Genetic resources of *Curcuma* and *Zingiber* from Northeast India: diversity, characterization and utilization

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Dedicated to dear Papa

Mr. Prahlad Chandra Das

and Maa

Mrs. Ranjuma Das

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I do hereby declare that the research findings of this thesis is the result of research work carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Latha Rangan.

As per the general norms of reporting research findings, due acknowledgements have been made wherever the research findings of other researchers have been cited in this thesis.

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CERTIFICATE

It is certified that the work described in this thesis entitled "***Genetic resources of Curcuma and Zingiber from Northeast India: diversity, characterization and utilization***" by Archana Das for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for the award of degree in any form.

CERTIFIED **Date:** 3rd June 2011

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ABBREVIATIONS

Abs.	Absorbance
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AO	Acridine orange
AOAC	Association of Official Agricultural Chemists
BA	Benzylamino purine
bp	Base pair
C	Carbon
CBD	Convention of biodiversity
CC	Column chromatography
CCD	Charge coupled device
CDA	Czapek dox agar
$CDCl_3$	Carbon deuterium chloride
cm	Centi meters
cfu	Colony forming unit
CITIS	Convention of international trade in endangered species
cm^{-1}	Centimeter inverse (wave number)
CO_2	Carbon di oxide
CTAB	Cetyl trimethylammonium bromide
DMEM	Dulbecco's Modified Eagle's Medium
DMRT	Duncan's multiple range test
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene Diamine Tetra-acetic Acid
EB	Ethidium bromide
ETEC	Enterotoxic
FAM	6-carboxyfluorescein
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FTIR	Fourier transform infra-red spectroscopy
GU	Gauhati University
H	Hydrogen
HCl	Hydrochloric acid
HeLa	Henritta Lacks
HL-60	Human promyelocytic leukemia cells
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectroscopy
IAA	Indole acetic acid
IBA	Indole buteric acid
IC_{50}	Inhibiting concentration at which half of the cells are killed
ISM	Indian system of medicine
ISSR	Inter simple sequence repeats
JOE	6-carboxy 4,5 dichloro 2,7 dimethoxy fluorescein
KBr	Potassium iodide
KCl	Potassium chloride
kDa	Kilo-dalton
Kn	Kinetin
LB	Luria Bertani medium

Abbreviations

LC	Liquid chromatograph
LSD	Least square difference
M	Molarity
MBN	1 bromo naphthalene
MDR	Multiple drug resistant
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
MM	Multiplication and maintenance medium
MS	Murashige and Skoog
MSR	Murashige and Skoog medium devised for rice
MTCC	Microbial Type Culture Collection
MTT	Methyl-thiazolyldiphenyl-tetrazolium bromide
m/v	Mass by volume
m	Meter
mm	Millimetre
MHz	Megahertz
N	Normality
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NB	Nutrient broth medium
NE	Northeast
NCBI	National Center for Biotechnology Information
NCSS	National centre for cell science
NED	1-naphthyl ethylene diamine
NMR	Nuclear magnetic resonance
NRRL	Northern regional research laboratory
NTSYS	Numerical Taxonomy System of Multivariate Statistical Programs
’N	Degree North
OPA	Operon Primers
OQ	Ortho quinol or hydroxyl quinolene
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween 20
PCA	Principal co-ordinate analysis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Para-dichloro banzene
PGR	Plant growth regulators
pH	Power of hydrogen
PIC	Polymorphic Information Content
PMSF	Phenyl methane sulphonyl fluoride
POPGENE	Population genetics
ppm	Parts per million
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNAse	Ribonuclease enzyme
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium-dodecyl sulphate- polyacrylamide gel electrophoresis
SERS	Surface enhanced Raman Spectroscopy
SPSS	Statistical Package for the Social Sciences

Abbreviations

TAE	Tris- acetic acid- EDTA
TBE	Tris- boric acid- EDTA
TDZ	Thiadiazuron
TLC	Thin layer chromatography
U	Unit
UK	United Kingdom
UPGAMA	Unweighted pair group method using arithmetic averages
USA	United States of America
UV	Ultra violet
WHO	World health organisations
wt	Weight
ZM	*Zingiber moran*
ZOI	Zone of inhibition
^{0}C	Degree Centigrade
λ	Lambda
g	Grams
h	Hours
mg/L	Milligram per liter
nm	Nanometrer
v/v	Volume by volume
µg	Micrograms
µl	Microliter
µM	Micromolar

ABSTRACT

This study is an attempt to collect, maintain, characterize and observe the pharmacological properties of genetic resources of *Curcuma* and *Zingiber* from Northeast part of India so as to open a door for further in-depth studies regarding biochemical and molecular aspects and their biological applications in near future.

The doctoral thesis work was focused on two significant genera *Curcuma* and *Zingiber* and the indigenous species *C. amada* and *Z. moran* in particular were collected, identified with taxonomic tools and properly maintained. In addition, ploidy level of *C. amada* and *Z. moran* was also studied and protocols were optimized for chromosome study of these two species. Efficient *in vitro* regeneration protocols were developed for four selected members of these two important genera, using different growth media, growth regulators and culture conditions and cyto-genetic fidelity was confirmed by RAPD and SDS-PAGE profiling and also by chromosome counting in regenerated plants. Intra and interspecific genetic variations and relationship was investigated using different molecular marker systems (RAPD, ISSR and AFLP) among 9 *Curcuma* species and 10 different ecotypes of endemic *Z. moran*, from Northeast India. The results hold promising potential for future identification and analysis of the members of family Zingiberaceae.

Antimicrobial spectrum of the crude rhizome extracts of selected *Curcuma* and *Zingiber* species was studied using techniques like disc-diffusion, viability assay, growth rate and micro-Raman spectroscopy against various human pathogenic indicators. All rhizome extracts revealed potent antibacterial and antifungal activities inferring the immense therapeutic significance as antimicrobial agents. *Z. moran* hydrodistilled oil showed best result among all and hence was further characterized chemically using techniques like TLC, CC, NMR, FTIR, and HRMS. Finally 3 bioactive compounds (Camphene, Citral and Linalool) were isolated and identified from the *Z. moran* rhizome essential oil. The components were further characterized by evaluating the antitumerous property against cancerous HeLa cell lines. Three of the components revealed a significant cytotoxic and anti-cell proliferation effect on the malignant cells. This establishes their broad pharmacological effects against common pathogens and cancer cells. Characterization of the essential oil of such endemic species could bring immense possibilities in the pharmaceutical industry in near future as a pharmaceutical agent.

Introduction

Introduction

General introduction

Systematic identification, conservation and cataloguing of plant genetic resources and indigenous genetic wealth has gained prominence in the post convention on biodiversity (CBD) scenario. There is an ever increasing demand for herbal products in almost every field of life. Medicinal plants are more important than others because of their huge requirement in medicine and need to treat dreaded diseases. The use of plants, plant extracts and plant-derived chemicals in the treatment of diseases, in supplementing foods and in making cosmetics is firmly rooted in the past and still developing. Many drugs used in contemporary medicine have been derived from plants and were originally discovered through the traditional use by indigenous people. Progress in science and technology boosts the further development of medicinal plants as valuable sources of drugs and drug leads. Modern analytical methods, biotechnological approaches (genomics, proteomics and metabolomics etc.) are nowadays applied in medicinal plant research and contribute towards the advancement of the field. However, a relatively low yield of active components and difficulties in standardization are the bottlenecks in medicinal plant exploitation. Efforts have been made worldwide to enhance the production of bioactive component using a biotechnological approach. Presently, the major thrust is on identification and conservation of medicinally and economically viable and useful plants.

The family Zingiberaceae is one of the most important herbaceous plant groups with immense medicinal, economic and aesthetic values. It demands a lot of studies for its proper and optimum utilization for the benefit of mankind. But taxonomic identification of such plant groups is still a major hurdle. Moreover, due to continuous exploitation at the rural level, most of the members are getting endangered and in the verge of extinction. Fortunately, the members of Zingiberaceae are found to grow naturally in Northeast (NE) India. These important germplasm must be protected and conserved. This study is an attempt to collect, maintain and characterize the selected members of Zingiberaceae found wildly in the NE part of India so as to open a door for

further studies regarding biochemical and molecular aspects in near future. Subjects of present study were two selected Zingiberaceae plants from NE India traditionally used in medicines

The Northeast region of India

The Northeastern (NE) region of India covering nearly 2, 62,379 sq. km. area has been divided into two biogeographic zones – Eastern Himalaya and North East India, based on floristic composition, the naturalness of the flora and the local climate (Rodgers and Panwar 1988; Aranyak 2009). The Eastern Himalaya comprising of Arunachal Pradesh and Sikkim is more mesic due to high degree of precipitation resulting from direct confrontation of monsoon laid wind blowing from Bay of Bengal by abruptly raising hills. The NE India biogeographic zone (Assam, Nagaland, Manipur, Meghalaya, Mizoram Sikkim and Tripura) is most significant one and represents the transition zone between the Indian, Indo-Malayan, Indo-Chinese biogeographic regions as well as a meeting place of Himalayan mountains with that of Peninsular India (Rao 1994). The NE Region of India lies between 22^{o} N and $29^{o}5'$ N latitude and 88^{o} E and $97^{o}30'$E longitudes, and shares international border with Bhutan, China, Myanmar and Bangladesh. The region is geographical 'gateway' for much of India's flora and fauna, and as a result, the region is one of the richest in biological values with vegetation types ranging from Tropical rain forest in the foothills to Alpine meadows and cold deserts. The NE region of India contains more than one-third of the country's total biodiversity. The region represents important part of Indo Myanmar bio-diversity hotspot, one of 25 global biodiversity hotspots recognized (www.biodiversityhotspots.org). An estimate of species richness in NE Indian states as surveyed by Forest Survey of India (FSI) is given in figure I.

The flora of NE India comprises of at least 7,500 flowering plants, 700 orchids, 58 bamboos, 64 citrus, 28 conifers, 500 mosses, 700 ferns and 728 lichen species (Chaterjee et al. 2006). About one third of the flora of NE India is endemic to this region. The eastern Himalaya supports one of the world's richest alpine floras with high level of endemism (WWF and ICIMOD 2010). According to WWF and ICIMOD, the temperate broad-leafed forest type in eastern Himalaya is among the most species rich temperate

forest in the world. Nearly 50% of total flowering plants that were recorded from India hail from NE region of India (Rao 1994).

