 mg/l) in combination with triiodebenzoic acid or 2,4-dichlorophenoxyacetic acid (0.1 mg/l), green shoot primordia were seen to differentiate from the surface of the callus. On transfer of regenerating cultures to half MS media supplemented with Kn, shoot primordia developed into well developed shoots. When shoots were transferred to medium devoid of phytohormones, complete rooted plants were obtained.

Regeneration of *Kaempferia galanga* by callusing and somatic embryogenesis was initiated by Vincent *et al.*, 1991 and 1992) from the leaf base-derived callus cultures. Lakshmi and Mythili (2003), obtained plantlets via somatic embryogenesis in callus derived from rhizome bud explants. They induced callus in MS medium supplemented with 1 mg/l 2,4 -D and 0.5 mg/l BAP. Somatic embryogenesis was observed when the calluses were subcultured onto a medium containing 0.5 mg/l 2,4-D, 0.2 mg/l BAP and 16.336 mg/l tryptophan (80 μM). Plantlets were formed when the embryogenic calluses were subcultured in MS liquid or solid medium without hormones and also in media containing 3 mg/l and 4 mg/l BAP alone or 2 mg/l BAP with 0.5 mg/l kinetin or 0.5 mg/l IAA. They also encapsulated somatic embryos in calcium alginate beads to study the regeneration potential of such synthetic seeds. Swapna *et al.* (2004), cultured leaf and rhizome explants of *Kaempferia* on MS medium with various combinations of indole-3-acetic acid, benzyl amino purine, napthalene acetic acid, 2-4-dichlorophenoxy acetic acid and kinetin at concentrations ranging from 0.5 to 2.5 mg/l. They observed high-frequency organogenesis and multiple shoot regeneration was induced from rhizome explants on MS medium supplemented with 0.5 mg/l of IAA and 2.5 mg/l of BAP. Rahaman *et al.* (2004) regenerated plantlets through somatic embryogenesis from leaf-base derived callus culture of *Kaempferia galanga* using different concentrations and combinations of growth regulators. The highest percentage of callus

induction was observed on MS medium supplemented with 2, 4-D (1.5 mg/l) and BA (1.0 mg/l). Globular embryos regeneration of plantlets was obtained on MS medium supplemented with BA (2.0 mg/l) and NAA (0.1 mg/ l). Chirangini *et al*. (2005) observed callus induction on the medium supplemented with 2.85 µM IAA from which microshoots was regenerated on 2.69 µM NAA and 2.22 µM benzyladenine-enriched medium.

2.10.5 Direct regeneration of plantlets

Hosoki and Sagawa (1977) reported direct regeneration of numerous adventitious shoots with roots by repeated subculture of rhizome buds of *Zingiber officiale* in media containing Murashigge and Skoog major salts and Ringe-Nitsch minor salts supplemented with 1ppm benzyladenine. They reported a maximum of 6 shoots per bud in *in vitro* culture. A maximum of 15 plantlets were obtained by Pillai and Kumar (1982), from single explants when cultured on Schenk and Hildebrandt liquid medium containing different concentrations of hormone. Shooting in the Wyad Giant variety of ginger was achieved in ¾ M S medium containing sucrose (6 %), coconut milk (20 %), ascorbic acid (100 mg/l), glutamine (400 mg/l), activated charcoal (250 mg/l) and BA (0.4 mg/l) Bhagyalakshmi and Singh (1988). They showed that meristem derived shoot exhibits consistent multiplication on ¾ strength of MS medium containing sucrose (3 %), ascorbic acid (100 mg/l), activated charcoal (100 mg/l) and BA (5 mg/l). They observed that incorporation of kinetin and NAA at various levels with or without BA and IBA, neither improved plantlet formation nor enhanced shoot multiplication. The efficacy of direct *in vitro* regeneration of ginger can be assessed by the comment- 'one explanted shoot tip produced more than 4 shoots within 3 weeks and a huge quantity of plantlets which is estimated to be about 750,000 or more can be regenerated from a single explant within one year on MS medium supplemented with BA (5 mg/ l) and NAA (0.5 mg/l)' (Inden *et al*., 1988). Tissue culture technique could significantly contribute towards maximizing the use of high quality rhizomes for *in vitro* propagation (Ikeda and Tanaba, 1989). They cultured leaf pseudostem and decapitated crown sections of ginger on MS medium containing various concentrations of BA and NAA. The

pseudostems cultured on solid medium supplemented with BA (1 µM/l) and NAA (0.6 µM/l) produced on an average 5 shoots and 15.3 roots. Whereas decapitated crown sections cultured in liquid medium with BA (11µM/l) produced on an average 10 shoots and 16.3 roots under 16 hr fluorescent light. Choi and Kim (1991), obtained maximum number of shoots by culturing shoot apex of ginger on MS medium containing NAA (0.5 mg/l) and BA (5.0 mg/l). Inflorescence of ginger cv. Maran was cultured by (Nirmal Babu *et al*., 1992) for regeneration of plants on MS medium containing BA 10 mg/l and 2,4-D (0.2 mg/l). The inflorescence produced vegetative bud without callus formation. BA (2.5 mg/l) and NAA (0.5 mg/l) was more effective to produce more number of shoots from rhizome buds of ginger when on MS medium (Dogra *et al.*, 1994). They found that the greatest umber roots were formed on MS medium containing supplemented with NAA (1 mg/l). Khatun *et al*. (2003), cultured aseptic shoot tips of ginger on MS media containing 3% sucrose, different concentrations and combinations of hormones along with 0.5% agar. They obtained 22 to 25 ginger plantlets from each shoot tip explant in the medium supplemented with 2.5 mg/l BAP and 0.5mg/l Kinetin.

Sit *et al.* (2005) regenerated ginger cv Garubathan by using sprouted bud in MS medium using different concentrations of BAP. They obtained maximum number of viable shoots and roots when the buds were treated with 0.1% $HgCl_2$ as surface sterilant for 15 minutes. It was observed that with the increase of BAP concentration, the multiplication rate increased of shoots increased up to a certain level and than declined. The rate of shoot multiplication was maximum at BAP 4 mg/l. Bhattacharya and Sen (2006) achieved *in vitro* regeneration of disease-free plantlets through tissue culture. They used Murashige and Skoog and Gamborg media supplemented with different concentrations and combinations of cytokinins. Media supplemented with 4 mg/l benzyl amino purine (8.33 shoots/explant) provided the best regeneration compared to kinetin and zeatin when they were used alone. Combination of 4 mg/l BAP and 3 mg/l Kn (9.60) resulted in maximum number of shoots in their experiment. Profuse rooting was observed in the same media.

Mohanty *et al.* (2008) developed a protocol for *in vitro* propagation of ginger (*Zingiber officinale*) cv. Suprava using dormant axillary buds from unsprouted rhizomes. The dormant axillary buds embedded in the rhizome nodes were induced to sprout when cultured on MS medium supplemented with 6-benzyladenine alone or with a combination of and indole-3-acetic acid. MS basal medium supplemented with BA (1 mg/l), IAA (1 mg/l) and adenine sulfate (100 mg/l) was found optimum for the *in vitro* multiplication of shoots producing shoots from a single explant within 30 days of culture. The multiplication rate remained unchanged in subsequent subcultures. Microropagation of *Zingiber officinale* Rosc. cv, Suprava and Suruchi using fresh rhizome sprouting bud was conducted by Behera and Sahoo (2009) on Murashige and Skoog's medium supplemented with different concentrations and combinations of BAP and NAA for shoot and root induction. The explants cultured on MS basal medium supplemented with 2.0 mg/l BAP + 0.5gm/l NAA showed the highest rate of shoot multiplication.

Direct *in vitro* regeneration of *Curcuma longa* was reported by Shetty *et al.* (1982) and Keshavachadra and Khader (1989). They cultured sprouting buds of turmeric on MS media. They found that MS medium containing kinetin (1 mg/l) and BA (1 mg/l) were best for multiplication. Winnar and Winnar (1989) obtained 8 multiple shoots from a single explant when sprouting buds were cultured on MS medium containing BA (1 mg/l). Balachandran *et al*. (1990) used rhizome buds of different *Curcuma* species for culture on MS medium. The medium was supplemented with different concentrations of bioregulators. They found that BA (3 mg/l) was optimum for shoot multiplication for all the species. Rhizome buds of several species of *Curcuma* were cultured, on MS medium with varying levels of BAP and kinetin by Balachandran *et al.* (1990), to produce multiple shoots. They found that a concentration of 3.0 mg/l BAP was optimum for shoot multiplication in all the species. Salvi *et al*. (2000), regenerated plantlets from immature inflorescence of *Curcuma longa* by direct shoot development on Murashige and Skoog's basal medium supplemented with BA (5 or 10 mg/l) in combination with 2,4-D or NAA and TDZ in

combination with IAA. Sunitibala *et al.* (2001) observed multiplication and callus induction starting from the rhizome buds and shoots on MS medium. A combination of naphthalene acetic acid (1.0 mg/l) with kinetin (1.0 mg/l) or NAA (1.0 mg/l) with 6-benzylaminopurine (2.0 mg/l) was optimum for rapid clonal propagation of turmeric. Salvi *et al.* (2002), proposed a protocol for *in vitro* propagation of turmeric cv `elite' using young vegetative buds from sprouting rhizomes. The shoot buds produced multiple shoots when cultured on MS solid medium supplemented with benzyladenine and 1-naphthalene acetic acid. The effect of various cytokinins on shoot multiplication was studied by culturing the shoot tips on MS liquid medium supplemented with benzyladenine, benzyladenine riboside, kinetin, kinetin riboside, zeatin, 6-dimethylallylaminopurine, adenine, adenine sulfate or metatopolin each at 10 M in combination with 1-naphthalene acetic acid (1 M). Significant differences were observed between the treatments. Liquid medium was more favourable than agar medium for shoot multiplication. Among the various concentrations of agar tested, 0.4% and 0.6% were the best and produced the highest number of shoots per explant. Among the different carbohydrates tested, sucrose, fructose, glucose, sugar cubes, maltose, levulose and market sugar were found to be equally effective for shoot multiplication and xylose, rhamnose, lactose and soluble starch were inhibitory. Rahaman *et al*. (2004), regenerated multiple shoots from rhizome derived cultures of turmeric on MS medium supplemented with BA (2.0 mg/l). They experimented rooting in half strength MS medium supplemented with various concentrations of AA, IBA and IAA. It was observed that 0.1–1.0 mg/l concentrations of any auxin was found to be effective but IBA was the best. Nayak (2004), achieved shoot multiplication and plant regeneration from freshly sprouted shoots of *Curcuma aromatica* on Murashige and Skoog's medium supplemented with BA alone (1–7 mg/l) or a combination of BA(1–5 mg/l) and Kn (0.5–1 mg/l). A concentration of 5 mg/l BA was found to be optimum for shoot multiplication and rooting of shoots. Prathanturarug *et al*. (2005) were successful in rapid micropropagation of *Curcuma longa* using bud explants pre-cultured in thidiazuron-

supplemented liquid medium. Naz *et al*. (2009) standardized a rapid propagation and acclimatization method of three different varieties of turmeric (Faisalabad, Kasur and Bannun) using rhizome bud explants on MS medium supplemented with different concentrations and combinations of cytokinin and auxins. The frequency of shoot induction was 70, 60 and 75 in Faisalabad, Kasur and Bannun varieties respectively. The number of shoots per explant increased with increased BAP concentration while shoot length decreased. Behera *et al.* (2010) developed a high frequency *in vitro* plantlet regeneration method for *Curcuma longa* L. (cv.Ranga) using fresh sprouting rhizome bud on semisolid Murashige and Skoog's medium supplemented with different concentration and combinations of BAP and NAA for shoot and root induction. Explants cultured on MS basal medium supplemented with 2.0mg/l BAP+0.5gm/l NAA showed highest rate of shoot multiplication. Singh *et al.* (2011) proposed a protocol for in vitro micropropagation of an elite genotype of turmeric (cv. suroma) using latent axillary bud explants from unsprouted rhizome, available throughout the year. MS media containing 3 mg/l 6-Benzyladenine and 1 mg/l Indole Acetic acid was found optimum for regeneration, multiplication and *in vitro* conservation of plantlets.

Geetha *et al.* (1997) standardised protocols for micropropagation of *Kaempferia galanga* by young sprouting buds on Murashige and Skoog basal medium supplemented with 0.5 mgl-' kinetin and 1.5% sucrose solidified with 0.7% agar. In regeneration of *Kaempferia galanga* using rhizomes as explants by Shirin *et al.* (2000), various concentrations of BAP and a range of auxins were used. They achieved *in vitro* plantlet production on 0.75 × MS medium supplemented with 12 μM BAP, 3 μM NAA and 3% sucrose. Rahaman *et al*. (2005), regenerated by using rhizome tips and lateral buds on MS medium supplemented with BA, Kn, NAA, IBA and IAA. 100 percent regeneration was obtained in MS medium supplemented with BA and NAA. Chirangini *et al*. (2005), induced microshoots of rhizomatous buds of *Kaempferia galanga* and *K. rotunda* when cultured on MS medium supplemented with plant growth regulators. Multiple shoots were

induced on MS medium containing 5.70 µM IAA alone and a combination of 0.57 µM IAA plus 4.65 µM kinetin in the case of *K. galanga*. On the other hand, the medium supplemented with 2.69 µM NAA plus 2.22 µM benzyladenine was the best for *K. rotunda*. Parida *et al.* (2010) worked on an efficient protocol for rapid multiplication and *in vitro* production of leaf biomass in *Kaempferia galanga*. They used different plant growth regulators like Benzyladenine, Indoleacetic acid, Indolebutyric acid, Napthaleneacetic acid and adenine sulphates for induction of multiple shoots using lateral bud of rhizome as explants. The highest rate of shoot multiplication (11.5 ± 0.6) shoot/ explant as well as leaf biomass production (7.4 ± 0.3) gram/explant was observed on Murashige and Skoog medium supplemented with Benzyladenine (1 mg/l) and Indoleacetic acid (0.5 mg/l). An protocol for multiple shoot induction of *Kaempferia galanga* using rhizome segment explants was established by Kochuthressia *et al.* (2012) on Murashige and Skoog medium supplemented with BA (2.0 mg/ml) and Kn (1.0 mg/ml) exhibited regeneration rate up to 10.85±1.34 shoot/explants.

2.10.6 Rooting of plantlets

In *Zingiber officinale*, spontaneous rooting were observed by Hosoki and Sagawa (1977); Sit *et al.* (2005); Bhattacharya and Sen (2006); Mohanty *et al.* (2008). Profuse rooting was observed in MS media supplemented with NAA (Nirmal Babu *et al.*, 1992; 1996 and Dogra *et al.*, 1994) while Behera and Sahoo (2009) observed that *in vitro* shootlets rooted best on to the half strength MS basal media supplemented with NAA.

Ikeda and Tanaba (1989) observed that pseudostems cultured on solid medium supplemented with BA and NAA produced an average number of roots. Whereas decapitated crown sections cultured in liquid medium with BA produced more number of roots. Choi (1991) reported that rooting of *in vitro* shootlets was enhanced by the addition of 2 gm activated charcoal per litre.

In *Curcuma longa* rooting was observed in MS media devoid of phytohormones (Salvi *et al.*, 2001) while Shetty *et al.* (1982), Keshavachadra and Khader (1989), Balachandran *et al.* (1990) and Nayak (2004) found that the plants rooted on the same medium with same hormonal

concentrations.

NAA for induction of roots were necessary (Salvi, 2000) and Behera *et al.* (2010) observed better rooting on half-strength MS basal media supplemented with NAA.

In *Kaempferia galanga*, Geetha *et al.* (1997) standardised protocols for production of multiple shoots and well developed roots in medium supplemented with 0.5mg/l naphthaleneacetic acid and 1.0 mg/l 6-benzylaminopurine. Swapna *et al.* (2004), observed simultaneous shooting and rooting on MS medium supplemented with IAA and BAP. Chirangini *et al*. (2005) observed that the microshoots produced roots irrespective of their method of regeneration while Kochuthressia *et al.* (2012) and Bhattacharya and Sen (2013) observed spontaneous rooting of shoots on the same medium used for shoot development.

2.10.7 Subculture of plantlets

Subculture of plantlets is an efficient method to increase the number of plantlets in very short time. During the process of subculture decrease in number of shoots per explant were observed in some cases while in others there were no decline in the number of shoots.

In tissue culture of *Zingiber officinale* decrease of proliferation rate on each subculture even after one year was not observed by Inden *et al*. (1988), Sharma *et al.* (1997) and Mohanty *et al*. (2008). But, Choi (1991) observed decrease in Shoot regeneration capability after third subculture while gradual decrease in the rate of plantlet production after second subculture was observed by Sit *et al.* (2005).

In *Curcuma longa* the regenerated shoots were further multiplied by sub culturing on fresh medium after 30 days (Naz *et al.*, 2009).

In *Kaempferia*, the number of shoots per explants gradually increased when the primary cultures were subcultured at two weeks interval (Rahaman *et al.*, 2005). But a constancy in the rate of multiplication and leaf biomass production of *Kaempheria* remained unchanged in subsequent subcultures when cultured on same media formulations (Parida *et al.*, 2010). Bhattacharya and Sen (2013) reported that the number of plantlets declined after one or two subcultures.

2.10.8 Microrhizome production

Microrhizomes are very convenient for packing and transportation besides its

advantage in germplasn conservation and exchange (Parthasarathy and Sasikumar, 2006). Thus this process has gained special importance in the biotechnology of Zingiberous crops.

In *Zingiber officinale*, Sharma *et al.* (1995) produced microrhizomes of from tissue culture derived shoots by transferring them to liquid MS medium supplemented with 8 mg/l BAP and 75 g/l sucrose. Microrhizome formation started after 20 days of incubation in stationary cultures at 25+1° in the dark. Rout *et al.* (2001) experimented cultural variations such as photoperiod, carbohydrate, nutrient composition, and growth regulators were tested for the maximum yield of microrhizomes. Among the different photoperiods used, a 24 hour photoperiod helped in the formation of more rhizomes when compared with other photoperiods. They achieved shoot multiplication of *Zingiber officinale* cv. V3S8 by meristem culture on MS basal medium supplemented with BA, IAA and adenine sulfate and 3% (w/v) sucrose. *In vitro* rhizome formation from *in vitro*-raised shoots was achieved on MS medium supplemented with BA, IA A, and 3-8% (w/v) sucrose after 8 week of culture

Shirgurkar *et al.* (2001) produced *in vitro* microrhizome in *Curcuma longa*. They found that half strength MS basal medium supplemented with 80 g/l sucrose was optimal for microrhizome production. Cytokinin like BAP had an inhibitory effect on microrhizome production with a total inhibition at concentration of 35.2 M. The microrhizome production depended on the size of the multiple shoots used. Sunitibala *et al.* (2001) incubated plantlets in a medium containing different concentrations of sucrose supplemented with NAA (0.1 mg/l) and Kn (1.0 mg/l) at 27 +/- 2 degrees C under an 8 h photoperiod for induction of rhizomes. *In vitro* rhizome formation was observed in media containing 6 and 8% sucrose. Microrhizomes were induced at the base of the *in vitro* derived shoots upon transfer to medium containing various combinations and concentrations of sucrose and BA and grown under varying photoperiods Nayak (2004). MS basal medium with mg/l BA, 60 g/l sucrose and an 8 hour photoperiod was optimum for induction of microrhizomes within 30 days of culture. Microrhizome formation was found to be controlled by the concentrations of BA and sucrose as well as photoperiod during

culture. Islam *et al*. (2004) published an improved *in vitro* microrhizome induction system in *Curcuma longa* L. Freshly sprouted axillary buds were used as initial explants and multiplied through established *in vitro* systems. Multiplied shoots were excised and subcultured on hormone free medium for four weeks to induce microrhizome. Effects of light, sucrose and growth regulators on *in vitro* microrhizome production have been studied. Nine per cent sucrose was found to be the most suitable for microrhizome production, when incubated in the dark. Various concentrations of BA and Kn, and NAA were tested. BA (12.0 µM) and NAA (0.3 µM) were found suitable for the induction of microrhizomes.

Nayak and Naik (2006), produced microrhizomes from tissue culture Rajan *et al.* (2005), reported *in vitro* microrhizome production on MS medium supplemented with BAP, NAA and ancymidol along with 10% sucrose. They derived shoots of *Curcuma longa* by transferring them to the liquid medium of Murashige and Skoog supplemented with 13.3mM BA and 6% sucrose, and culturing with a reduced photoperiod of 4 hours daily. The number of buds per microrhizome varied from 1-4 and the weight varied from 50 mg to 580 mg. They observed that factors such as concentration of sucrose and BA in the medium, as well as photoperiod and their interaction, were found to have a significant effect in the induction of microrhizomes. In their observation sucrose was most effective in rhizome formation, followed by photoperiod and BA in the medium.

Microrhizome formation was observed in *Kaempferia galanga* within one month of microshoot culture incubation in the medium supplemented with 6-9% sucrose with either 22.2 µM benzyladenine or 23.25 µM kinetin. These microrhizomes produced shoots when transferred to fresh microshoot induction medium within 2-3 weeks of incubation. The microshoots produced roots irrespective of their method of regeneration Chirangini *et al*. (2005).

2.10.9 Hardening of plantlets

One of the major obstacles in the application of tissue culture methods for plant propagation has been the difficulty in successful transfer of plantlets from the laboratory to the field (Wardle *et al.*, 1983). The reasons for such a difficulty appear to be related to the dramatic change in the

environmental conditions. The environment of the culture vessel is one of low light intensity, with very high humidity (generally 100%) and poor root growth, while the greenhouse and/or field conditions are typified by very high light intensity, low humidity and microflora (Desjardins *et al*., 1987). Several workers have developed protocols to overcome some of these constraints. These reasons for such a difficulty appear to be related to the dramatic change in the environmental conditions.

In hardening of regenerated tissue cultured plantlets of the family Zingiberaceae hardening of regenerated plantlets can be achieved very easily. Different hardening materials like peat moss and river sand, sterilized potting soil, vermiculite and soil, soil and compost, soil, sand, soil with mycorrhiza, soil without mycorrhiza, organic soil and sand etc have been used (table 2.5). The success rate of hardening depends upon the hardening material and the condition of the regenerated plantlet. High rate of survival of regenerated plantlets have been achieved in field in all the cases.

The *Zingiber officinale* plantlets were successfully transferred to a pot mixture containing peat: sponge rock: vermiculite in ratio of 2:1:1 (Hosoki and Sagawa, 1977), vermiculite medium supplemented with a solution containing half of normal concentration of MS medium (Inden *et al*., 1988), vermiculite medium (Choi, 1991), garden soil, sand and vermiculite in equal proportions (Samsudeen *et al.*, 2000) and garden soil and sand in the proportion of 1:1 (Bhattacharya and Sen, 2006). Varied survival rate of the hardened plantlets were reported. Low (Hosoki and Sagawa, 1977), 80% (Nirmal Babu *et al.*, 1992), 85% (Samsudeen *et al.,* 2000), 94 % (Bhattacharya and Sen, 2006) and 95% (Behera and Sahoo, 2009) survival was reported under field conditions. Khatun *et al*. (2003), achieved 100% and Mohanty *et al.* (2008) achieved 96% survival rate in field when the plantlets were directly transferred to the fields.

Keshavachadra and Khader (1989) transferred five weeks old culture of the rooted *Curcuma longa* to pots covered with polythene bags and kept under shade. Two weeks later the plants were well established. The *in vitro* raised plantlets of *Curcuma* were hardened in sterilized soil in paper cups (Salvi, 2000; Salvi *et al.*, 2002)

and Sand: Soil: Peat in ratio of 1:1:1 (Naz *et al.*, 2009). Hardened plantlets were successfully transferred to the field with 70 % (Rahaman *et al*., 2004), 90 % (Salvi *et al*., 2001), 90 to 95% (Salvi, 2000, Salvi *et al.*, 2002), high percentage (Zapata *et al.*, 2003), 90% (Gayatri and Kavyashree, 2005), 70-80% (Naz *et al.,* 2009) and 95% (Behera *et al.*, 2010) survival rate.

Geetha *et al*. (1997) hardened regenerated *Kaempferia* plantlets in mixture of Sand, garden soil and vermiculite (1:1:1). Hardened plantlets of *Kaempferia galaga* produced by callus culture showed normal storage roots and were acclimatized and subsequently transferred to field with 90-95% (Geetha *et al.*, 1997) and 85% survival (Rahaman *et al*., 2004) while regenerated plants by rhizome bud proliferation were hardened and established on the field with 85% success (Rahaman *et al.*, 2005). Field survival of 80-90% (Chirangini *et al*., 2005) and 95 % was observed by (Parida *et al.*, 2010).

2.10.10 Pathogen and nematode elimination

The elimination of nematode infection from the rhizomes of ginger has been reported by de Lange *et al*. (1987). Pathogen free *in vitro* derived ginger plants were free from ginger yellows (*Fusarium oxysporum* f. sp. *zingiberi*) were developed by Sharma *et al.* (1997). The well-developed rhizomes obtained from the tissue-cultured plants did not rot during storage of up to 6 months, thus indicating that the method is also effective in checking storage rot caused by *F. oxysporum* f. sp. *zingiberi.* Bhattacharya and Sen (2006) transferred rhizome pieces of ginger to PDA to observe fungal growth on the medium, visual observations on the presence of ginger yellows symptoms and detection of the number of rotted rhizomes after storage on river sand were performed to detect the presence or absence of the pathogen in tissue culture-derived clones.

Gayatri and Kavyashree (2005), isolated root rot disease tolerant clones of turmeric variety Suguna by using continuous *in vitro* selection technique against pure culture filtrate of *Pythium graminicolum*. Callus was challenged with pure culture filtrate of *P. graminicolum* to isolate viable callus within 30 days of culture, which was further subjected to pure culture filtrate treatment. After three cycles of treatment, they obtained, four cell lines which are tolerant to culture filtrate

was isolated through continuous *in vitro* selection and subcultured on regeneration medium fortified with BAP (4mg/l) along with the control non-selected callus to obtain complete plantlets through discontinuous *in vitro* selection technique. The data obtained from their experiment revealed a ratio of 225:49 tolerant: susceptible *in vitro* clones retrieved from tolerant callus.

2.11 Study of molecular diversity

Study of molecular diversity among the regenerated plantlets can provide suitable tool for crop improvement. Several strategies can be used to assess the genetic fidelity of in vitro derived clones, but most have limitations. Using the polymerase chain reaction (PCR) in conjugation with short primers of arbitrary sequences (Williams *et al*., 1990). Randomly amplified polymorphic DNA (RAPD) markers were shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al.*, 1991 and Roy *et al*., 1992).

2.10.1 Diversity study in zingibers

Nayak *et al.* (2005) assessed the genetic diversity of 16 cultivars of ginger using cytological and molecular markers. The variation among 16 promising cultivars on differential rhizome yield was proved to have a genetic basis using different genetic markers such as, 4C nuclear DNA content and random amplified polymorphic DNA. A significant variation of 4C DNA content was recorded in ginger at an intraspecific level with values ranging from 17.1 to 24.3pg. RAPD analysis revealed a differential polymorphism of DNA indicating genetic variations that can be significant in ginger improvement programs. Lee *et al*. (2007) reported isolation and characterization of eight polymorphic microsatellite markers for *Zingiber officinale.* A total of 34 alleles were detected across the 20 accessions, with an average of 4.3 alleles per locus. Values for observed and expected heterozygosities ranged from 0 to 1.0 and from 0.23 to 0.67, respectively. The heterozygote deficits were observed at three loci. At the significance threshold ($P< 0.05$) of the eight loci, seven were found to have deviated from Hardy–Weinberg equilibrium, whereas significant linkage disequilibria were observed between 10 pairs of loci. Their data indicates the existence of moderate level of genetic diversity among the ginger accessions genotyped with eight markers.

Syamkumar and Sasikumar (2007)

Table 2.5: Hardening material and survival rate of regenerated plantlets of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*

Potting mixtire	Survival rate	Reference
Zingiber officinale		
Peat: sponge rock: vermiculite (2:1:1)	Low	Hosoki and Sagawa, 1977
Vermiculite medium supplemented with 1/2 MS	—	Inden *et al.,* 1988
Vermiculite	—	Choi, 1991
Garden soil and sand (1:1)	80%	Nirmal Babu *et al.,* 1992
Garden soil, sand and farm yard manure (1:1:1)	80 %	Nirmal Babu *et al.,* 1996
Garden soil, sand and vermiculite (1:1:1)	85%	Samsudeen *et al.,* 2000
Not used i.e. direct field transfer	100%	Khatun *et al.,* 2003
Garden soil and sand (1:1)	—	Sit *et al.,* 2005
Garden soil and sand (1:1)	—	Sit and bhattacharya, 2007
Garden soil and sand (1:1)	94 %	Bhattacharya and Sen, 2006
Not used i.e. direct field transfer	96%	Mohanty *et al.,* 2008
Vermiculite	95%	Behera and Sahoo, 2009
Curcuma longa		
Sterilized soil	90 to 95%	Salvi, 2000; Salvi *et al.,* 2002; Salvi, 2001
Sterile soil and agrolite (1:1)	High	Zapata *et al.,* 2003
Not used i.e. direct field transfer	70 %	Rahaman *et al*., 2004
—	90%	Gayatri and Kavyashree, 2005
Sand : Soil : Peat (1:1:1)	70-80%	NAZ *et al.,* 2009
Vermiculite	95%	Behera *et al.,* 2010
Kaempferia galaga		
Sand, garden soil and vermiculite (1:1:1)	90-95%	Geetha *et al.,* 1997
Garden soil, compost and sand (2:2:1)	90%	Rahaman *et al.,* 2004
Soil and organic manure	85%	Rahaman *et al.,* 2004
—	85%	Rahaman *et al.,* 2005
Not stated in abstract	80-90%	Chirangini *et al*., 2005
Soil, cowdung and sand mixture (1:1:1)	95 %	Parida *et al.,* 2010
Garden soil and sand (1:1)	94	Bhattacharya and Sen, 2013

performed molecular genetic fingerprints of 15 *Curcuma* species using Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers to elucidate the genetic diversity/ relatedness among the species. Thirty-nine RAPD primers yielded 376 bands of which 352 were polymorphic and out of the 91 bands produced by the 8 ISSR markers, 87 were polymorphic. Dendrograms were constructed based on the unweighted pair group method using arithmetic averages. The maximum molecular similarity observed between two of the *Curcuma*

species namely *Curcuma raktakanta* and *Curcuma montana* is suggestive of the need for relooking the separate status given to these two species.

Jan *et al.* (2011) performed molecular genetic fingerprints of indigenous turmeric genotypes of Pakistan using Randomly Amplified Polymorphic DNA marker to elucidate the genetic diversity among them. Ten decamer-primers generated 95 RAPD fragments, of which 92 fragments were polymorphic with 96.84% of polymorphism. Amplified fragment sizes ranged from 200 to 3640 bp. Pair-wise Nei and Li's similarity coefficient value ranged from 0.00 to 0.71 for 20 genotypes of turmeric.

Singh *et al*. (2012) examined the genetic diversity among turmeric accessions from 10 different agro-climatic regions comprising 5 cultivars and 55 accessions. They used random amplified polymorphism DNA and inter simple sequence repeat to assess the genetic diversity in turmeric genotypes. RAPD analysis of 60 genotypes yielded 94 fragments of which 75 were polymorphic with an average of 6.83 polymorphic fragments per primer. Number of amplified fragments with RAPD primers ranged from 3 to 13 with the size of amplicons ranging from 230 to 3000 bp in size. The polymorphism ranged from 45 to 100 with an average of 91.4%. The 6 ISSR primers produced 66 bands across 60 genotypes of which 52 were polymorphic with an average of 8.6 polymorphic fragments per primer. The number of amplified bands varied from 1 to 14 with size of amplicons ranging from 200 to 2000 bp. The percentage of polymorphism using ISSR primers ranged from 83 to 100 with an average of 95.4%. Nei's dendrogram for 60 samples using both RAPD and ISSR markers demonstrated an extent of 62% correlation between the genetic similarity and geographical location. The result of Nei's genetic diversity generated from the POP gene analysis shows relatively low genetic diversity in turmeric accessions of South eastern ghat, Western undulating zone with 0.181 and 0.199 value whereas highest genetic diversity (0.257) has been observed in Western central table land.

Techaprasan *et al*. (2009) studied genetic variation and species authentication of 71 *Kaempferia* accessions found indigenously in Thailand were examined by determining chloroplast *psbA-trnH* and

partial *petA-psbJ* spacer sequences. Ten closely related species (*Boesenbergia rotunda*, *Gagnepainia godefroyi*, *G. thoreliana*, *Globba substrigosa*, *Smithatris myanmarensis*, *S. supraneanae*, *Scaphochlamys biloba*, *S. minutiflora*, *S. rubescens*, and *Stahlianthus* sp) were also included. After sequence alignments, 1010 and 865 bp in length were obtained for the respective chloroplast DNA sequences. Intraspecific sequence variation was not observed in *Kaempferia candida*, *K. angustifolia*, *K. laotica*, *K. galanga*, *K. pardi* sp nov., *K. bambusetorum* sp nov., *K. albomaculata* sp nov., *K. minuta* sp nov., *Kaempferia* sp nov. 1, and *G. thoreliana*. In contrast, intraspecific sequence polymorphisms were observed in various populations of *K. fallax*, *K. filifolia*, *K. elegans*, *K. pulchra*, *K. rotunda*, *K. marginata*, *K. parviflora*, *K. larsenii*, *K. roscoeana*, *K. siamensis*, and *G. godefroyi*.