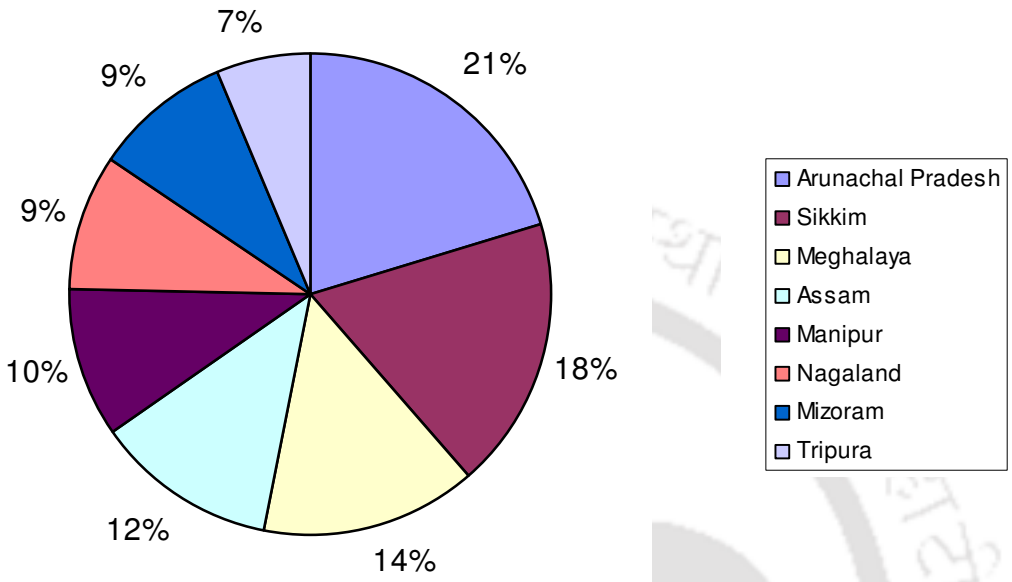


Figure I: Species richness among the NE Indian States

The region is habitat of many botanical curiosities and botanical rarities. According to the Indian Red data book published by the Botanical Survey of India, 10% of the total flowering plants in the country are endangered. Of the 1500 endangered floral species, 800 are reported from NE India (Chatterjee et al. 2006). The region supports a rich biodiversity spanning from tropical rainforests to alpine scrubs. Takhtajan (1969) describes the region as the 'cradle of flowering plants' because of its diversified angiosperms. This rich biodiversity has a significant role in the maintenance of the ecosystem. Besides, the rich biodiversity of the region is used ethnologically by locals for various socio-economic and developmental purposes. However, a large part of the NE India is botanically under-explored or even unexplored There is a great paucity of documentation of medicinal plants of NE states which demands identification and conservation for their optimal utilization. Research in the dwindling forest cover and its flora and fauna has either been ignored or accorded least priority. Scientific investigations and research programmes on the rich flora of NE region is still in a budding stage (Jamir et al. 1999; Sharma et al. 2001). India has strengthened its hold on

biodiversity conservation by implementing the Indian Forest Act, 1927; the Wildlife (Protection) Act, 1972; the Forest (Conservation) Act, 1980; the Environment (Protection) Act, 1986; the Biodiversity Act, 2002; the Biodiversity Rule, 2004, etc. India became a party to Convention on International Trade in Endangered Species (CITES) since 1976. India is also a signatory of the Convention on Biological Diversity (CBD) since 1992. A network of protected areas- biosphere reserves, sanctuaries, national parks, arboreta, botanical gardens etc. have been established throughout the country, of which this region had its share. The region has four biosphere reserves, 48 sanctuaries, 14 national parks, and two world heritage sites (Yumnum 2008). But lack of awareness at the grassroot level hampers the process of biodiversity conservation. Due to continuous exploitation of the medicinal plants by the local folk, most of the plants are either extinct or on verge of getting endangered. Especially the members of Zingiberaceae which comprise of promising ethno medicinal resources found to grow wild throughout the NE region are facing the threat of extinction.

The family Zingiberaceae

The family Zingiberaceae, commonly known as the ginger family is a unique plant family comprising of perennial aromatic forest plants. It is one of the renowned plant groups for its immense pharmacological significance. The family Zingiberaceae comprises of more than 1500 species of rhizomatous herbs all over the world (Saowaluck et al. 2009) and is well known for its multipurpose medicinal properties. It finds place among the few largest families of angiosperms, and is an important natural resource that provides many useful products for food, spices, medicines, dyes, perfumes and aesthetics. Broadly, the members of Zingiberaceae are mostly rhizomatous herbs, naturally occurred in the Indo-Malaysia subkingdom, comprise of highly evolved monocotyledons with floral characters that appear to converge with those of orchids, though they are not homologous. These are found to grow mostly in damp of humid shady places of the forest floor. Some species can expose to the sun and are well adapted on high elevation. The members of Zingiberaceae are distributed in tropical and subtropical areas with the center of distribution in Southeast Asia. The pantropical Zingiberaceae is the largest family in the order Zingiberales with about 52 genera and more than 1500 species (Sirirugsa 1999;

Kress et al. 2002). The worldwide distribution of the family Zingiberaceae is presented in figure II.

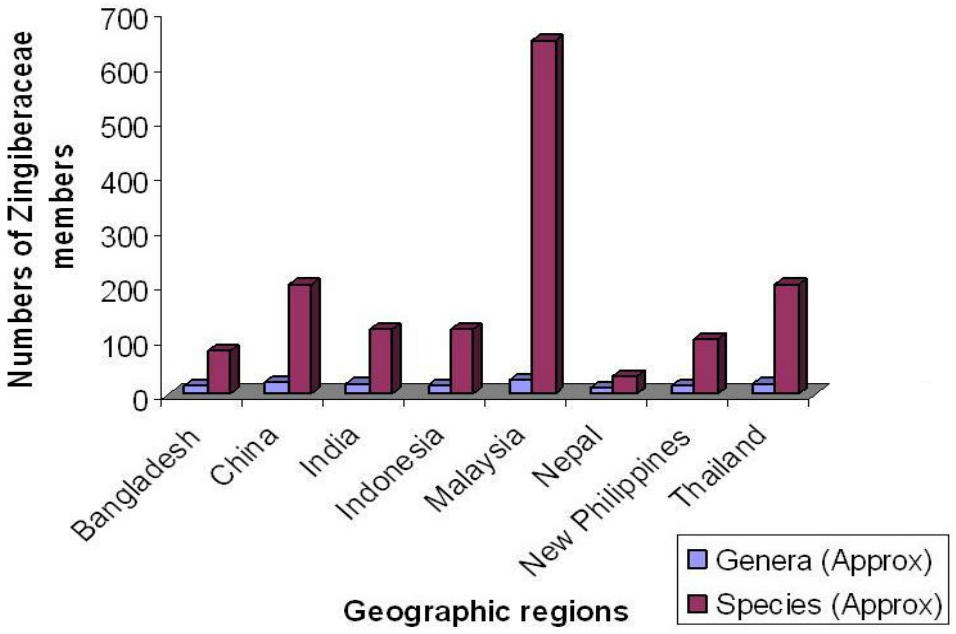


Figure II: Distribution of members of the family Zingiberaceae (Kress et al. 2002)

The genera *Curcuma* and *Zingiber*

Curcuma and *Zingiber* are two most important and well known among all the genera of family Zingiberaceae. The members of these two genera have paramount importance as vegetables, spice, condiments, starch, medicine, dyes, cosmetics and ornamentals. The genus *Curcuma* comprises over 80 species of rhizomatous herbs that are adapted to environments from sea level to elevations as high as 2000m in the Western Ghats and Himalayas (Sasikumar 2005). The geographical distribution of this genus ranges from India to Thailand, Indochina, Malaysia, Indonesia and northern Australia (Apavatjrut et al. 1999). Roughly 100-150 species have been identified worldwide in the genus *Zingiber* (Saowaluck and Paisooksantivatana 2010). The genus is distributed in tropical and sub-tropical Asia and it has been under cultivation in India, China and Southeast Asian countries for a long time (Theilade 1999; Wu and Larsen 2000). *Zingiber* is a multipurpose genus grown for medicine, for flavoring foods, and beverages and as an

ornamental plant. Plants of this genus are of rhizomatous annual herbs rich in volatile oils and are used as sources of foodstuffs, spices and traditional medicines.

Morphology of the two genera: *Curcuma* and *Zingiber*

Morphologically genus *Curcuma* is highly variable in different taxonomical important traits (Apavatjrut et al. 1999). Rhizomes of *Curcuma* are branched, fleshy and aromatic. Roots often bear conical or ellipsoidal tubers. Leaves are basal and blade is broadly lanceolate or oblong or rarely linear and narrow. The genus can be easily recognized by its large compound spike inflorescence bearing prominent spiral bracts which laterally fuse to form pouches. Each pouch subtends to a cincinnus of two to ten flowers that contain a single versatile anther. The terminal bracts form a sterile cluster called 'coma' very long and often brightly colored. It has two distinct flowering times as well as habits. Early flowering (April-May) species develop laterally from the rhizome before development of leafy shoot. Late flowering (August-September) species usually develop terminally from the leafy shoots (Sirirugsa 1999). The plants vary from 50-200 cm in height. *Curcuma* species are mostly triploid and do not produce seeds. They reproduce asexually by rhizomes. The identification of the members of this genus has traditionally been achieved using morphological data. However, *Curcuma* species exhibit large morphological variations both inter and intra species, but in some cases, especially early flowering group shows a very similar pattern of morphology between them which leads to confusion in their identification (Apavatjrut et al. 1999).

Genus *Zingiber* is terrestrial, can withstand a range of habitats. Mostly grow on hot and humid regions. Members of this genus possess sympodial and creeping rhizomes which are fibrous and strongly aromatic. Leafy stem is generally erect and developed from the rhizome and composed of leaf-sheaths. Leaves are clustered at the base or scattered along a distinct stem, alternate, sessile or petiolate, usually caudate, acuminate, linear and narrow. The inflorescence is racemose with flowers on a separate peduncle. The spike is dense cone like usually develop during the vegetative growth of the plant. Each cluster of flower is subtended by a bract. Flowers are usually with white corolla but labellum is brightly colored and scented. The plants acquire a height of 50-100 cm.

Zingiber species propagate asexually by the rhizomes and polyploidy is found to be absent in this genus.

Taxonomic hierarchy of the two genera

The taxonomic hierarchy of the two genera (Takhtajan's classification, 1969) is presented in the following box:

Kingdom	Plantae
Subkingdom	Trachiobionta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae
Subfamily	Zingiberoideae
Tribe	Zingibereae
Genus	***Curcuma***

Kingdom	Plantae
Subkingdom	Trachiobionta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberidae
Order	Zingiberales
Family	Zingiberaceae
Subfamily	Zingiberoideae
Tribe	Zingibereae
Genus	***Zingiber***

Applications/ importance of the two genera

Curcuma and *Zingiber* are the two significant genera under the family Zingiberaceae from medicinal, aesthetic and commercial aspects. The members possess many biological properties and have been used in traditional system of medicine since long back. *C. longa,* commonly known as turmeric plant is widely used as a spice, coloring agent, and is well known for its medicinal properties (Luthra et al. 2001). It is the best studied in this genus for its various bioactive substances which demonstarte germicidal, aromatic, carminative, antioxidant, anti-inflammetory, antihelmintic, antiplatelet, cholesterol lowering, antibacterial, antifungal, anticancerous and neuro protective activities (Cao et al. 2001; Cao and Komatsu 2003; Sasaki et al. 2004). Turmeric is the processed underground rhizome used as spice, herbal medicine, dyeing agent and cosmetics since Vedic age (Salvi et al. 2000). Turmeric illustrates its clinical applications over time which was partly overshadowed in the past by its common use as commercial dyestuff and ingredient of curries. However, its medicinal values have long been recognized in traditional cultures of Southeast Asia. The finest Indian arrowroot is derived from *C. angustifolia* Roxb. has been used as a source of starchy food from centuries (Das et al.

1999). Young rhizome of *C. zedoaria* is eaten as vegetable soup; also rhizomes are used in relief of stomachache and as a carminative. *C. xanthorhiza* is extensively used in traditional medicines viz. remedies for bloody diarrhoea, dysentery, fevers, hemorrhoids, stomach disorder, infected wounds and skin eruptions (Sirirugsa 1999). *C. amada* is used as perfumery to flavor curries and possess many bioactive properties like antioxidant, antibacterial, anti fungal, insecticidal etc. Some other members of *Curcuma* are *C. aeruginosa, C. aromatica, C. caesia, C. elata, C. parviflora, C. alismatifolia* etc. have received considerable attention as cut flowers and tropical glasshouse ornamentals besides their hidden medicinal properties.