2.12 Somaclonal variations

Somaclonal variation involves all forms of variation among regenerated plants derived from tissue culture (Larkin and Scowcroft, 1981; Jain *et al*., 1998; Jain and De Klerk, 1998) such as:

i) physical and morphological changes in undifferentiated callus,

ii) differences in the ability to organize and form organs in vitro,

iii) changes manifested among differentiated plants, and

iv) chromosomal changes.

Somaclonal variation has been reviewed at length (Scowcroft and Larkin, 1988), and has proven useful in plant improvement (Jain *et al*., 1998; Jain and De Klerk, 1998) and could be of much interest to the horticultural breeders. In Chrysanthemum, little variation is observed in plants derived from shoot tips (Khalid *et al*., 1989). Most of the variation is observed in plants originating from protoplasts, which is termed as protoclonal variation (Kawata and Oono, 1997; Jain, 1997; Jain and De Klerk, 1998). Plants regenerating from unorganized callus vary more than those from organised callus, whereas no or hardly any variation occurs when plants are regenerated directly without anintermediate callus phase (Bouman and De Klerk, 1996). Exploitation of somaclonal variation through callus culture might become a source for new cultivars if this method is combined with strategic and efficient *in vitro* selection pressures (Gudin and

Mouchotte, 1996). The selected somaclones should be genetically stable in seed and vegetatively propagated crops for routine induction of genetic variability through tissue culture, and this aspect should be thoroughly checked before using them in regular crop improvement programs. Somaclonal variation is unpredictable in nature and can be both heritable (genetic) and non-heritable (epigenetic) in regenerated plants. DNA methylation causes genetic instability in somaclones, which probably comes from epigenetic changes (Jain, 2001). Since somaclonal variation can broaden the genetic variation in number of crop plants, a broader range of plant characteristics can be altered, including plant height, yield, no. of flower/plant, early flowering, resistance to diseases, insects and pests and salt. The reduction, and even the total loss of regeneration ability, is a general phenomenon observed during undifferentiated cell culture. The somaclonal variation creates problem for micropropagators by the production of off-types in clonally propagated plants. This can be controlled by reducing the subcultures and the age of the cultures, depending on the plant species. Hirochika *et al*. (1996) reported that certain types of retrotransposons are activated as the tissue cultures get older and the regenerated plants show an increase in retrotransposon copy numbers leading to off-types.

Plantlets derived from *in vitro* culture might exhibit somaclonal variation (Larkin and Scowcroft, 1981) which is often heritable (Breiman *et al*., 1987). Other reports claim that useful morphological, cytological, and molecular variations may be generated *in vitro* (Larkin *et al*., 1989). Any system which significantly reduces or eliminates tissue culture generated variations can be of much practical utility. The variations may be due to several factors (Vasil, 1987 and 1988), such as genotypes used (Breiman *et al*., 1987), pathways of regeneration, and parameters employed for assessing the effect of *in vitro* culture, such as gross morphology and cytology (Swedlund and Vasil, 1985), field assessment, and molecular studies (Breiman *et al*., 1989; Chawdhury *et al*., 1994; Shenoy and Vasil, 1992).

Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones, but most have limitations (table 2.6). Karyological analysis, for

example, cannot reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al*., 1993). Isozyme markers provide a convenient method for detecting genetic changes, but are subject to ontogenic variations. They are also limited in number, and only DNA regions coding for soluble proteins can be sampled. Using the polymerase chain reaction (PCR) in conjuction with short primers of arbitrary sequence (Williams *et al*., 1990), randomly amplified polymorphic DNA (RAPD) markers were shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al*., 1991 and Roy *et al*., 1992). RAPD markers have been used successfully to assess genetic stability among somatic embryos in spruce species (Isabel *et al*., 1993 and 1996) and among micropropagated plants of poplar (Rani *et al*., 1995).

Balachandran *et al.* (1990) reported morphologically uniform *in vitro* raised plantlets. Rout *et al.* (1998), used random amplified polymorphic DNA markers to evaluate the genetic stability of micropropagated plants of *Zingiber officinale* cv. V3S18. They used fifteen arbitrary decamers to amplify DNA from *in vivo* and *in vitro* plant material to assess the genetic fidelity. All RAPD profiles from micropropagated plants were monomorphic and similar to those of field grown control plants. No variation was detected within the micropropagated plants.

The genetic stability of micropropagated clones of ginger was evaluated by Mohanty *et al.* (2008) at regular intervals of 6 months up to 24 months in culture using cytophotometric estimation of 4C nuclear DNA content and random amplified polymorphic DNA analysis. Cytophotometric analysis revealed a unimodal distribution of the DNA content with a peak corresponding to the 4C value (23.1 pg), and RAPD analysis revealed monomorphic bands showing the absence of polymorphism in all fifty regenerants analyzed, thus confirming the genetic uniformity.

Random Amplified Polymorphic DNA analysis of eight regenerated plants of turmeric 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 (Salvi *et al.,* 2001). The

chances of somaclonal variations are more in case of callus regenerated plants than through direct *in vitro* regeneration. Micropropagated turmeric showing stable drug yielding potential also proved to have genetic basis of stability as revealed by RAPD based molecular profiling (Singh *et al*., 2011).

Parida *et al.* (2010) confirmed the genetic fidelity of the *Kaempferia galanga* regenerants by using random amplified polymorphic DNA marker.

2.13 Antioxidant studies

2.13.1 Reactive oxygen species

Oxygen is the vital for aerobic life processes. However, about 5% or more of the inhaled O_2 is converted to reactive oxygen species (ROS) such as O_2^-, H_2O_2, and ·OH by univalent reduction of O_2 (Bandyopadhyay *et al.*, 1999). Thus cells under aerobic condition are always threatened with the insult of ROS, which however are efficiently taken care of by the highly powerful antioxidant system of the cell without any untoward effect. When the balance between ROS production and antioxidant defense is lost 'oxidative stress' results which through a series of events, deregulates the cellular function leading to various pathological conditions. The free radical mediated oxidative stress results in oxidation of membrane lipoproteins, glycoxidation and reduction of DNA resulting cell death. Free radicals have been implicated as the cause of several diseases such as liver cirrhosis, atherosclerosis, cancer, diabetes etc. and compounds that can scavenge free radicals have great potential in ameliorating these disease processes (Wilson, 1998). Many plant extracts and phytochemicals have been shown to have antioxidant/free radical scavenging properties (Larson, 1988

Table 2.6: Somaclonal variations *in vitro* regenerated plantlets of of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*

Plant species	*In vitro* regeneration	Technique used	Somaclonal variation	Reference
Zingiber officinale cv. V_3S_{18}	—	RAPD	Not found	Rout *et al.,* 1998
Curcuma longa	Callus mediated	RAPD	Found	Salvi *et al.*, 2001
Curcuma longa cv 'elite'	—	RAPD	Not found	Salvi *et al.*, 2002
Zingiber officinale	—	4C nuclear DNA content and RAPD analysis	Not found	Mohanty *et al.,* 2008
Kaempferia galanga	Rhizome buds	RAPD	Not found	Parida *et al.,* 2010
Curcuma longa	—	RAPD	Not found	Singh *et al.,* 2011
Manihot esculenta	Apical meristems	RAPD	Not found	Angel *et al.,* 1996

and Tripathi *et al.,* 1996).

The different forms of reactive oxygen species are all capable of causing oxidative damage to proteins, DNA, and lipids.

2.13.2 Antioxidants

Free radicals that may cause damage or dysfunction in either living or nonliving systems could presumably be prevented from exerting their harmful effects by several means. Either physical or chemical techniques could, in principle be employed to limit the potential damage.

2.13.2.1 Preventive antioxidation

Living organisms employ several such strategies. One approach is simply to avoid oxygen altogether. Many microorganisms live in environments that are either totally anoxic or limited in oxygen concentration. Other life forms eschew sunlight and occupy permanently dark environments, such as the ocean depths, subsurface layers of the soil, or caves. The surface of many animals that do live in presence of sunlight and oxygen are either dark in colour or highly reflective, or both, presumably at least in part because of the potentially toxic effects of light.

2.13.2.2.Chemical antioxidation

The most common and useful approach used is preventing damage to autooxidizable materials is to incorporate chemical additives in the formulation to deactivate the species that initiate or promote destructive oxidation reactions. Autooxidation reactions are normally initiated by species capable of producing free radicals, which then undergo rapid subsequent reactions with molecular oxygen leading to damage. Protective additives may be light absorbing compounds, metal ion complexing agents, free radical scavengers, peroxide destroying compounds or singlet oxygen quenchers.

2.13.3 Antioxidant studies

2.13.3.1 Antioxidants in plants

Plants possess efficient antioxidant defense systems (Blokhina *et al*., 2003; Bhattacharjee, 2005; Smirnoff, 2008; Inze and Motagu, 2004; Arora *et al*., 2002; Mittler, 2002) to scavenge the ROS and protect the plants from destructive reactions. A regulated balance between oxygen radical production and their destruction is required to maintain metabolic efficiency and functions under both optimal and stress conditions. There are many sites of ROS production in the plant cell such as mitochondria, chloroplasts, glyoxysomes and cytosol,

which are highly controlled and tightly coupled to prevent release of intermediate products (Inze and Motagu, 2004). Under stress conditions if the production of ROS exceeds plants' capacity to detoxify them, a breakdown in control and coupling occurs. This leads to a process, dysfunction leaking ROS. The main cellular component susceptible to damage by free radicals are proteins (oxidation), lipids (peroxidation of unsaturated fatty acids in membranes which leads to membrane permeability), carbohydrates (oxidation produces dicarbonyls which react with the damaged proteins by crosslinking and condensation reactions) and nucleic acids (purine and pyrimidine bases are potential targets for oxidative damage leaving strands intact but modifications of sugar residues may lead to strand breakage).

Plants have evolved different phytochemicals and enzymes as antioxidant defense to maintain growth and metabolism. Antioxidants are produced in leaves and protect the plant from damage by quenching free radicals. Plants with high antioxidative capacity are more tolerant to herbicide-induced photooxidative stresses than plants with low antioxidative capacity (Arora *et al*., 2002). Plants contain antioxidative system in all subcellular compartments including the apoplastic space which show large changes during the life cycle of plant germination, emergence of young foliage, expansion, maturity, senescence and death. These defence systems are composed of:

1. hydrophilic (ascorbate, glutathione, phenolic compounds, flavooids, cucurmin) and lipophilic (α-tocopherol, carotenoids, lycopene) metabolites with antioxidative properties.
2. protective enzymes dealing directly with toxic oxidants (superoxide dismutase, peroxidases and catalases) and enzymes helping to maintain the pool of antioxidants in their reduced state (monodehydroascorbate reductase, dehydroascorbate reductase, glutathione reductase).

High local concentrations of antioxidants play a major role in deactivation of O_2, whereas high activities of protective enzymes are mainly responsible for detoxification of long living oxy-products such as $O_2^{\cdot -}$ and H_2O_2. Protective enzymes and antioxidants are constitutively present

in plants and the capacity of this system is not constant but responds to intrinsic and extrinsic factors such as environmental factors or developmental determinants (Inze and Motagu, 2004).

2.13.4 Antioxidant studies in Zingibers

The rhizomes of tropical zingibers like *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* are rich various secondary metabolites. Ginger and turmeric contain curcumin as well as less oxygenated curcumin derivatives. Katiyar *et al.* (1996) showed that water or organic solvent extract of ginger possesses antioxidative property, which inhibits tumour promotion in mouse skin. Thus zingiber extract is postulated to probably contain anti-inflammatory agents with antioxidant activity. *Curcuma* is rich in antioxidants in both *in vitro* and *in vivo* systems (Halim, 2002). *Kaempferia galanga* is also a potential sources of antioxidants and/or cytotoxic agents against tumour cells (Zaeoung *et al.*, 2005). Chan *et al.* (2008) performed an extensive work to study the total phenolic content, Ferrous ion-chelating abilities and ascorbic acid equivalent antioxidant capacity of leaves of 26 *Zingiber* species belonging to nine genera and three tribes. Of the 26 species, leaves of *Etlingera* species had the highest total phenolic content and ascorbic acid equivalent antioxidant capacity. Eleven of the 14 species had significantly higher total phenolic content and/or ascorbic acid equivalent antioxidant capacity in leaves than in rhizomes. In terms of Ferrous ion-chelating ability, six of the eight species clearly showed higher values in leaves than in rhizomes. The most outstanding was the Ferrous ion-chelating value of *Alpinia galanga* leaves which was more than 20 times higher than that of rhizomes. Of the five species of *Etlingera*, leaves of *Etlingera elatior* displayed the strongest tyrosinase inhibition activity, followed by leaves of *Etlingera fulgens* and *E. maingayi*. Values of their inhibition activity were significantly higher than or comparable to the positive control. Besides promising tyrosinase inhibition ability, leaves of these three *Etlingera* species also have high antioxidant activity and antibacterial properties.

Ginger is rich in secondary metabolites like oleoresin, essential oil and different polyphenols (Bhagyalaxmi and Singh, 1988). Ginger, the rhizome

of *Zingiber officinale* is one of the most common constituents of diets worldwide and is reported to possess antioxidant properties. The pungent phenolic constituent of ginger, [6]-gingerol, inhibited LPS-induced NOS expression and production of NO and other RNS species in macrophages and blocked peroxynitrite-induced oxidation and nitration reactions *in vitro* (Ippoushi *et al.*, 2003). These results suggest that [6]-gingerol is a potent inhibitor of NO synthesis and also an effective protector against peroxynitrite-mediated damage.

Anti-inflammatory activities of silica gel chromatography fractions of ginger have also been tested using an *in vitro* PGE2 assay. Results showed that most of the fractions containing gingerols and/or gingerol derivatives were excellent inhibitors of LPS-induced PGE2 production (Jolad *et al.*, 2004).

Bhattacharya *et al.* (2009), investigated the free radical, hydroxyl radical and nitric oxide scavenging activity along with lipid peroxidation capability of different solvent fractions of ginger. Out of the 34 fractions studied, 10 fractions showed free radical scavenging activity ranging from 5.88% to 80%. 5 different peaks were obtained. The maximum NO generation activity was observed in the benzene fraction The liver protective function was maximum in diethyl ether and ethyl acetate (1:1) fraction. The bioactive fraction of diethyl ether and ethyl acetate (1:1) showed the reactivity against Natural Product-Polyethylene Glycol reagent (NP/PEG) from which it may be concluded that zinger flavonoids have some contributory roles in scavenging free radical activity.

Methanol extracts, water extracts and volatile oils of the fresh rhizomes of *Zingiber officinale* have been assessed for free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical by Zaeoung *et al.* (2005). 6-shogaol, 6-dehydrogingerdione (or 1-dehydrogingerdione) and 6-gingerol were isolated from the methanol extract of *Zingiber officinale*.

Curcumin, the active principle of turmeric, is commonly used as a coloring agent in foods, drugs and cosmetics, and has a wide range of effects. Curcumin is known to act as an antioxidant, antimutagen and anticarcinogen (Anto *et al.*, 1994). Curcumin was the most potent compound for free radical scavenging activity and also has therapeutic

properties for some human diseases (Srimal and Dhawan, 1973). Bleomycin (BLM) is an antibiotic and radiomimetic glycopeptide that is routinely used in cancer chemotherapy as an antineoplastic agent. BLM is mutagenic in diverse genetic assays (Povirk and Austin, 1991) and is thought to exert its genotoxic effects through free radical production and the induction of oxidative damage to DNA (Lown and Sim, 1977). Butkhup and Samappito (2011) studied the antioxidant activity of *Cucurma longa*.

Anticancer activity of the rhizomes of turmeric was evaluated *in vitro* using tissue culture methods and *in vivo* in mice using Dalton's lymphoma cells grown as ascites form. Turmeric extract inhibited the cell growth in Chinese Hamster Ovary (CHO) cells at a concentration of 0.4 mg/ml and was cytotoxic to lymphocytes and Dalton's lymphoma cells at the same concentration. The active constituent was found to be 'curcumin' which showed cytotoxicity to lymphocytes and Dalton's lymphoma cells at a concentration of 4 micrograms/ml that indicated that turmeric extract and curcumin reduced the development of animal tumours (Kuttan *et al.*, 1985)

Sacchetti *et al.* (2005) evaluated the essential oils *Curcuma longa* by means of GC and GC–MS. Antioxidant and radical-scavenging properties were tested by means of 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and b-carotene bleaching test. In the DPPH assay major effectiveness, with a radical inhibition ranging from 59.6 ± 0.42 to 64.3 ± 0.45%, while in the b-carotene bleaching test *Curcuma longa* (72.4 ± 0.51%) were obtained.

Methanol extracts, water extracts and volatile oils of the fresh rhizomes of *Curcuma longa,* was assessed for free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and cytotoxic activity against MCF7 (breast adenocarcinoma) and LS174T (colon adenocarcinoma) cell lines by Zaeoung *et al.* (2005). Methanol extract of *Curcuma longa* exhibited the most pronounced radical scavenging activity with an EC 50 value of 9.7 µg/ml, whereas the water extracts and volatile oils showed weak activity. All volatile oils and the methanol extract of *Curcuma longa* showed strong activity against MCF7 and LS174T with IC50 less than 50 µg/ml. ar-Turmerone, curcumin, demethoxycurcumin and bisdemethoxycurcumin were isolated from the methanol extract of *Curcuma*

longa was the most potent compound for free radical scavenging activity. Demethoxycurcumin was found to be the most active compound against LS174T with an IC50 value of 0.8 µg/ml and 6-shogaol was the most potent compound against MCF7 with an IC50 value of 1.7 µg/ml. Roy and Raychaudhury (2004) studied the antiradical activity of *in vitro* regenerated *Curcuma longa*.

Tewtrakul *et al.* (2005) extracted volatile oil of dried rhizome of *Kaempferia galanga* obtained by water distillation was determined for its chemical components using gas chromatography and mass spectrometry (GC-MS). The major chemical constituents were identified as ethyl-*p*-methoxycinnamate (31.77%), methylcinnamate (23.23%), carvone (11.13%), eucalyptol (9.59%) and pentadecane (6.41%), respectively.

Rajendra *et al.* (2011), studied the antioxidant activities of the extracts of *Kaempheria galanga*. The results revealed the presence of sterols, Triterpenoids and resins in petroleum ether extract, sterols, Triterpenoids, Flavanoids and resins in chloroform extract, Steroids, Triterpenoids, alkaloids, Flavanoids, carbohydrates, resins and proteins in methanolic extract. The water extract showed the presence of saponins, carbohydrates and proteins. However the tannin content was not detected from any of the rhizome extracts.

Chapter 3
MATERIALS AND METHODS

3.1 Collection of germplasm

Selected places of Jalpaiguri and Darjeeling district of West Bengal like Lataguri, Dhupguri, Mohitnagar, Gorubathan etc. were visited for the germplasm collection of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*. The field work was carried on during the harvest. Essential field data related to different genera and cultivars were recorded in data sheets during the field study (Refer Appendix B) which included date and time of collection, habit, habitat and area of the vegetation. Plant materials thus collected were planted in the experimental garden for further study.

3.2 Maintenance of germplasm

The collected samples of the genera under study were planted in separate pots placed at experimental garden of Department of Botany for future use. A part of the rhizomes were kept in the green house of the department for regeneration and genetic diversity studies.

3.3. *In vitro* culture studies

3.3 1. Media for in vitro culture

The *Z. officinale, C. longa* and *K. galanga* sprouts were cultured on Murashige & Skoog (Murashige and Skoog, 1962) basal media (Refer Appendix C for composition) and Gamborg B5 (Gamborg *et al*., 1968) media (Refer Appendix D for composition). The media were supplemented with vitamins, sucrose, agar and plant growth regulators. Sucrose was used as the carbon source at the rate of 30 g/l, and agar-agar was use to solidify the media at the rate of 8 g/l. Filter sterilized benzyl amino purine (BAP 1, BAP 2, BAP 3, BAP 4 and BAP 5 mg/l) (Sigma, Cat# B3408) were added to the media after

autoclaving.

3.3.2 Sucrose for in vitro culture

To assess ideal percentage of sucrose required for growth of *Z. officinale*, *C longa* and *K galanga*, the sprouts were cultured on Murashige & Skoog (Murashige and Skoog, 1962) basal media supplemented with vitamins, agar and cytokinins. Sucrose as the carbon source was added at the rate of 10, 20, 30 and 40 g/l. Benzyl amino purine was added to the media at the rate of 3 and 4 mg/l.

3.3.3 Cytokinins on regeneration

Efficacy of three important cytokinin like benzyl amino purine, kinetin and zeatin were tested for the regeneration of *Z. officinale*, *C. longa* and *K. galanga.* The sprouts were cultured on Murashige and Skoog (Murashige and Skoog, 1962) basal media supplemented with vitamins, sucrose, agar and cytokinins in different concentrations and combinations. Cytokines viz. benzyl amino purine, kinetin and zeatin were added at concentrations of 1, 2, 3, 4 and 5 mg/l. Combinations of benzyl amino purine and kinetin were experimented in the following concentrations:-

BAP 1 mg/l + Kinetin 1, 2, 3 and 4 mg/l
BAP 2 mg/l + Kinetin 1, 2, 3 and 4 mg/l
BAP 3 mg/l + Kinetin 1, 2, 3 and 4 mg/l
BAP 4 mg/l + Kinetin 1, 2, 3 and 4 mg/l

Sucrose was used as the carbon source at the rate of 30 g/l and agar-agar was use to solidify the media at the rate of 8 g/l. Plant growth regulators were added to the media after autoclaving.

3.3.4 Secondary culture

Subcultures to obtain maximum number of healthy shoots were experimented by inoculating sets of explants in media. The regenerated juvenile plantlets were collected from the culture vessels and were subcultured in the Murashige & Skoog (Murashige and Skoog, 1962) media supplemented with same concentrations of sucrose, vitamins, agar and plant growth regulators as used in the media of primary culture. The MS media were supplemented with cytokinines like benzyl amino purine at a concentration of 3 and 4 mg/l for all the three genera. In addition to this the *C. longa* and *K. galanga* explants were cultured on MS media supplemented cytokinins like benzyl amino purine at different combinations and concentration as shown below:-

BAP 3 mg/l + Kin 3 mg/l
BAP 3 mg/l + Kin 4 mg/l
BAP 3 mg/l + Kin 5 mg/l

Sucrose was used as the carbon source

at the rate of 30 g/l and agar-agar was use to solidify the media at the rate of 8 g/l. Plant growth regulators were added to the media after autoclaving.

3.3.5 Preparation of explants

3.3.5.1 Sprouting of explants

* The rhizomes of *Zingiber officinale* cv Gorubathan, *Curcuma longa* cv Local (collected from Lataguri) and *Kaempferia galanga* cv local were washed thoroughly under running tap water to remove the soil from their surface.
* They were treated with Diathane M 45 (1% in water) for 1 hour and were kept on trays filled with sand for sprouting.
* The rhizomes sprouted within 2-4 weeks and the sprouts were used as explants.

3.3.5.2 Sterilization of explants and inoculation

* The young sprouts (1 to 2 cm) of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* were washed under running water to remove the sand of the germinating tray.
* The sprouts were dipped in the solution of 1% Extran for 10 minutes.
* Sprouts were washed several times with double distilled water to remove the traces of extran.
* The explants were taken to laminar air flow cabinet and were dipped in solution of Mercuric chloride [0.1 to 0.5 % (w/v)] (Merck India, Cat# 17524) for 10 minutes.
* The treated explants were washed several times with sterile double distilled water and dipped in 70% Ethyl alcohol for 1 minute.
* The traces of Ethyl alcohol were removed by washing them with sterile double distilled water for 5 times.
* The open ends of the explants were cut off before inoculating in the culture media.
* Under aseptic condition of laminar air flow cabinet, the 1 to 2 mm of the basal region of the sprouts were gently placed on the gelled agar media of culture vessels.

3.3.6. Growth conditions

The inoculated culture vessels were kept at a temperature of 25 ± 2^{o}C. Photoperiod of 16 hours with a light intensity of 2000-2500 Lux were provided by cool white fluorescent tubular lamps.

3.3.7. Subculture of plantlets

The plantlets regenerated from primary and secondary cultures were taken out of the culture tubes without causing much damage to the plantlets. The shoots were separated from each other and the dry or brown parts were cleaned under aseptic conditions. They were reinoculated in the media containing the same concentration and combinations of plant growth regulator.

3.3.8 Hardening of plantlets

* Healthy, *in vitro* grown plantlets of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* with good number of roots were selected for hardening. The plantlets were taken out and washed carefully to remove all traces of agar sticking to the roots.
* The individual plantlets were separated from each other and were then transplanted into polycups containing a mixture of garden soil and sand (1:1).
* They were kept covered with plastic bags with holes to provide 60-70% relative humidity.
* The plastic bags were removed after 7-10 days and were sprinkled with water on every alternate day.
* Subsequently they were transferred to bigger pots and then to the garden.

3.3.9 Detection of disease free rhizomes

Three diagnostic tests were performed to detect the presence or absence of the pathogen:-

1. Rhizome pieces of these clones were transferred to PDA media (Potato -40%, Dextrose-2.5% and Agar-1.5%) and observed for 8 to 10 days for fungal growth on the medium;

2. Visual observations on the presence of symptoms were recorded throughout the growing season;

3. Rhizomes harvested from the tissue culture-derived plants were stored in river sand, and the number of rotted and healthy rhizomes was recorded after 6 months of storage.

3.3.10. In vitro regeneration of different turmeric cultivars

3.3.10.1. Media for regeneration

Regeneration potential of *Curcuma longa* cultivars was assessed. The sprouts were cultured on Murashige & Skoog (Murashige and Skoog, 1962) basal media. The media were supplemented with vitamins, and cytokinins. Sucrose was used as the

carbon source at the rate of 30 mg/l. The pH of media were adjusted to 5.7 on electronic pH meter by adding 0.1 (N) NaOH or HCl to the media. The culture media were solidified with agar -agar at a concentration of 8 g/l. The media were sterilized at 121 ^{0}C for 20 minutes at 1.08 Kg/cm^2 pressure. Sterilized media were transferred to Laminar air flow cabinet and after a few minutes filter sterilized plant growth regulators benzyl amino purine (BAP 1, BAP 2, BAP 3, BAP 4 and BAP 5 mg/l) were added. The media were poured into sterile culture vessels.

3.3.10.2. Preparation of explants of turmeric cultivars

* The rhizomes of different turmeric cultivars were washed several times under running tap water to remove the soil from their surface.
* They were treated with Diathane M 45 (1% in water) for 1 hour and were kept on trays filled with sand for sprouting.
* The rhizomes sprouted within 2-4 weeks and the sprouts were used as explants.

3.3.10.3 Sterilization of explants and inoculation

* The young sprouts (1 to 2 cm) of the genera under study were washed under running water to remove the soil of the germinating tray.
* The sprouts were dipped in the solution of 1% Extran for 10 minutes.
* Sprouts were washed several times with double distilled water to remove the traces of extran.
* The explants were taken to laminar air flow cabinet and were dipped in solution of Mercuric chloride [0.1 to 0.5 (w/v)] for 10 minutes.
* The treated explants were washed several times with sterile double distilled water and dipped in 70% Ethyl alcohol for 1 minute.
* The traces of Ethyl alcohol were removed by washing them with sterile double distilled water for 5 times.
* The open ends of the explants were cut off before inoculation in the culture media.
* 1 to 2 mm of the basal region of the sprouts was gently placed on the solidified agar media of culture vessels under aseptic condition on laminar air flow cabinet.

3.3.10.4 Growth conditions

Please refer to section 3.3.6 for details.

3.4 Diversity studies

3.4.1 Isolation of genomic DNA from leaves of Curcuma longa

The leaves of all the 12 cultivars of *C. longa* were collected from the experimental garden of the laboratory. All these samples were washed to remove dirt and other debris. After cleaning, all these samples were taken for PCR based diversity studies. Considering various factors, method of Bousquet *et al.* (1990) was opted with slight modifications. Isolation of DNA from the leaves was carried out with following procedures:

* Sliced leaf samples (6gm) of turmeric were ground to fine powder in prechilled mortar and pestle with liquid nitrogen.
* The ground samples were carefully transferred to prewarmed (at 65ºC) freshly prepared 18 ml CTAB DNA extraction buffer (Refer Appendix E for composition) contained in an Oakridge tube (Tarsons, Cat#541040).
* The Oakridge tubes containing ground samples dipped in DNA extraction buffer were incubated at 65ºC in a water bath for 2 hours with occasional mixing by gentle swirling.
* An equal volume of chloroform (Merck India, Cat#822265):isoamyl-alcohol(Merck India, Cat#8.18969.1000) (24:1) was added to the incubated Oakridge.
* The contents of the tubes were mixed by inversion for 15 min.
* The tubes were spin at 6,500 RPM at 25ºC for 15 min in a centrifuge (REMI make, Model No.C-24) at 25^0C. The upper aqueous layer was transferred to a fresh Oakridge tube.
* 0.6 volume of ice cold Isopropanol (Merck India, Cat#17813) was added to the upper aqueous phase.
* The tubes were kept overnight at -20ºC.
* After overnight incubation, the mixture was centrifused at about 6,500 rpm (5,000 xg) for 30 minutes at 4ºC.
* The supernatant was discarded and the pellet was washed in 70% ethanol.
* The pellet was dried in vacuum and dissolved in 500µl of 1X TE buffer (pH 7.4) (Refer Appendix E for composition).
* An equal volume of equilibrated phenol (pH 8.0) (Sigma,

Cat#P4557-400ML), was added to the tube and was mixed well.

* The tubes after adding phenol were centrifuged at 13,000 rpm (10,000 xg) for 15 minutes.
* The upper phase was collected.
* The upper phase was dissolved in equal volume of C:I and mixed well for 5 min.
* The mixture was centrifuged at 10,000 RPM at 20ºC for 15 min at room temperature.
* The upper phase was collected in a fresh tube.
* 1/10 volume 3M Sodium acetate (SIGMA, Cat#S-9513) was added to the upper phase and mixed well.
* Double volume of ice cold ethanol (BDH Cat#10107) was added to the mixture and keep 1 hour at -20ºC.
* After incubation the mixture was centrifuged (REMI make) at 13,000 RPM at 4ºC for 30 min.
* The supernatant was discarded and the pellet was washed in 70% ethanol.
* Finally, the pellet was dried in vacuum and dissolve in T E buffer.

3.4.2 Purification of DNA

Crude DNA contains Major contaminants like RNA, protein and polysaccharides. It is essential to remove them as they hamper the further downstream processing.

CTAB buffer eliminates polysaccharides from DNA to a large extent, but in isolated DNA from the leaves of Zingiberaceous plants starch residues are found intact with the isolated DNA even after CTAB and phenol: chloroform treatment. Therefore peg purification of DNA is necessary for complete elimination of polysaccharides. The RNA was removed by treating the sample with RNase enzyme. Extraction with phenol: chloroform following RNase treatment was also employed for eliminating RNA and most of proteins. Following protocols were used to purify DNA.

3.4.2.1 PEG purification of isolated DNA

* 30% W/V solution of PEG [Refer Appendix E for composition] was added to 1000µl of 1X TE buffer (pH 7.4) containing DNA in a microcentrifuge tube. The tube was incubated at 0ºC for 14 hrs.
* The incubated mixture was centrifuged at 12,000 rpm (REMI make, Model No.C-24) at 4ºC for

30 min.

* The pellet obtained was resuspended in 500µl of 1X TE buffer.
* To this resuspended pellet, 0.1 volume of 3M sodium acetate (pH 5.5) and 2.5 volume of ice cold absolute ethanol was added for DNA precipitation.
* The mixture of solution was incubated overnight at -20ºC and after incubation it was centrifuged at 13,000 rpm (13,500 xg) for 30 minutes at 4ºC.
* Transparent DNA pellet was obtained which was washed with 70% ethyl alcohol, air dried and finally dissolved in 500µl of 1XTE (pH 7.4) buffer

3.4.2.2 RNaseA treatment

* RNaseA (50µg/ml) (SIGMA, Cat#R-4875) was added to the genomic DNA dissolved in 500µl of 1X TE buffer (pH 7.4) and it was incubated at 37ºC for 1 hr in a Dry water bath (GeNei™ make, Cat#107173).
* Equal volume of Chloroform: Isoamyl alcohol (500µl) was then mixed to each sample and centrifuged at 10000 RPM for 10 min (sometimes C:I and P:C:I was repeated twice to remove traces of phenols mixed with DNA pellets) at room temperature.
* The aqueous phase was then transferred to a fresh microcentrifuge tube (Tarsons, Cat#500010).
* Each sample was mixed with 1/10th volume of 3M sodium – Acetate and double volume of chilled Ethanol and stored overnight in -20°C for DNA to precipitate.
* The tubes with DNA sample were centrifuged at 13000 RPM for 30 minutes at 4°C. The supernatant was discarded; the pellets were washed with 80% Ethanol and completely dried as described above.
* The completely dried DNA pellets were dissolved in 100µl 1X TE buffer and stored at -20°C for quantification.

3.4.3 Quantification of Curcuma longa DNA

Reliable measurements of DNA concentration are important for many applications in molecular biology including amplification of target DNA by polymerase chain reaction and complete digestion of DNA by

restriction enzymes. DNA quantification is generally carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods were employed in the present study.