Zingiber is the next well known genus with huge medicinal and commercial significance. *Z. officinale, Z. zerumbet, Z. casuamonar, Z. purpurium, Z. spectabile* are some important members of this genus. *Z. officinale* is the most studied plant commonly known as the ginger, has great commercial and therapeutic value. It is typically consumed as a fresh paste, dried powder, slices preserved in syrup, candy or for flavoring tea. In India and China, fresh ginger is used to prepare vegetables and meat dishes and as a flavoring agent in beverages and many other food preparations. The rhizome of the plant has been used as a medicine in Asian, Indian and Arabic herbal traditions since ancient times (Altman and Marcussen 2001). It has been used in treatment of nausea, cold, migraine headaches, arthritis, rheumatological conditions and muscular discomfort, atherosclerosis, high cholesterol, ulcers, depression and impotence (Liang 1992; Bordia et al. 1997; Grant and Lutz 2000). Recently anticancer property of ginger has also been investigated (Shukla and Singh 2007). Rhizomes of *Z. zerumbet* are used to treat stomachache, as tonic, stimulant and depurative. It is also used as spice ginger. Flower buds are eaten as vegetables in many Asian countries. The clear liquid in the cones is an excellent hair conditioner, hence also known as 'shampoo ginger'. Rhizomes of *Z. purpuriumm* are used to treat fevers and intestinal disorders. Most species of this genus have beautiful inflorescence and luxurious foliages that have immense commercial value in floriculture as a versatile ornamental flower used as cut flower, pot and landscape plant (Maciel and Criley 2003). Apart from known species of *Zingiber,* there are a lot more wild varieties that are regularly used by the local folk in various home remedies which need to work on.

Present research in *Curcuma* and *Zingiber*

Plants which have not been identified as yet through pharmacology, folk medicine, Ayurveda, Unani and Chinese systems of medicine, Homeopathy and Ethnopharmacology are being investigated for their medicinal usage and may be proved so in due course of time (Chaturvedi et al. 2007). With an ever-increasing global inclination towards herbal medicines there is not only an obligatory demand for a huge raw material but also of right stage when the active principles are available in optimum quantities at the requisite time. In order to meet such demand, intervention of biotechnology, can play a major role. With tools and techniques like systematic collection and identification, *in situ* conservation, micro propagation, molecular markers, etc can go a long way in understanding, identification, utilization and successive conservation of the natural resources in demand. The Genera *Curcuma* and *Zingiber* are being studied by various workers in different countries on various aspects till date. The genera comprise most common and popular plants like turmeric and ginger. However, most of other members are also of much commercial and therapeutic significance. Studies on *in vitro* regeneration, genetic diversity, biochemical analysis and bioactivity of the essential oils of these two genera have been reported by many researchers from India and other countries. But, research in NE Indian members of *Curcuma* and *Zingiber* are still in a nascent stage and demands to be studied in order to explore and conserve the rich germplasm.

Collection and maintenance of germplasm

The flora of NE India, because of climatic advantage, is rich in Zingiberaceae species which are found to grow in wild state with many unique and endemic varieties. Very little efforts have been made in proper collection and maintenance of the germplasm of these Zingiberaceae members (Kalita 2005). Researchers have collected some species and maintained randomly as and when they need. A novel approach has been made by Dr. Sabu from CU, Kerala and his group in collecting and maintaining the Zingiberaceae members from all over India. However, till date there is no report of proper collection, identification and maintenance of the Zingiberaceae members from NE India. Due to difficulties in collection during the monsoon season, these plants have been neglected and

the scientific and systematic studies are also least attempted. Identification of the species also poses difficulties due to short reproductive phase. Some of the species descriptions are without Latin diagnosis or type specimen, therefore the legitimate status of many species is suspicious and remains unclear (Per. Comm., Dr. Sarma GU). The local folk use to harvest the medicinally significant members for the family and thus most of them are getting endangered. Accurate identification of species is fundamental to both basic and applied research and forms the foundation for all biology. Therefore, systematic approach of collection thus will be helpful in planning conservation strategies, scientific studies and optimum utilization of the elite germplasm of NE India.

In vitro regeneration studies

Plant regeneration through a rapid multiplication protocol could be used extensively for plants with a very slow rate of propagation. Tissue culture techniques also minimize the dependency on environmental factors and other constraints of cultivation (Sasikumar 2005). Tissue culture has been successfully used in the two genera viz, *Curcuma* and *Zingiber* for rapid multiplication and disease free plantlet production. *In vitro* regeneration studies on *Curcuma* and *Zingiber* are available in some commercial and cultivated species like *C. longa, C. domestica, C. aromatica; Z. officinale, Z. zerumbet* etc. (Sharma and Singh 1997; Borthakur et al. 1999; Shirin et al. 2000; Mello et al. 2001; Salvi et al. 2002; Rehman et al. 2004; Loc et al. 2005). These studies mainly emphasize on *in vitro* multiplication and production of micro rhizome from the commercial species (Nayak 2000; Rout et al. 2001; Jeanette et al. 2004). Other members of the two genera are also attempted by workers from different parts of the world (Prathanturarug et al. 2004; Chithra et al. 2005; Tyagi et al. 2006; Anish et al. 2008). But the elite members of *Curcuma* and *Zingiber* from NE Indian flora are yet to be studied and therefore in need of a better tissue culture protocol so that these could be cultivated and commercialized. Little efforts have been made by some workers to study the wild but medicinally important *Curcuma* and *Zingiber* species of NE India (Barthakur and Bordoloi 1992; Borthakur et al. 1999).

Molecular analysis of the regenerated plants gives a confirmation of the genetic purity and thereby brings success to the regeneration process. Also these can promote *in*

vitro production and conservation of rare and endangered species. Moreover, karyological studies also confirm the cytogenetic fidelity of the regenerated plants. Reports are lacking in use of these method of testing the stability in the Zingiberaceae members. Ploidy status of *in vitro* grown regenerants of *Curcuma longa* has been reported earlier by Panda et al. (2007) through estimation of nuclear DNA content after 6 months of culture. However no reports are known on cytogenetic assessment of micropropagated plants in any member of *Zingiber*. It is a strong attribute to confirm that the tissue culture raised plantlets did not develop any kind of polyploidy or difference in chromosome number in spite of long *in vitro* conditions.

Genetic diversity studies

The knowledge of genetic variability is a pre requisite to study the evolutionary history of a species, as well as for other studies like intraspecific variations, genetic resources conservation etc (Islam et al. 2007). Studying genetic diversity and relationship using molecular markers would therefore be useful for any kind of future analysis using the members of the family Zingiberaceae. Studies are available in genetic diversity and relationships among the members of Zingiberaceae from all over the world (Kress et al., 2002; Jatoi et al. 2006; Jatoi et al. 2008; Syamkumar and Sasikumar 2007; Hussain et al. 2008; Das et al. 2011). Mostly, studies are based on the phylogenetic relationship, hybrid detection and variability of economically important varieties of *Curcuma* and *Zingiber* (Jiang et al. 2006; Anuntalabhochai et al. 2007; Jatoi et al. 2008). Researchers have used various types of markers like isozyme markers, morphological traits, DNA based markers, protein markers etc (Jiang et al. 2006; Syamkumar and Sasikumar 2007; Kavitha et al. 2010). The molecular markers are far more informative in detecting the variances at genetic level than the morphological ones. Among these, DNA based markers have proved efficient and more discriminating. Markers like Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), Inter Simple Sequence Repeats (ISSR), Amplified Fragment Length Polymorphism (AFLP) etc. are extensively used to characterize the genetic variation existing in the two genera *Curcuma* and *Zingiber* (Jatoi et al. 2006; Syamkumar and Sasikumar 2007; Hussain et al. 2008; Kavitha et al. 2010). However, the use of three different marker systems simultaneously

is not reported in case of Zingiberaceae members, which could serve as a more precise and powerful way of executing the diversity both inter and intraspecific at molecular level. Therefore, the molecular approach of studying genetic diversity is of significance especially towards protection and utilization of bioresources from NE India.

Phytochemical studies

There have been a great thrust of exploring natural compounds existing in plants and plant products and analyze them for the better use in human welfare. People have been searching from time immemorial the naturally occurring useful products to utilize and pertain in every step of civilization. In the scientific world too, research and exploration is as to extract, analyze and execute the plant originated natural products for the betterment of mankind. Zingiberaceae, being the best known medicinal family among angiosperms, have been worked on extensively especially in the medicinal purposes. However, most of the members of this unique family are still to be invaded. The Zingiberaceae plants suffer from the lack of scientific interest as they exhibit very short vegetative phase and occur in inaccessible habitats. However, much works have been reported on the chemical investigation of many commercial members from various part of the globe (Richmond and Villar 1997; Leela et al. 2002; Jirovetz et al. 2003; Jiang et al. 2005). Among them, commercial species like *C. longa, Z. officinale* etc are most explored. Other members of these genera like *C. amada, C. aromatica, C. caesia, C. zedoaria, Z. petiolata, Z. zerumbet, Z. corallinum* etc. have also been investigated chemically and the rhizome oil and their application in medicines and other uses (Jain and Mishra 1964; Behura et al. 2002; Singh et al. 2002; Mustafa et al. 2005; Yang et al. 2009; Bhuiyan et al. 2009). Phenolic pigments (curcuminiods) and essential oils are the main constituents in *Curcuma* plants. Essential oils are considered as the active constituents which have reported to possess antitumor, antimicrobial and antiviral activities (Nie et al. 2003; Xia et al. 2004). Major components of found in essential oil of the genus *Curcuma* are tumerone, ar-tumerone, phellandrene, ar-curcumine, curdione, curzerenone, curcumol, 1, 8-cineole, germacrone, myrcene, pinene etc. (Zheng et al. 1997; Wang et al. 2001; Singh et al. 2002; Raina et al. 2005; Purakayashtha et al. 2006).

The members of the genus *Zingiber* are reported to possess diarylheptaniods in their rhizome essential oils. Major constituents present in the essential oil of this genus are zerumbone, zingiberene, zingiberol, bisabolene, farnesene, gingerol, linalool, shogaol, methyl kaempferol, etc. (Zancan et al. 2002; Chien et al. 2008). The components of the *Z. officinale* essential oil are found to include immuno-modulatory, anti-tumerogenic, anti-inflammatory, anti-apoptotic, anti-hypergycemic, anti-lipidemic and anti-emetic actions (Ali et al. 2008).

In the NE Indian flora, there are many unexplored members of *Curcuma* and *Zingiber* with paramount medicinal significance still waiting for proper scientific study. These plants are regularly used by the local folk for various medications and ritual use. In this study, *Z. moran* is such an endemic and untouched species from NE India.

Bioactivity studies

Northeast India is one of the biggest biological diversities in the word. Indian people have been using the Zingiberaceae plants to treat various diseases since long. Several studies are reported on various bioactivity assays of the rhizome extract of different gingers revealing their broad pharmacological effects against common pathogens and other biological attributes (Sacchetti et al. 2005; Indu et al. 2006; Sabulal et al. 2006; Tewtrakul and Subhadhirasakul 2007). Among all, the genera *Curcuma* and *Zingiber* have been extensively worked on by many researches across world (Kishore and Dwivedi 1992; Briggs Goldman 2002). In recent years, several reports have been published concerning the composition and/or the biological properties (antimicrobial, antioxidant, anticancer and a stimulated effect on the immune system) of Zingiberaceae extracts (Ekwenye and Elegalam 2005; Chen et al. 2008). Zingiberaceae members contain many essential oils like terpenes, alcohols, ketones, flavanoids, carotenoids, gingeroles and phytoestrogens (Habash et al. 2000; Suhaj 2006). Less polar compounds like curcuminoids, gingeroles etc have been reported to have great antifungal, antioxidant, insecticidal and anti-inflammatory activities (Sirat 1994; Sirat et al. 1996). *C. longa* and *Z. officinale* are reported to possess multiple pharmacological activities, viz. antioxidation (Sacchetti et al. 2005; Mohanty et al. 2004), antimicrobial (Singh et al. 2002; Khattak et al. 2005), antiatherosclerotic, anti-inflammatory, antidepressant,

antiplatelet and immune activation activities (Ashraf et al. 2005; Lantz et al. 2005; Lee 2006). The essential oil of *C. longa* and *Z. officinale* are considered as one of the major bioactive compounds found in nature (Kim et al. 2003; Sacchetti et al. 2005). Rhizome and tuberous root of *C. longa* are used as two Chinese medicines, e.g. *Jianghuang* and *Yujin*, respectively. The two cultivated and common species *C. longa* and *Z. officinale* are also reported to possess anticancerous properties (Katiyar et al. 1996; Manju and Nalini 2005; Shukla and Singh 2007). Keeping in view the increasing demand for the herbal medicines and bioactive products at a global level, there is a strong need of scientific study on the indigenous Zingibers from NE India to extort the hidden medicinal and bioactive properties of the plants which are very much popular among the local folk for therapeutic use. The characterization of the essential oil of such endemic members of the family could bring immense possibilities in the pharma industry in near future. Present study aims to give comprehensive information about the biological activity and therapeutic value for some of the most commonly used medicinal plants (and plant constituents) of Zingiberaceae. This knowledge can be used to further develop the medicinal varieties in India in a rational way.