3.4.3.1 Spectrophotometric measurement

* Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260nm as well as 280nm by taking 600µl 1X TE buffer in a cuvette.
* DNA (6µl diluted in 594µl of 1X TE) was taken in the cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm.
* DNA concentration was estimated by employing the following formula:

$$\text{Amount of DNA (ng/µl)} = \frac{OD_{260} \times 50 \times dilution\ \ factor}{1000}$$

* The quality of DNA was judged from the OD values recorded at 260nm and 280nm. The DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification using RAPD and ISSR markers and PCR-RFLP techniques.

3.4.3.2 Gel analysis

* Agarose gel (0.8%, gelling temperature 36°C) (SIGMA, Cat#A9539) was casted in 0.5X TBE (Tris-Borate-EDTA) buffer (Refer Appendix E for composition) containing 0.5µg/ml Ethidium bromide (Himedia, Cat#RM813) on gel platform (100X70mm) (Tarsons, Cat#7024).
* Sample DNA (5µl) mixed with 3 µl of 6X gel loading dye (Refer Appendix E for composition) was loaded.
* Lambda DNA/*Eco*RI/*Hin*dIII double digest (1µl) [GeNei™, Cat#106000] was loaded as molecular marker to determine the molecular size of the adjacent genomic DNA.
* The gel was run at 40V for 1hr in a Mini Submarine Gel Electrophoresis Unit (Tarsons, Cat#7030) with Electrophoresis Power Supply Unit (Tarsons, Cat#7090).
* After the run time was over the gel was visualized under UV light on a UV Transilluminator (GeNei™, Cat#SF850).

* The DNA quality was judged by presence of a single compact band at the corresponding position to λ DNA/*Eco*RI/*Hin*dIII double digest indicating high molecular weight of the DNA.
* The quantity of the DNA was estimated by comparing the sample DNA with the control by eye judgment.
* The pure DNA thus obtained was used for various fingerprinting studies.

3.4.4 Gel photography:

The gel was placed over the transilluminator (Bangalore GeNei ™) and photograph was taken using using an indigenously built gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58 mm YA2, Marumi, Japan). The software used was EOS utility.

3.4.5 RAPD analysis of Curcuma longa cultivars

3.4.5.1 Primer used

Polymerase Chain Reaction (PCR) amplifications were performed with the genomic DNA of the twelve different cultivars of *Curcuma longa* by decamer primer. A total of 25 random 10 mer primers (GeNei™) were screened for RAPD analysis (table 3.1).

Table 3.1: List of RAPD primers used

Primer ID	Sequence (5'-3')
OPA01	CAGGCCCTTC
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA11	CAATCGCCGT
OPA17	GACCGCTTGT
OPA20	GTTGCGATCC
OPB01	GTTTCGCTCC
OPF09	CCAAGCTTCC
OPG19	GTCAGGGCAA
OPH04	GGAAGTCGCC
OPN04	GACCGACCCA
OPN13	AGCGTCACTC
OPN19	GTCCGTACTG
OPA 02	TGCCGAGCTG
OPA 06	GGTCCCTGAC
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
MGL01	GCGGCTGGAG
MGL02	GGTGGGGACT
MGL03	GTGACGCCGC
MGL04	GGGCAATGAT
MGL05	CTCGGGTGGG

3.4.5.2 RAPD-PCR amplification

3.4.5.2.1 PCR mix for RAPD

In a sterile 0.2ml thin wall PCR tube (Tarsons, Cat#500050); 25µl of PCR mixture was taken. For reaction mixture, the components were added in the following order:

* Pyrogen free water- To a final volume of 25µl
* PCR master Mix 2X (GeNei™ Cat# 610602200031730 Pl. No. MME22): 12.5µl
* Primer -1.25µl (0.25 µM)

* Template DNA -2µl (25ng)
* One negative control was prepared. A tube with PCR mix but without DNA.

3.4.5.2.2 PCR amplification

PCR was performed in thermal cycler **(**Perkin-Elmer make**).** Amplification programme consisted of one initial cycle of denaturation at 94ºC for 4 min, primer annealing at 37ºC for 1min., primer extension at 72ºC for 2min; followed by denaturation temperature at 94ºC for 1 minute, annealing temperature at 37ºC for 1 minute, extension temperature at 72ºC for 2 minutes and final denaturation at 94ºC for 1min., primer annealing at 37ºC for 1min. and primer extension at 72ºC for 10 min (table: 3.2). Total of 45 cycles were run for amplification of DNA samples isolated from *Curcuma longa* using 10 mer RAPD primers.

3.4.5.3 Agarose-gel electrophoresis of RAPD-PCR products:

To check the efficiency of RAPD-PCR products, 12µl of PCR products mixed with 4µl of 6X Gel loading dye (Refer Appendix E for composition) were examined through electrophoresis on horizontal gel containing in 1.5% (w/v) agarose (Sigma cat No A9414) gel and 7µl Ethedium Bromide (3,8-Diamino-5 -ethyl-6-phenylphenanthridinium bromide) i.e. EtBr (Sigma cat No. E1510).

For preparation of agarose gell, 1.5 g of agarose was dissolved in 100 ml of double H_2O and warmed until agarose powder got dissolved completely. After few minutes, 7µl of EtBr (0.5µg/ml) was added to it and mixed gently, then it was poured over the gel casting tray with comb inserted to it. After the gel was solidified, the comb was removed and the gel was shifted to the gel loading tray (Tarsons) containing 0.5X TBE (Tris Borate EDTA) buffer (pH-8). A DNA ladder (λ DNA/*Eco*RI/ *Hin*dIII double digest) (GeNei^{TM}, Cat#106000) and 100 bp DNA ladder (GeNei^{TM}, Cat#612652670501730) were used as a molecular size marker. Simultaneously, 12µl of PCR products were mixed with 4µl of 6X Gel loading dye of Bromophenol Blue solution individually in all 12 (11 + 1control) samples, mixed thoroughly and loaded gently in the individual wells of the gel. Negative control was loaded in the

Table 3.2: PCR cycle for RAPD analysis of *Curcuma longa* cultivars

Cycle	Denaturation	Primer annealing	Primer extension
1	94ºC for 4min.	37ºC for 1min	72ºC for 2min.
2-44	94ºC for 1min.	37ºC for 1min.,	72ºC for 2min.
45	94ºC for 1min.	37ºC for 1min.	72ºC for 10 min.

last well. The power pack (Bangalore GeNei ™) was set at 50V for 2.4 hrs. All PCR reactions were run at least thrice.

3.4.5.4 Gel photography RAPD-PCR products

The agarose gel after electrophoresis of RAPD-PCR products was placed over the UV transilluminator (Bangalore GeNei™, Cat#107161) and photograph was taken using using an indigenously built gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58 mm YA2, Marumi, Japan). The software used was EOS utility.

3.4.6 ISSR (Inter Simple Sequence repeat) analysis of Curcuma longa

3.4.6.1Primer used

A total of 15 ISSR primers were screened for 12 different cultivars of *Curcuma* which are listed in table.3.3.

Table 3.3: List of primers used for ISSR analysis

Primer ID	Sequence (5'-3')
UBC810	(GA)8T
UBC815	(CT)8G
UBC818	(CA)8G
UBC822	(TC)8A
UBC824	(TC)8G
UBC825	(AC)8T
UBC841	(GA)8YC
UBC856	(AC)8YA
UBC873	(GACA)4
UBC807	(AG)8T
UBC808	(AG)8C
UBC811	(GA)8C
UBC813	(CT)8T
UBC834	(AG)8YT
UBC836	(AG)8YA

3.4.6.2 ISSR-PCR amplification

In a 0.2ml sterile PCR tube (Tarsons, Cat#500050), 25µl of PCR mixture was taken, for which the components were added in the following order:

* Pyrogen free water- To a final volume of 25µl
* PCR master Mix 2X (GeNei™ Cat# 610602200031730 Pl. No. MME22): 12.5µl
* Primer -1.25µl (0.25 µM)
* Template DNA -2µl (25ng)
* One negative control tube was prepared. PCR mix without DNA.

The PCR reactions were performed on a Perkin-Elmer Thermocycler. The amplification cycle consisted of the following specifications:

Amplification program (table 3.4) consisted of one initial cycle of denaturation at 94ºC for 5 min, primer annealing at 52ºC for 1min., primer extension at 72ºC for 2min; followed by denaturation temperature at 94ºC for 45 seconds, annealing temperature at 52ºC for 1 minute, extension temperature at 72ºC for 1 minutes and final denaturation at 94ºC for 45 seconds, primer annealing at 52ºC for 1min. and primer extension at 72ºC for 7 min. Total of 35 cycles were run for

Table 3.4: PCR cycle for ISSR analysis of *Curcuma longa* cultivars

Cycle	Denaturation	Primer annealing	Primer extension
1	94ºC for 5min.	52ºC for 1min.	72ºC for 2min.
2-34	94ºC for 45 seconds	52ºC for 1min.	72ºC for 1min.
35	94ºC for 45 seconds	52ºC for 1min.	72ºC for 7 min.

amplification of DNA samples isolated from *Curcuma* using ISSR primers.

3.4.6.3 Agarose-gel electrophoresis of ISSR-PCR products

To check the efficiency of ISSR-PCR products, 12µl of PCR products mixed with 4µl of 6X Gel loading dye (Refer Appendix E for composition) were examined through electrophoresis on horizontal gel containing in 1.5% (w/v) agarose (Sigma cat No A9414) gel and 7µl Ethedium Bromide (3,8-Diamino-5 -ethyl-6-phenylphenanthridinium bromide) i.e. EtBr (Sigma cat No. E1510).

For preparation of agarose gell, 1.5 g of agarose was dissolved in 100 ml of double H_2O and warmed until agarose powder got dissolved completely. After few minutes, 7µl of EtBr (0.5µg/ml) was added to it and mixed gently, then it was poured over the gel casting tray with comb inserted to it. After the gel was solidified, the comb was removed and the gel was shifted to the gel loading tray (Tarsons) containing 0.5X TBE (Tris Borate EDTA) buffer (pH-8). A DNA ladder (λ DNA/*Eco*RI/ *Hin*dIII double digest) (GeNei™, Cat#106000) and 100 bp DNA ladder (GeNei™, Cat#612652670501730) were used as a molecular size marker. Simultaneously, 12µl of PCR products were mixed with 4µl of 6X Gel loading dye of Bromophenol Blue solution individually in all 12 (11 + 1control) samples, mixed thoroughly and loaded gently in the individual wells of the gel. Negative control was loaded in the last well. The power pack (Bangalore GeNei ™) was set at 50V for 2.4 hrs. All PCR reactions were run at least thrice.

3.4.6.4 Gel photography of ISSR-PCR products

The agarose gel after electrophoresis of ISSR-PCR products was placed over the UV transilluminator (Bangalore GeNei™, Cat#107161) and photograph was taken using using an indigenously built gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58 mm YA2, Marumi, Japan). The software used was EOS utility.

Table 3.5: List of *trn*L-*trn*F primers used

Primer	Sequence (5'-3')
Tab c	CGAAATCGGTAGACGCTACG
Tab f	ATTTGAACTGGTGACACGAG

3.4.7 Fingerprinting data analysis

Each polymorphic band was regarded as a binary character and was scored as 1 (presence) or 0 (absence) for each sample and assembled in a data matrix. A similarity matrix on the basis of band sharing was calculated from the binary data using Dice coefficient (Nei & Li, 1979). Similarities were graphically expressed using the group average agglomerative clustering to generate dendrograms. The analysis was done using the software package NTSYSpc (version 2.0) (Rohlf, 1998).

Correspondence analysis (2D and 3D plot) of right vectors from the binary data was performed to graphically summarize associations among the varieties. Analysis was done through a batch file following the software package NTSYSpc.

3.4.8 PCR of *trn*L-*trn*F region of *Curcuma longa*

3.4.8.1 Primers used

Tab c-f in (Taberlet *et al*., 1991) region of the *Curcuma longa* genome was amplified. The primer sequences were used on the basis of the known sequence from the Taberlet region of the other plant species (table 3.5). A schematic representation of the primer location is shown in figure 3.2.

3.4.8.1.1 PCR mix for amplification of trnL-trnF region

In a 0.2ml sterile PCR tube (Tarsons, Cat#500050), 25µl of PCR mixture was taken, for which the components were added in the following order:

* Pyrogen free water- To a final volume of 25µl
* PCR master Mix 2X (GeNei™ Cat#

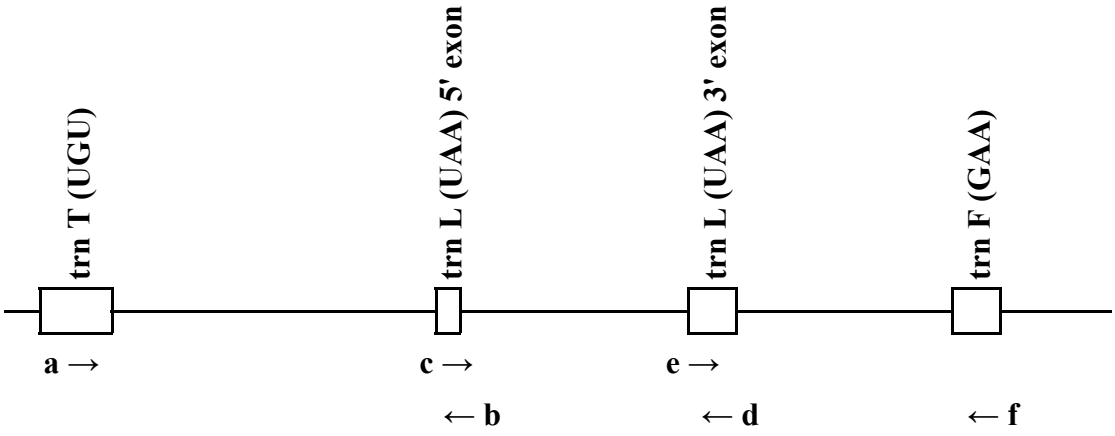


Figure 3.1: Schematic representation of the Tab c-f primer location

610602200031730 Pl. No. MME22): 12.5µl

* Primer -1.25µl (0.25 µM)
* Template DNA -2µl (25ng)
* One negative control tube was prepared. PCR mix without DNA.

3.4.8.1.2 PCR amplification of trnL-trnF region

PCR was performed in thermal cycler **(**Perkin-Elmer make**).** Amplification programme consisted of one initial cycle of denaturation at 95ºC for 5 min, primer annealing at 50ºC for 45s, primer extension at 72ºC for 2min; followed by denaturation temperature at 95ºC for 45 seconds, annealing temperature at 50ºC for 45s, extension temperature at 72ºC for 2 minutes and final denaturation at 95ºC for 45 seconds, primer annealing at 50ºC for 45s and primer extension at 72ºC for 7 min (table:3.6). Total of 35 cycles were run for amplification of DNA samples isolated from *Curcuma* using *trnL-trnF* primers.

3.4.8.2 Agarose-gel electrophoresis of trnL-trnF region-PCR products

To check the efficiency of PCR products, 10µl of PCR products mixed with 3µl of 6X Gel loading dye (Refer Appendix E for composition) were examined through electrophoresis on horizontal gel containing in 1.8% (w/v) agarose (Sigma cat No A9414) gel and 7µl Ethedium Bromide (3,8-Diamino-5 -ethyl-6-phenylphenanthridinium bromide) i.e. EtBr (Sigma cat No. E1510).

3.4.8.3 Gel photography of trnL-trnF region-PCR products

The agarose gel after electrophoresis of PCR products was placed over the UV transilluminator (Bangalore GeNei™, Cat#107161) and photograph was taken using using an indigenously built gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58 mm YA2, Marumi, Japan). The software used was EOS utility.

3.4.9 Sequence analysis

Two (1 forward and 1 reverse) sequences were received from the Chromous Biotech, Bangalore. The resultant sequences were individually compared with the equivalent sequences from a range of *Curcuma*

Table 3.6: PCR cycle for amplification of *trn*L-*trn*F region

Cycle	Denaturation	Primer annealing	Primer extension
1	95ºC for 5min.	50ºC for 45s	72ºC for 2min.
2-34	95ºC for 45 seconds	50ºC for 45s	72ºC for 2min.
35	95ºC for 45 seconds	50ºC for 45s	72ºC for 7 min.

longa and other members of Zingiberaceae present in sequence banks using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990) obtained from National Centre for Biotechnology Information (NCBI) web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.4.9.1 Sequence submission in public domain

The sequences of *Curcuma longa* were documented with the help of Sequin Application Version 12.30 Standard Release [Nov 13, 2012] for Database submission to GenBank providing necessary information's like, definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.).

3.4.10. Somaclonal variations among the in vitro regenerated plantlets

3.4.10.1 In vitro *culture*

The standard protocol of micropropagating *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* plants through shoot bud explant culture were used for the regeneration of plantlets. In all the genera, plantlets were developed from subculture of shoots derived from its single explant of primary culture. After two subcultures about 50 plantlets of each genus were regenerated. For hardening plantlets with good number of roots were selected. They were taken out and washed carefully to remove all traces of agar sticking to the roots. The individual plantlets were then transplanted into polycups containing a mixture of garden soil and sand (1:1). They were kept covered with plastic bags with holes to provide 60-70% relative humidity. The plastic bags were removed after 7-10 days and were sprinkled with water on every alternate day. Finally they were transferred to bigger pots.

3.4.10.2. Isolation of genomic DNA from the in vitro *regenerated plantlets*

The genomic DNA of *in vitro* leaves was isolated using Genelute Plant Genomic DNA kit (Sigma Cat# G2N-70) as follows:

* 100mg leaves of hardened *in vitro* regenerated plantlets were ground into fine powder `in a small mortar and pestle using liquid nitrogen.
* The ground plant tissue was then lysed using 350µl of lysis solution (Part A) and 50µl of lysis solution (Part B) and mixed thoroughly by

inversion and incubated at 65ºC for 10 min.

* After incubation 130µl precipitation solution was added, mixed by inversion and incubated on ice for 5 min to pellet debris. The supernatant was transferred to blue filtration column and centrifuged at 12,000 rpm (13,500 xg) for 1 min.
* To the filtrate 700µl of binding solution was added and mixed thoroughly by inversion. The mixture was then transferred to binding column (prepared by adding 500µl of column preparation solution to the binding column and spinning for 1 min and discarding the flow-through) and centrifuged for 1 min at 12,000 rpm (13,500 xg) and the flow through was discarded. The process was repeated with the remaining mixture. The column was then transferred to a new collection tube.
* Then 500µl of wash solution (containing ethanol) was added to the column and centrifuge at 12,000 rpm (13,500 xg) for 1 min. The column was then transferred to a new collection tube. The process was repeated where the spinning time was increased to 3 minutes.
* Finally 100µl of elution solution (pre-warmed at 65ºC) was added to the column and centrifuged at 12,000 rpm (13,500 xg) for 1 min. The filtrated contained the pure genomic DNA.

3.4.10.3 RAPD analysis

Please refer to section 3.4.5 for details.

3.4.10.4 ISSR analysis

Please refer to section 3.4.6 for details.

3.5 Antioxidants studies

3.5 1 Extraction of plant material

* Rhizomes of *Z. officinale*, *C. longa* or *K. galanga* were collected from the experimental garden at full maturity.
* The rhizomes were thoroughly washed under running tap water to remove the dirt and garden soil.
* The dry scaly leaves were removed and the dry epidermal layers were peeled off.
* The rhizome pieces were washed with distilled water.
* 30 g of the sliced rhizomes of each genera were taken and were crushed in a mechanical grinder.
* The crushed rhizome was subjected to soxhalation and exhaustively extracted with methanol for 8

hours.

* The extracts were evaporated at 50°C to make to a final volume of 3 ml.

3.5.2 Preparation of solvent fractions

* Column chromatography was performed using a glass column apparatus (local made) filled with silica [Merck, 200-400 mesh] at a height of 50cm.
* 3 ml of the extracts were poured on the silica column.
* A series of non polar to polar solvents (table:3.7) like hexane, benzene, chloroform, diethyl ether, ethyl acetate, acetone, ethanol, methanol and water in different ratio were passed through the silica column to obtain the fractions of *Zingiber* and *Curcuma*. In case of *Kaempferia* non polar to polar solvents (table:3.7) like chloroform, diethyl ether, ethyl acetate, acetone, ethanol, methanol and water in different ratio were passed through the silica column to obtain the fractions.
* The solvent fractions of *Zingiber*, *Curcuma* and *Kaempferia* thus obtained were vacuum evaporated at low temperature to make them dry.
* The dried fractions were dissolved in 10 ml methanol.
* The methanol insoluble fractions if any were dissolved in the solvent by which it was extracted.
* All the fractions were stored in airtight tubes at 25 ± 2°C.

3.5.3 Free radical scavenging activity

Free radical scavenging activities of different fractions were tested in models for different radicals' viz. antiradical activity, hydroxyl radical and nitric oxide. Effect on lipid peroxidation was evaluated on goat liver homogenate. The reaction mixtures of different assays are given below.

3.5.3.1 Assay of antiradical activity with DPPH

Antiradical activities of different fractions of *Z. officinale, C. longa* and *K. galanga* were measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH brought about by the sample.

3.5.3.1.1 Reaction mixture of the extracts

Reaction mixtures were prepared by adding100 µl of the *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* extracts to 2900 µl, DPPH

Table 3.7: Solvents for silica gel column chromatography of *Z. officinale*, *C. longa* and *K. galanga*

Fraction	Solvent I	Quantity	Solvent II	Quantity	Total
1	Hexane	200 ml	------	------	200 ml
2	Hexane	150 ml	Benzene	50 ml	200 ml
3	Hexane	100 ml	Benzene	100 ml	200 ml
4	Hexane	50 ml	Benzene	150 ml	200 ml
5	Benzene	200 ml	------	------	200 ml
6	Benzene	150 ml	Chloroform	50 ml	200 ml
7	Benzene	100 ml	Chloroform	100 ml	200 ml
8	Benzene	50 ml	Chloroform	150 ml	200 ml
9	Chloroform	200 ml	------	------	200 ml
10	Chloroform	150 ml	Diethyl ether	50 ml	200 ml
11	Chloroform	100 ml	Diethyl ether	100 ml	200 ml
12	Chloroform	50 ml	Diethyl ether	150 ml	200 ml
13	Diethyl ether	200 ml	------	------	200 ml
14	Diethyl ether	150 ml	Ethyl acetate	50 ml	200 ml
15	Diethyl ether	100 ml	Ethyl acetate	100 ml	200 ml
16	Diethyl ether	50 ml	Ethyl acetate	150 ml	200 ml
17	Ethyl acetate	200 ml	------	------	200 ml
18	Ethyl acetate	150 ml	Acetone	50 ml	200 ml
19	Ethyl acetate	100 ml	Acetone	100 ml	200 ml
20	Ethyl acetate	50 ml	Acetone	150 ml	200 ml
21	Acetone	200 ml	------	------	200 ml
22	Acetone	150 ml	Ethanol	50 ml	200 ml
23	Acetone	100 ml	Ethanol	100 ml	200 ml
24	Acetone	50 ml	Ethanol	150 ml	200 ml
25	Ethanol	200 ml	------	------	200 ml
26	Ethanol	150 ml	Methanol	50 ml	200 ml
27	Ethanol	100 ml	Methanol	100 ml	200 ml
28	Ethanol	50 ml	Methanol	150 ml	200 ml
29	Methanol	200 ml	------	------	200 ml
30	Methanol	150 ml	Water	50 ml	200 ml
31	Methanol	100 ml	Water	100 ml	200 ml
32	Methanol	50 ml	Water	150 ml	200 ml
33	Water	200 ml	------	------	200 ml

solution. The mother stocks of the extracts were at a concentration of 3 mg/µl.

3.5.3.1.2 Measurement and calculation

Antiradical activity was measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH brought about by the sample (Vani *et al.*, 1997 and Ravishankar *et al.*, 2002). A fresh solution of DPPH was prepared by adding 0.00394g of DPPH to 100 ml of methanol. 100 µl of the fractions (3 mg/µl) were added to 2900 µl of DPPH solution. The reaction mixtures were kept undisturbed for 30 minutes. Decrease in the absorbance in presence of the different fractions was noted after 30 min in a Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK). Percentages of DPPH scavenging activity were calculated as: - {1-(optical density of sample/optical density of control) x 100}

3.5.4 Determination of IC_{50}

The fractions showing maximum antiradical responses were diluted to different concentrations and their antiradical activities were observed. IC_{50} was calculated from graphical presentation of concentration verses radical scavenging activity. Antiradical activity was measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH. A stock solution of DPPH (100µm) in methanol was prepared. 100 µl of the fractions (3 mg/µl) were added to 2900 µl of DPPH solution. The reaction mixtures were kept undisturbed for 30 minutes. Decrease in the absorbance in presence of the different fractions was noted after 30 min in a Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK). Percentages of DPPH scavenging activity were calculated as: - {1-(optical density of sample/optical density of control) x 100}

3.5.5Assay of hydroxyl radical scavenging activity

Hydroxyl radical scavenging assay was measured according to Elizabeth and Rao (1990). Scavenging activity was measured by studying the competition between deoxyribose and the test compounds for hydroxyl radical generated from the Fe^{+3}/ascorbate/ EDTA/H_2O_2 system. The hydroxyl radical attacks deoxyribose, which results in thiobarbituric acid reacting substance (TBARS) formation. The reaction mixture contained deoxyribose (2.8 mM), $FeCl_3$ (0.1mM), EDTA (0.1mM), H_2O_2(1mM), ascorbic acid (0.1mM), KH_2PO_4 – KOH buffer (20 mM) and various concentrations of extract (The reaction mixture of contained 3, 30 and 300 mg/ml of fresh rhizome of *Z. officinale*, *C. longa* and *K. galanga* in a final volume of 1 ml. The reaction mixtures were incubated for 1 hour at 37°C. Deoxyribose degradation was measured as TBARS at 532 nm. Percentage inhibition was calculated as:-

{1-(optical density of sample/optical density of control) x 100}

3.5.6 Assay of nitric oxide scavenging activity

Nitric oxide scavenging activity was measured following Bagul *et al*. (2005). Sodium nitroprusside (10mM) in phosphate buffered saline was mixed with 3, 30 and 300 mg/ml of fresh rhizome of *Z. officinale*, *C. longa* and *K. galanga* dissolved in methanol. The mixture was incubated at room temperature for 150 minute. The same reaction mixture without the sample

but equivalent amount of solvent served as control. After incubation, 0.5 ml Griess reagent (1% sulphanilamide, 2% H_3PO_4 ad 0.1% naphthylene diamine dihydrochloride) was added to the mixture. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylene diamine was read at 546 nm. Percentage inhibition was calculated as :-

{1-(optical density of sample/optical density of control) x 100}.

3.5.7 Determination of lipid peroxidation activity

Inhibition of lipid peroxidation activity was worked out following the protocol of Ohkawa *et al.* (1979). The same reaction mixture without the extracts was used as control. The lipid peroxide formed was measured by TBARS. Incubation mixtures (0.4 ml) were treated with sodium dodecyl sulphate (SDS-8.1%, 0.2 ml), thiobarbituric acid (TBA-0.1%, 1.5 ml) and acetic acid (20%, 1.5 ml, pH -3.5). The total volume was made up to 4 ml with distilled water. The mixture was kept in water bath at 100^{o}C for 1 hour. After 1 hour the incubated mixture was cooled to room temperature. 1 ml of distilled water and 5 ml of mixture of n -butanol and pyridine (15:1 v/v) were added to the mixtures and shaken vigorously. The mixtures were centrifuged. The absorbance of the organic layer was measured at 532 nm. Percentage inhibition was calculated as {1-(optical density of sample/optical density of control) x 100}. The percentage inhibition of lipid peroxidation was determined by comparing the result of the test compounds with those of controls.

3.5.8 Detection of compounds

The bioactive fractions were analyzed on Silica Gel 60 F_{254} –precoated TLC plates (0.25 mm thickness). 1 mg. of component was dissolved in 1 ml. methanol; 10 µl was used for TLC. Compounds were separated through hexane-diethyl ether (40:60) solvent and detected under UV light (254 nm) and ammonia vapour. For spraying, Bartons reagent, Berlin blue reagent, Iodine reagent, NP/PEG reagent and Phenol reagents were used.

3.5.9 Comparative study of antiradical activity of the genera

3.5.9.1 Extraction of plant material

Rhizomes of *Z. officinale*, *C. longa* and *K. galanga* were collected from the experimental garden at full maturity. The rhizomes were thoroughly washed

under running tap water to remove the dirt and garden soil. The dry scaly leaves were removed and the dry epidermal layers were peeled off. The rhizome pieces were washed with distilled water.

* 3 g of the sliced rhizomes of each genera were taken and were crushed in a mortar and pestle.
* The crushed rhizomes were separately extracted with 10 ml of diethyl ether : ethyl acetate (3:1), diethyl ether : ethyl acetate (1:1) and acetone for 48 hours at room temperature.
* The extracts were evaporated at 50^{o}C.
* The dry extracts were dissolved in methanol to make to a final volume of 1ml.
* All the fractions were stored at 25 ± 2^{o}C.

3.5.9.2Assay of comparative antiradical activity of Zingiber officinale, Curcuma longa and Kaempferia galanga

Comparative antiradical activities of different fractions of *Z. officinale*, *C. longa* and *K. galanga* were measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH brought about by the sample. Reaction mixtures were prepared by adding 300 µl of the extracts (1 mg/µl) Antiradical activity was measured by a decrease in absorbance at 517 nm of methanolic solution of coloured DPPH brought about by the sample (Vani *et al.*, 1997 and Ravishankar *et al.*, 2002). A fresh solution of DPPH was prepared by adding 0.00394g of DPPH to 100 ml of methanol. 300 µl of the fractions (1 mg/µl) were added to 2700 µl of DPPH solution. The reaction mixtures were kept undisturbed for 30 minutes. Decrease in the absorbance in presence of the different fractions was noted after 30 min in a Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK). Percentages of DPPH scavenging activity were calculated as: {1-(optical density of sample/optical density of control) x 100}

3.5.10 Assay of antiradical activity of turmeric with DPPH

Antiradical activity of the rhizome of *Curcuma longa* cultivars were subjected to DPPH to detect their degree of free radical scavenging activity.

3.5.10.1 Preparation of rhizome extracts

Rhizomes of different *Curcuma longa*

cultivars were collected from the experimental garden at full maturity. The rhizomes were thoroughly washed under running tap water to remove the dirt and garden soil. The dry scaly leaves were removed and the dry epidermal layers were peeled off. The rhizome pieces were washed with distilled water.

20 g of dry rhizomes of different turmeric varieties were cut into small pieces and crushed in a mechanical grinder. The crushed plant materials were divided into two parts, dipped in methanol and water. The extracts dipped in methanol and water was kept undisturbed for 48 hours. The extracts were centrifuged at 6000 RPM for 10 minutes. The supernatants were collected. The supernatants were diluted to make a concentration of 1 mg/µl.

3.5.10.2 Measurement and calculation

Reaction mixtures were prepared by adding 100 µl extracts (1 mg/µl) of the *Curcuma longa* cultivar. Activity was measured by absorbance at 517 nm of methanolic solution of coloured DPPH (Vani *et al.,* 1997 and Ravishankar *et al.,* 2002). A fresh solution of DPPH was prepared by adding 0.00394g of DPPH to 100 ml of methanol. 100 µl of the fractions (1 mg/µl) were added to 2900 µl of DPPH solution. The reaction mixtures were kept undisturbed for 30 minutes. Decrease in the absorbance in presence of the different fractions was noted after 30 min in a Spectrophotometer. Percentages of DPPH scavenging activity were calculated as {1-(optical density of sample/optical density of control) x 100}.

Chapter 4
RESULTS AND DISCUSSION

4.1 Germplasm collection

Collection of samples from different places of Darjeeling (Gorubathan), Jalpaiguri (Lataguri, Dhupguri and Mohitnagar) and other districts of West Bengal resulted in the collection of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* (table 4.1). In all the cases the rhizomes were collected from the fields just after harvest. The rhizome samples were planted in separate plots at the experimental garden of Department of Botany, University of North Bengal and were maintained for the downstream experiments.

4.2 Micropropagation studies

4.2.1 Explant selection

In Micropropagation studies rhizome sprouts measuring 1-2 cm were used as explants for *in vitro* regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* (figure 4.1). The rhizome sprouts have active meristems and can grow into plantlets. These properties of the sprouts were considered for using them as explant. Further, addition of culture medium containing inorganic and organic nutrients along with ambient growth conditions enhances the growth. The superiority of using rhizome buds has been expressed in regeneration of *Z. officinale* (Balachandran *et al.*, 1990; Shirgurkar *et al.*, 2001; Sit *et al.*, 2005; Bhattacharya and Sen, 2006), *C. longa* (Nadagouda *et al.*, 1978; Kuruvinshetti and Iyer, 1981; Shetty *et al.*, 1982; Sunitibala *et al.*, 2001; Salvi, *et al.*, 2002; Rahaman *et al.*, 2004; Gayatri and Kavyashree, 2005; Behera *et al.*, 2010) and *K. galanga* (Shirin *et al.*, 2000; Swapna *et al*., 2004; Chirangini *et al.*, 2005; Bhattacharya and Sen, 2013).

4.2.2 Establishment of aseptic culture

The *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* explants responded within 2-3 weeks

Table 4.1: List of germplasm collected for the study.

Sample ID	Name	Collected from
Z	*Zingiber officinale* Rosc. cv Gorubathan	Gorubathan
T 1	*Curcuma longa* L. cv Local	Lataguri
T 2	*Curcuma longa* L. cv Local	Dhupguri
T 3	*Curcuma longa* L. cv Prova	Central Plantation Crops Research Institute, Mohitnagar, Jalpaiguri
T 4	*Curcuma longa* L. cv Suguna	"
T 5	*Curcuma longa* L. cv TC Assam	"
T 6	*Curcuma longa* L. cv Allepy	"
T 7	*Curcuma longa* L. cv Kasturi	"
T 8	*Curcuma longa* L. cv CLS2A	"
T 9	*Curcuma longa* L. cv Suvarna	"
T 10	*Curcuma longa* L. cv Roma	"
T 11	*Curcuma longa* L. cv Sudarshana	"
T 12	*Curcuma longa* L. cv PTC 13	"
K	*Kaempferia galanga* L. cv Local	Zonal Horticulture Department, Mohit-nagar, Jalpaiguri

of inoculation in the culture medium. Bacterial, fungal contamination and browning of explants occurred within the 1st three weeks of culture inoculation. 28%, 37% and 32% of *Z. officinale*, *C. longa* and *K. galanga* explant respectively were discarded after 21 days of culture. In ginger a high degree of contamination was reported by Hosoki and Sagawa (1977). The difficulty in establishment of contamination-free *in vitro* cultures of *Curcuma longa* was also reported by several groups (Nadgauda *et al*., 1978; Yasuda *et al*., 1988; Balachandran *et al*., 1990; Dekker *et al*., 1991; Shirgurkar *et al*., 2001; Sunitibala *et al*., 2001; Salvi *et al*., 2002; Rahman *et al*., 2004).

In the present study 0.1% to 0.5% mercuric chloride followed by 70% ethanol was used to surface sterilize the explants. Different authors have reported, differential response of mercuric chloride used for different durations, to get contaminant free cultures. Raju *et al.* (2005) was successful using 0.1 per cent $HgCl_2$ for 15 minutes, while, Rahman *et al.* (2004) used 0.1 per cent $HgCl_2$ for 14

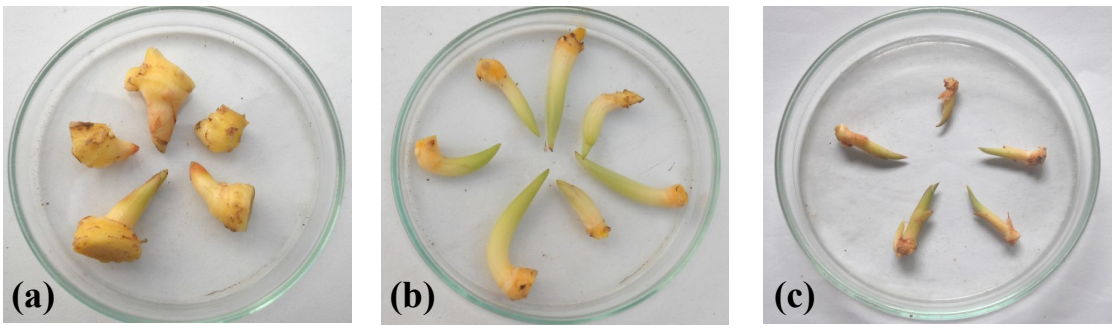


Figure 4.1: Explants used for *in vitro* studies (a) *Zingiber officiinale.* (b) *Curcuma longa.* (c) *Kaempferia galanga*

minutes to establish aseptic cultures in turmeric. These findings are in conformity with the results obtained by de Lange *et al.* (1987) in ginger regeneration. In our experiments with medicinal zingibers surface sterilization with 0.2% mercuric chloride for ten minutes followed by 70% ethanol for one minute showed extremely low rate of contamination. Similar results were observed in *Z. officinale* (Sit *et al.*, 2005 and Bhattacharya and Sen, 2006) *C. longa* (Naz *et al.*, 2009) and *K. galanga* (Bhattacharya and Sen, 2013) tissue culture. Though early sprouting and less browning of the explant buds occurred when the explants were treated with lower concentration of mercuric chloride but the percentage of bacterial and fungal contaminations were very high. Almost similar effect of mercuric chloride was observed in regeneration of the three genera- *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*. It was observed that, the number of days required for shoot and root initiation increased with the increase in the concentration of mercuric chloride. This may be due to cell death of explants during surface sterilization with higher concentrations of mercuric chloride. Concentrations of mercuric chloride above 2mg//l delayed sprouting while concentrations below it were not be able to properly sterilize the inoculums. So, surface sterilization with 0.2% mercuric chloride for ten minutes followed by 70% ethanol for one minute was considered best for all the genera under study.

4.2.3 Initiation of shoots and roots

Shoot and root initiation are influenced by the quantity of growth regulators supplied to the medium, if other growth conditions are kept constant (table 4.2 and figure 4.2). In regeneration of *Z. officinale*, *C. longa* and *K. galanga* by using rhizome bud as an explant at least 21±4, 19±2 and 25±3 days respectively were required

Table 4.2: Days required for shoot and root initiation. Results are average of 30 replicates.

BAP (mg/L)	Duration of initiation (Days)	
	Shoot	Root
Zingiber officinale		
1	60±2	68±6
2	45±4	51±5
3	38±4	42±5
4	24±3	38±7
5	21±4	36±6
Curcuma longa		
1	56±4	61±4
2	43±3	47±5
3	32±3	36±5
4	21±2	28±4
5	19±2	27±6
Kaempferia galanga		
1	64±5	71±5
2	48±6	53±4
3	43±4	49±7
4	28±2	36±4
5	25±3	34±3

for shoot initiation while 36±6, 27±6 and 34±3 days respectively were required for initiation of root respectively. In ginger plantlets regeneration a maximum number of days required for shoot and root initiation was 60±2 and 68±6 respectively. In turmeric plantlets regeneration a maximum number of days required for shoot and root initiation was 56±4 and 61±4 respectively, while in *K. galanga* it was 64±5 and 71±5 respectively. In the cultures of *C. longa*, Rahman *et al*. (2004) observed shoot initiation around 3 weeks after inoculation.

In this study, it was observed that with the increase of concentrations of growth regulator (BAP), the duration for shoot and root initiation decreased. At the same time, in all the cases the time duration required for the initiation of roots were more than that of the time required for the initiation of shoots.

4.2.4 Micropropagation medium

Medium formulations form one the important basis for tissue culture experiments. Regeneration of plantlets varied with the medium and plant growth regulators (table 4.3). Regeneration of *Zingiber officinale*, using Murashige & Skoog medium supplemented with 4 mg/l BAP showed the maximum rate of shooting (8.33) and rooting (20.06) per explant, whereas, in the Gamborg B5 medium maximum shooting (4.67) was observed when the medium was supplemented with 4 mg/l BAP and maximum rooting was observed (5.80) with 2 mg/l BAP. In regeneration of *Curcuma longa*, Murashige & Skoog medium supplemented with 3 mg/l BAP showed the maximum rate of shooting (9.08) and rooting (13.39) per explant, whereas, in the Gamborg B5 medium, maximum shooting (6.19) was observed when the medium was

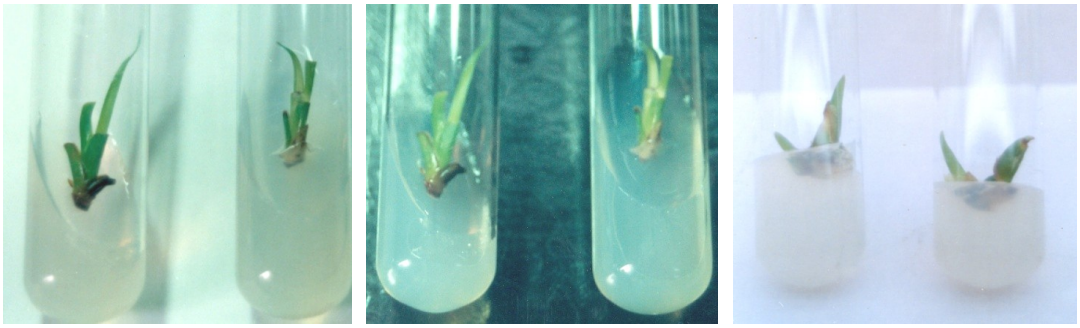


Figure 4.2: Initiation of shoot bud formation after 6 weeks of inoculation. (a). *Zingiber officinale*. (b) *Curcuma longa*. (c) *Kaempferia galanga*

Table 4.3: Effect of culture medium on regeneration of *Z. officinale*, *C. longa* and *K. galanga.* Average of 20 replicates.

BAP (mg/l)	*Zingiber officinale*		*Curcuma longa*		*Kaempferia galanga*	
	Shoots*	**Roots#**	**Shoots***	**Roots#**	**Shoots***	**Roots#**
Murashige and Skoog medium						
1	2.73±0.25	5.87±0.25	3.50±0.19	6.31±0.41	1.91±0.12	2.84±0.36
2	4.66±0.05	8.40±0.33	6.11±0.43	8.64±0.28	2.03±0.10	4.63±0.41
3	6.26±0.57	13.20±0.18	9.08±0.57	13.39±0.36	3.17±0.11	6.15±0.44
4	8.33±0.41	20.06±0.22	8.41±0.28	12.48±0.14	4.33±0.11	6.83±0.23
5	6.50±0.34	18.21±0.17	7.80±0.71	10.56±0.29	3.00±0.12	5.65±0.21
Gamborg B5 medium						
1	1.45±0.25	3.48±0.47	2.81±0.32	4.36±0.32	1.56±0.26	3.24±0.19
2	2.40±0.33	5.80±0.28	4.63±0.40	5.15±0.46	1.85±0.34	4.61±0.24
3	3.20±0.16	4.73±0.25	6.19±0.53	5.65±0.18	2.64±0.27	5.16±0.30
4	4.67±0.25	3.00±0.43	6.55±0.14	6.13±0.27	3.28±0.14	4.84±0.49
5	3.47±0.34	2.40±0.32	5.63±0.23	4.14±0.18	2.78±0.11	4.02±0.38

Shoots*= Shoot per explant and Roots#= Roots per explant

supplemented with 3 mg/l BAP and maximum rooting (6.13) with 4 mg/l BAP. The response of *Kaempferia galanga* to MS medium supplemented with 4 mg/l BAP showed the maximum rate of shooting (4.33) and rooting (6.83) per explant, whereas, in the Gamborg B5 maximum shooting (3.28) was observed when the medium was supplemented with 4 mg/l BAP and maximum rooting (5.16) with 3 mg/l BAP. The results reveal that Murashige and Skoog medium showed comparatively better results than Gamborg B5 in regeneration of plantlets in *Z. officinale*, *C. longa* and *K. galanga*. Therefore, Murashige and Skoog medium was considered for all downstream tissue culture experiments. Superiority of Murashige and Skoog medium over Gamborg B5 medium was also reported by Bhattacharya and Sen (2006) in their experiments to regenerate pathogen-free ginger plantlets.

4.2.5 Sucrose on regeneration

Regenerated *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* plantlets subcultured in MS medium supplemented with different percentage of sucrose and BAP showed variable number of shoots and roots (table 4.4). The three genera under study, showed better regeneration in the medium supplemented with 3% sucrose and 3 to 4 mg/l BAP. *Z. officinale* explants produced maximum number of shoots in the medium supplemented with 3% sucrose and 4 mg/l BAP (8.33), while the maximum shoot height was observed in the medium supplemented with 4% sucrose and 4 mg/l BAP (6.03). The rate of shooting in *Z. officinale* is depicted in the figure 4.3. In

regeneration of *C. longa* explants, maximum number of shoots were observed in the medium supplemented with 3% sucrose and 3 mg/l BAP (9.08), while the maximum shoot height was observed in the medium supplemented with 3% sucrose and 4 mg/l BAP (5.54). The explants of *K. galanga*, produced maximum number of shoots in the medium supplemented with 3% sucrose and 4 mg/l BAP (4.33), while the maximum shoot height was observed in the medium supplemented with 3% sucrose and 4 mg/l BAP (5.54). The rate of rooting increased with the concentrations of sucrose. Profuse rooting was observed in the medium supplemented with high percentage of sucrose. The experiments revealed that the quantity of sucrose in the medium has a profound effect on the rate of regeneration as well as on the growth of the plantlets. The plantlets turned pale or white in the medium supplemented with 1% and 2% sucrose, this may be due to deficiency of elementary carbon. Though sucrose have important role in

Table 4.4: Effect of sucrose on regeneration of *Z. officinale*, *C. longa* and *K. galanga*.
(Average of 20 replicates after 10 weeks of inoculation).

BAP (mg/l)	Sucrose (%)	Regenerated plantlets		
		Shoots*	**Height****	**Roots*****
		Zingiber officinale		
3 mg/l	1	1.53±0.34	1.73±0.07	very low
	2	2.60±0.43	2.79±0.08	low
	3	6.26±0.57	4.61±0.13	profuse
	4	4.87±0.25	5.21±0.21	profuse
4 mg/l	1	2.06±0.25	1.15±0.05	very low
	2	3.73±0.34	3.33±0.14	low
	3	8.33±0.41	5.78±0.17	profuse
	4	5.20±0.43	6.03±0.16	profuse
		Curcuma longa		
3 mg/l	1	2.34±0.65	1.85±0.32	Low
	2	5.67±0.34	2.71±0.42	Low
	3	9.08±0.57	4.11±0.65	Profuse
	4	8.87±0.23	3.98±0.13	Profuse
4 mg/l	1	3.65±0.44	2.64±0.11	Very low
	2	6.48±0.28	4.32±0.48	Low
	3	8.41±0.28	5.54±0.29	Profuse
	4	7.78±0.77	5.28±0.37	Profuse
		Kaempferia galanga		
3 mg/l	1	1.58±0.45	2.34±0.17	Very low
	2	2.73±0.56	3.68±0.54	Very low
	3	3.17±0.11	4.31±0.16	Low
	4	2.98±0.14	4.22±0.33	Profuse
4 mg/l	1	1.86±0.32	2.32±0.18	Very low
	2	3.14±0.24	3.47±0.52	Very low
	3	4.33±0.11	3.97±0.19	Profuse
	4	4.04±0.43	3.68±0.19	Profuse

Shoots*= Number of shoots per explant, Height**= Plantlet height (cm) and Roots***= Number of shoots per explant

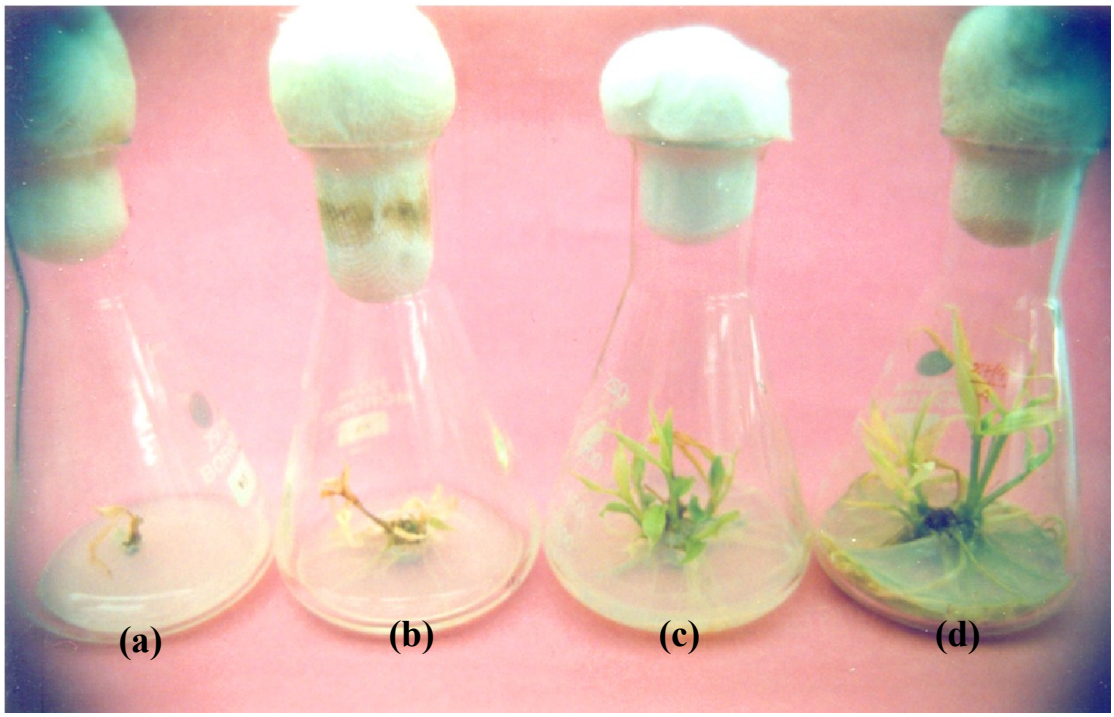


Figure 4.3: Response of *Zingiber officinale* explants to MS media supplemented with BAP 4 mg/l and different percentages of sucrose. (a) 1% sucrose. (b) 2% sucrose. © 3% sucrose. (4) 1% sucrose.

regeneration but show reduced rate of shooting at higher concentrations.

4.2.6 Plant growth regulators on regeneration

4.2.6.1 Cytokinin on regeneration

Cytokinins like Benzyl amino purine, Kinetin and Zeatin were added to the medium at different concentrations to observe their effects on regeneration. It has been observed that the growth regulators trigger variable responses (table 4.5). In micropropagation of *Zingiber officinale* (figure 4.4a) the maximum numbers of plantlets/explant (8.33) were observed in the medium supplemented with BAP at a concentration of 4 mg/l while the maximum height (6.90 cm) was observed with BAP at a concentration of 5 mg/l. In the medium supplemented with kinetin, the maximum numbers of plantlets/explant (5.40) as well as maximum height (6.06 cm) was observed at 4 mg/l. The highest numbers of plantlets/explant (7.20) regenerated with zeatin were at 5 mg/l, whereas, the maximum height (4.67 cm) was at zeatin concentration 4 mg/l.

In regeneration of *Curcuma longa* (figure 4.5a), the maximum numbers of plantlets/explant (9.08) were observed in the medium supplemented with BAP at a concentration of 3 mg/l while the maximum height (6.47 cm) was found with BAP at a concentration of 5 mg/l). In the medium supplemented with

Table 4.5: Effect of different concentrations of cytokinins Average of 20 replicates

Plant growth regulator	***Zingiber officinale***		***Curcuma longa***		***Kaempferia galanga***	
	Plantlets*	**Height#**	**Plantlets***	**Height#**	**Plantlets***	**Height#**
			Benzyl amino purine			
1	2.73±0.25	2.51±0.26	3.50±0.19	2.53 ± 0.28	1.91 ± 0.12	2.67 ± 0.61
2	4.66±0.50	3.55±0.27	6.11±0.43	3.21 ± 0.47	2.03 ± 0.10	3.05 ± 0.07
3	6.26±0.57	4.61±0.13	9.08±0.57	4.11 ± 0.65	3.17 ± 0.11	4.31 ± 0.16
4	8.33±0.41	5.78±0.17	8.41±0.28	5.54 ± 0.29	4.33 ±0.11	3.97 ± 0.19
5	6.50±0.34	6.90±0.54	7.80±0.71	6.47 ± 0.32	3.00 ±0.12	3.40 ± 0.11
			Kinetin			
1	1.86±0.34	2.11±0.28	2.58 ± 0.23	2.20 ± 0.36	2.15 ± 0.20	1.81 ± 0.04
2	2.66±0.34	3.19±0.14	4.39 ± 0.26	3.31 ± 0.39	3.21 ± 0.31	2.26 ± 0.18
3	3.73±0.25	4.20±0.10	6.11 ± 0.47	5.04 ± 0.48	3.44 ± 0.08	2.56 ± 0.12
4	5.40±0.25	6.06±0.38	6.85 ± 0.44	5.81 ± 0.36	2.13 ± 0.21	2.74 ± 0.08
5	4.86±0.34	4.99±0.20	6.59 ± 0.79	5.02 ± 0.35	1.94 ± 0.16	2.82 ± 0.21
			Zeatin			
1	1.93±0.34	1.15±0.15	3.26 ± 0.32	1.34 ± 0.38	2.25 ± 0.10	1.99 ± 0.23
2	2.46±0.25	2.13±0.19	4.59 ±0.49	1.93 ± 0.49	3.58 ± 0.41	2.21 ± 0.06
3	3.80±0.49	2.97±0.21	6.22 ± 0.43	2.55 ± 0.19	2.28 ± 0.31	2.42 ± 0.11
4	5.33±0.10	3.87±0.07	5.90 ± 0.76	3.58 ± 0.27	2.27 ± 0.38	2.48 ± 0.11
5	7.20±0.65	4.67±0.06	5.43 ± 0.42	4.43 ± 0.23	1.63 ± 0.34	2.44 ± 0.22

Plantlets*=Plantlets per explant; Height#=Height of plantlets (cm)

kinetin both maximum numbers of plantlets/explant (6.85) and height (5.81 cm) were achieved at 4 mg/l. The highest numbers of plantlets/explant (6.22) regenerated with zeatin were at 3 mg/l whereas the maximum height (4.43 cm) was at zeatin 5 mg/l.

The maximum numbers of *Kaempferia galanga* (figure 4.6a) plantlets/explant (4.33) were observed in the medium supplemented with BAP at a concentration of 4 mg/l while the maximum height (4.31cm) was found with BAP 3 mg/l. In the medium supplemented with kinetin the maximum numbers of plantlets/explant (3.44) were observed at 3 mg/l and the maximum height (2.82cm) at 5mg/l. The highest numbers of plantlets/ explant (3.58) regenerated with zeatin were at 2 mg/l whereas the maximum height (2.48cm) was at zeatin 4 mg/l. Callusing of the explants was observed in the medium supplemented with 4 and 5 mg/l BAP.

Among the cytokinins tried, BAP gave better results compared to Kinetin and Zeatin in regeneration of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*. BAP was more effective than kinetin and zeatin in all the cultures when they were used alone. Our result finds similarity with several workers. BAP was found to be very much effective in the generation of ginger tissue culture (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Ikeda and Tanaba, 1989; Kackar *et al.,* 1993; Sit *et al.*, 2005; Bhattacharya and Sen, 2006), turmeric tissue culture (Winnar and Winnar, 1981; Shetty *et al.*,1982; Keshavachandran and Khader, 1989; Balachandran *et al.,* 1990; Rahman *et*

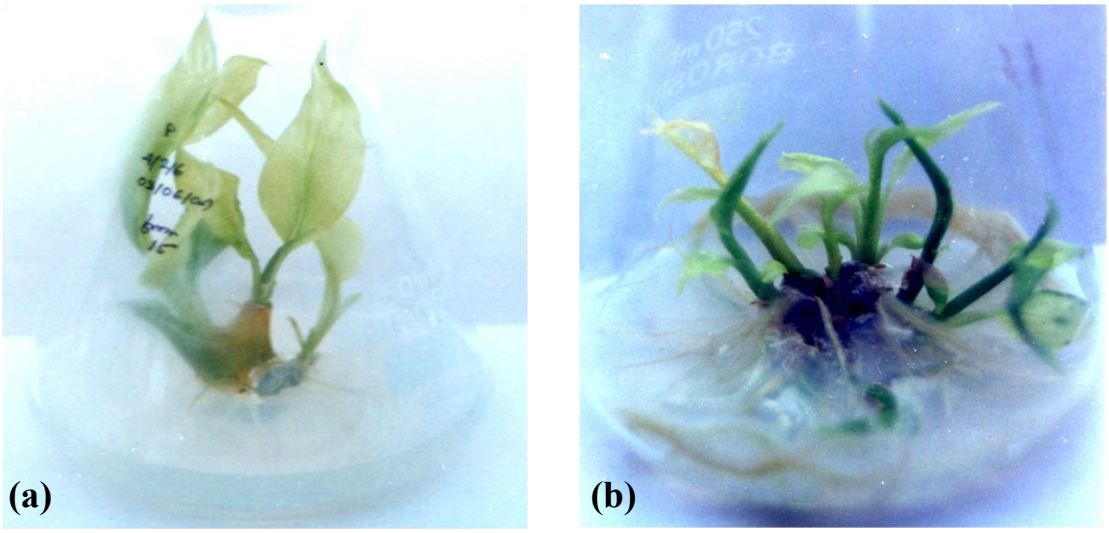


Figure 4.4: Shooting of *Zingiber officinale* plantlets in MS media (a) Media supplemented with BAP 3 mg/l. (b) Media supplemented with BAP 3 mg/l + kinetin 2 mg/l

al., 2004; Dipti *et al.*, 2005 and Panda *et al*., 2007) and in tissue culture of *K. galanga* (Lakshmi and Mythili, 2003 and Bhattacharya and Sen, 2013).

Lower concentrations of cytokinin produced less number of shoots from the explants while it declined above a critical level. The decline in the rate of shooting above a critical level of cytoikinins may be due to some inhibitory effect produced by higher concentrations of cytokinins. Similar results showing gradual increase and decline in the number of shoots after a certain level of cytokinins was observed in ginger tissue culture (Balachandran *et al.*, 1990 and Sit *et al.*, 2005, Bhattacharya and Sen, 2006).

4.2.6.2 Combinations of cytokinins on regeneration

The regeneration of plantlets varied considerably with different concentrations and combinations of BAP and Kinetin (table 4.6). In *Zingiber officinale* (figure 4.4b) culture, maximum number of plantlets/ explant were obtained in the medium supplemented with 4 mg/l BAP + 3 mg/l Kinetin (9.60) followed by 4 mg/l BAP +2 mg/l Kinetin (8.94), while the maximum plantlet height was obtained in the medium supplemented with 4 mg/l BAP + 3 mg/l Kinetin (8.59) followed by 3 mg/l BAP + 4 mg/l Kinetin (8.38). Similar results were obtained by Khatun *et al*., (2003). However, other combinations like BAP and NAA (Inden *et al*., 1988; Choi 1991; Choi and Kim, 1991; Dogra *et al*., 1994; Behera and Sahoo, 2009), BAP and 2, 4-D (Nirmal Babu *et al*., 1992) and BAP, IAA and adenine sulfate (Mohanty *et al*., 2008) were

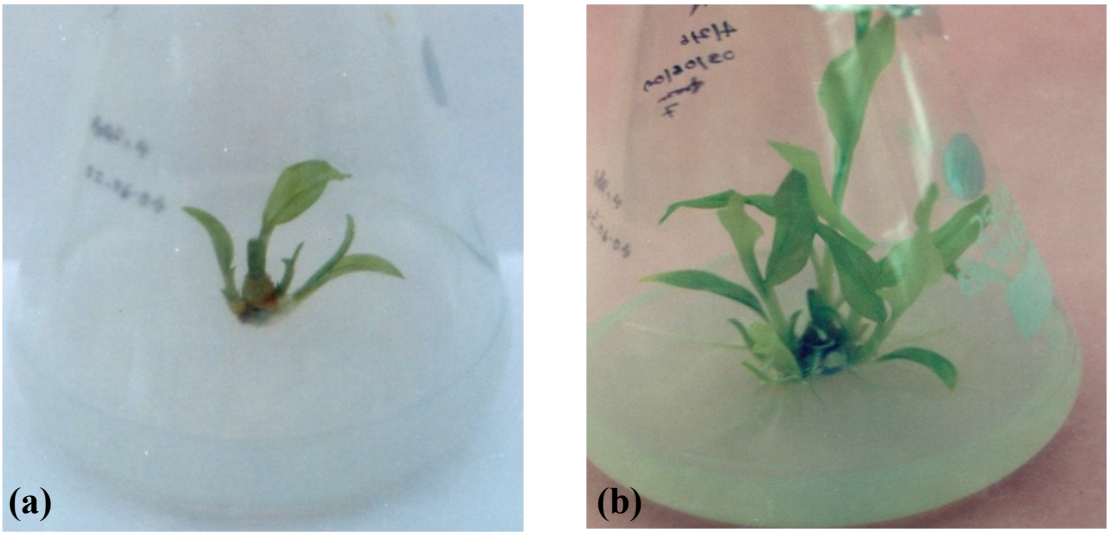


Figure 4.5: Shooting of *Curcuma longa* plantlets in MS media (a) Media supplemented with BAP 2 mg/l. (b) Media supplemented with BAP 3 mg/l + kinetin 2 mg/l

also effective.

In *Curcuma longa* (figure 4.5b) culture the maximum number of plantlets were obtained in the medium supplemented with 3 mg/l BAP + 4 mg/l Kinetin (9.20) followed by 3mg/l BAP + 3 mg/l Kinetin (8.64), while the maximum plantlet height was obtained in the medium supplemented with 4 mg/l BAP + 4 mg/l Kinetin (8.36 cm) followed by 3 mg/l BAP + 4 mg/l Kinetin (8.19 cm). Similar results showing high efficacy of BAP and Kinetin were observed by Shetty *et al.* (1982); Keshavachadra and Khader (1989). Other combinations of plant growth hormones like BAP and NAA (Yasuda *et al*., 1988; Behera *et al*., 2010), BAP and IAA (Singh *et al*., 2011), BAP and NAA or NAA and kinetin (Sunitibala *et al*., 2001), IBA (Rahaman *et al*., 2004) and Thidiazuron (Prathanturarug *et al*., 2005) were also effective.

In *Kaempferia galanga* (figure 4.6b) culture the maximum numbers of plantlets were obtained in the medium supplemented with 3 mg/l BAP + 4 mg/l Kin (6.52) followed by 2 mg/l BAP + 4 mg/l Kin (6.06), while the maximum plantlet height was obtained in the medium supplemented with 2 mg/l BAP + 4 mg/l Kin (8.23 cm) followed by 3 mg/l BAP + 3 mg/l Kin (8.15 cm).

Regeneration of plantlets gradually increased with increase in hormone concentrations while it declined above a certain concentrations. Similar results were observed by Lakshmi & Mythili (2003). Combinations of BAP and IAA (Swapna *et al*., 2004 and Parida *et al*.,

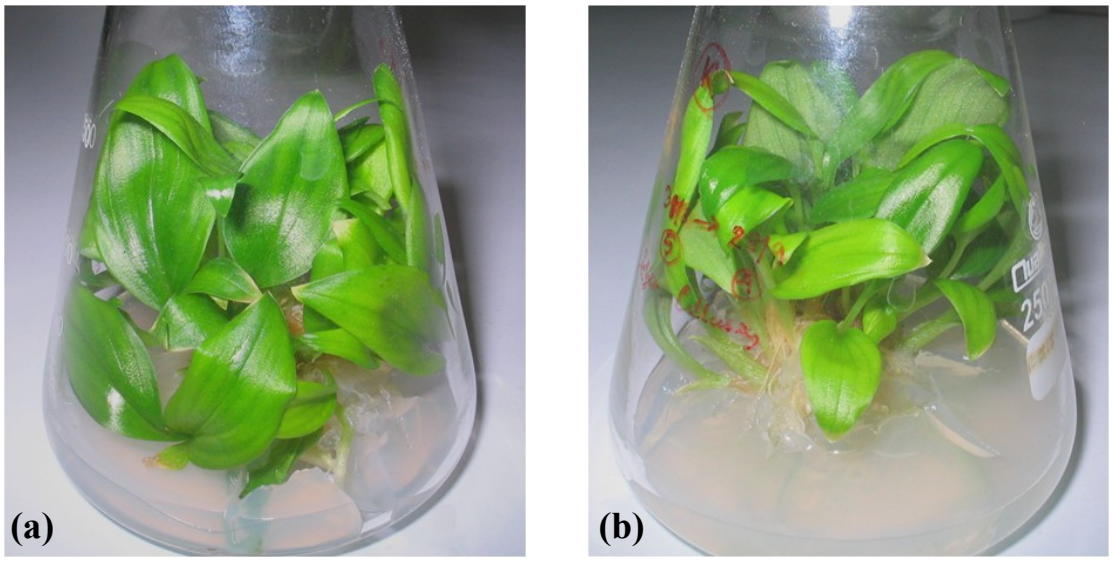


Figure 4.6: Shooting of *Kaempferia galanga* plantlets in MS media (a) Media supplemented with BAP 4 mg/l. (b) Media supplemented with BAP 4 mg/l + kinetin 3 mg/l

Table :4.6. Effect of different combinations and concentrations of BAP and Kinetin on *in vitro* regeneration of *Z. officinale*, *C. longa* and *K. galanga.* The values *are* average of 20 replicates

Plant growth regulator (mg/l)		*Zingiber officinale*		*Curcuma longa*		*Kaempferia galanga*	
BAP	**Kinetin**	**Plantlets***	**Height#**	**Plantlets***	**Height#**	**Plantlets***	**Height#**
1	1	2.13±0.25	1.99±0.13	2.38 ±0.31	2.13±0.29	2.56± 0.11	0.71± 0.12
	2	3.06±0.41	3.56±0.35	2.80± 0.37	3.32±0.55	2.73± 0.12	1.62± 0.36
	3	5.33±0.34	5.78±0.55	3.63± 0.57	5.44±0.32	3.38± 0.41	2.21± 0.16
	4	5.66±0.41	6.27±0.40	4.13± 0.60	6.15±0.43	3.93± 0.30	2.74± 0.12
2	1	3.26±0.25	2.72±0.12	2.59± 0.21	2.76±0.40	3.13± 0.35	4.31± 0.24
	2	5.73±0.52	3.55±0.12	3.31± 0.43	3.82±0.32	4.33± 0.27	5.41± 0.12
	3	7.13±0.25	5.98±0.16	4.12± 0.56	5.97±0.31	4.51± 0.15	6.81± 0.16
	4	6.93±0.47	7.02±0.20	5.40± 0.44	6.88±0.46	6.06± 0.24	8.23± 0.20
3	1	5.87±0.19	4.04±0.07	6.70± 0.47	4.02±0.43	4.76± 0.11	6.28± 0.12
	2	7.13±0.23	4.38±0.50	7.46± 0.34	4.91±0.50	5.75± 0.72	7.14± 0.38
	3	7.40±0.43	7.07±0.69	8.64± 0.66	6.25±0.36	6.00± 0.29	8.15± 0.16
	4	8.53±0.41	8.38±0.13	9.20± 0.47	8.19±0.39	6.52± 0.22	7.41± 0.21
4	1	8.40±0.30	4.59±0.22	7.90± 0.45	6.18±0.41	5.48± 0.39	5.86± 0.16
	2	8.94±0.57	7.53±0.28	8.48± 0.41	7.08±0.29	5.76± 0.20	6.39± 0.28
	3	9.60±0.59	8.59±0.19	7.53± 0.52	8.12±0.31	5.53± 0.26	7.09± 0.09
	4	7.40±0.16	7.09±0.61	7.19± 0.50	8.36±0.20	5.43± 0.45	6.26± 0.26

Plantlets*=Plantlets per explant; Height#=Height of plantlets (cm)

2010), BAP and NAA (Shirin *et al*., 2000; Rahaman *et al*., 2004; Rahaman *et al*., 2005 and Chirangini *et al*., 2005) and IAA and kinetin (Chirangini *et al*., 2005) were also found good.