Scope and Significance

1. Studies in systematic characterization of *Curcuma amada* and *Zingiber moran*, two promising species of the family Zingiberaceae from NE India has immense pharmacological, economic, and environmental significance,
2. Karyological studies like counting of chromosomes in *C. amada* and *Z. moran* from various ecological groups allows more reliable interpretation of the resulting data and in ploidy level check. Chromosome number study in the above two species carries significance and possibilities for further research in cytogenetic and molecular aspects.
3. *In vitro* regeneration studies would expedite the propagation rate and generation of superior clones of such medicinally significant plants. Also, *in vitro* studies hold ground for future research like genetic engineering and molecular improvement of the species.
4. Genetic diversity studies among different species of *Curcuma* and ecotypes of *Z. moran*, reveals information regarding intra and interspecific biodiversity among them and thereby signifies the need of conserving and maintaining the essential germplasm.
5. Antimicrobial assay using the rhizome essential oils of wild and cultivated gingers depicts their strong biological efficacy and hence opens a door for further research on the traditionally important medicinal Zingibers.
6. Chemical characterization of the endemic *Zingiber moran* holds immense possibilities in medicinal, economic and pharmacological fields as it contains bioactive compounds. It sheds light on the principal bioactive compounds Camphene, Citral and Linalool.
7. Anticancerous cytotoxicity study reveals an excellent property of *Z. moran* rhizome oil and therefore signifies its potential use in pharma-industries.

Specific Objectives

On the background of the literature reviewed, the following specific objectives were defined:

1. Systematic germplasm collection, maintenance and identification of pharmacologically important members of Zingiberaceae from NE India.

2. Optimization of somatic chromosome study of two selected members: *Curcuma amada* and *Zingiber moran* from NE India to check their ploidy level.

3. Standardization of micropropagation protocols in the genera *Curcuma* and *Zingiber* to enhance propagation rate and large scale cultivation in field.

4. Standardization of DNA isolation protocol and investigation genetic variability among different species of *Curcuma* and ecotypes of *Zingiber moran* from NE India.

5. Rhizome essential oil extraction of important gingers and antimicrobial assay of crude rhizome extracts for testing bio-efficacy.

7. Chemical characterization of *Zingiber moran* rhizome essential oil for identifying bioactive compounds.

8. Investigation of anticancerous property of the bioactive compounds isolated from *Z. moran* rhizome essential oil.

Chapter 1

Chapter 1

Systematic germplasm collection, identification, maintenance and chromosome-study of two selected genera of Zingiberaceae from Northeast India

1.1 Introduction

Variety is the spice of life and basic characteristic of life is its unlimited diversity. Nature has myriads of life forms on this planet among which variations are of ubiquitous occurrence. It is particularly so in the mega centers of diversity in the tropics which harbor approximately two-thirds of the biota and where many species of economic importance presumably had their origin. As a matter of fact, variations between individuals of the species were observed over the millennia and were considered as real 'hot spots' of evolution (Sabu 1992). The area under study, NE India is such an important biodiversity hot spot holding lots of natural resources. The immense variety of the climatic, edaphic and altitudinal variations in India have resulted in a great range of ecological habitats, among which NE India takes the pride of being in premium place.

Maintenance of biodiversity is one of the most important current concerns of mankind, as wild species and domestic breeds and strains are disappearing at an alarming rate, and an increasing number of these require human intervention to guarantee their survival (Frankham et al. 2002). As genetic diversity is the basis of evolutionary potential of species to respond to environmental changes, this becomes an essential pillar in conservation biology. Most populations of endangered species are commonly subdivided in different breeding groups, either in different fragments of habitats, natural reserves, arboreta or zoos, or in different breed or strains in case of domestic plants and animals which are in turn subdivided into smaller reproductive units more or less interconnected. Thus characterization and management of genetic diversity has to be made considering idiosyncratic population structures or 'metapopulation', a term widely used in conservation biology to designate a group of population with some possible genetic flow

among them (Hanski and Gilpin 1997). Conserved populations of domestic species could be useful because of their better adaptation to specific environment or disease, or because of the presence of possession of specific traits of cultural, historical and scientific value. Moreover native breeds are an important part of our landscape and culture and therefore, there is a need for maintaining them. The family Zingiberaceae being the largest one to hold medicinal plants is fortunately found in the regime of NE India naturally. Ironically, many members are found to be endemic to this locality and are lagged behind without proper recognition. The green cover of the region is shrinking due to various socio-geographic reasons resulting in greater loss of the important flora. In this study, an attempt has been made to collect, identify and maintain the elite genotypes of important members of the family Zingiberaceae from NE India.

1.2 Literature review

Nowhere in India conservation of natural environment and resources is as challenging as in NE region which is a transitional zone of Indo-China, Indian and Indo-Malayan flora and fauna. The ecology of the region where mountain and flood plains dominate the topography, is under tremendous pressure from a burgeoning population, unabated conversion of forest lands to agricultural land, illegal felling of trees to cater to the growing demands for timber, coal mining activities and unregulated expansion of tea plantation areas (Aranyak 2009). The flora of NE India consists of numerous Zingiberaceae species having medicinal, ethno botanical, aesthetic and other economic values. However, the work on this family is regularly constrained by difficulties in species identification (Sasikumar 2005). *Curcuma* and *Zingiber*, two well known genera of this family have immense medicinal value, which finds extensive use in the indigenous system of medicine (Velayudhan et al. 1999; Mridula and Jayachandran 2001; Matsuda et al. 2004; Kalita 2005). NE India houses the vast majority of *Curcuma* and *Zingiber* species, some of which are endemic to the region (Kalita 2005). These species are mostly neglected due to their inaccessible natural habitat and difficulties in collection in dense tropical forests during monsoon season which is the favorable period of collection. The species are gradually facing the fear of being extinct in NE region as a result of ruthless uprooting of the rhizomes by the tribal from their wild habitats for its extreme medicinal

value and use in spices. Sustainable management has so far not succeeded further aggravating the extinction. Thus characterization of the elite and endemic genotype especially *Curcuma* and *Zingiber* species will be useful for planning strategies for their conservations and optimal utilization. An extensive literature survey on collection, identification and maintenance programme in economically important Zingiberaceae species found in India has been reviewed in this chapter with special reference to *Curcuma* and *Zingiber*, to gather the available information to form the guidelines to initiate management programme in these two selected genera. These plants are much desirable in a conservation or management programme with their rhizomes having high medicinal value. Moreover, Zingiberaceae members are well adapted to the weather and climatic conditions of NE India though there is a need to domesticate the wild varieties for cultivation under different production systems. Almost all of the species from *Curcuma* and *Zingiber* possess essential aromatic oils and medicinal importance as established at the grass root level (Tushar et al. 2010). Conservation and maintenance of such species would lead to improved production of drug yielding plants in the field of pharmacognosy. Essential oil content and composition in *Curcuma* and *Zingiber* species also vary considerably and can be attributed to a number of factors such as genotype, climatic conditions and soil fertility. However, as the plants are vegetatively propagated, the genetic factors are least expected to influence the oil content and compositions. Thus, its growth and productivity depends mainly on the availability of geographical and edaphic factors. Variation in rhizome morphology and oil content of these two genera is of great potential in management and conservation programs. Unfortunately these resources have been neglected without assessment. Since the species are found to grow wild in inaccessible habitats, efforts towards domestication through identification of elite genotypes have been very limited. Collection and maintenance of these medicinally important species is the prior step to make optimum use of them and also to grow them as an economic crop.

Scientific investigations and research programmes on the rich flora of NE region is still in a budding stage (Jamir et al. 1999; Sharma et al. 2001). Reports are available in extensive study in economically important species like *Curcuma longa*, *C. amada*, *C. zedoaria*, *Zingiber officinale*, *Z. zerumbet*, etc (Araujo and Leon 2001; Chattopadhyay et

al. 2004; Sasikumar 2005; Jatoi et al. 2007; Hussain et al. 2008). Phytochemical, pharmacological, toxicological, genetic diversity, tissue culture and bioactivity studies are available on both the genera *Curcuma* and *Zingiber* from all over the world including India (Rao 1994; Behura et al. 2002; Prakash et al. 2005; Bharalee et al. 2005; Policegoudra and Aradhya 2007; Purakayashtha et al. 2006; Syamkumar and Sasikumar 2007; Jatoi et al. 2008). However, there are still many endemic members of Zingiberaceae lying in the lap nature which await proper identification, maintenance and conservation. Such species are getting endangered and very soon be lost with the unique germplasm which need to be recognized. However, no attempts are made to collect and maintain such endemic species. *Z. moran* is one such promising endemic medicinal plant regularly used by the local folk for various ethno medicinal uses. This is the first report to collect, identify and maintain this elite germplasm in its natural habitat leaving a ground for its extensive future study.

Genome size and chromosome numbers are important cytological characters that significantly influence various organismal traits. However, geographical representation of these data is seriously unbalanced, with tropical and subtropical regions being largely neglected. The family Zingiberaceae contains many taxa of economic, medicinal, ornamental and cultural importance, *Curcuma* and *Zingiber* probably being the best known. Several taxonomic and biological problems have hindered satisfactory systematic treatment of the two genera. Original descriptions of many species are vague and inaccurate, and type specimens are often lacking or fragmentary. Proper preservation of the specimens is extremely difficult, exacerbating the limited amount of type material, leading to ambiguous name assignment and usage. In addition, high intra and interpopulation variation has led to debate concerning species concepts and boundaries. As a result, one species has often been described repeatedly under different names where as the same name has been applied to different taxonomic entities. Some species may hybridize in the wild and the crosses may become naturalized (Skornickova and Sabu 2005b; Skornickova et al. 2007). Frequent cultivation of *Curcuma* and *Zingiber* spp. and targeted selection of peculiar morphotypes have further contributed to taxonomic complexity of the group. Moreover, polyploidy has played a significant role in evolution

and diversification of various members of Zingiberaceae (Mukherjee 1970; Lim 1972a, b; Poulsen 1993; Chen and Chen 1984; Takano 2001).

In the course of studies in the Zingiberaceae flora of India the use of cytological data has been proven necessary to the satisfactory understanding of the taxonomy and evolution of certain species. Until now this flora has been cytologically neglected. A comparison of the incidence of polyploidy in the Zingiberaceae flora of NE India with that in other Zingiberaceae floras should be especially pertinent to studies on the geobotanical significance of polyploidy. Although only two species were included in this study from the Zingiberaceae flora of NE India, still they represent the most frequently encountered genera of the family. The chromosome counts have been obtained from material collected during field studies in the monsoons of 2008-2010. No report on other work accomplished in these two species has been published elsewhere.