In general plantlet regeneration was relatively less in the medium supplemented with low BAP and Kinetin combination. Good results were obtained when the combined concentrations of BAP and Kinetin were more than 5 mg/l. The plant growth regulators worked better in combinations than that when used alone. The plantlets rooted in the same medium and they were moderate to profuse in most of the combinations. The rate of rooting was found to be proportional to the number of plantlets. Similar results of proportional rooting to the number of plantlets were observed by (Khatun *et al*., 2003) in ginger tissue culture**.** Rout *et al*. (2001) observed that in ginger regeneratoion, the medium having kinetin alone or in combination with Adenine sulphate showed a low rate of shoot multiplication and inhibited shoot elongation as compared to BA alone or BAP + Adenine sulphate.

4.2.7 ***Primary and secondary cultures***

The effects of plant growth regulators on regeneration were not the same in primary and secondary cultures (figure 4.7). Comparatively less variations were observed in number of shoots/ explant in the primary and secondary cultures of *Curcuma longa* than *Zingiber officinale* and *Kaempferia galanga*.

In all the cases of *Zingiber officinale* and *Curcuma longa* regeneration the least number of plantlets were produced in the primary culture and the cultures showed maximum regeneration in the 2nd subculture. The regeneration potential decreased from 3rd subculture of *Zingiber officinale* and *Curcuma longa.*

In case of *Kaempferia galanga* regeneration the least number of plantlets were produced in the primary culture and it declined after the 1st or 2nd subcultures. The regeneration potential decreased in 2nd subculture (Kinetin 3 mg/l, BAP 2mg/l + Kinetin 4 mg/l, BAP 3 mg/l + Kinetin 3 mg/l and BAP 3 mg/l + Kinetin 4 mg/l) and 3rd subculture (BAP 4 mg/l).

The increase in the number of plantlets in the secondary cultures can be due to the adaptations of the explants to the culture medium and diminishing effect of sterilant. The gradual decline in the rate of regeneration with subculture can be due to ageing of the explants

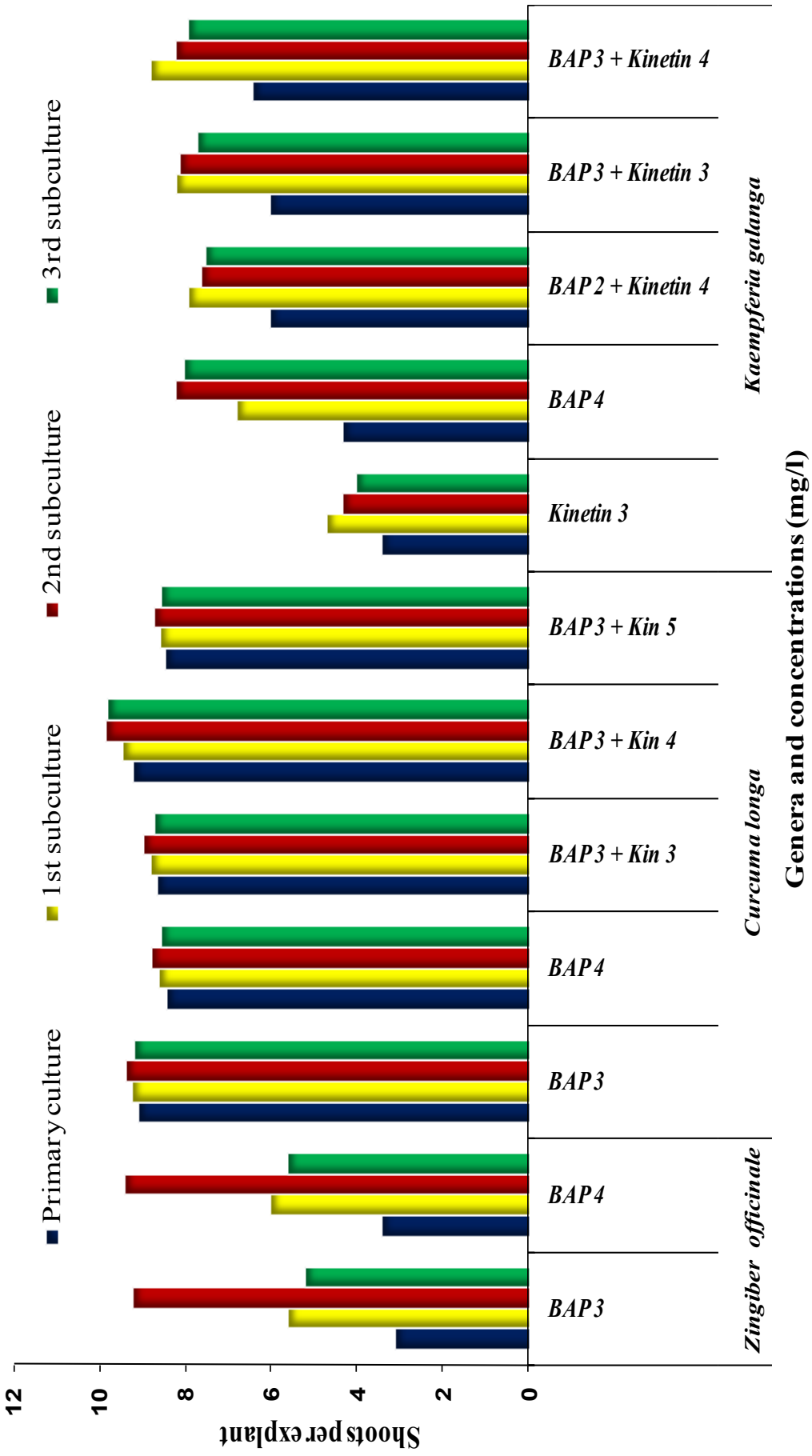


Figure 4.7: Responses in primary and subsequent subcultures

and decline in their totipotency. Mohanty *et al*. (2008) reported that the multiplication rate remained unchanged in subsequent subcultures of ginger, but, in most of the experiments with Zingibers it declined after a few subcultures.

4.2.8 ***Hardening of plantlets***

The ultimate success of *in vitro* propagation lies in the successful establishment of plant in the soil. *In vitro*-derived plantlets with a well-developed root and shoot system were successfully transferred to pots in potting mixture (figure 4.8), and after hardening were transferred to the field. The regenerated plants *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* showed a survival percentage of 94%, 91% and 94% the polycups respectively. 100% survival rates were observed in the field environment. High survival rate of 80% (Nirmal Babu *et al.*, 1992), 85% (Samsudeen *et al.,* 2000), 94% (Bhattacharya and Sen, 2006) and 95% (Behera & Sahoo, 2009) were previously reported in *Zingiber officinale.*

Khatun *et al*. (2003), achieved 100% and Mohanty *et al*. (2008) achieved 96% survival rate in field when the plantlets were directly transferred to the fields.

Survival rate of 90 to 95% (Salvi, 2000, Salvi *et al.*, 2002), 90% (Salvi *et al.*, 2001), high percentage (Zapata *et al.*, 2003), 70% (Rahman *et al.*, 2004), 90% (Gayatri and Kavyashree, 2005), 70-80% (Naz *et al.*, 2009); 95% (Behera *et al.*, 2010) survival rate.

Geetha *et al*. (1997) hardened regenerated *Kaempferia galanga* plantlets in mixture of Sand, garden soil and vermiculite (1:1:1). Hardened plantlets of *Kaempferia galaga*

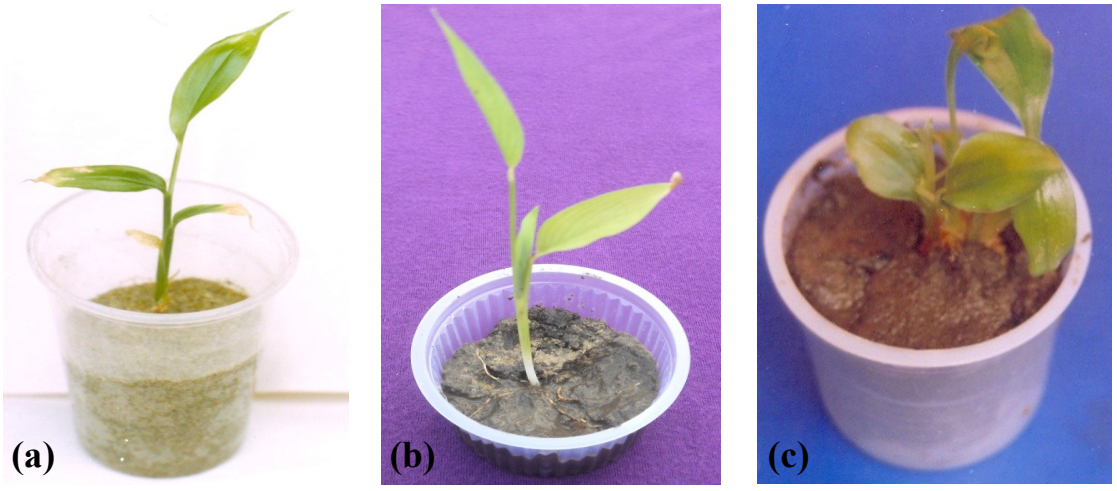


Figure 4.8: Hardened plantlets. (a) *Zingiber officinale*. (b) *Curcuma longa*. (c) *Kaempferia galanga.*

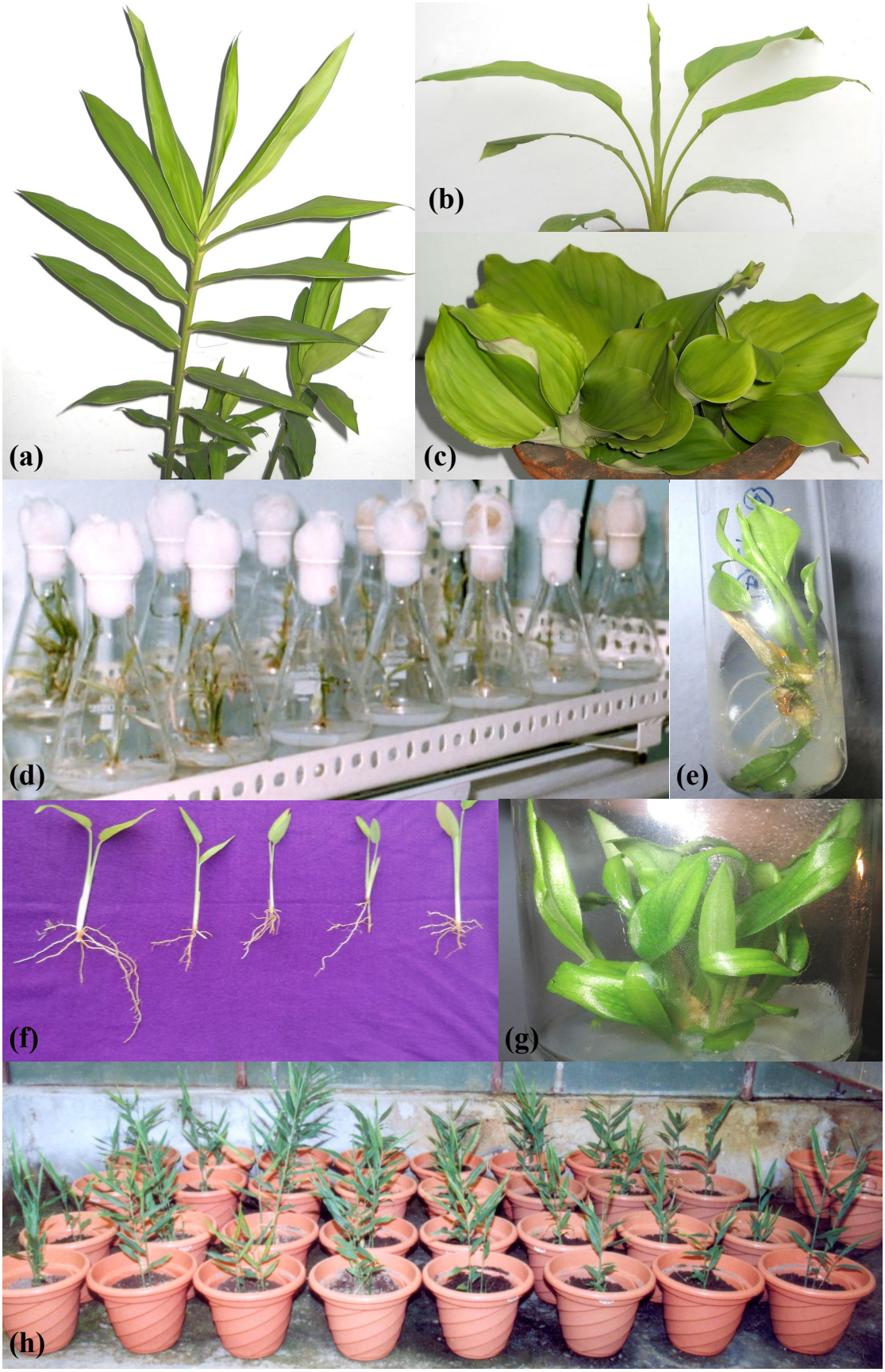


Figure 4.9: Hardened plantlets of (a) *Zingiber officinale*, (b) *Curcuma longa* & (c) *Kaempferia galanga*. (d) Regeneration of *C. longa cultivars*. (e) Regenerated plantlet of *Z. officinale* in primary culture. (f) Rooted plantlets of *C. longa* prior to hardening. (g) Secondary culture of *K. galanga* (h). Hardened plantlets of *Z. officinale*.

produced by callus culture showed normal storage roots and were acclimatized and subsequently transferred to field with 90-95% (Geetha *et al.*, 1997) and 85% survival (Rahaman *et al.*, 2004) while regenerated plants by rhizome bud proliferation were hardened and established on the field with 85% success (Rahaman *et al.*, 2005). Field survival of 80-90% (Chirangini *et al.*, 2005) and 95% was observed by (Parida *et al.*, 2010).

4.2.9 Production of disease free plantlets

In vitro-derived plants raised under field conditions did not show any disease symptoms until maturity. The rhizomes obtained from field planted tissue culture derived plantlets on storage on sand for 6 months did not show any visible root-for or shoot-rot whereas, conventionally obtained *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* rhizomes stored on sand for 6 months showed infection about 61%, 46% and 53% respectively. Rhizome pieces of micropropagated and conventional plants were transferred onto PDA to examine the infection, if any. Mycelia growths were not observed on medium after 8 – 10 days of incubation containing the micropropagation derived rhizomes. While, 79%, 74% and 58% of the pieces of conventional *Z. officinale*, *C. longa* and *K. galanga* rhizomes respectively showed mycelia growth.

4.2.10 Regeneration of Curcuma longa cultivars

Cytokinin (Benzyl Amino Purine) showed variable effects on the shooting of twelve different *Curcuma longa* cultivars (figure 4.10). The mean number of shoots formed per explant were 3.075 (BAP 1 mg/l), 4.983 (BAP 2 mg/l), 6.891 (BAP 3 mg/l), 6.358 (BAP 4 mg/l) and 5.816 (BAP 5 mg/l). Out of the twelve cultivars two cultivars (local from Dhupguri and Suguna) showed maximum number of plantlets per explant at BAP concentration of 2mg/l, nine (cultivar local from Lataguri, Prova, TC Assam, Kasturi, CLS 2A, Suvarna, Roma, Sudarshana and PTC 13) at the level of 3mg/l BAP concentration and the rest one (Allepy) at level of 4 mg/l BAP concentration while minimum number of shoots per explant were produced in eleven (cultivars local from Lataguri, Prova, Suguna, TC Assam, Allepy, Kasturi, CLS 2A, Suvarna, Roma, Sudarshana and PTC 13) at 2 mg/l BAP concentration and rest one

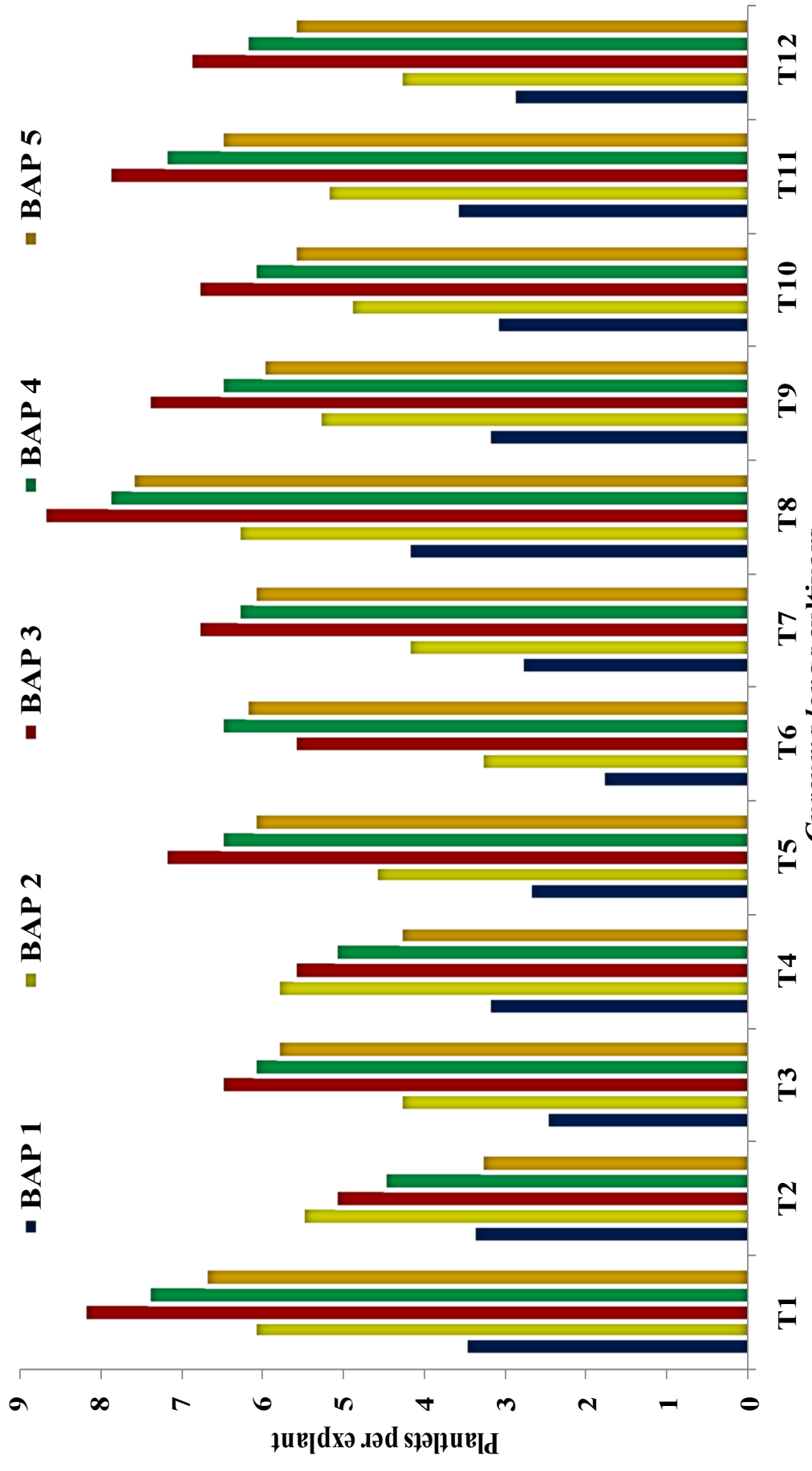


Figure 4.10: Effect of BAP on regeneration of different *Curcuma longa* cultivars (please refer table 4.1 for the species and varieties name)

(cultivar local from Dhupguri) at the level of 5mg/l BAP concentration.

Maximum regeneration potential was observed in the cultivar CLS-2A (8.7 shoots/explant) followed by the cultivar local from Lataguri (8.2 shoots/explant) with BAP 3 mg/l. The lowest regeneration potential was observed in the cultivar Allepy (1.8 shoots/explant) followed by the cultivar Prova (2.5 shoots/explant) at BAP 1 mg/l.

The highest regeneration rate was observed at BAP concentration 3 mg/l in most of the cultivars. Similar to our results, Panda *et al*. (2007) reported that, MS medium supplemented with 3.0 mg/l BAP is the best hormonal concentration for multiple shoot production in *Curcuma longa* (cv-Roma). Behera *et al.* (2010) observed that explants of *Curcuma longa* cv. Ranga) explants cultured on MS basal medium supplemented with 2.0mg/l BAP+0.5gm/l NAA showed highest rate of shoot multiplication. Highest shoot proliferation of turmeric was observed at BAP 1 mg/l (Winnar and Winnar, 1981) BAP 3 mg/l (Dipti *et al.*, 2005, Balachandran *et al.*, 1990).

The rate of shoot formation increased gradually up to a certain level of BAP concentration and then declined. This confirms the inhibitory effect of BAP at higher concentrations.

4.3 Diversity studies

4.3.1 DNA extraction, purification and quantification

DNA from the leaves of *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* were isolated using the standard protocol of Bousquet *et al*. (1990) with minor modifications. The DNA-CTAB complex gave a very good network of whitish precipitate of nucleic acid which was used for further downstream processing. The agarose gel analysis of the DNA thus obtained showed distinct and clear bands.

DNA extracted from plant tissue includes contaminants like RNA, protein, polysaccharides etc. which severely hampers the downstream process. Thus, Purification of DNA is very essential. The RNA was removed by treating the sample with RNase enzyme. Extraction with phenol: chloroform following RNase treatment was also employed for eliminating most of proteins. CTAB buffer eliminated polysaccharides from DNA to a large extent, but in isolated DNA from the leaves of Zingiberaceous plants starch residues were found intact

with the isolated DNA even after CTAB and phenol: chloroform treatment. So, peg purification of DNA was necessary for complete elimination of polysaccharides.

Spectophotometric and agarose gel analysis methods were used to quantify the DNA extracted from *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga*. Spectrophotometric analysis of the DNA showed A_{260}/A_{280} ratio in between 1.82 to 2.00 (table 4.7). For PCR based molecular documentation methods, quantification of DNA was done based on the formula mentioned in the materials and methods section.

By gel analysis the quality of DNA was judged by the presence of single compact and clear band at the corresponding position of the molecular marker λ DNA/*Eco*RI/ *Hin*dIII double digest. The quantity of DNA was estimated by comparing the sample DNA with the control by eye adjustment. The combination of the above three steps (extraction, purification and quantification) allowed the extraction of sufficient amount of pure DNA from the leaves for PCR amplification.

Table 4.7: List of different cultivars of *Curcuma longa* showing their purity

Sample ID	A_{260}/A_{280} (Purity)
T 1	1.98
T 2	1.82
T 3	1.88
T 4	1.86
T 5	1.78
T 6	1.90
T 7	1.96
T 8	1.89
T 9	1.79
T 10	2.00
T 11	1.92
T 12	1.84

4.3.2 RAPD analysis of C. longa

The genomic DNA of twelve different cultivars of *Curcuma longa* were analyzed using 25 different primers having 10mer length. Of the 25 primers screened 14 resulted in producing distinct and scorable bands (table 4.8). The amplification profiles of the total genomic DNA from the 12 cultivars of *C. longa* using 14 primers resulted in production of 170 bands ranging in between 151 and 1767bp of which only 10 were monomorphic, while rest were polymorphic (table 4.8). The percentage of polymorphism was found to be 94.11%. The number of bands generated by each RAPD primes ranged in between 09 (OPA02, OPN04 & OPN13) and 19 (OPA01). The cause for the high level of polymorphism could be intra-specific variation as reported by Nayak *et al*. (2006), who found similar outcome from RAPD analysis of 17 cultivars of turmeric. Jan *et al*. (2011) and Singh *et al*. (2012),

observed 96.84% and 91.4% polymorphism respectively in RAPD of different accessions of turmeric. Nayak *et al*. (2005) observed 69.66% polymorphism in 16 cultivars of ginger with RAPD analysis.

A representative of RAPD profiles of the 12 cultivars of *C. longa* generated using OPA04, where all the bands generated are polymorphic and OPA07 which also depicts two monomorphic band is presented in figure 4.11. The similarity matrix obtained using the Dice coefficient of similarity (Nei and Li, 1979) depicted in table 4.9. Similarity coefficient among the 12 cultivars ranged from 0.560 to 0.857. The lowest similarity was observed between *C. longa* cv local-Dhupguri and Roma and *C. longa* cv local-Dhupguri and Sudarshana while the highest value was recorded between *C. longa* cv Suguna and *C. longa* cv TC Assam. The dendrogram constructed on the basis of the data obtained from RAPD analysis is depicted in figure 4.12 Based on the similarity indices, the dendrogram showed two cultivars of *C. longa* cv Suguna and TC- Assam clustered together sharing a node at

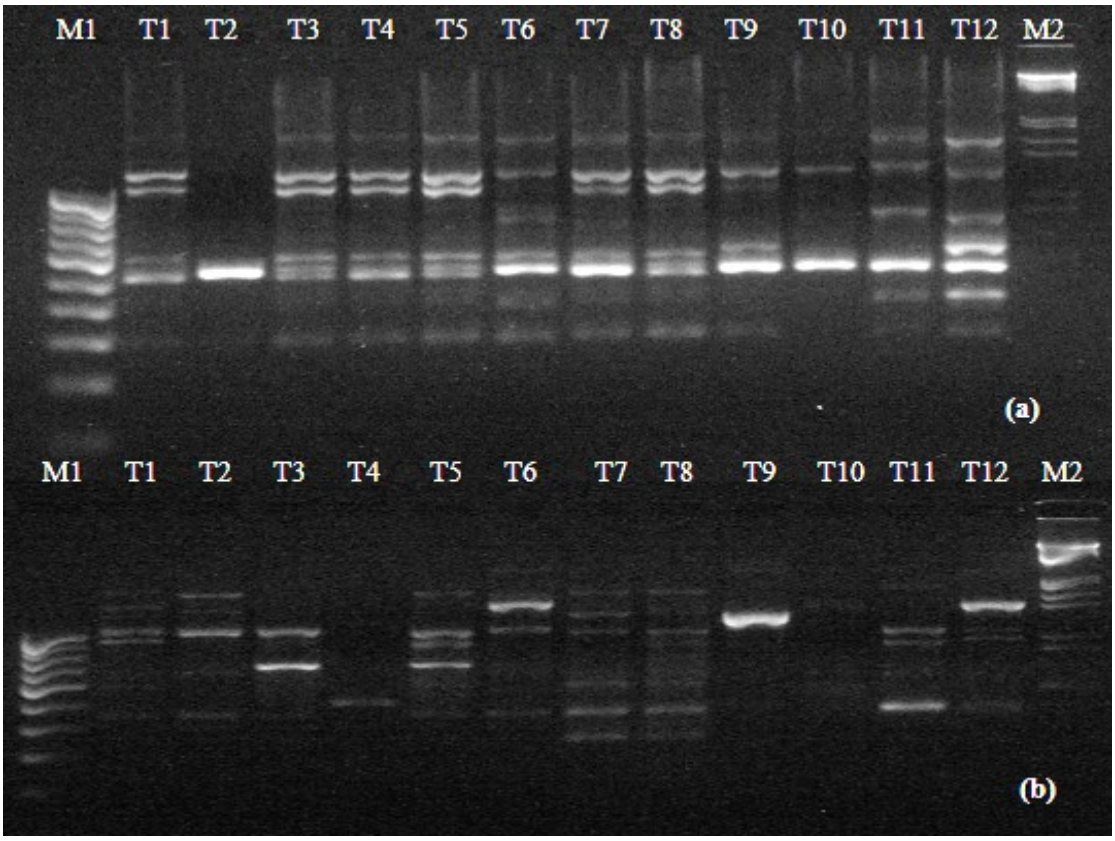


Figure 4.11: A representative RAPD profile of 12 cultivars of *Curcuma longa* amplified with primers (a) OPN04 & (b) OPA07. Lane M1: 100bp molecular marker; Lane T1-T12 different cultivars of *C. longa* under study (please refer table 4.1 for the cultivar name); Lane M2: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder

Table 4.8.Total number and size of amplified bands, number of monomorphic and polymorphic bands generated and percentage of polymorphism generated by the RAPD primers.

Primer ID	Sequence (5'-3')	Total bands amplified	Monomorphic bands	Polymorphic bands	Percentage of polymorphism	Band size (bp)
OPA01	CAGGCCCTTC	19	0	19	100%	151-1159
OPA02	TGCCGAGCTG	9	2	7	77.77%	257-1201
OPA03	AGTCAGCCAC	10	0	10	100%	349-1317
OPA 06	GGTCCCTGAC	13	0	13	100%	357-1465
OPA07	GAAACGGGTG	14	0	14	100%	386-1767
OPA09	GGGTAACGCC	15	1	14	93.33%	238-1408
OPA20	GTTGCGATCC	10	2	8	80%	647-1514
OPB01	GTTTCGCTCC	12	0	12	100%	443-1642
OPF09	CCAAGCTTCC	11	1	10	90.90%	646-1511
OPG19	GTCAGGGCAA	16	2	14	87.5%	372-1369
OPH04	GGAAGTCGCC	11	0	11	100%	581-1476
OPN04	GACCGACCCA	9	0	9	100%	335-1410
OPN13	AGCGTCACTC	9	2	7	77.77%	354-1350
OPN19	GTCCGTACTG	12	0	12	100%	412-1512
TOTAL		**170**	**10**	**160**	**94.11**	

Table: 4.9: The similarity matrix obtained using Dice coefficient of similarity among the 12 cultivars of *C. longa*, based on RAPD profiling

	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**
T1	1.000											
T2	0.824	1.000										
T3	0.791	0.703	1.000									
T4	0.725	0.615	0.802	1.000								
T5	0.780	0.670	0.813	0.857	1.000							
T6	0.637	0.593	0.626	0.714	0.769	1.000						
T7	0.670	0.626	0.703	0.769	0.802	0.725	1.000					
T8	0.758	0.692	0.725	0.747	0.802	0.703	0.846	1.000				
T9	0.736	0.670	0.835	0.791	0.824	0.725	0.714	0.758	1.000			
T10	0.604	0.560	0.637	0.681	0.692	0.681	0.670	0.626	0.736	1.000		
T11	0.582	0.560	0.659	0.593	0.626	0.615	0.604	0.626	0.714	0.626	1.000	
T12	0.670	0.582	0.637	0.659	0.758	0.813	0.648	0.670	0.714	0.714	0.648	1.000

For details of sample ID (T1 to T12) please refer table 4.1

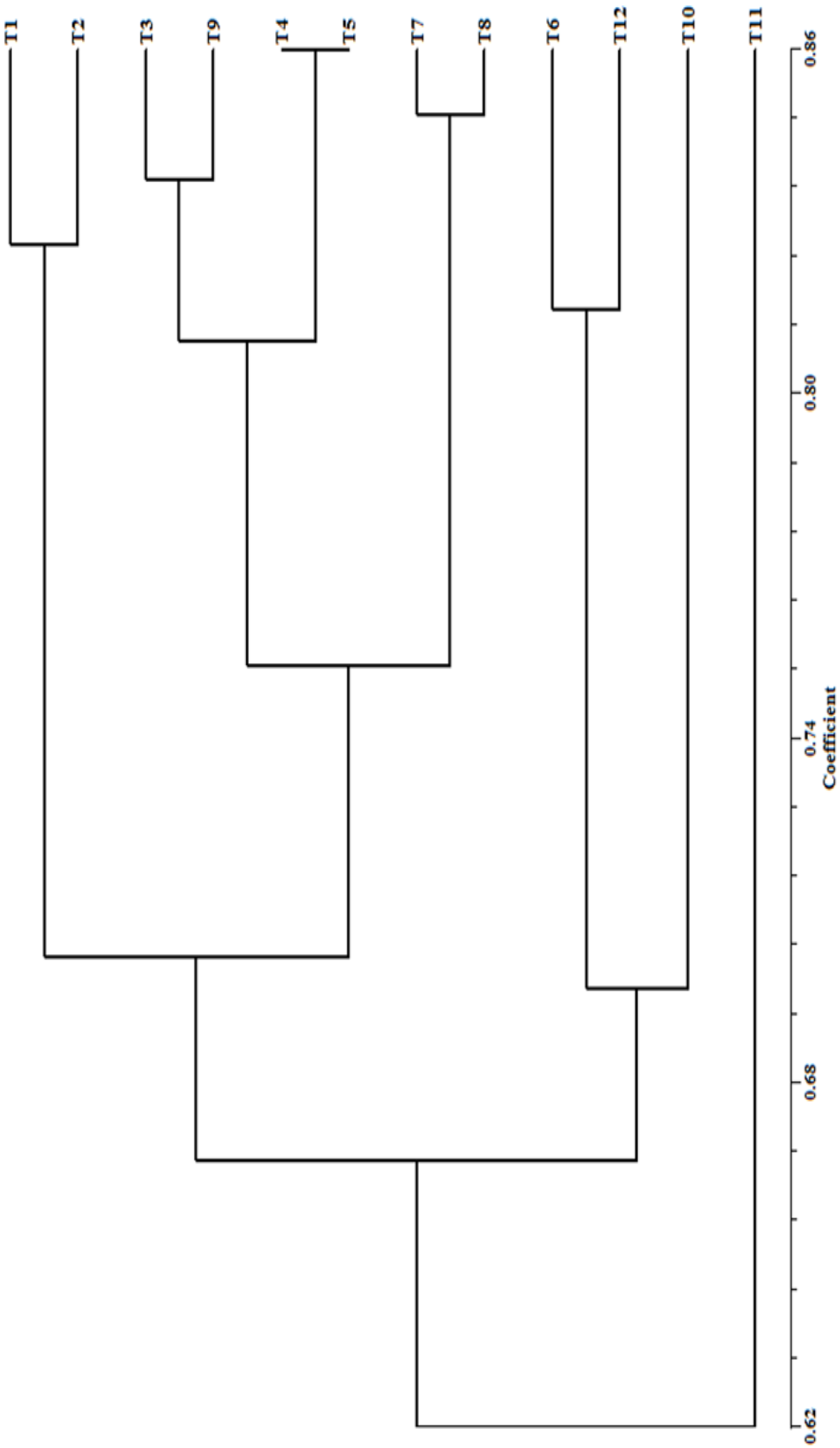
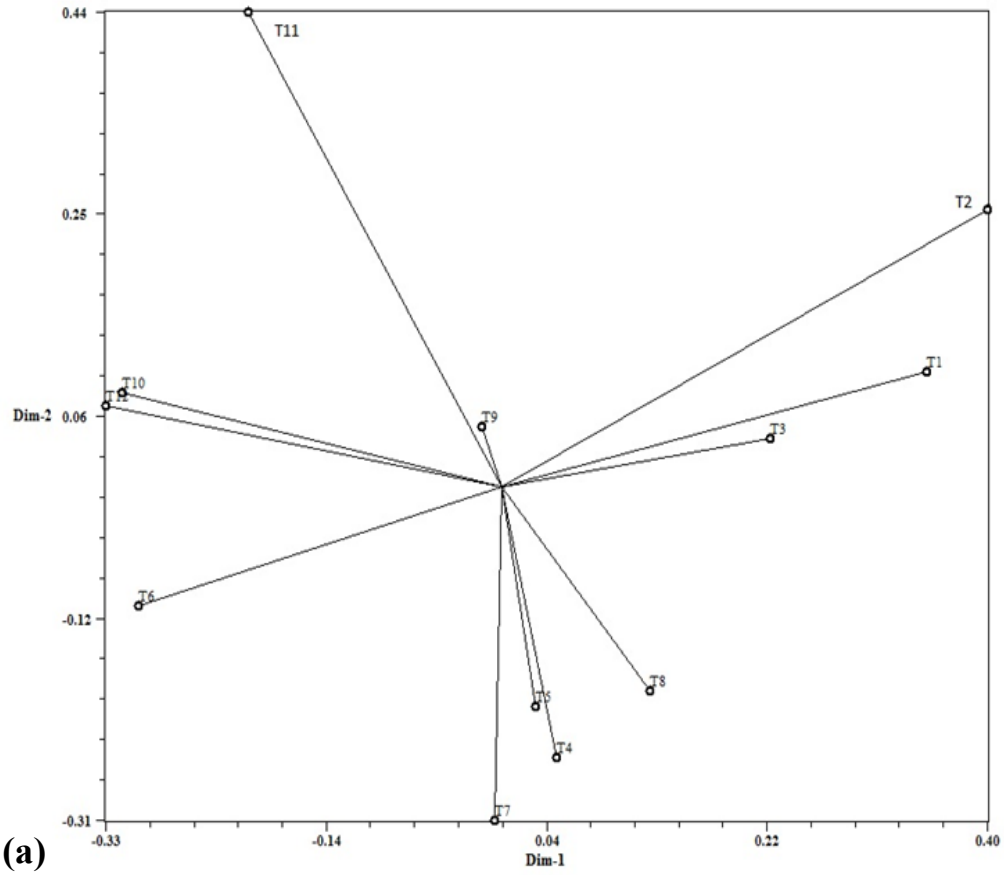
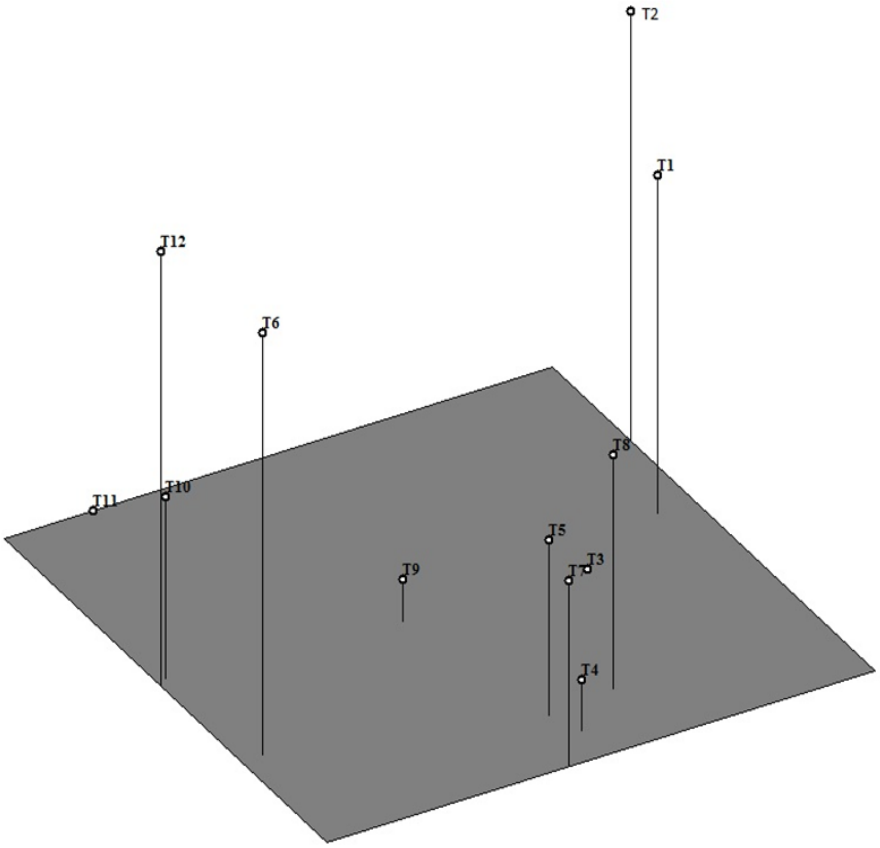


FIgure 4.12: Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationships among the 12 cultivars of *C. longa*. For details of sample ID (T1 to T12) please refer table 4.1



(a)



(b)

Figure 4.13: Principal coordinate analysis of 12 cultivars of *Curcuma longa* on RAPD analysis data. (a) 2-dimensional plot and (b) 3-dimensional plot. For details of sample ID (T1 to T12) please refer table 4.1

85.7%. Similar clusters were also observed between *C. longa* cv Kasturi and *C. longa* cv CLS 2A; *C. longa* cv Prova and *C. longa* cv Suvarna; *C. longa* cv Local-Lataguri and *C. longa* cv Local-Dhupguri and two south Indian varities, *C. longa* cv Allepy and *C. longa* cv PTC13 sharing nodes with similarity of 84.6%, 83.5%, 82.4% and 81.3% respectively. Three distinct groups viz. group I comprising *C. longa* cv Local-Lataguri, Local-Dhupguri, Prova, Suvarna, Suguna, Kasturi and CLS 2A; group II comprising *C. longa* cv Allepy, PTC13 and Roma and group III comprising *C. longa* cv (Sudarshana) were noted. The position of *C. longa* cv Sudarshana distinc from other cultivars indicate its genetic divergence.

4.3.3 ISSR analysis of C. longa

Phylogenetic relationship among 12 cultivars of *Curcuma longa* collected from different places were analyzed by DNA based techniques. Fifteen ISSR primers were initially used, out of which only 8 primers were able to produce distinct, scorable bands (table 4.10) and were selected for further study. Among the primers used, the primer UBC818 produced only 02 bands while UBC824 and UBC873 amplified the highest number of bands i.e. 12. A total of 75 amplified bands

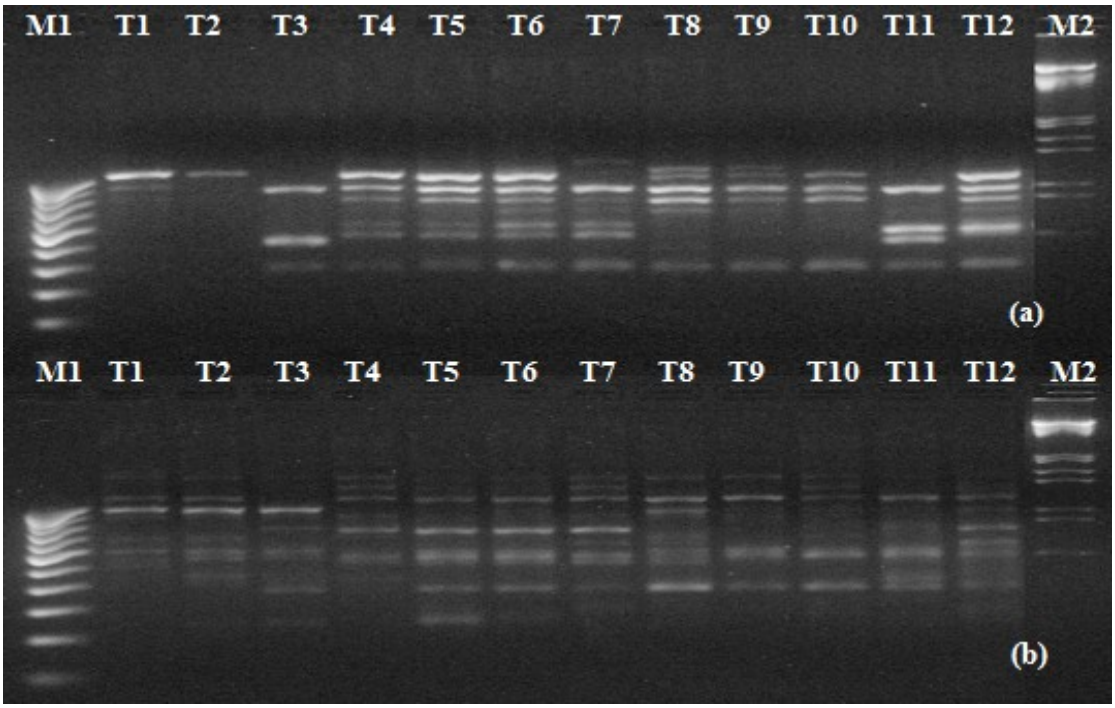


Figure 4.14: ISSR banding patterns of 12 cultivars of *Curcuma longa* generated by (a) UBC 815 primer and (b) UBC873. Lane M1: λ DNA/*Eco*RI/*Hin*dIII double digest DNA ladder; Lane T1-T12 different cultivars of *C. longa* under study (please refer table 4.1 for the cultivar name); Lane M2:100bp molecular marker

were produced by the 8 primers of which 69 were polymorphic. The frequency of polymorphism was found to be 92%. The band size ranged between 274bp to 1758bp. A representative of ISSR profile of the 12 cultivars of turmeric generated with the primers UBC815 and UBC 873 are depicted in figure 4.14. Singh *et al.* (2012) observed 95.4% polymorphism with ISSR analysis of *Curcuma longa* accessions collected from different agroclimatic conditions.

The similarity matrix obtained using the Dice coefficient of similarity (Nei and Li, 1979) depicted in table 4.11. Similarity coefficient among the 12 cultivars ranged between 0.523 to 0.904. The lowest similarity was observed between *C. longa* L. cv Local -Dhupguri and *C. longa* L. cv Allepy, while the highest value was recorded between *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam. The dendrogram constructed on the basis of the data obtained from ISSR analysis is depicted in figure 4.15. The dendrogram showed that based on the similarity indices, two cultivars of *C. longa* i.e. Suguna and TC Assam formed a cluster sharing a node at 90.4%. Clustering above a similarity of 80% was also formed in between Local -Lataguri and Local-Dhupguri (88.0%) and CLS 2A and Suvarna (88.0%). Three major groups viz. group I comprising *C. longa* cv (Local-Lataguri and Local-Dhupguri), group II comprising *C. longa* cv (Prova, Sudarshana and PTC 13) and group III

Table 4.10: Total number and size of amplified bands, number of monomorphic and polymorphic bands generated and percentage of polymorphism generated by the ISSR primers.

Primer ID	Sequence (5'-3')	Bands amplified			Percentage of polymorphism	Band size (bp)
		Total	Monomorphic	Polymorphic		
UBC810	(GA)8T	10	0	10	100%	512-1234
UBC815	(CT)8G	9	0	9	100%	434-1758
UBC818	(CA)8G	2	1	1	50%	490-656
UBC822	(TC)8A	8	2	6	75%	600-1550
UBC824	(TC)8G	12	1	11	91.66%	387-1642
UBC825	(AC)8T	11	0	11	100%	355-1567
UBC856	(AC)8YA	11	1	10	90.90%	274-1500
UBC873	(GACA)4	12	1	11	91.66%	368-1153
TOTAL		**75**	**6**	**69**	**92.00%**	

Table 4.11: The similarity matrix obtained using Dice coefficient of similarity among the 12 cultivars of *C. longa*, based on ISSR profiling

	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**
T1	1.000											
T2	0.880	1.000										
T3	0.738	0.714	1.000									
T4	0.690	0.619	0.714	1.000								
T5	0.595	0.523	0.666	0.904	1.000							
T6	0.547	0.523	0.666	0.809	0.809	1.000						
T7	0.547	0.571	0.666	0.714	0.666	0.714	1.000					
T8	0.666	0.690	0.595	0.642	0.595	0.690	0.785	1.000				
T9	0.690	0.666	0.619	0.666	0.619	0.714	0.666	0.880	1.000			
T10	0.690	0.666	0.666	0.761	0.714	0.761	0.666	0.785	0.857	1.000		
T11	0.619	0.595	0.738	0.690	0.642	0.690	0.642	0.619	0.690	0.738	1.000	
T12	0.571	0.595	0.642	0.642	0.595	0.642	0.595	0.619	0.738	0.690	0.714	1.000

For details of sample ID (T1 to T12) please refer table 4.1

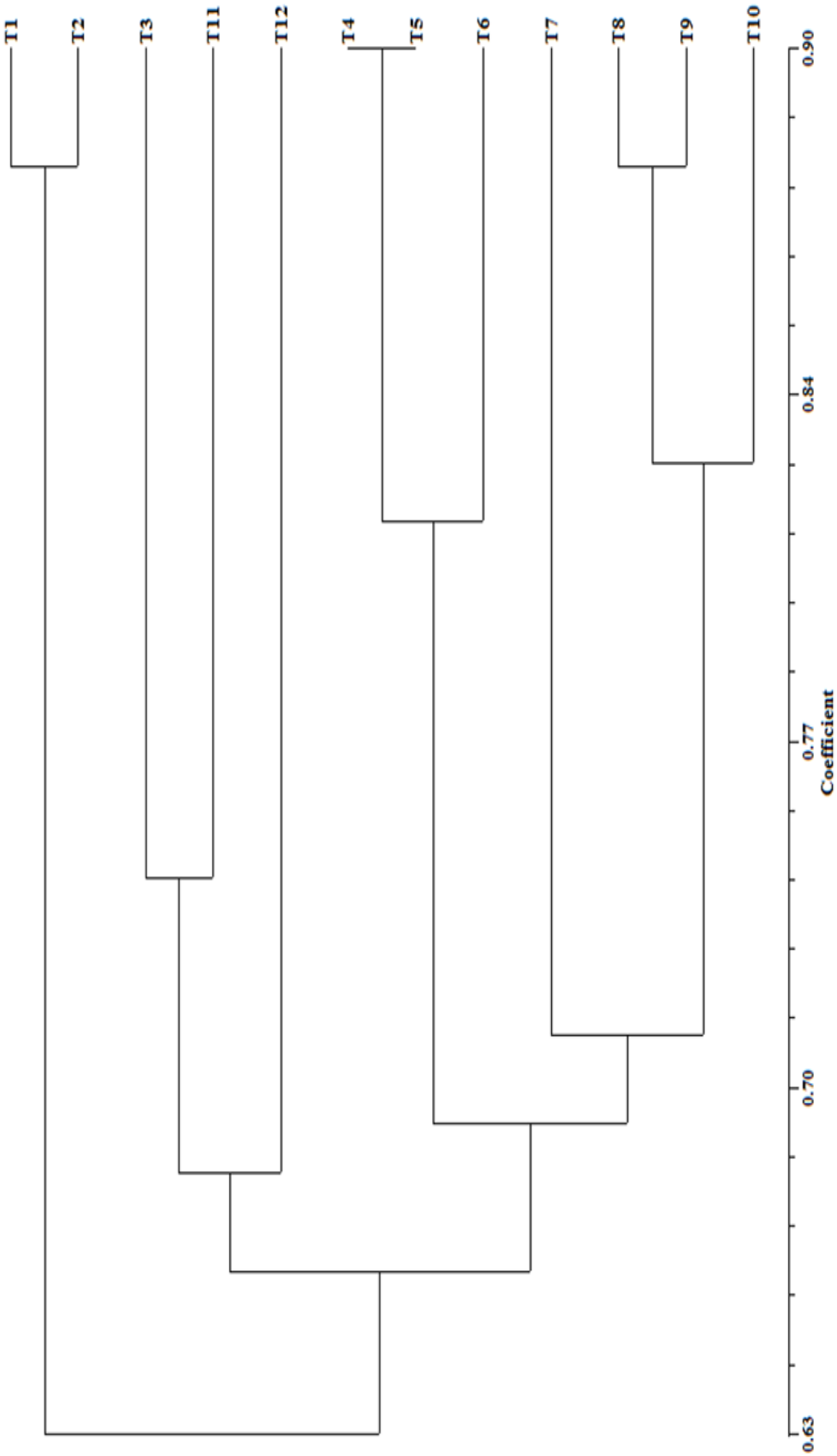
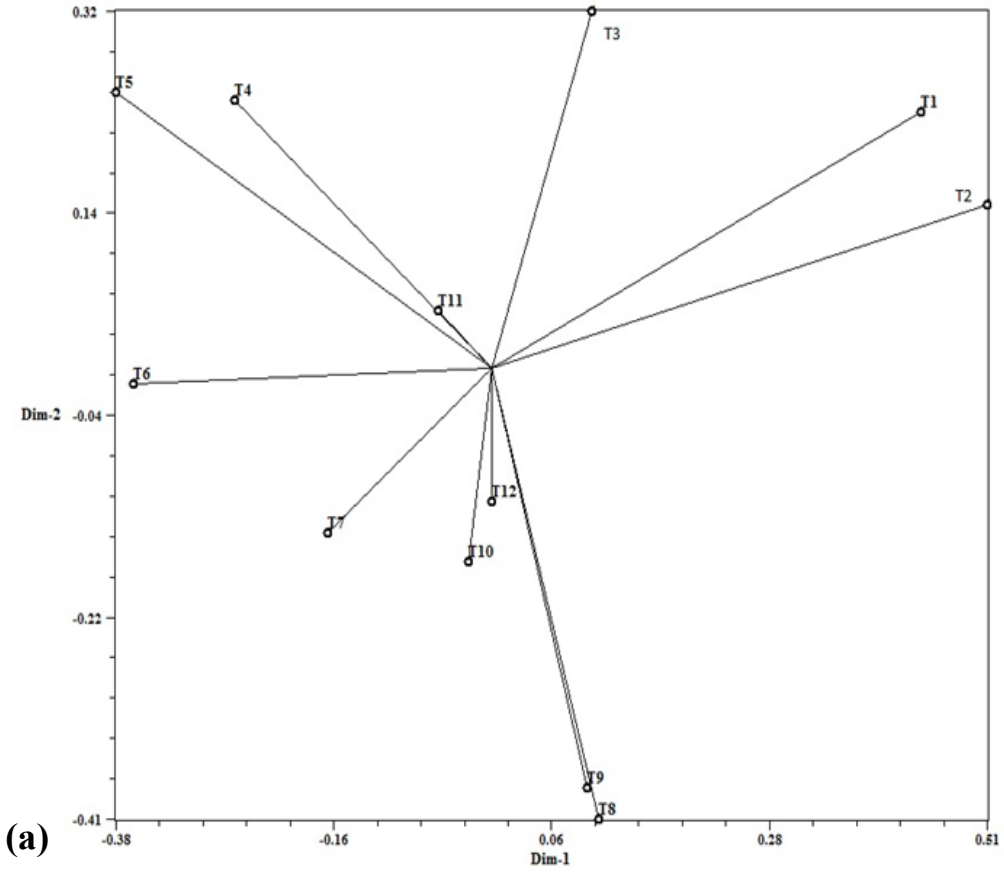
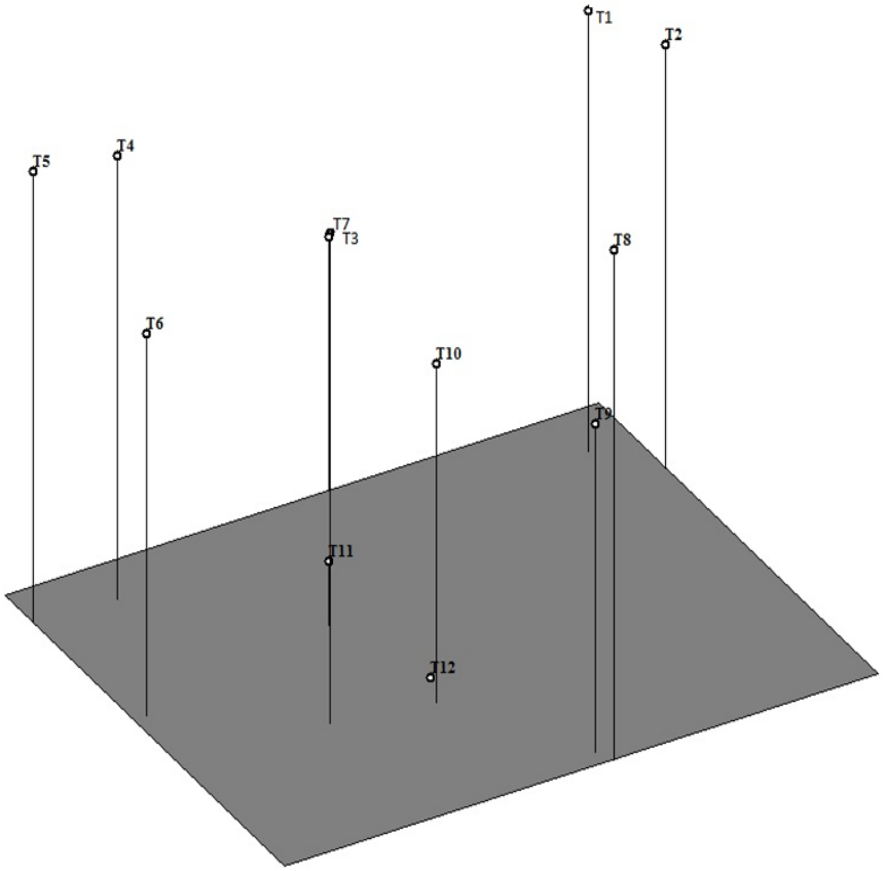


Figure 4.15: Dendrogram generated from the cluster analysis of ISSR markers of 12 *C. longa* cultivars
For details of sample ID (T1 to T12) please refer table 4.1



(a)



(b)

Figure 4.16: Principal coordinate analysis of 12 cultivars of *C. longa* based on ISSR analysis data. (a) 2-dimensional plot and (b) 3-dimensional plot. For details of sample ID (T1 to T12) please refer table 4.1

comprising *C. longa* cv (Suguna, TC Assam, Allepy, Kasturi, CLS 2A, Suvarna and Roma) were noted. Of these the first group with two local cultivars of *C. longa* formed a distinct clade from rest of the cultivars.

4.3.4 Combined RAPD and ISSR based analysis of C longa

Similarity coefficients of the 12 cultivars of *Curcuma longa* based on 170 RAPD and 75 ISSR loci ranged in between 0.571 and 0.872 (table 4.12). The highest similarity was found between the cultivars *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam while the lowest was noted between *C. longa* L. cv Local-Dhupguri and *C. longa* L. cv Sudarshana. The highest similarity of the cultivars *C. longa* L. cv Suguna and *C. longa* L. cv TC Assam were also noted in the results of RAPD and ISSR when calculated separately.

Cluster analysis performed from the combined data sets of both RAPD and ISSR markers generated a dendrogram as illustrated in figure 4.17. The dendrogram showed that, based on the similarity indices, two cultivars of *Cucuma longa* i.e. Suguna and TC Assam formed a cluster sharing a node at 87.2%. Clustering above a similarity of 80% was also formed in between Local-Lataguri and Local-Dhupguri (84.2%) and Kasturi and CLS 2A (82.7%). Three major groups viz. group I comprising *C. longa* cv (Local-Lataguri and Local-Dhupguri), group II comprising *C. longa* cv (Prova, Suguna, TC Assam, Allepy, Kasturi, CLS 2A, Suvarna, Roma and PTC 13) and group III comprising *C. longa* cv (Sudarshana) were noted. Of these the first and the third group with two local cultivars of *C. longa* and the cultivar Sudarshana respectively formed two distinct clade from rest of the cultivars.

Detection of high polymorphism is of considerable significance. Amplification of large number of polymorphic bands indicate that the primer sets used in this study could be of significance for assessment of genetic diversity in turmeric cultivars. The high genetic diversity among the local and other cultivars also confirms the correlation between the geographical distance and genetic similarity between the individuals.

4.3.5 Comparative account of the DNA fingerprinting study

A detailed study of DNA fingerprinting showed that among various techniques used in this study like RAPD and ISSR markers. Both RAPD and ISSR markers proved to be efficient

Table 4.12: The similarity matrix obtained using Dice coefficient of similarity among the 12 cultivars of *C. longa*, based on ISSR profiling

	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**	**T12**
T1	1.000											
T2	0.842	1.000										
T3	0.774	0.706	1.000									
T4	0.714	0.616	0.774	1.000								
T5	0.721	0.624	0.766	0.872	1.000							
T6	0.609	0.571	0.639	0.744	0.781	1.000						
T7	0.631	0.609	0.691	0.751	0.759	0.721	1.000					
T8	0.729	0.691	0.684	0.714	0.736	0.699	0.827	1.000				
T9	0.721	0.669	0.766	0.751	0.759	0.721	0.699	0.796	1.000			
T10	0.631	0.593	0.646	0.706	0.699	0.706	0.669	0.676	0.774	1.000		
T11	0.593	0.571	0.684	0.624	0.631	0.639	0.616	0.624	0.706	0.661	1.000	
T12	0.639	0.586	0.639	0.654	0.706	0.759	0.631	0.654	0.721	0.706	0.669	1.000

For details of sample ID (T1 to T12) please refer table 4.1

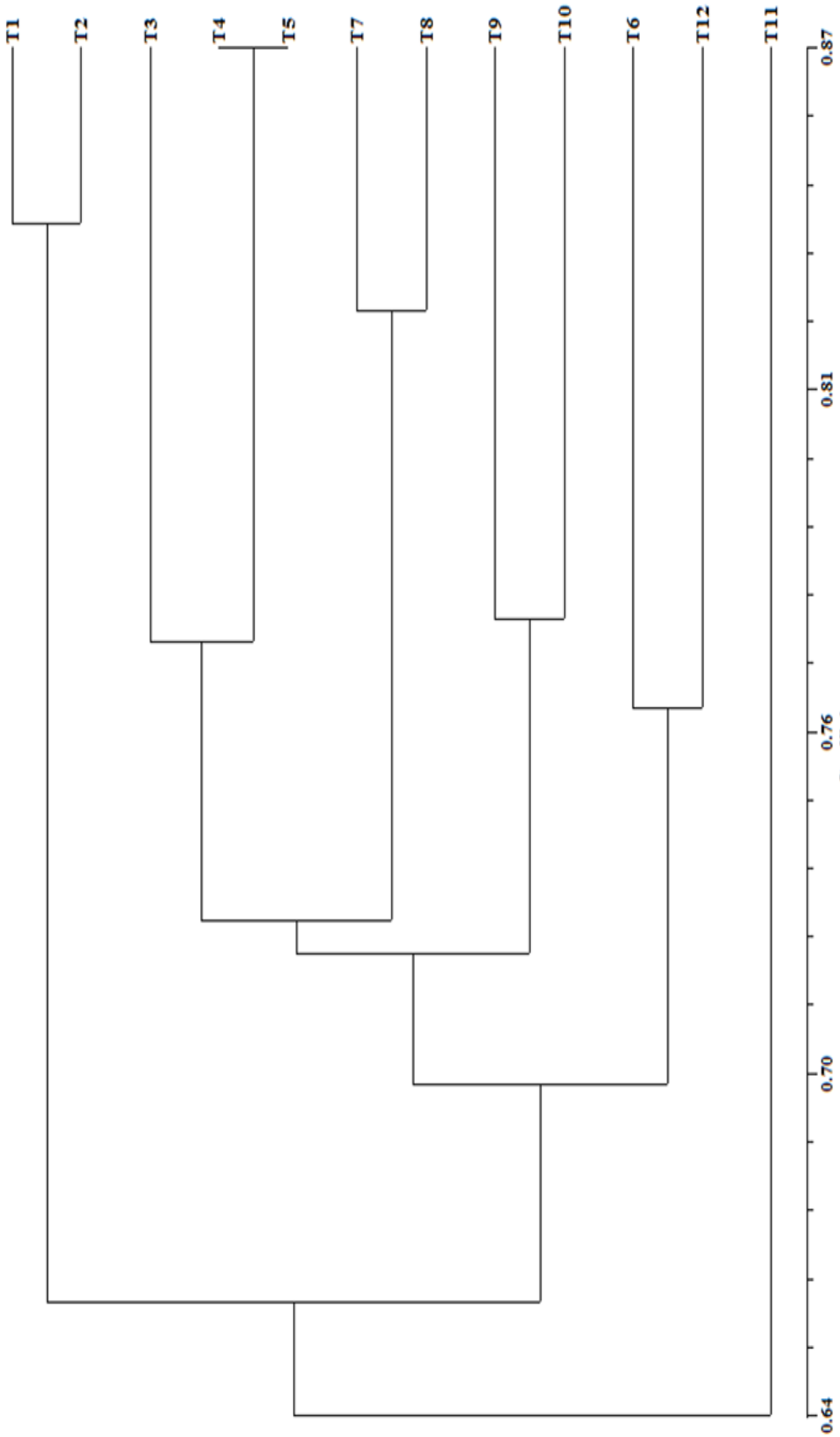
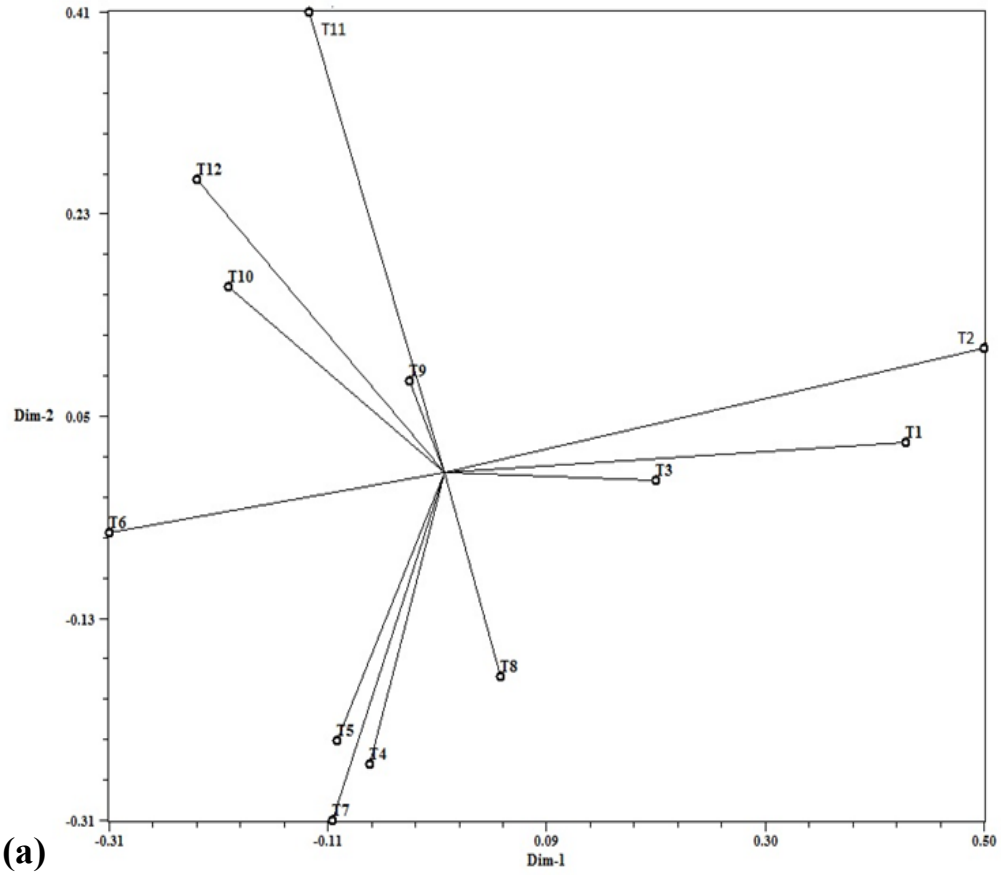
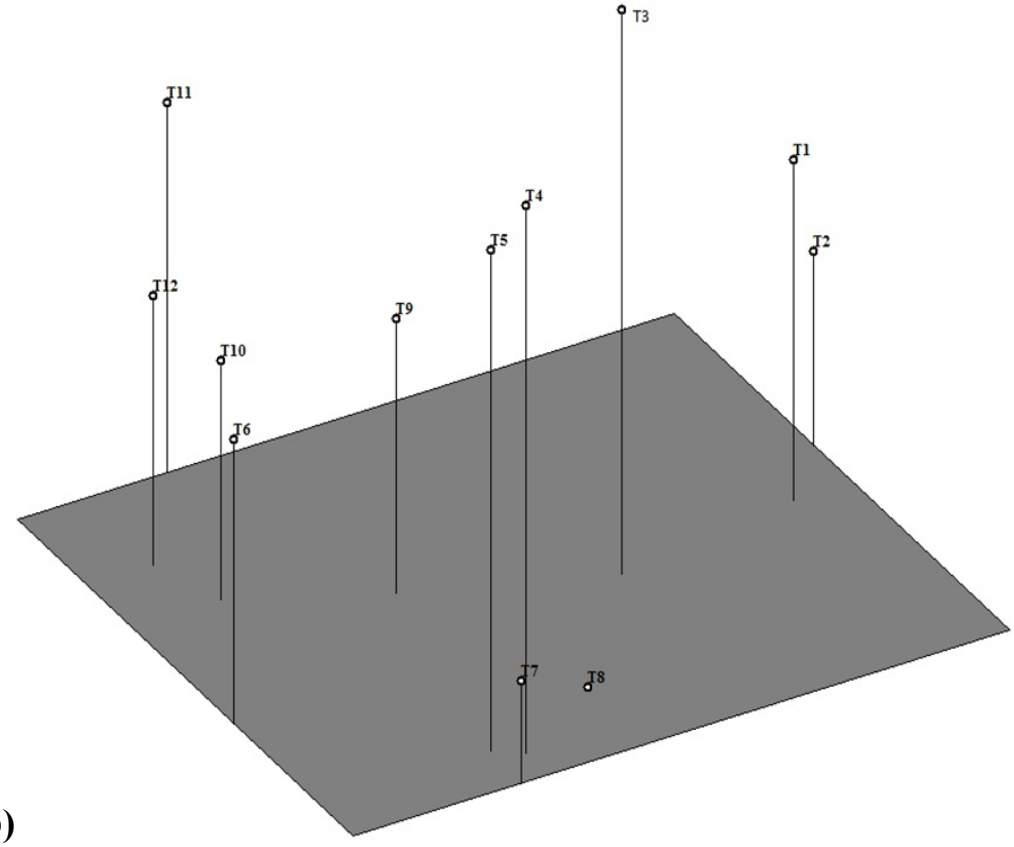


Figure 4.17: Dendrogram constructed on the basis of data obtained from the combined RAPD and ISSR analysis. For details of sample ID (T1 to T12) please refer table 4.1



(a)



(b)

Figure 4.18: Principal coordinate analysis of 12 cultivars of *C. longa* based on combined RAPD and ISSR analysis data. (a) 2-dimensional plot and (b) 3-dimensional plot. For details of sample ID (T1 to T12) please refer table 4.1

revealing 94.11% and 92% polymorphism respectively among the twelve cultivars of *Curcuma longa* under study. These molecular techniques revealed a quick and effective means to establish the genetic relationships between the cultivars on the basis of their molecular differences under the light of the statistical analysis using software like NTSYSpc. The fourteen RAPD primers (table 4.8) and eight ISSR primers (table 4.10) revealing 94.11% and 92% polymorphism respectively may prove to be promising markers obtained from this study. These markers may provide a cheap, rapid and effective means to evaluate the genetic diversity among different *C. longa* cultivars . As flowering is rare in Zingibers so this method can also be useful for cultivar identification.