Curcuma is found to possess natural polyploids in many of its species. Six different chromosome counts (2n=22, 42, 63, 70, 77 and 105) were reported in the genus *Curcuma* (Skornickova et al. 2007). *C. amada* being a significant member have still not studied well. Only one report regarding chromosome study is available on this species (Skornickova et al. 2007). The genus *Zingiber* is found to be comparatively with less frequency of polyploidy. Studies on Karyotyping and chromosome counts in this genus have revealed less report of existence of polyploidy compared to the other members of the family. Moringa et al. (1929) and Suguira (1936) reported the chromosome number of ginger as 2n=22. A more detailed study was made by Raghavan and Venkatasubban (1943) on cytology of the three important *Zingber* species (*Z. officinale*, *Z. casuamonar* and *Z. zerumbet*) and found the chromosome number to be 2n = 22 in all cases. But based on difference in ideogram morphology they concluded that chromosome morphology of *Z. officinale* was different from the other two species. They found the existence of two B chromosome in certain types of gingers in addition to the normal set 2n = 22 (Darlington and Janaki-Ammal 1945). Chakravorty (1948) also found chromosome number 2n=22 in *Z. officinale* and concluded that in view of normal pairing of 11 bivalents in species like *Z. zerumbet* and *Z. casuamonar*; *Z. mioga* having a chromosome set of 2n=55 which is considered as a pentaploid. Sharma and Bhattacharya (1959) reported the widespread occurrence of an inconsistency in chromosome numbers in several species of

Zingiberaceae including *Z. officinale*. Sato (1960) carried out Karyotyping studies of 24 species belonging to 13 genera of the family Zingiberaceae and concluded that the basic number of the genus *Zingiber* is n = 11, and of *Z. mioga* with 2n = 55 is a pentaploid. Ramachandran (1969) studied the cytology of five species of *Zingiber* (*Z. macrostachyum, Z. roseum, Z. weihtianum, Z. zerumbet and Z. officinale*) and found a diploid number of 2n = 22 in all species. He found evidence of hybridity involoving interchanges and inversions in ginger. Mohanty (1970) studied the cytology of Zingiberales and reported 2n = 22 for *Z. spectabile* and *Z. cylindricum* and concluded that the genus *Zingiber* is closer to *Hedychium*. Das et al. (1999) investigated various cultivars of *Zingiber* and found that all of them possess a somatic chromosome number of 2n = 22. An asymmetrical karyotype of "1B" was found in all the cultivars except in cultivars of Bangkok and Jorhat, which have a karyotype asymmetry of "1A". The karyotype of various cultivars exhibited only minor differences. However, in this study, ploidy level of *Z. moran* has been investigated for the first time.

1.3 Materials and Methods

1.3.1 Collection of germplasm

The plants used in this study were *Curcuma amada* and *Zingiber moran* and were collected from different places of NE India (Assam, Meghalaya, Nagaland, and Arunachal Pradesh) during April-October. The plants were collected as a whole along with rhizome and the reproductive parts were kept in polyethene bags with some retaining soils with the rhizomes to keep it alive till the replanting in greenhouse conditions. Mature rhizomes were collected separately. The flowers of Zingiberaceae plants are very delicate and ephemeral and cannot be studied in usual herbarium stage. Hence after specimens were collected, the complete plant was cut into pieces and each piece was numbered. Rhizomes and roots were also collected in form of small pieces and detailed information about colour and odor were noted down. The flowers are succulent and the floral parts are very fragile that gets distorted as and when preserved in the form of herbarium. Dissection was also a problem due to delicate, tender nature of the floral parts. Therefore, flowers were dried separately along with the inflorescence and kept in

packet. In addition to the above data, the field data ecology, associated smell and the colour of the rhizome, leaves, flowers, fruits and aerial colour and other details of the floral parts were noted.

1.3.2. Identification and maintenance of germplasm

The collected plants were then maintained as both live specimen and as herbariums in the departmental green house of Indian Institute of Technology Guwahati (IITG), Assam. Herbaria are essential for the study of plant taxonomy, geographic distributions, and the stabilizing of nomenclature. The specimens in a herbarium are often used as reference material in describing plant taxa; some specimens may be types. To preserve their form and color, collected plants were spread flat on sheets of newsprint and dried in between absorbent paper. The specimens are then mounted on sheets of stiff white paper, labeled with all essential data such as date and place found, description of the plant, altitude, and special habitat conditions. The sheet was then placed in a protective case. As a precaution against insect attack, the pressed plant is frozen or poisoned and the case disinfected.

A botanical specimen consists of the whole plant, complete with roots, stem, leaves, flowers and if possible, with fruits. Since Zingiberaceae species were large herbs, a portion of a twig with leaves and flowers were collected as a representative specimen for herbarium. The size of the specimen used for preservation was set according to the size of the herbarium sheet (17x11 inches) used for mounting. After collection, the specimen was tied with a tag number. Most of the plants wilted very rapidly after being cut or dug out of the ground. To avoid this, the dug out plants and cut twigs were kept in polythene bags along with some soil retained with the rhizome. This could keep the plants fresh for more than 6 hrs. The flowers of *C. amada* were too large to be pressed with the leaves and were kept separately in small polythene bags. Two specimens from each were collected for every species. Soon after collection, pressing of the specimens was carried out with a ventilated drying press. While pressing, care was taken so that the leaves are upside down to show the ventral side. Rhizomes were difficult to press satisfactorily and hence were dried separately with tags. The specimens were examined in every 24 hrs. The newspapers were replaced with new ones in every week. Next, the plant twig was mounted to a herbarium sheet and was labeled. A label consisted of

collection number, Latin name, family name, vernacular name, habit, habitat, locality, date of collection and collector's name. Then the herbarium sheets were stored under a cover, called species cover. Collected specimens were identified by studying morphological traits following taxonomic rules and with the help of herbarium of Gauhati University.

1.3.3. Ploidy check by chromosome study

1.3.3.1. Collection and storage of root tips

Mature rhizomes of *C. amada* and *Z. moran* were allowed to sprout on moist soil in pots for 5-10 days in the departmental green house. When the roots arose, 1-1.5 cm of root tips were collected from the sprouted rhizomes. *Z. moran* roots were collected between 9.30-10.00 am and *C. amada* roots were collected between 11.00-11.30 am respectively. The root tips were then washed for few minutes with tap water and finally with distilled water and soaked on a filter paper. A total of 10 replicates of each plant species from different places were collected and fixed for the study.

1.3.3.2. Pre-treatment and fixation

The collected roots of *Z. moran* were pre-treated with saturated aqueous solution of ρ-dichloro benzene (PDB) and kept at 16 ^{o}C for 3 h. Those of *C. amada* were pretreated with a 1:1 mixture of saturated PDB solution and 2 mM 8-hydroxyquinoline at 4-5 °C for 4 h. The pre-treated materials were washed with distilled water for several times, soaked on a filter paper. The root tips were then fixed in freshly prepared Carnoy's fluid (Ethyl alcohol and glacial acetic acid in 3:1 ratio), incubated for 6 hours at room temperature. After six hours, the root tips were washed with 70 % alcohol for 3-4 times and preserved in 70 % alcohol for future use.

1.3.3.3. Hydrolysis and staining

Fixed root tips were hydrolyzed in 1 N HCl at 58 ^{o}C. Six different times (1, 2, 3, 4, 5 and 6 min) were tried for optimization of hydrolysis. Thereafter, the root tips were immersed for 2 hours in two different staining solutions, (A) Acetic acid-orcein 45% (Darlington

and Lacour 1979); (B) Acetic acid-Carmine 50% (Darlington and Lacour 1979; Harandi and Ghaffari 2001). The root tips turned black once stained.

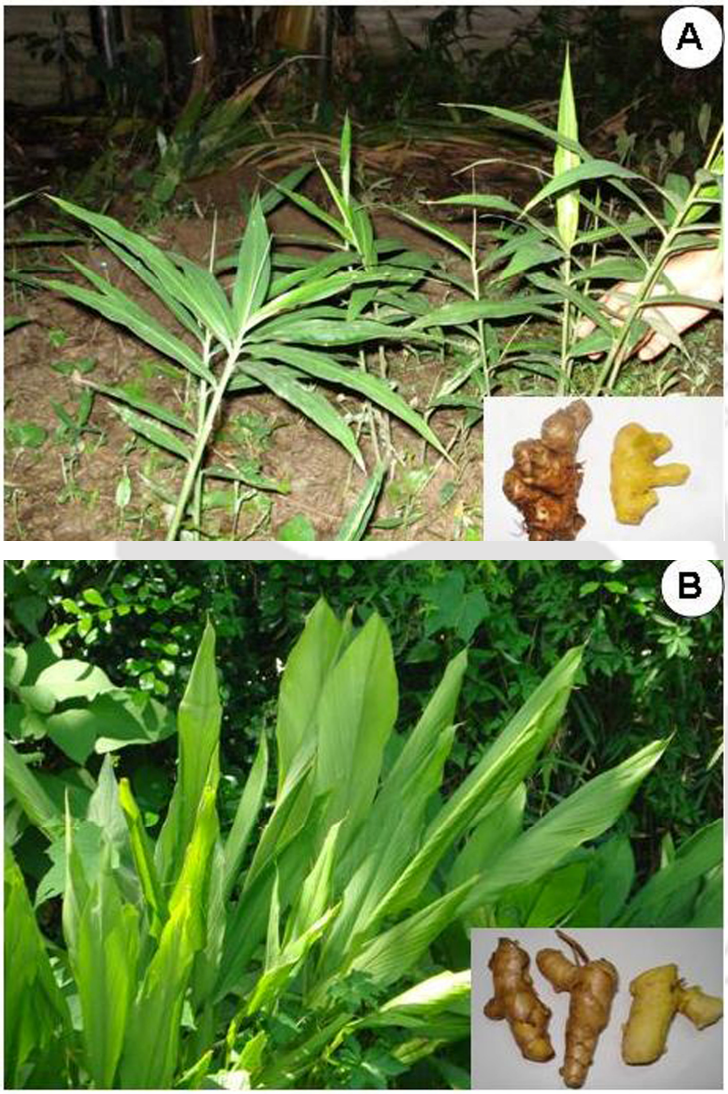


Figure 1.1: Live specimen of *Z. moran* (**A**) and *C. amada* (**B**) in natural habitat

1.3.3.4. Slide preparation, squash and observation

The root tips stained previously were taken out on a clean microscopic slide using a brush. The root was cut gently into small pieces towards the root tip, with a razor blade and dissecting needle. Few drops of 45 % glacial acetic acid was added onto it and macerated subsequently. A cover slip was placed over the tissue trying not to get air bubbles under the cover slip. The cover slip was pressed down firmly by the thumb. Excess of acids were soaked with filter paper. The glass was heated over the flame for few times just to soften the tissue and then squashed by pressing the slide firmly between sheets of blotting paper. With a small cork stick or pencil headed eraser the cells were spread into a monolayer. After the cells were spread uniformly, the slide was observed under the compound light microscope. Three replicates of each slide for each species were tested to avoid the potential errors of chromosome counting as well as taxonomic ambiguity.