Thus, DNA fingerprinting study of *Curcuma longa* can prove to be effective and promising in assessing genetic variations among the cultivars commonly found in North Bengal.

4.3.6 Sequencing of PCR product

A sample (*Curcuma longa* cv Local-Lataguri) was sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequencing resulted in forward and reverse sequence of 730bp and 750bp. In the present study the nucleotide BLAST was performed for each of the sequence obtained to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 99 to 100% identity with the *Curcuma* sequence already available in the GenBank. The forward sequence showed a maximum of 100% identity with *Curcuma longa*, *Curcuma roscoeana*, *Curcuma colorata*, *Curcuma purpurascens* and *Curcuma soloensis* while the reverse sequence showed a maximum of 99% identity with *Curcuma longa*, *Curcuma colorata*, *Curcuma purpurascens*, *Curcuma xanthorrhiza*, *Curcuma soloensis*, *Curcuma zedoaria*, *Curcuma ochrorhiza*, *Curcuma heyneana*, *Curcuma elata*, *Curcuma aromatica*, *Curcuma aurantiaca*, *Curcuma australasica*, *Curcuma aeruginosa*, *Curcuma alismatifolia*, *Stahlianthus involucratus*, *Curcuma roscoeana*, *Curcuma phaeocaulis*, *Curcuma amada*, *Curcuma petiolata*, *Curcuma*

Table 4.13: List of the GenBank accessions for TrnL-TrnF region of *C. longa*

Sl. No	Plant species	GenBank accession number
1	*Curcuma longa* cv. local-Lataguri	KC404804
2	*Curcuma longa* cv. local-Lataguri	KC404823

gracillima, *Curcuma thorelii*, *Paracautleya bhatii*, *Curcuma harmandii*, *Smithatris* suprIaneanae, *Roscoea bhutanica* and *Scaphochlamys kunstleri*. After authentication, the sequences were submitted to the GenBank (Refer Appendix: G). The list of GenBank accession number and the sequence are given in table 4.13.

4.3.7 Somaclonal variation among the in vitro raised plantlets

A total of 10 primers (which already successfully amplified the genomic DNA of all the 12 cultivars) were used to screen somaclonal variations, out of which 6 were RAPD and rest 4 were ISSR primers.

All the RAPD and ISSR primers produced distinct and scorable bands (table 4.13) The number of bands varied with the plant material. In case of *Z. officinale*, RAPD and ISSR primers produced respectively 58 and 35 scorable bands. The RAPD bands ranged from 204 to 1582bp, while the ISSR bands from 305 to 1647bp. In *C. longa*, RAPD and ISSR produced 62 and 38 scorable bands respectively. The RAPD bands ranged from 151-1767bp, while the ISSR bands from 274-1758bp. RAPD and ISSR produced 55 and 33 scorable bands

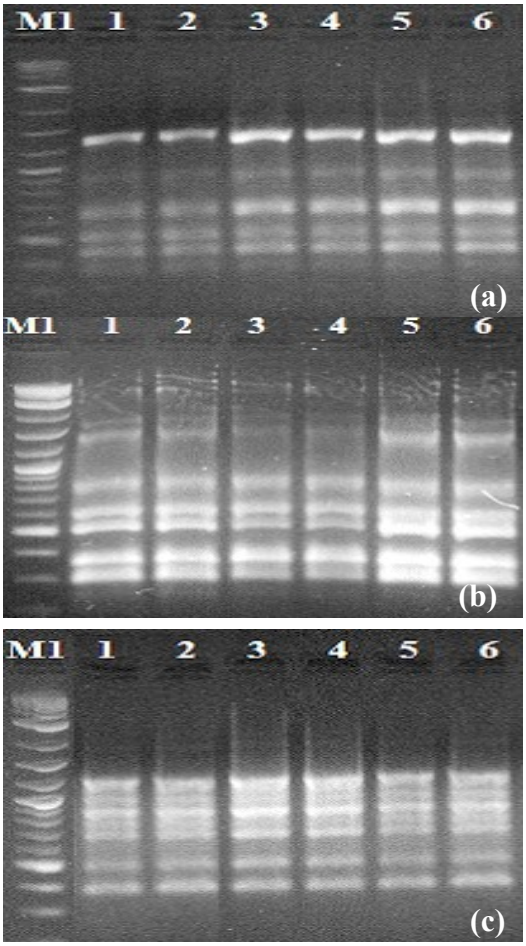


Figure 4.19: DNA fingerprinting patterns of *in vitro* raised (a) *Z. officinale* (b) *C. longa* and (c) *K. galanga* by using RAPD primers. Parent plant (lane1), micropropagated plants (lanes 2-6) and molecular weight markers 0.1-10 kb DNA ladder (M1).

Table 4.13: PCR amplicons obtained from RAPD and ISSR markers in *in vitro* raised plantlets of *Z. officinale*, *C. longa* and *K. galanga*.

	Primer details	*Z. officinale*	*C. longa*	*K. galanga*
RAPD	No of primer used	6	6	6
	Total bands	58	62	55
	No. of monomorphic bands	58	62	55
	Band size (bp)	204-1582	151-1767	185-1604
ISSR	No of primer used	4	4	4
	Total bands	35	38	33
	No. of monomorphic bands	35	38	33
	Band size (bp)	305-1647	274-1758	220-1647

respectively in *K. galangal*. The RAPD bands ranged from 185-1604bp, while the ISSR bands from 220-1647bp. A representative of RAPD and ISSR profile is depicted in figure 4.19. All the bands generated in *Z officinale*, *Curcuma longa* and *Kaempferia galanga* were found to be monomorphic across all the *in vitro* raised plantlets and the parent plant analyzed irrespective of whether RAPD primers or ISSR markers were used. This uniformity in the banding pattern confirms the genetic fidelity of the *in vitro* raised *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* plantlets.

Though both RAPD primers and ISSR markers helps in detecting polymorphism, ISSR markers dominates the RAPD primers. ISSR markers showed high polymorphism and have good reproducibility because of the presence of large number of SSR region as compared to RAPD (Ray *et al*., 2006). Moreover, ISSR markers are much larger in size than RAPD which are decamers and hence has higher annealing temperature. Higher annealing temperature is considered to result in greater consistency where as lower annealing temperature might produce artefact amplicons because of non-specific amplification (Bornet and Branchard, 2001). Both the fingerprinting techniques have their own advantages and disadvantages. Thus, keeping this in mind both RAPD primers and ISSR markers were used to screen the clonal stability of *in vitro* raised plantlets of the genera under study. This analysis showed that there was virtually no genetic variation among the micropropagated plantlets of *Z officinale*, *C longa* and *K galanga*. Therefore, it can be concluded that *in vitro* raised plants avoided the genomic aberrations and did not lead to any somaclonal variation.

4.4 Antioxidant studies

4.4.1 Free radical scavenging activity with DPPH

The solvent fractions of *Zingiber officinale* (figure. 4.20), *Curcuma longa* (figure 4.21) and *Kaempferia galanga* (figure 4.22) exhibited different levels of antiradical activities. Out of the 34 solvent fractions of *Z. officinale* 33 solvent fractions were dissolved in methanol. The hexane fraction had both methanol soluble and methanol insoluble parts. The methanol insoluble part was dissolved in hexane. 33 solvent fractions of *C. longa* and 25 solvent fractions obtained from *K. galanga* were finally dissolved in

methanol.

10 *Zingiber officinale* fractions [Hexane (dissolved in hexane), Benzene, Chloroform, Chloroform : diethyl ether (1:1), Chloroform : diethyl ether (1:3), Diethyl ether, Diethyl ether : ethyl acetate (3:1), Diethyl ether : ethyl acetate (1:1), Diethyl ether : ethyl acetate (1:3), Ethyl acetate and Ethyl acetate : acetone (1:1)] showed DPPH scavenging activity ranging from 5.88% to 80%. Five different peaks were obtained when the inhibition percent of different fractions were plotted (figure. 4.20). Diethyl ether & ethyl acetate (1:1) showed the maximum inhibition percent (80%) of antiradical scavenging activity. In similar experiments, antioxidative activities of water and organic solvent were observed by Katiyar *et al.* (1996) and Bhattacharya *et al*. (2009). Antioxidant properties of ginger were also reported by Ippoushi *et al.* (2003), Jolad *et al.* (2004) Zaeoung *et al*. (2005), Chan *et al*. (2008).

31 *Curcuma longa* fractions [Hexane, Hexane : benzene (3:1), Hexane : benzene (1:1), Hexane : benzene (1:3), Benzene, Benzene : chloroform (3:1), Benzene : chloroform (1:1), Benzene : chloroform (1:3), Chloroform, Chloroform : diethyl ether (3:1),

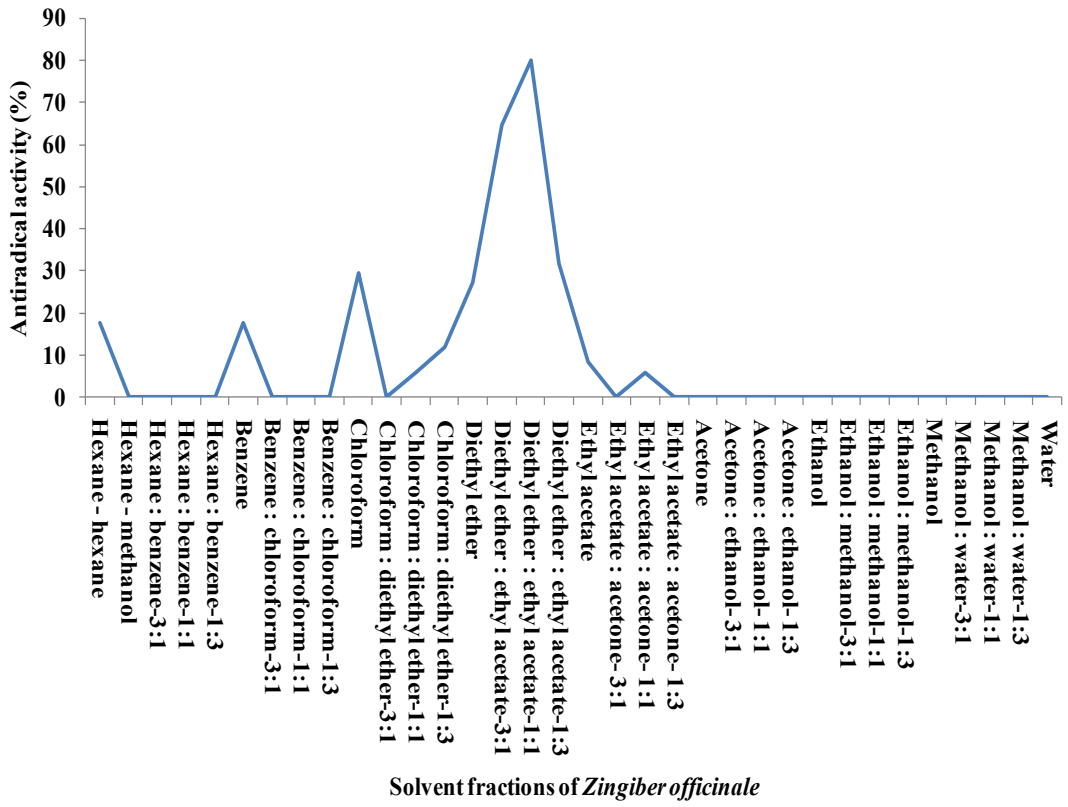


Figure 4.20: Free radical scavenging activity of different solvent fractions of *Z. oficinale* by DPPH

Chloroform : diethyl ether (1:1), Chloroform : diethyl ether (1:3), Diethyl ether, Diethyl ether : ethyl acetate (3:1), Diethyl ether : ethyl acetate (1:1), Diethyl ether : ethyl acetate (1:3), Ethyl acetate, Ethyl acetate : acetone (3:1), Ethyl acetate : acetone (1:1), Acetone, Acetone : ethanol (3:1), Acetone : ethanol (1:1), Acetone : ethanol (1:3), Ethanol, Ethanol : methanol (3:1), Ethanol : methanol (1:1), Ethanol : methanol (1:3), Methanol, Methanol : water (3:1), Methanol : water (1:1), and Methanol water (1:3)] showed DPPH scavenging activity ranging from 3.75% to 77.75%. Six different peaks were obtained when the inhibition percent of different fractions were plotted (figure. 4.21). Diethyl ether & ethyl acetate (3:1) showed the maximum inhibition percent (77.75%) of antiradical scavenging activity. Seven fractions [Benzene: Chloroform (1:1), Chloroform: Diethyl ether (1:3), Diethyl ether, Diethyl ether: Ethyl acetate (3:1), Diethyl ether: Ethyl acetate (1:1), Diethyl ether: Ethyl acetate (3:1) and Acetone: Ethanol (1:1) showed activity more than 10 percent. Antioxidant activity of *Curcuma longa* has been observed by Srimal and Dhawan (1973), Nagabhushan and Bhide (1992), Anto

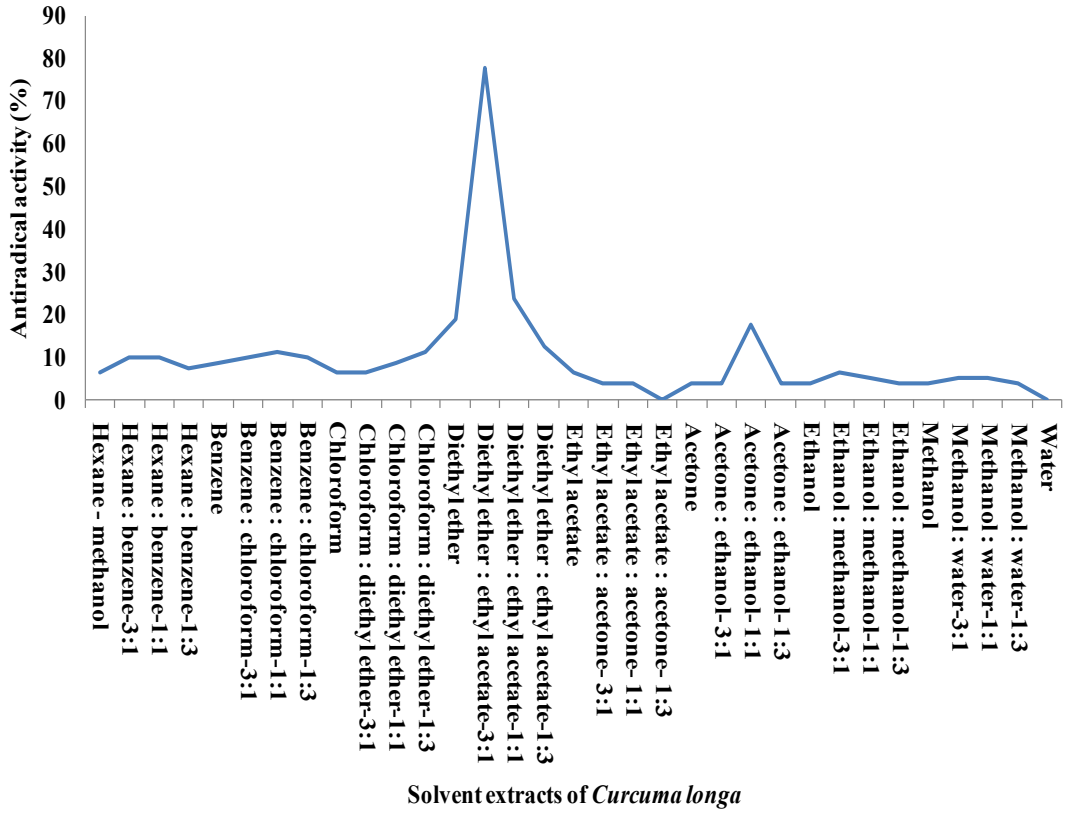


Figure 4.21: Free radical scavenging activity of different solvent fractions of *C. longa* by DPPH

et al. (1994), Halim (2002) Roy and Raychaudhury (2004) Sacchetti *et al.* (2005), Zaeoung *et al.* (2005) and Butkhup and Samappito (2011).

25 *Kaempferia galanga* fractions showed DPPH scavenging activity ranging from 9.52% to 66.66%. Except the water fraction all other fractions [Chloroform, Chloroform : diethyl ether-3:1, Chloroform : diethyl ether-1:1, Chloroform : diethyl ether-1:3, Diethyl ether, Diethyl ether : ethyl acetate-3:1, Diethyl ether : ethyl acetate-1:1, Diethyl ether : ethyl acetate-1:3, Ethyl acetate, Ethyl acetate : acetone- 3:1, Ethyl acetate : acetone- 1:1, Ethyl acetate : acetone- 1:3, Acetone, Acetone : ethanol- 3:1, Acetone : ethanol- 1:1, Acetone : ethanol- 1:3, Ethanol, Ethanol : methanol-3:1, Ethanol : methanol-1:1, Ethanol : methanol-1:3, Methanol, Methanol : water-3:1, Methanol water-1:1, Methanol : water-1:3 and Water] showed DPPH scavenging activity greater than 42.85%. Three peaks were obtained when the inhibition percent of different fractions were plotted (figure 4.22). The acetone fraction showed the maximum inhibition percent (66.66%) of antiradical scavenging activity. Similar activities were observed by Zaeoung *et al.* (2005), Tewtrakul *et al.*

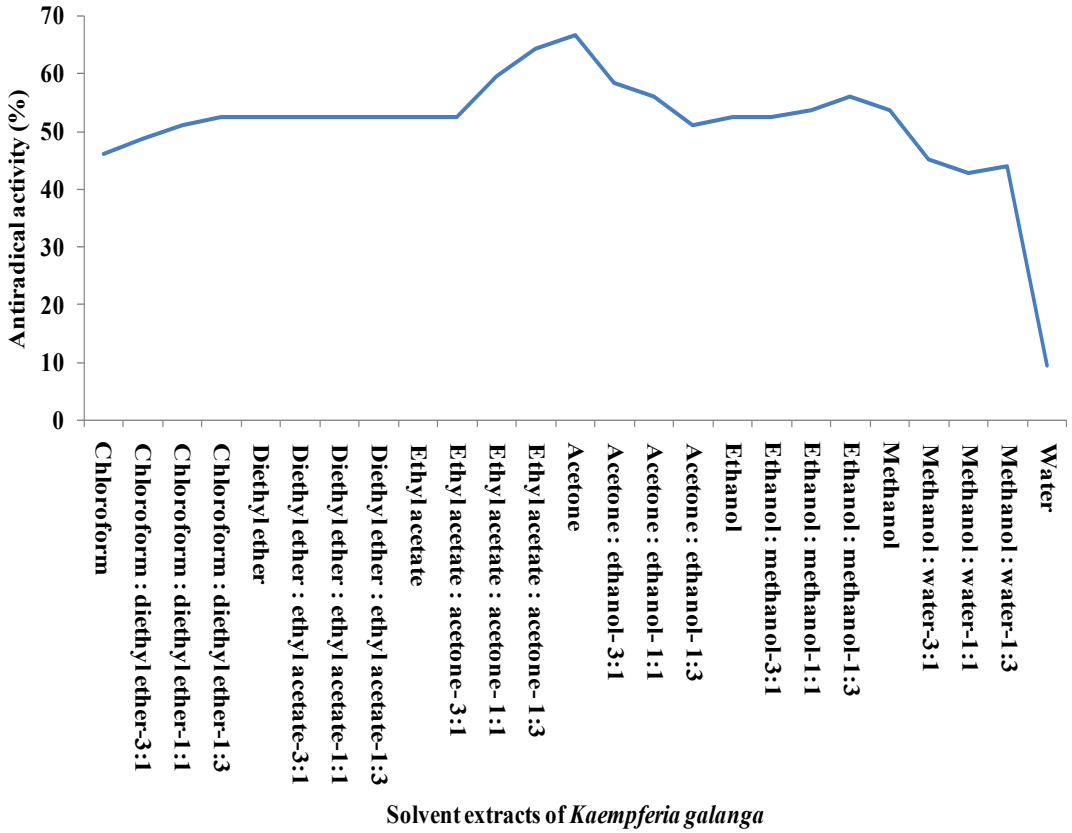


Figure 4.22: Free radical scavenging activity of different solvent fractions of *K. galanga* by DPPH

(2005) and Rajendra *et al*. (2011), in their studies with the extracts of *Kaempheria galanga*.

4.4.2 IC_{50} of the fractions

The fractions showing the maximum antiradical activity was diluted to different concentrations and the diluted fraction was subjected to DPPH assay for antiradical activity. Diethyl ether & ethyl acetate (1:1) fraction of *Z. officinale* (80% antiradical activity), Diethyl ether & ethyl acetate (3:1) fraction of *C. longa* (77.5% antiradical activity) and Acetone fraction of *K. galanga* (66.66% antiradical activity) were used to determine the IC_{50}. The IC_{50} differed considerably in the fractions studied. The bioactive fractions of *Z. officinale*, *C. longa* and *K. galanga* showed a concentration dependent DPPH radical scavenging activity with IC_{50} of 8 mg/ml, 55.80 mg/ml and 280 mg/ml respectively at fresh weight basis.

4.4.3 Assay of hydroxyl radical scavenging activity

Degradation of deoxyribose mediated by hydroxyl radicals generated by the Fe^{3+}/ ascorbate/EDTA/H_2O_2 system was also inhibited by the 6, 5 and 4 bioactive fractions of *Z. officinale*, *C. longa* and *K. galanga* respectively (figure 4.23). Concentrations dependent hydroxyl radical scavenging activity was observed. In all the cases the hydroxyl radical scavenging activities increased with the increase in concentrations of the extracts.

In *Zingiber officinale*, the chloroform fraction (43.75%) showed maximum hydroxyl radical scavenging activity followed by hexane fraction (39.13%) insoluble in methanol and diethyl ether & ethyl acetate (1:3) fraction (39.13%) at a concentration of 300 mg/ml. The minimum hydroxyl radical inhibition activity was recorded in hexane fraction insoluble in methanol and Diethyl ether: Ethyl acetate (1:1) fraction at a concentration of 3 mg/ml.

In *Curcuma longa*, the diethyl ether fraction (40.00%) showed maximum hydroxyl radical scavenging activity followed by Benzene: Chloroform (1:1) fraction (36.00%) and diethyl ether: ethyl acetate (1:1) fraction (32.00%) at a concentration of 300 mg/ ml. The minimum hydroxyl radical inhibition activity was recorded in Diethyl ether: Ethyl acetate (3:1) fraction (8.00%) at a concentration of 3 mg/ml.

In *Kaempferia galanga*, the acetone fraction (40.00%) showed maximum hydroxyl radical scavenging activity

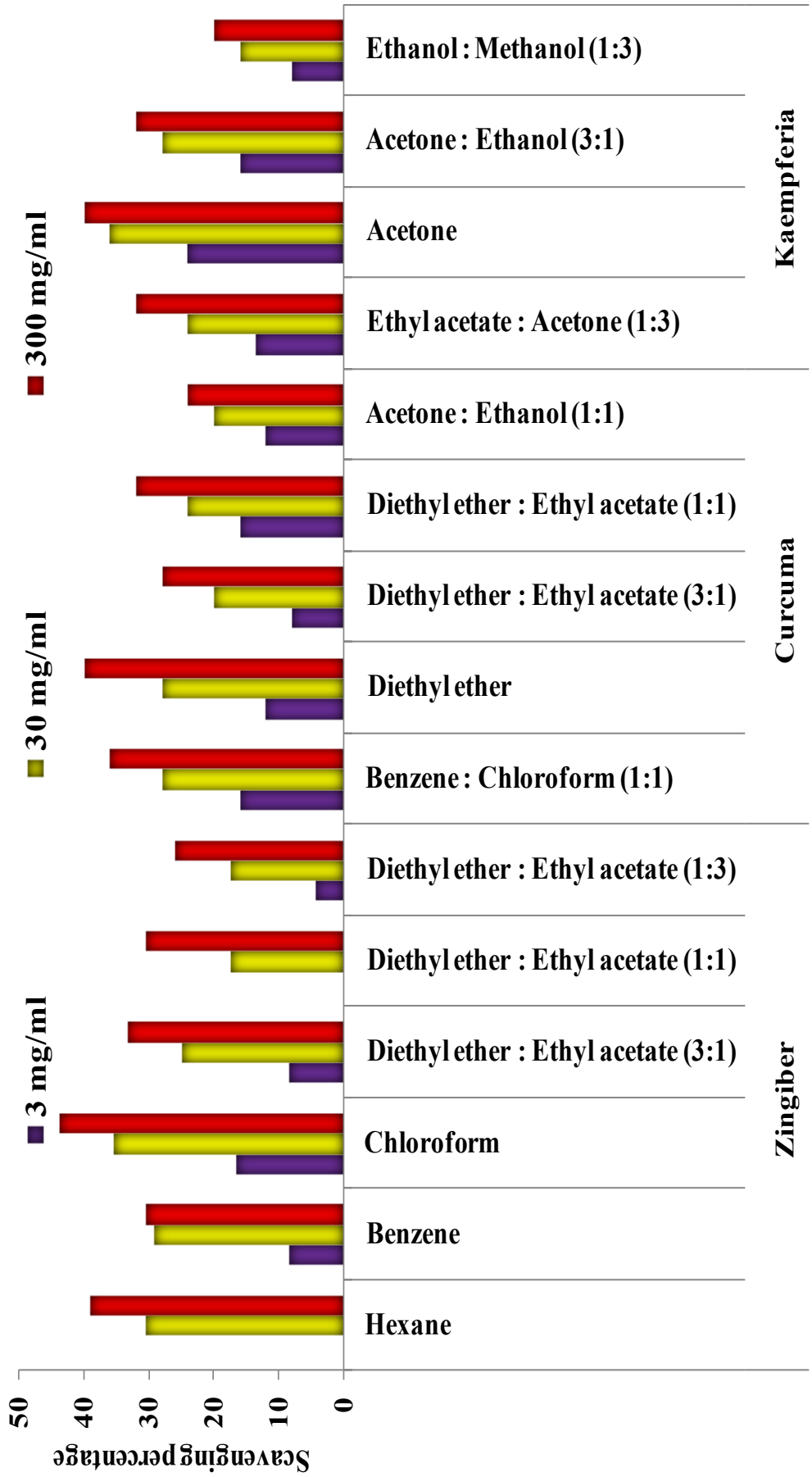


Figure 4.23: Hydroxyl radical scavenging activity of different solvent fractions of *Z. officinale*, *C longa* and *K. galanga*..

followed by Ethyl acetate : Acetone (1:3) fraction (32.00%) and Acetone : Ethanol (3:1) fraction (32.00%) at a concentration of 300 mg/ml. The minimum hydroxyl radical inhibition activity was recorded in Ethanol: Methanol (1:3) fraction (8.00%) at a concentration of 3 mg/ml.

4.4.4 Assay of nitric oxide scavenging activity

Results indicate that, all the bioactive *Zingiber officinale*, *Curcuma longa* and *Kaempferia galanga* fractions have NO scavenging activity (figure 4.24). The scavenging activity increased with decrease in concentrations in all the bioactive fractions other than the hexane fractions *Z. officinale*.

In *Z. officinale* the hexane fraction (47.72%) was most active in NO scavenging activity followed by chloroform fraction (43.75%) and hexane fraction (43.18%) at concentrations of 3 mg/ml, 300 mg/ml and 30 mg/ml respectively. the minimum NO scavenging activity was observed in Diethyl ether: Ethyl acetate (1:1) fractions (11.36%) at concentrations of 3mg/ml and 30 mg/ml. Similar results were obtained by Ippoushi *et al.* (2003) and Bhattacharya *et al*. (2009). These results suggest that ginger extracts are potent inhibitor of NO synthesis.

In *C. longa* the benzene: chloroform (1:1) fraction (42.22%) was most active in NO scavenging activity followed by diethyl ether: ethyl acetate (3:1) fraction (33.33%) and diethyl ether fraction (28.88%) at a concentration of 300 mg/ml. The minimum NO scaveging activity was observed in diethyl ether: ethyl acetate (1:1) fraction (6.66%) at a concentration of 3 mg/ml. Similar NO scavenging activity of *Curcuma longa* has been observed by Ippoushi *et al.* (2003).

In *K. galanga* the acetone fraction (41.30%) was most active in NO scavenging activity followed by Ethyl acetate: Acetone (1:3) fraction (36.95%) and Acetone: Ethanol (3:1) fraction (28.26%) at a concentration of 300 mg/ml. The minimum NO scaveging activity was observed in Acetone: Ethanol (3:1) fraction (17.39%) at a concentration of 3 mg/ml.