1.4 Results and Discussion

1.4.1. Plant material

The studied plants were collected during the monsoon season which is the optimum time of their vegetative and reproductive growth (Figure 1.1). The plants were found to grow in hot and humid conditions and prefer shady places. *C. amada* grows both in alluvial and hill soils, where as *Z. moran* is only found to grow in alluvial soils predominantly. *C.* amada grows from the month of April to August and *Z. moran* was found to grow from May till October. Flowering occurs in the month of April-May just before the vegetative growth in *C. amada*; but in *Z. moran*, reproductive growth was found in late vegetative phase in the month of October when the plant starts dying. *C. amada* species were collected from places with moderate climate with high humidity, habitat of the plant is mainly the hilly slopes and moist grasslands. The rhizome is soft, creeping, light yellow in color, with characteristic strong smell of raw mango. The leaves are distichously arranged, much similar to that of *C. longa* but are distinctly different with the characteristic raw mango aroma. Flowers pinkish white, born in a raceme, bisexual, zygomorphic and epigynous. Flowers are very short lived and fruits were either absent or sterile. The *Z. moran* species was restricted to shady humid hilly places of few districts of

Assam, Nagaland and Arunachal. The rhizome of this species is off white in color, with strong pungent smell, smaller in size and is less fibrous than the common ginger. The immature rhizomes are reddish white and the scale leaves are much darker. Flowers are solitary, born in a racemose spike which is dense cone like usually develop during the vegetative growth of the plant. Corolla are light yellow with a black-yellow spotted labellum. Flowers are bisexual, zygomorphic and epigynous. Fruits absent.

1.4.2. Identification and maintenance

The collected plants of *C. amada* and *Z. moran* were identified following the taxonomic identification keys.

***Curcuma amada*:**

Key to the Kingdom: Presence of chlorophyll, autotrophic, whole body is divided into stem, root and leaves. ------- **Plantae**

Key to the Division: Plants with flowers and seeds, ovules enclosed within the ovary ---------- **Angiosperms**

Key to the Sub-division: Leaves simple with parallel venation, Flowers mostly trimerous, one cotyledon. ----- **Monocotyledons**

Key to the Order: Inflorescence racemose, flowers bisexual, zygomorphic, creeping horizontal or tuberous rhizomes. **-------- Zingiberales**

Key to the Family: Flowers zygomorphic, epigynous, only one fertile stamen, other stamens transform into staminodes. ------- **Zingiberaceae**

Key to the Sub-family: All parts aromatic, leaves distichously arranged in two rows, sometimes tufted or single, sheaths open on side opposite lamina, lateral staminoids usually petaloid or represented by a teeth at the base of the labellum, aromatic oil present ------ **Zingiberoideae**

Key to the Genus: Inflorescence central appear with leaves with a terminal plume of sterile bracts, peduncle enclosed within leaf sheaths, several flowers forming a spike, each flower or each cluster of flowers is subtended by 2 bracts, reddish or pinkish white in color, nectaries present. ----- ***Curcuma***

Key to the Species: Ovary inferior, rhizomes with sessile branched tubers, possess characteristic flavor of raw mango. ------- ***amada.***

Floral diagram:

Floral formula: $\% \; {}_{+}\male Br_2P_{3+3}A_{2+4}\underline{G_{(3)}}$

***Zingiber moran*:**

Key to the Kingdom: Presence of chlorophyll, autotrophic, whole body is divided into stem, root and leaves. ----------**Plantae**

Key to the Division: Plants with flowers and seeds, ovules enclosed within the ovary -------- **Angiosperms**

Key to the Sub-division: Leaves simple with parallel venation, Flowers mostly trimerous, one cotyledon. ----- **Monocotyledons**

Key to the Order: Inflorescence racemose, flowers bisexual, zygomorphic, creeping horizontal or tuberous rhizomes. **--------- Zingiberales**

Key to the Family: Flowers zygomorphic, epigynous, only one fertile stamen, other stamens transform into staminodes. -------- **Zingiberaceae**

Key to the Sub-family: All parts aromatic, leaves in two rows, nectaries present. ------ **Zingiberoideae**

Key to the Genus: Flowers solitary, each cluster of flowers is subtended by a bract. -------- ***Zingiber***

Key to the Species: Ovary inferior, petals yellow, bracts green, labellum is yellow-black, spotted, rhizome with characteristic odor, dirty or off-white in color, particularly cultivated by the 'Moran' tribe of NE India. ------ ***moran***

Floral diagram:

Floral formula:

$$\% \; {}_{+}\delta P_{3+3}\, A_{2+4}\, \underline{G}_{(3)}$$

Hence the systematic position of the two species is identified as:

Kingdom: Plantae	Kingdom: Plantae
Division: Angiosperms	Division: Angiosperms
Sub division: Monocotyledons	Sub division: Monocotyledons
Order: Zingiberales	Order: Zingiberales
Family: Zingiberaceae	Family: Zingiberaceae
Genus: *Curcuma*	Genus: *Zingiber*
Species: ***amada***	Species: ***moran***

Collected plants were replanted under green house conditions in pots prepared with alluvial soil and sand mixture (3:1) and maintained as live specimen. Figure 1.2 depicts the *Z. moran* plant maintained in greenhouse. These were also maintained as herbarium specimen for future reference (Figure 1.3).

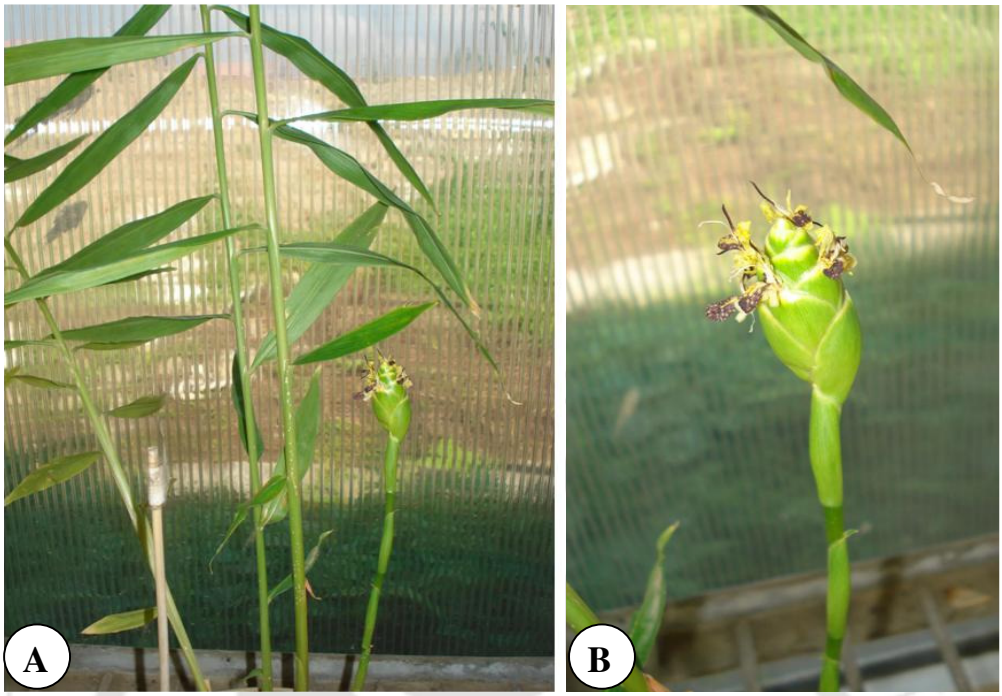


Figure 1.2: Live specimen of *Z. moran* (**A**) in green house;
An inflorescence of *Z. moran* (**B**)

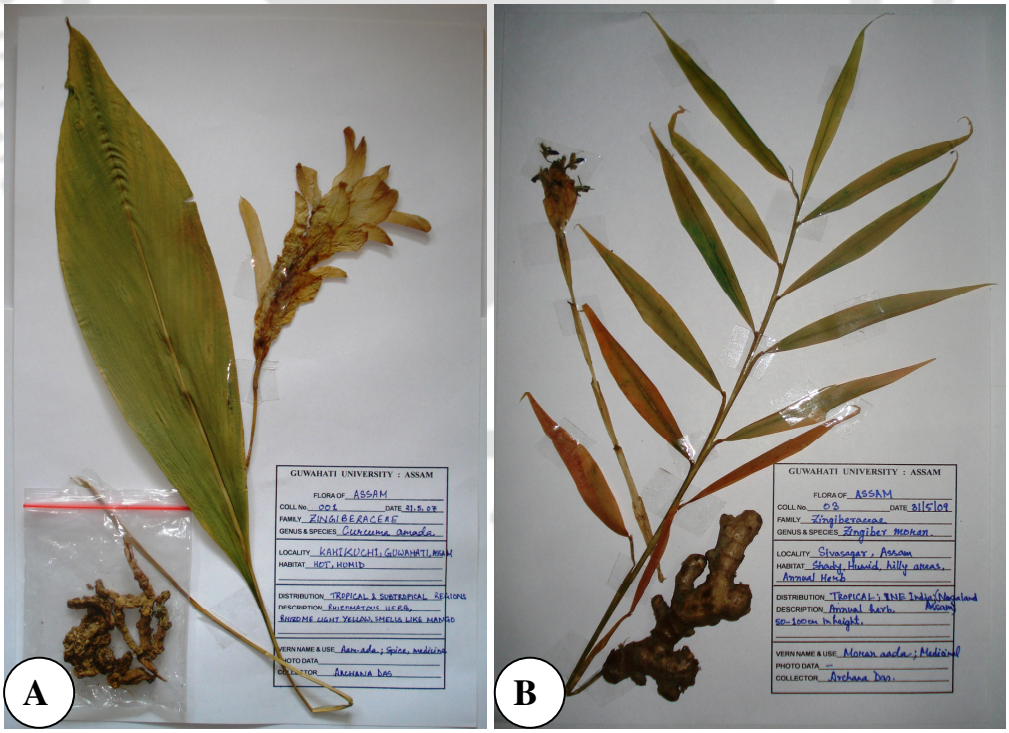


Figure 1.3: Herbarium specimen of *C. amada* (**A**) and *Z. moran* (**B**)

1.4.3. Chromosome counts

1.4.3.1. Collection of root tips

To check the ploidy level of the collected species, chromosome counts of *C. amada* and *Z. moran* were carried out. The best time of the day to collect root tips for karyological study is 10 am - 12 pm where there are more cell metaphases. However the time was optimized for both the plants under study and accordingly growing root tips were collected between 9.30- 10.00 am for *C. amada* and 11.00 – 11.30 am for *Z. moran.* Both the periods were found to give adequate numbers of metaphases in respective plants. However, the midday period is widely followed in the field of cytology for the family Zingiberaceae (Lim 1972b; Newman 1990; Nair and Sasikumar 2009). Midday is known to be at a peak of cell division in many plants and thus will yield the highest number of metaphases when fixed for cytological observation. Late-morning period was also preferred by some workers (Chen 1992; Augsonkitt et al. 2004).

1.4.3.2. Pretreatment

Pre-treatment is a necessary step in cytological studies of members of Zingiberaceae. Different workers have used various pre-treatments and staining chemicals in their studies (Chen 1992; West and Cowley 1993; Rai et al. 1997; Das et al. 1998; Joseph et al. 1999; Nair and Sasikumar 2009). This helps to block the mitosis cycle at metaphase stage by inhibiting spindle fiber formation revealing more number of cells in metaphase. PDB and 1-bromonapthalene (MBN) were found better in treating the Zingiberaceae plants by Chen (1992). However, in this study it was found that PDB alone was effective in treating *Z. moran* and a mixture of PDB and 8-hydroxyquinolene (OQ) gave a slightly higher percentage of metaphase cells for *C. amada*. Our result matches with the findings of Rai et al. (1997) and Das et al. (1998). Literature on cytological study says that PDB and OQ are preferred for the pre-treatment of *Curcuma* and *Zingiber* species.

1.4.3.3. Hydrolysis and staining

Hydrolysis of the root tips is carried out to soften the root tissue. The roots of monocots are harder and larger size and therefore, it is an important step to soften the pretreated roots for a specified period in 1N HCl. In the present study, the time was optimized as 5

min for *C. amada* and 3 min for *Z. moran* respectively at 58 ^{o}C. Later, the roots were allowed to cool down at room temperature and subjected to dye (2% aceto-orcein or aceto carmine) in a test tube and heated over a flame for few seconds. The heating for a few seconds in the acid-dye mixture intensified the staining of the chromosomes and gave a clearer general picture of the chromosome structure. Both the stains tested, were found to give better results in the two plants under study. However, Feulgen has also proved to be an effective in staining Zingiberaceae (Lim 1972a; Newman 1990; West and Cowley 1993). Orcein is another stain giving fruitful results in many members of Zingiberaceae (Nair and Sasikumar 2009).

1.4.3.4. Slide preparation, squash and observation

The preparation of a good slide depends on the proper handling of the root material, cutting, maceration and squashing. Care was taken so that the tip of the root is not hurt. Adding of the acid onto the root tip slowly macerate it and also decolorize the cytoplasmic material leaving only the chromosomes stained. The squashing and spreading of the cells uniformly is a crucial step which results in proper visualization of the slide. The slides revealed well-spread metaphases under low power (10x) and high power (40x) magnifications in compound light microscope. Some cells were found to be in anaphase and late prophase stages also.

Out of 10 varieties studied, *C. amada* confirmed the chromosome number of 2n = 42 where as *Z. moran* revealed the chromosome number of 2n=22 without any polyploidy (Figure 1.4). The slides showing the chromosome counts are shown in figure 1.3. The occurrence of different ploidy levels in *Curcuma* was highlighted in early cytological studies (Suguira 1931; Suguira 1936; Raghavan and Venkatasubban 1943; Venkatasubban 1946; Chakravorty 1948; Sharma and Bhattacharya 1959). Considering the widely accepted basic chromosome number n=21 (Ramachandran 1961; Prana 1977; Islam 2004), this chromosomal variation roughly corresponds to three euploid levels (2n, 3n and 4n) plus several aneuploids. The ploidy level of *C. amada* matches with the findings of other workers (Das et al. 1999). However *Z. moran*'s chromosome number is a new count. Chromosome structures of the species studied are metacentric.

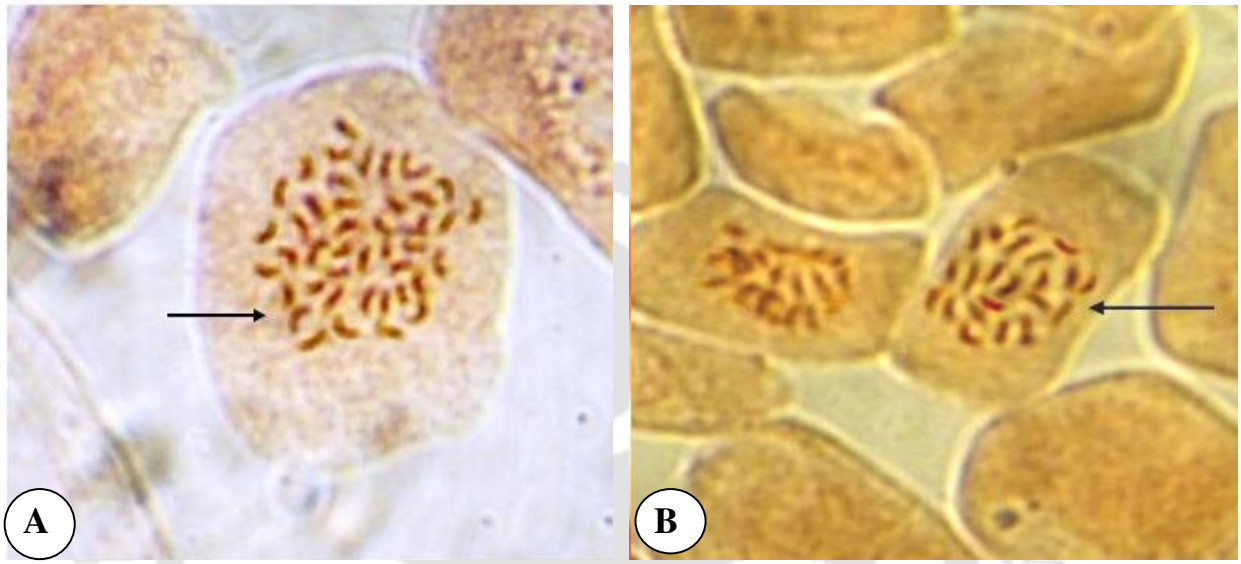


Figure 1.4: Slides showing chromosome number of *C. amada*, 2n=42 (**A**); *Z. moran* 2n=22 (**B**)

1.5 Conclusion

The genera *Curcuma* and *Zingiber* of family Zingiberaceae are found throughout south and South-east Asia with a few species extending to China, Australia and the South Pacific. The highest diversity is concentrated in India and Thailand, with many species in each area, followed by Burma, Bangladesh, Indonesia and Vietnam. Due to the lack of a comprehensive taxonomic revision, there is still little consensus on the number of species that could be recognized. The destruction of forests, in many parts of the tropical region has lead to an increased disappearance of native plants proportionately. Moreover, the native people who have been living in these areas and accumulated a compendium of folk knowledge about the usefulness of plants for curing various diseases are also getting faded with generations. Scientific collection and identification of the local medicinal plants that have been used by the folk people traditionally would definitely secure the species and also will make the tribal people aware, whose socio-economic life is interwoven with the forests from where they derive all their material requirements, including their healthcare needs and foodstuffs.

The chromosome number of individuals is sometimes found to be different to the number of species because of factors, such as chromosome fission and misdivision of the paired chromosomes at meiosis. This leads to chromosomal variations including polyploidy, which is a common phenomenon found among the members of Zingiberaceae. Therefore, chromosome count is supposed to be a reliable approach of studying the ecotypes of the same species in order to find the minute genetic variations existing among them. This further helps in species and variety identification associated with systematic studies. In the current study, *C. amada* species collected from different regions of NE India showed a chromosome number of 2n=42 denoting absence of the variation in ploidy level. It thus appears to be a natural diploid as the basic chromosome number in the genus *Curcuma* is known to be 21 (Ramachandran 1961). *Z. moran* shows no polyploidy in all the ecotypes studied a feature common to the genus *Zingiber*. This could be concluded that as the species is a strictly vegetatively propagated one, the planting material was confined to a few districts of Assam and Nagaland; therefore, the karyotype of the cultivars remains relatively asymmetrical because of the lack of recombination and evolution by sexual processes.

Chapter 2

Chapter 2

Micro propagation of two important genera *Curcuma* and *Zingiber* from Northeast India and assessment of genetic stability of regenerated plants by biomolecular techniques

2.1 Introduction

Medicinal and aromatic plants form the essential components of biodiversity. There has been gradual and recently rapid loss of medicinal plant genetic resources because of many factors. At present 90% collections of medicinal plants are from the wild and the 70% of plants collections involve destructive harvesting (Mishra 2009). Also, majority of the medicinal plants come under herbaceous category and thereby easily harvested by people. The family Zingiberaceae is one of the largest families comprising almost all the members with utmost medicinal values. It has 52 genera and about 1500 species distributed throughout tropical Asia especially in Indochina peninsula (Sirirugsa 1998). In NE India, the members of Zingiberaceae are found to grow naturally due to the favourable climate. However, as a result of over exploitation, most of the members fall under the critically endangered or vulnerable category. Moreover, being rhizomatous the plants have a slower rate of propagation. Even the seed bearing members too are observed to propagate through rhizomes. Keeping in view all these factors, their demand in the pharma industry and traditional use, there is a strong need to increase the productivity and conservation of such plant species. But as these plants are herbaceous and propagate by vegetative means, there is less possibilities of hybrid production and cloning. Therefore, rapid multiplication through tissue culture techniques may definitely play a role in propagation and conservation of such important herbaceous plants. *In vitro* techniques could be used for conservation of medicinally significant members of *Curcuma* and *Zingiber* species, as proven in case of many Zingiberaceae species having condiment and spice value (Ashmore 1997).

In vitro culture technique has been recognized as an efficient tool for rapid clonal multiplication. Plant regeneration *in vitro* and re-introduction into the original or favourable habitats is one strategy for conservation of important plant species. Tissue culture is the technique of maintaining plant tissue in an artificial medium *in vitro* under controlled condition. Propagation of plants through tissue culture has become an important and popular technique. The continuous supply of sterile plantlets helps to overcome the contamination problem and reduce the time for sterilization process. Tissues from various organs such as stem and leaf of the axenic plantlets can be induced to form callus. Moreover, callus tissue can serve as an experimental system to investigate the biological activities using specific bioassays. However, many factors contribute to the ability of a specific tissue to form callus such as medium and plant growth regulators. At present, researchers aim to produce substances with antitumor, antiviral, hypoglycemic, anti-inflammatory and antimicrobes through tissue culture technology. However, establishment of tissue culture system is required prior to further exploration of the biosynthetic capabilities of various *in vitro* cultures.

Monitoring the degree of genetic integrity between *in vitro* raised plants using molecular markers is desirable to reduce the chances of inclusion of variable genotypes. Several strategies can be used to assess the genetic stability of *in vitro* derived plants such as karyological analysis, isozyme markers, DNA based markers etc. (Isabel et al. 1993). Among different molecular markers used, random amplified polymorphic DNA (RAPD) is the cheapest and appears to be powerful tool for the detection of genetic variability and analysis of genetic fidelity of *in vitro* propagated plants and have been well established in many plants (Cassells et al. 1997; Salvi et al. 2001; Rout et al. 2001; Hussain et al. 2008). Polypeptide profiling (SDS-PAGE) is a useful biochemical marker system with functional/expressed gene diversity and has been more popularly applied to seeds analysis for diagnostics and estimating out-crossing rates (Ferreira et al. 2000). The members of Zingiberaceae are known to show polyploidy in general. Therefore, checking the chromosome numbers of the regenerated plants could also establish the cytogenetic stability. The karyological study to check the ploidy status of *in vitro* grown plants was reported earlier by Panda et al. (2007).

Curcuma and *Zingiber* are two major genera of the family Zingiberaceae and used as one of the main cash crops in NE region. Besides some cultivated species of *Curcuma* and *Zingiber*, there are many other promising species from these two genera which are left unexplored. Many species also possess variety of medicinal properties and have been used by the tribal folk in traditional system of medicine over centuries. Such species need to be worked on. In fact, there are immense possibilities of growing such species commercially if *in vitro* tissue culture techniques can be applied. Cultivated species also await some better regeneration techniques to meet the demands and increase productivity. It will not only speed up the multiplication rate but also will enhance conservation and optimum utilization of the germplasm. Hence the present study is based on developing a better micropropagation technique in some wild and cultivated species from *Curcuma* (*C. amada*, *C. longa*) and *Zingiber* (*Z. moran* and *Z. zerumbet*) found in NE India leading to their *in situ* conservation and proper utilization.

The chapter has been divided into three objectives, first, micropropagation by direct organogenesis from axillary buds using various phytohormones individually and synergistically. The varying concentrations of cytokinins viz; Benzyl aminopurine (BA), Kinetin (Kn) alone and in combination with auxins viz, indole 3-buteric acid (IBA) and naphthalene acetic acid (NAA) were used for regeneration, multiplication and rooting response. Secondly, to establish optimum culture conditions to enhance the regeneration rate and mass multiply. The effect of two different growth media, Murashige and Skoog (MS) (Murashige and Skoog 1962) and a modified MS medium (MSR) along with varying concentrations of agar and carbon source was investigated for multiplying *Curcuma* and *Zingiber* species from rhizomatous bud. A comparison of initiation and multiplication of rhizome in MS media and MSR media with different hormone combination were also attempted in this study. The use of MSR media instead of liquid medium is a significant step in developing a standard protocol for rapid multiplication of species studied. The third objective was to produce a disease free material for conservation and further studies. In addition, we performed biomolecular techniques like RAPD analysis, SDS-PAGE analysis and karyological analysis of the micropropagated plants for cytogenetic purity assessment. The overall aim of the chapter was to develop

an efficient regeneration protocol from mature rhizomes for two species of *Curcuma* and *Zingiber* from NE India.