4.4.5 Determination of lipid peroxidation inhibition activity

The bioactive fractions of *Z. officinale*, *C. longa* and *K. galanga* fractions protected hepatocytes from damage due to lipid peroxidation induced in

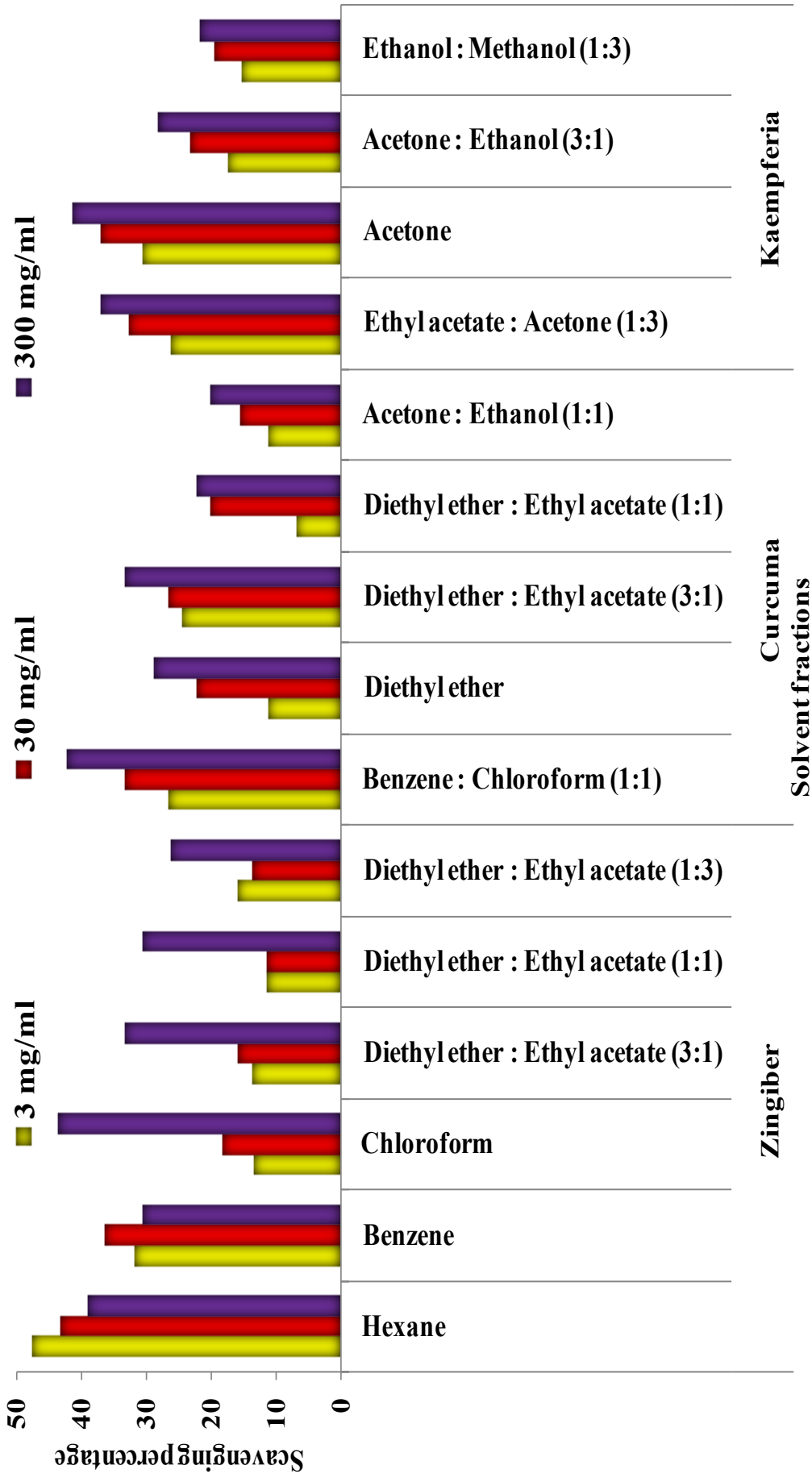


Figure 4.24: Nitric oxide scavenging activity of different solvent fractions of *Z. officinale*, *C longa* and *K. galanga*.

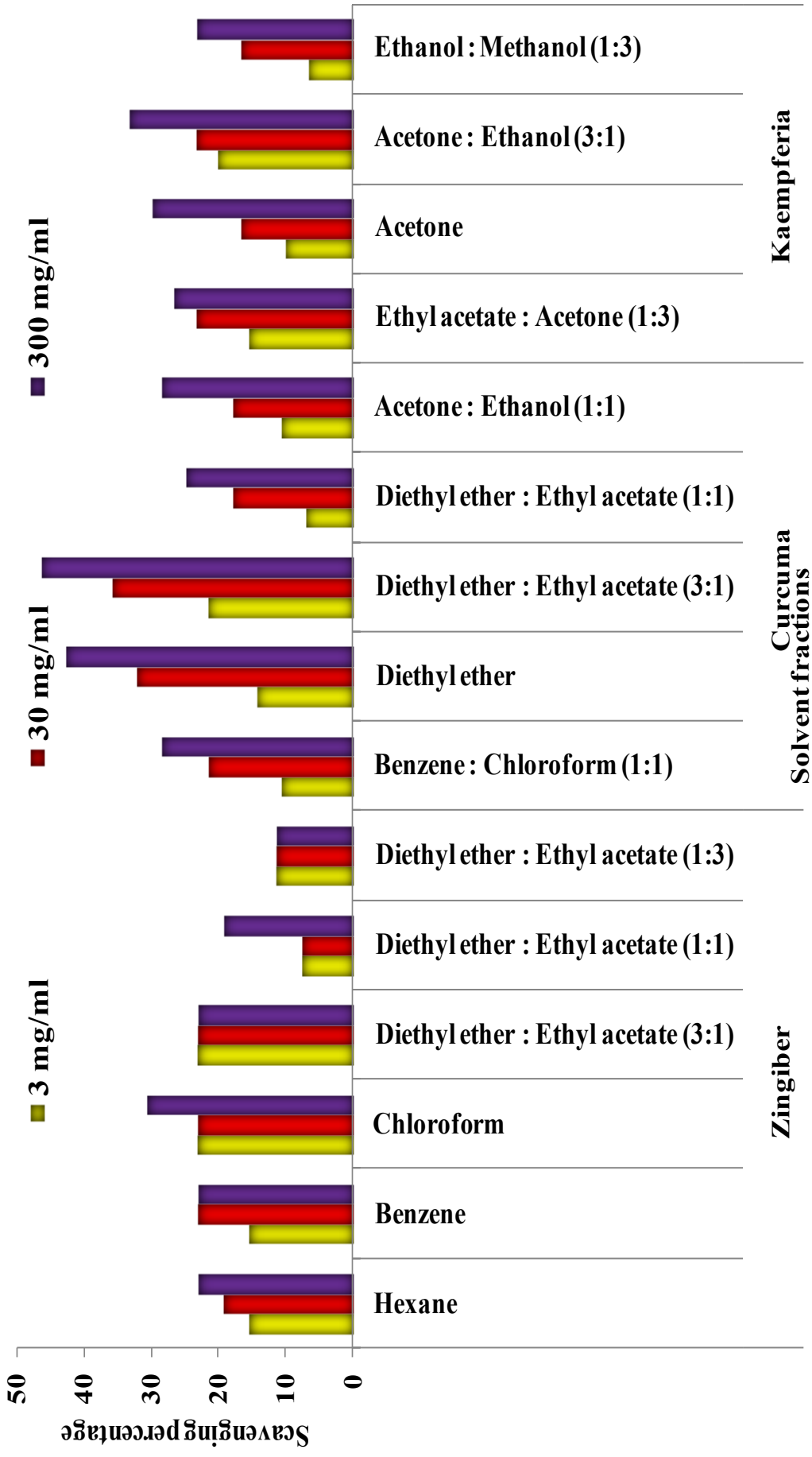


Figure 4.25: Lipid peroxidation inhibition activity of different solvent fractions of *Z. officinale*, *C longa* and *K. galanga*.

goat liver homogenate by ferric-ADP and ascorbate in a dose dependent manner (figure 4.25).

In *Z. officinale* the maximum protective function was recorded in chloroform fraction (30.76%) followed by Hexane, Benzene and Diethyl ether : Ethyl acetate (3:1) fractions (23.07%) and Diethyl ether : Ethyl acetate (1:1) fraction (19.23%) at 300 mg/ml. the minimum activity was observed in Diethyl ether : Ethyl acetate (1:1) fraction (7.69%) at concentrations of 3 mg/ml and 30 mg/ml. Diethyl ether: ethyl acetate (1:3) showed similar hepatocyte protective activity in all the concentrations. Similar results were observed by Jolad *et al.,* (2004) and Bhattacharya *et al*. (2009).

In *C longa* the maximum protective function was recorded in Diethyl ether : Ethyl acetate (3:1) fraction (46.42%) followed by Diethyl ether fractions (42.85%) at a concentration of 300 mg/ml and Diethyl ether : Ethyl acetate (3:1) fraction (35.71%) at concentration of 30 mg/ml. The minimum activity was observed in Diethyl ether: Ethyl acetate (1:1) fraction (7.69%) at concentrations of 3 mg/ml and 30 mg/ml.

In *K. galanga* the maximum protective function was recorded in Acetone: Ethanol (3:1) fraction (33.33%) followed by acetone fraction (30.00%) and Ethyl acetate : Acetone (1:3) fractions (26.66%) at a concentration of 300 mg/ml. The minimum activity was observed in Ethanol : Methanol (1:3) fraction (6.66%) at concentrations of 3 mg/ml.

4.4.6 Comparison of antiradical activity

Comparative study of antiradical activities of the genera were conducted (figure 4.26). The Diethyl ether: ethyl acetate (1:1) and Diethyl ether: ethyl acetate (3:1) fractions of *Z. officinale* and *C. longa* are more potential than the two fractions of *K. galanga*. The most potential fractions are Diethyl ether: ethyl acetate (1:1), Diethyl ether: ethyl acetate (3:1) and Acetone in *Z. officinale* (80.88%), *C. longa* (61.76%) and *K. galanga* (13.23%) respectively. The maximum inhibition was recorded in Diethyl ether: ethyl acetate (1:1) fraction of *Z. officinale* (80.88%) while the lowest inhibition was shown by Diethyl ether: ethyl acetate (3:1) fraction of *K. galanga* (4.41%). In general the Diethyl ether: ethyl acetate (1:1) and Diethyl ether: ethyl acetate (3:1) fractions of *Z. officinale* and *C. longa* are much more active in

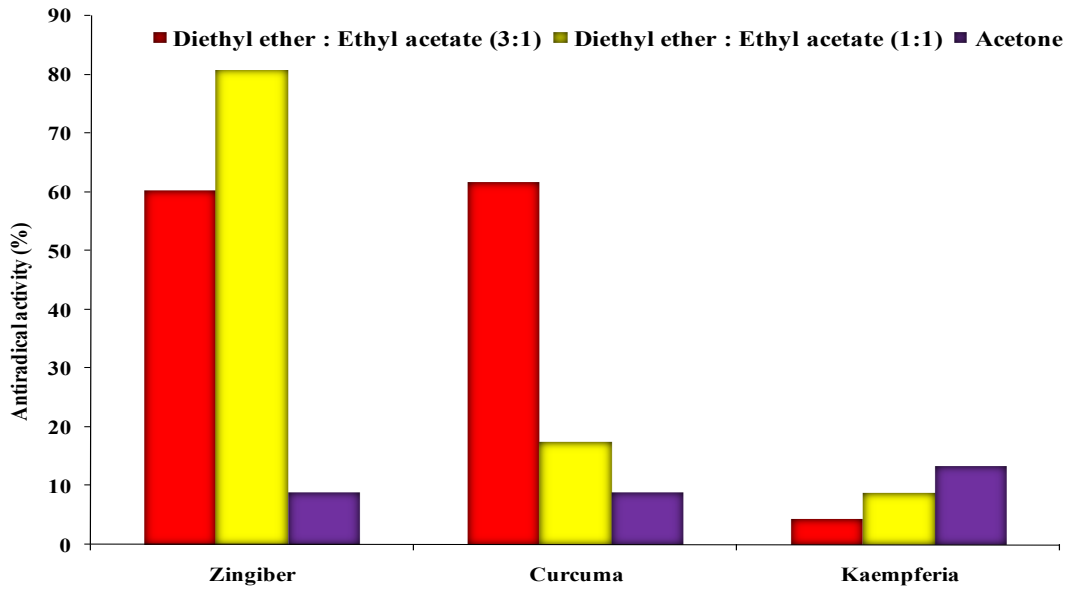


Figure : 4.26: Comparative study of free radical scavenging activity of different solvent fractions of *Z. officinale*, *C. longa* and *K. galanga* by DPPH

scavenging free radicals in comparison to the *K. galanga* fractions.

4.4.7 Detection of compounds

When the distribution of polyphenols was analyzed in different fractions Ginger, it was observed that phenolic compounds were distributed from chloroform to aqueous fraction whereas gingeroles and arbutin related compounds were mainly restricted in diethyl ether: ethyl acetate fractions. The bioactive fraction, di ethyl ether & ethyl acetate (1:1) showed the sensitivity against NP/PEG from which it may be concluded that ginger flavonoids have some contributory roles in scavenging antiradical activity. Polyphenols and flavonoids have already been reported to act as potent antioxidants in *Alpinia nutans* (Habsah *et al*., 2003), *Galium fissurense* (Delioram 2003) and many other medicinal plants.

4.4.8 Antiradical activities of C. longa cultivars

The antiradical activities in the methanol and water extracts of different *Curcuma longa* cultivars were variable (figure 4.27). In all the varieties the methanol soluble fraction showed more antiradical activity than the water soluble fraction. The arithmetic means of the antiradical activities of the ten varieties in their methanol and water soluble fractions were 43.03% and 26.60% respectively. The cultivars like Suguna, T. C. Assam Suvarna and Roma, were above the

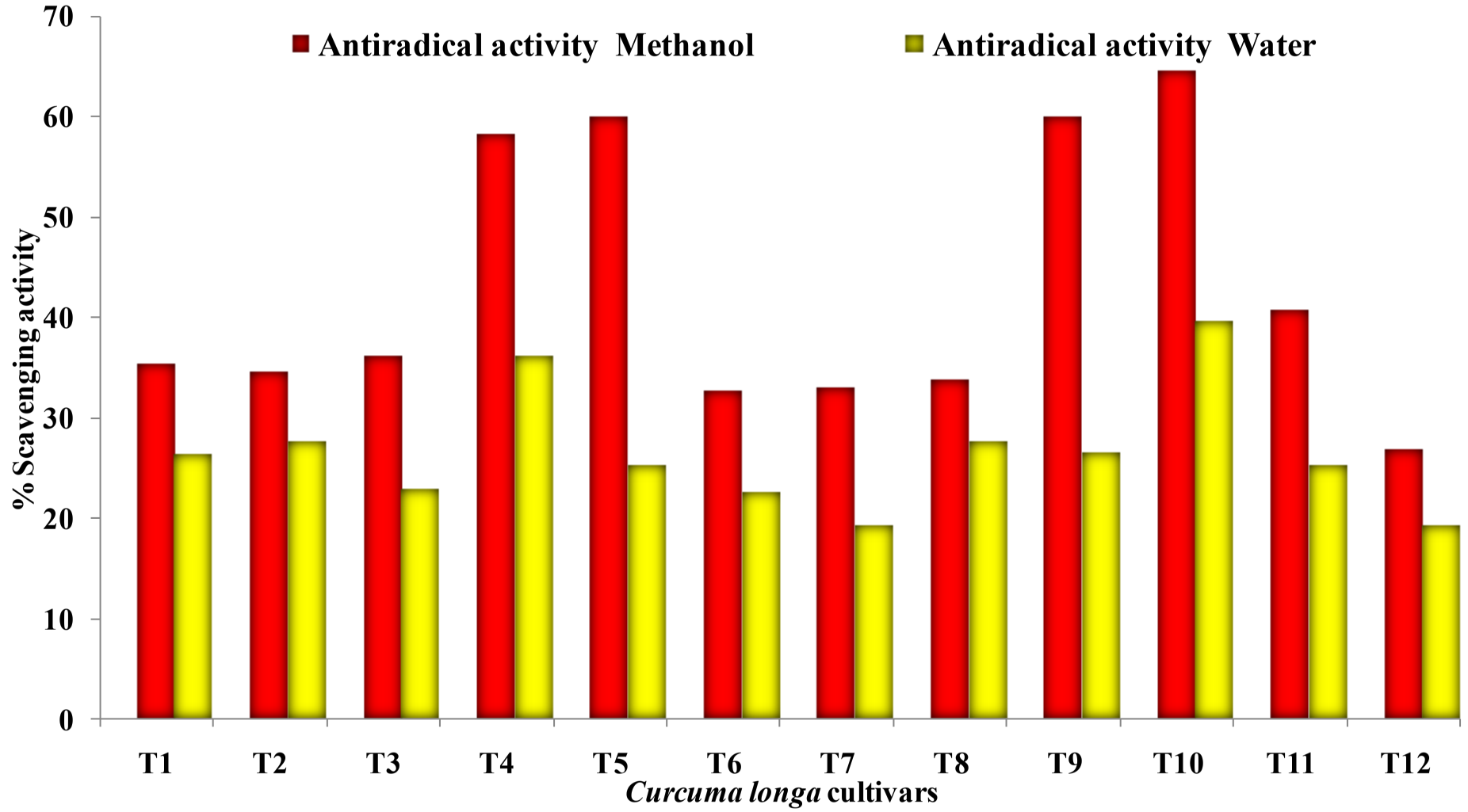


Figure : 4.27: Antiradical (% Scavenging activity of DPPH) of different *Curcuma longa* cultivars (please refer table 4.1 for the species and varieties name)

average in antiradical activities in their methanol soluble fraction while the local cultivar collected from Dhupguri, Suguna, CLS 2A and Roma were above the average in antiradical activities of their water soluble fraction. Antiradical activities of four cultivars viz. Local collected from Lataguri (26.50%), T C Assam (25.31%), Suvarna (26.52%) and Sudarshana (25.38%) were marginally lower than the average antiradical activities. The maximum antiradical observed in methanol solvent fraction of Roma (64.61%) followed by Suvarna (60.00%) and T. C. Assam (60.00%) cultivars, while the minimum activity was observed in the cultivar PTC13 (26.92%). The maximum antiradical observed in water solvent fraction was in cultivar Roma (39.75%) followed by the cultivar Suguna (27.71%) while the minimum activity was observed in Kasturi (19.27%) and PTC 13 (19.27%). The maximum and minimum antiradical activity was showed by the cultivar Roma and PTC13 respectively.

CONCLUSION

The study area is situated in Darjeeling district and adjoining plains. This region is well known for its majestic beauty and is an ideal place for health. It serves as one of the richest and interesting botanical regions in the whole of Indian subcontinent and thus, has been a central point of natural and floristic attraction for tourists and nature lovers. Its rich flora and fauna is of paramount significance for the nature lovers and biologists. A wide range of vegetation structures with extremely rich plant and animal diversity has developed due to the extreme climatic, edaphic, and physiographic variations. Many ancient floristic elements have survived in this flora while some have differentiated to different races through the ages. The floral elements of the region include various members of the family Zingiberaceae, of which *Curcuma longa*, *Kaempferia galanga* and *Zingiber officinale* are cultivated in this region.

The rhizomatous plants of Zingiberaceae are vegetative propagated, carrying the pathogen from generation to the other. A large quantity of the crop is lost in the field every year due to pathogens. Improvement of these plants by conventional hybridization techniques are not possible due to lack of flowering and seed set. Most crop improvement programs are restricted to selection of improved cultivars. Therefore extensive study was required in the field to explore the idea of using *in vitro* techniques for improvement of the crop. *In vitro* regeneration by using rhizome buds of *Z. officinale, C. longa* and *K. galanga* as an explant has the potentiality to produce huge quantity of plantlets within a very short period. The plants after hardening can be well maintained in field condition and the lines if superior can be further multiplied by the conventional technique. Moreover, micropropagated plants are free from diseases or pests

and show superiority in different aspects. Standardization of mass propagation protocol of the genera through *in vitro* technique has been achieved.

Identification of potential cultivar by morphological studies has some limitations. Slight variations within the cultivars cannot be made out by the study of floral morphology. So employing molecular techniques is important. DNA fingerprinting using RAPD and ISSR primers may prove to be very useful in this process of identification. Keeping this limitation in mind, molecular documentation of the local cultivars of *Curcuma longa* was worked out. The knowledge of the gene sequences of the local cultivars and their relationships with other established cultivars of turmeric will certainly help in judging its origin and relatedness with other members.

Medicinal plants have been the subjects of man's curiosity since time immemorial. Approximately 80% of the people in the world's developing countries rely on traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts. Almost every civilization has a history of medicinal plant use. Interest in phytomedicine has exploded in the last few years. The resurgence of public interest in plant-based medicine coupled with rapid expansion of pharmaceutical industries has necessitated an increased demand for medicinal plant research. Assessment of antiradical activities of the local cultivars is important in this perspective. Chromatographic separation of antioxidant rich fractions has been achieved. Fractionation of the extracts with polar and non polar solvents has significance in understanding the *in vitro* mechanism of action of these formulations. This may open new field in the pharmaceutical industry and pave the way to treat several diseases.

Proper utilization of natural resources is important to boost the local economy too. Sustainable and eco-friendly use of resources by modern techniques can achieve the result and pave the way for development.

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INDEX

APPENDIX-A

Thesis related publication till January, 2014

Bhattacharya, M., & Sen, A. (2006). Rapid *in vitro* multiplication of disease-free *Zingiber officinale* Rosc. *Indian journal of plant physiology*, *11*(4), 379-384.

Bhattacharya, M., & Sen, A. (2013). *In vitro* regeneration of pathogen free *Kaempferia galanga* L.-a rare medicinal plant. *Research in Plant Biology*, *3*(3), 24-30.

Bhattacharya, M., Mandal, P., & Sen, A. (2009). *In vitro* detection of antioxidants in different solvent fractions of Ginger (*Zingiber officinale* Rosc.). *Indian Journal of Plant Physiology*, *14*(1), 23-27.

APPENDIX-B

Datasheet for collection of germplasm and recording field data

Collection Data sheet

Sample no.:

A. Collection information :

1. Collection site :
2. Date :
3. Time :
4. Scientific name :

Local name :

B. Habits:

1. Tree/Shrub/Herb :
2. Flowering time :

Propagation:

C. Habitat and area of the vegetation:

1. Rainfall :
2. Altitude :
3. Temperature :
4. Topography :
5. Vegetation type :

Management :

Collected by
Malay Bhattacharya
Research Scholar
Molecular Genetics Laboratory
Department of Botany
University of North Bengal

APPENDIX-C

Composition of Murashige and Skoog medium (Hi media Cat# PT018)

Macroelements	
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Microelements	
$CoCl_2,6H_2O$	0.025
$CuSO_4,5H_2O$	0.025
FeNaEDTA	36.70
H_3BO_3	6.20
KI	0.83
$MnSO_4,H_2O$	16.90
$Na_2MoO_4,2H_2O$	0.25
$ZnSO_4,7H_2O$	8.60
Vitamins	
Glycine	2.00
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thyamine HCl	0.10

To it was added 3% sucrose (Hi media Cat# RM134), 0.332 mg/l $CaCl_2$ (Merck India Cat# 61764405001730). pH was adjusted to 5.6±0.1 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 20 minutes at 121ºC and 15psi and cooled and plant growth regulators are added (if any) as per requirement.

Note: In case of solid media agar (Hi media Cat#RM026) is added at the rate of 0.8%.

APPENDIX-D

Composition of Gamborg B5 (Hi media Cat#)

Macroelements	
$(NH_4)_2SO_4$	134mg/l
$CaCl_2H_2O$	150mg/l
$MgSO_4.7H_2O$	246mg/l
KNO_3	2,528mg/l
Microelements	
H_3BO_3	3.0mg/l
$CoCl_2.6H_2O$	0.025mg/l
$CuSO_4.5H_2O$	0.025mg/l
$FeSO_4.7H_2O$	27.8mg/l
$MnSO_4.H_2O$	10mg/l
KI	0.75mg/l
$Na_2MoO_4.2H_2O$	0.25mg/l
$NaH_2PO_4.H_2O$	150mg/l
$ZnSO_4.7H_2O$	2.0mg/l
$Na_2EDTA.2H_2O$	37.2mg/l
Vitamins	
i-Inositol	100mg/l
Pyridoxine HCl	1.0mg/l
Nicotinic Acid	1.0mg/l
Thiamine HCl	10.0mg/l

To it was added 3% sucrose (Hi media Cat# RM134). pH was adjusted to 5.6±0.1 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 20 minutes at 121ºC and 15psi and cooled and plant growth regulators are added (if any) as per requirement.
Note: In case of solid media agar (Hi media Cat#RM026) is added at the rate of 0.8%.

APPENDIX-E

Buffers and chemicals used for DNA fingerprinting studies

*** CTAB- buffer**

100mM Trizma Base (Sigma, Cat# T1503) (pH-8.0)
20mM EDTA (Merck India, Cat# 60841801001730) (pH-8.0)
1.4 M NaCl (Merck India, Cat#60640405001730)
2% (w/v) CTAB (Hexadecyl cetyl trimethyl ammonium bromide) (Sigma, Cat# H6269)
12.11g of molecular grade Trizma base was dissolved in 400 ml double distilled water, pH was adjusted to 8.0 and was divided into two parts of equal volume. To one part 7.44g EDTA was added and to the other part 81.8g NaCl and 20g CTAB. Both the parts were than mixed and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.
Note: Add 1% PVP (Polyvinylpyrrolidone) (Sigma, Cat #P5288) and 0.3% β-mercaptoethanol (Sigma, Cat# M3148) just before use.

*** 5X TBE (Tris-borate-EDTA) buffer**

Trizma base (Sigma, Cat# T1503) = 27 gm
Boric acid (Sigma, Cat# 15663)= 13.75 gm
0.5M EDTA (pH 8.0)=1.86 gm
All the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 1X TE:**

Tris- Cl (pH 8.0) (i.e. 10Mm) =0.6055gm
EDTA (pH 8.0) (i.e. 1mM) =0.186 gm
Both the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 3M Sodium Acetate (Sigma, Cat# S9513):**

The required amount of sodium acetate i.e.12.31 g was dissolved in 50ml double distilled water prior to autoclaving. The solution was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

*** 6X gel loading buffer:**

TYPE 3: Stored at 4ºC.
0.25% Bromophenol blue (Sigma, Cat# B0126)
0.25% Xylene cyanol FF (Sigma, Cat# X4126)
30% Glycerol (Merck India, Cat#61756005001730) in water

*** RNase A:**

The RNase A enzyme (Sigma, Cat# R4875) was dissolved at a concentration of 10mg/ml in 0.01M sodium acetate (Sigma, Cat# S9513) (pH 5.2). The solution was heated at 100ºC for 15 minutes in a water bath and allowed to cool slowly to room temperature. The pH was adjusted by adding 1/10 volume of 1M Tris- Cl (pH 7.4) and stored at -20ºC for further use.
Note: Both 0.01M sodium acetate and 1M Tris-Cl were prepared and autoclaved at 121ºC and 15 psi for 20 mins prior to use.

APPENDIX-F

Chemicals and buffers used for antioxidant profiling

* 2-deoxy-D-ribose (Himedia, Cat# RM452)
* Acetone (Merck India, Cat# 60001405001730)
* Aluminium Chloride (Sd Fine, Cat# 37073) (10%)
* Ammonia (Merck India, Cat# 17500)
* Ascorbic acid (Himedia, Cat# CMS1014)
* Benzene (Merck India, Cat# 60178325001730)
* Butanol (Merck India, Cat# 17419)
* Butylated hydroxytoluene (BHT) (SD fine chem Limited, Cat# 38067)
* Cathechin (Sigma, Cat#C0567)
* Chloroform (Merck India, Cat# 82226505001730)
* Diethyl ether (SD fine chem Limited, #38132)
* DPPH (Himedia, Cat# RM2798)
* Ethyl acetate (SD fine chem Limited, 20108)
* Ethylenediamine tetra acetic acid (EDTA) (Merck India, Cat# 60841801001730))
* Ferric chloride (Himedia, Cat# RM1379)
* Ferrous sulphate (Merck India, Cat# 62840005001046)
* Folin Ciocalteu's reagents (SRL, Cat# 062015)
* Gallic acid (Himedia, Cat# RM233)
* Glacial acetic acid (SD fine chem Limited, Cat# 37013)
* Hexane (SD fine chem Limited, Cat# 38485)
* Hydrochloric acid (Merck India, Cat# 61762505001730)
* Hydrogen peroxide (Merck India, Cat# 61765305001730) (2mM)
* Iodine solution (SD fine chem Limited, Cat# 25008AM)
* Methanol (Merck India, Cat# 60600905001730)
* Naphthyl ethylene diamine dihydrochloride (Himedia, Cat# RM1073)
* Orthophosphoric acid (SD fine chem Limited, Cat# 20173)
* Phosphate buffer (0.2M) pH 6.6
    * a. Potassium dihydrogen phosphate (Merck India, Cat# 60487305001730)
    * b. Dipotassium hydrogen phosphate (Merck India, Cat#61788005001730)
* Phosphate buffer (pH 7.4)
    * a. Potassium dihydrogen phosphate (Merck India, Cat#60487305001730)
    * b. Potassium hydroxide (Merck India, Cat#60503305001730)
* Potassium chloride (Merck India, Cat# 61779205001730)
* Potassium ferrocyanide (Merck India, Cat# 61843605001730) (1%)
* Quercetin (Himedia, Cat# RM6191)
* Silica gel (SD fine chem Limited, 200-300 mesh size)
* Sodium acetate (Sigma, Cat# S9513) (5%)
* Sodium Carbonate (Merck India, Cat# 61778705001730) (20%)
* Sodium hydroxide (Merck India, Cat# 6184305001730) (1mM)
* Sodium Nitrite (Himedia, Cat# RM417) (5%)
* Sodium nitroprusside (Merck India, Cat# 61761501001730)
* Sulphanilamide (Himedia, Cat# RM1558)
* Thiobarbituric acid (TBA) (Himedia, Cat# RM1594)
* Trichloro acetic acid (Qualigens, Cat# 28445) (10%)

APPENDIX-G

Sequence of TrnL-TrnF region of *C. longa* cv Local-Lataguri

GenBank accession number	Sequence
KC404804	CCTGCTAAGTGGTAACTTCCAAATTCAGAGAAACCCTGGAAT- TTAAAATGGGCAATCCTGAGCCAAATCCTTAGTTTGATAAACC TTAGTTTTATCAAACTAGAAAAAAAAAAGGATAGGTGCAGAG ACTCAATGGAAGCTGTTCTAACGAATGAAGTTGACTACGTTTC GTCGGTAGTTGGAATCCGTCTATCAAAATTACAGAAAAGATGT TCCTATATACCTAATACATACGTATACATACTGACATATCAAA TCAAACGATTAATCATGACTCGAATCCATTATATTATATGGAT AATTATAATATGAAAAATTCAGAATTAGAGTTATTGTGAATCC AGTCCGATGGAAGTTGAAAGAAGAATTGAATATTCAATTCAA TTATTAAATCATTCATTCCAGAGTTTGATAGATCTTTTGAAAA ACTGATTAATCGGACGAGAATAAAGAGAGAGTCCCATTCTAC ATGTCAATACCGACAACAATGAAATTTATAGTAAGAGGAAAA TCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTCCCTCTATCC CCAATAAAAAGGTAATTTTACTTCCTAAATATTTATCCTCCTTT TTTTTTTCATCAGCGATTCAGTTCAAACAAAATTCACTATCTTT CTCATTCACTCCACTCTTTCACAACACAAATGTATCCGAACTA AAATCCTTGGATCTTATCCCAATTTCGATAGATACAATACCTC TACAAATAAACATATATGGGCA
KC404823	TACGTTTCGTCGGTAGTTGGAATCCGTCTATCAAAATTACA- GAAAAGATGTTCCTATATACCTAATACATACGTATACATACTG ACATATCAAATCAAACGATTAATCATGACTCGAATCCATTATA TTATATGGATAATTATAATATGAAAAATTCAGAATTAGAGTTA TTGTGAATCCAGTCCGATGGAAGTTGAAAGAAGAATTGAATA TTCAATTCAATTATTAAATCATTCATTCCAGAGTTTGATAGATC TTTTGAAAAACTGATTAATCGGACGAGAATAAAGAGAGAGTC CCCTTTTACATGTCAATACCGACAACAATGAAATTTATAGTAA GAGGAAAATCCGTCGACTTTAGAAATCGTGAGGGTTCAAGTC CCTCTATCCCCAATAAAAAGGTAATTTTACTTCCTAAATATTTA TCCTCCTTTTTTTTTTCATCAGCGATTCAGTTCAAACAAAATTC ACTATCTTTCTCATTCACTCCACTCTTTCACAACACAAATGTAT CCGAACTAAAATCCTTGGATCTTATCCCAATTTCGATAGATAC AATACCTCTACAAATAAACATATATGGGCAAATAATCTCTATT ATTGAATCATTCACAGTCCGTATCATTATCCTTACGCTTACTAG TTAAATTTTTTACTACTTTTTAGTCCCTTTAATTGACATAGACA CAAACACTACACCAGGATGATGCATGGGAAATGGTCGGGAT