2.2 Literature Review

Consumption of herbal medicines is widespread and increasing. Traditionally, plants have been collected for medicinal use from wild areas. Harvesting from the wild, the main source of raw material, is causing loss of genetic diversity and habitat destruction. Domestic cultivation is a viable alternative and offers the opportunity to overcome the problems that are inherent in herbal extracts: misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. In natural product research, the presence of large amount of plant biomass is necessary to provide enough bioactive compounds from the plant tissue. This presents an unfeasible solution due to the lack of reliable and abundant supply of the plant material. Natural habitats for medicinal plant are disappearing fast and together with environmental instabilities, it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of using *in vitro* culture techniques as an alternative supply for the production of plant natural products. Recently, researchers are keen interested in applying biotechnological tools to enhance production as well as the conservation of such elite germplasm. Plant cell and tissue cultures have the potential of providing a low cost route to numerous plant derived natural products that are very expensive to synthesize chemically or that occur naturally at very low concentration. During the past few decades, tissue culture techniques have been manipulated for many purposes such as for the improvement of plants growth, secondary metabolites production and biological activities and transformation. Apart from conventional breeding methods, many reports are available in developing *in vitro* culture techniques especially in case of herbaceous medicinal plants like Zingiberaceae which propagate vegetatively. This also offers the opportunity to optimize yield and achieve a uniform, high quality product. Several reports of *in vitro* culture of species from the Zingiberaceae family have been published (Illg and Faria 1995; Sharma and Singh 1997; Borthakur et al. 1998; Shirin et al. 2000; Mello et al. 2001; Salvi et al. 2002; Rahman et al. 2004; Chithra 2005; Tyagi et al. 2006; Anish et al. 2008). However, very

few reports are there in wild members of this family. *C. amada, Z. zerumbet* and *Z. moran* are promising species with little or no tissue culture works till date (Barthakur and Bordoloi, 1992; Nayak 2002; Prakash et al. 2004; Stanly et al. 2010). In this chapter, a comparative study of *in vitro* regeneration has been carried out between *Curcuma longa* and *C.* amada; and between *Zingiber moran* and *Z. zerumbet.*

Commercially important plants of the family like *C. amada*, *C. longa*, *Z. casuamonar*, *Z. officinale* etc. have wide use in food, spices and medicines. The wild species viz; *Z. moran* and *Z. zerumbet* find extensive use in the indigenous system of medicine. *C. amada* is a unique spice, which morphologically resembles the ginger (*Z. officinale*) and is also called 'mango ginger' due to its characteristic raw-mango aroma. The rhizome is rich in essential oils, and more than 130 chemical constituents with biomedical significance have been isolated from it (Yang et al. 2009; Bhuyan et al. 2008). It is a popular vegetable, also used to prepare chutneys and in curries to flavor. Researchers have tried and reported various tissue culture works in members of *Curcuma*, *Zingiber* and other Zingiberaceae members. Prakash et al. (2004) reported successful regeneration of *C. amada* plantlets from rhizomes as well as leaf-sheath explants. Nayak (2000; Nayak and Nayak 2006) investigated for microrhizome production for *C. amada* and *C. aromatica* using micropropagation techniques. However, *in vitro* techniques for mass multiplication of this species have not been successful till date. *C. longa* (turmeric) indigenous to southern Asia is known for its aromatic, stimulant, carminative and anthelmintic properties (Sasikumar 2005; Kalita 2005). Curcuminoids, the biologically active principles from *Curcuma*, promises a potential role in the control of rheumatism, carcinogenesis and oxidative stress-related pathogenesis (Chan and Thong 2004). Turmeric is a sterile triploid plant, 2n=3x=63, (John et al. 1997) propagates vegetatively through underground rhizomes. Rate of rhizome multiplication in this plant is very low (only six-ten times) with yield ranging from 15 to 25 tons/hectare (Balachandran et al. 1990). A considerable amount (10-20%) of rhizomes of the total yield is required for the next cropping year (Shirgurkar et al. 2001). Maintenance of such a huge amount of seed-rhizomes for annual planting is expensive and labour intensive. Moreover, many diseases and pests, particularly soft rot of turmeric caused by *Pythium* spp. and bacterial wilt are consistently threatening the germplasm of this important

medicinal plant (Balachandran et al. 1990, Nayak 2000, Salvi et al. 2001 and 2002, Shirgurkar et al. 2001). *In vitro* regeneration studies on *C. angustifolia* was carried out by Shukla and Singh (2007) who found 1.87 shoots per explant using shoot meristem in MS medium with BA 3 mg/L. In *C. longa* a high frequency of shoot multiplication was found to investigate by Prathanturarug et al. (2003), using the only growth regulator TDZ. Slow multiplication rate, limited availability of high yielding genotypes, expensive field maintenance of planting material and high susceptibility of *Curcuma* species to rot diseases necessitates application of tissue culture technique as a solution to these problems (Khader et al. 1994; Nayak and Nayak 2006).

Direct regeneration of functional plantlet in tissue culture is a pre-requisite for any successful *in vitro* conservation programme as the regeneration through callus is known to induce somaclonal variations in *C. longa* (Salvi et al. 2001). Young inflorescence was reported to be used as explant for regeneration of *C. alismatifolia*, an ornamental species of the genus (Wannakrairoj 1997). Salvi et al. (2001) have also used the immature inflorescence of *C. longa* as explant for *in vitro* regeneration studies. But it was observed the need of growth regulators (BA, TDZ etc.) at a much higher concentration (5-10 mg/L) for the regeneration. Moreover, the inflorescence comes at a certain season of the plant and that too in very less numbers. Nadgauda et al. (1978) and Balachandran et al. (1990) reported the *in vitro* multiplication in *C. longa* using rhizome bud as explants, and micropropagation of *C. longa* through callus cultures from leaf (Salvi et al. 2001). Use of leaf-base as explants was also reported in *Kaempferia galanga*, a medicinally promising member of Zingiberaceae, to obtain efficient plat regeneration through somatic embryogenesis (Rahman et al. 2004). But such protocols were much time consuming and leaves too were not available for the whole year. Therefore, rhizomes with axillary buds appear to be the best explant for *in vitro* regeneration and multiplication studies for most of Zingiberaceae members. In *vitro* induction of microrhizome in *Curcuma* species was reported by Islam et al. (2004). *C. zedoaria,* another medicinally sound species was reported for micropropagation and multiplication by various workers (Loc et al. 2005; Stanly and Keng 2007). They found the use of liquid medium more effective over that of solid medium. Bharalee and his co workers (Bharalee et al. 2005) used rhizome bud explants for *in vitro* clonal propagation of *C. caesia* and *C. zedoaria*. Similar

investigations on *in vitro* clonal multiplication of *Curcuma* species through rhizome buds has been reported (Sit and Tiwari 1997; Salvi et al. 2002; Prakash et al. 2004) but the difficulty has been to establish good regenerating material in a suitable medium. Even attempts at using axillary bud meristem have faced problem at the multiplication stage.

The ginger plant *Z. officinale* Roscoe produces pungent, aromatic rhizomes that are valued worldwide as a spice and in herbal medicine. Ginger is grown primarily in tropical areas of Asia and has been used in medicine since ancient times for conditions including colds, fevers, digestive problems, and as an appetite stimulant, nausea, and vomiting (Mowrey and Clayson 1982; Grontved et al. 1988; Stewart et al. 1991; Wood et al. 1988; Borrelli et al. 2005; Chaiyakunapruk et al. 2006). There are few reports on the use of tissue culture for *in vitro* propagation of ginger. Hosoki and Sagawa (1977) reported a maximum of six shoots per bud from *in vitro* culture, with low survival under field conditions. Other reports are available on rapid clonal multiplication of ginger (Balachandran et al. 1990; Bhagyalakshmi and Singh 1988; Khatun et al. 2003), organ culture (Sharma and Singh 1995), somatic embryogenesis (Kackar et al. 1993; Guo and Zhang 2005), organogenesis protoplast culture and *in vitro* germplasm preservation(Babu et al. 1992; Geetha et al. 2000; Guo et al. 2007). Earlier many workers reported to regenerate plantlets through somatic embryogenesis using leaves as explants (Kacker et al. 1993; Sultana et al. 2009). Shoot tips and root explants of *Z. officinale* were also used for large scale multiplication of ginger. Khatun et al. (2003) formulated a successful large scale multiplication protocol yielding 22-25 plantlets per explant in *Z. officinale.* They observed the synergistic effect of cytokinin and auxins in the study. However, for other species of *Zingiber* many factors like liquid media, light treatments, sources of nutrients were found to play significant roles in regeneration and *in vitro* microrhizome production (Tyagi et al. 2006; Guo et al. 2007; Zheng et al. 2008).

Z. zerumbet (L.) Smith, popularly known as 'Shampoo ginger' as the cone shaped bracts contains a clear liquid which is an excellent natural hair conditioner (Nalawade et al. 2003). The species is medicinally significant wild member of the family Zingiberaceae and has been traditionally used for the treatment of fever, constipation and to relieve pain. It possesses antipyretic and analgesic (Somchit et al. 2005), anti-inflammatory properties (Somchit and Shukriyah 2003) and chemo preventive activities (Nakamura et al. 2004).

Like other Zingibers, *Z. zerumbet* also propagate vegetatively by underground rhizomes at a slow rate. Besides, plants are frequently infected with pathogens and have very low multiplication rate. Another inherent problem with *Zingiber* rhizome is to establish a good axenic culture. A rapid multiplication method is therefore necessary to provide disease-free planting material in enough quantity for their conservation and optimum utilization. However, except some chemical investigation this species has not been worked on its rapid multiplication aspect (Murakami et al. 2004; Chien 2008). There are several reports on the bioactivity of zerumbone, which was identified as a major compound of this plant, including anticarcinogenesis (Takada et al. 2005) and anti-inflammation (Murakami et al. 2003). A few reports are available on tissue culture studies of this species; Hsu et al. (1991) evaluated a mass propagation method using shoot tip explants and Stanley and Keng (2007) used rhizome buds for micropropagation of *Z. zerumbet*. In both cases, liquid culture proved to be more suitable for shoot multiplication. There was a low rate of multiplication in semi-solid media. However, maintaining liquid culture *in vitro* is not so desirable for large scale multiplication. Thus, a more efficient mechanism of rapid clonal multiplication of *Z. zerumbet* is required.

Z. moran is a wild relative of *Z. officinale* strictly found in certain places of NE India and hence is endemic to this region. This species is morphologically similar to the common ginger (*Z. officinale*) plant differing only in rhizome and floral parts. The rhizome of *Z. moran* looks like ginger, but much smaller in size, darker in color and with a characteristic strong aroma much different from ginger. The species has immense medicinal value and is regularly used in home-made therapies by the local folk since centuries. It is popularly known as “Moran aada” in local language of Assam. Originally found in a place called ‘Moran’ in Sivasagar district of Assam and hence the name of the plant. It is used as an excellent expectorant, carminative, diuretic, stimulant and many others (Yadav et al. 2004; Sabulal et al. 2006). Till date, there is no any scientific report on this endemic plant species. However, *Z. petiolatum*, a rare species from Thailand was studied for *in vitro* propagation of plantlets by Prathanturarug et al. in 2004. Vincent et al. (1992) reported an efficient method of regeneration and rapid multiplication of another threatened ginger *Bosenbergia pulcherrima* from India and conservation of the progenies as well. Present study is an effort to optimize the *in vitro* protocols for direct plantlet

regeneration, rapid multiplication and effective conservation of wild and domestic species of *Curcuma* and *Zingiber*. Effects of various growth regulators and culture conditions were also tested on proliferation and shoot multiplication of the four species under study. Like investigation was reported by Rout et al. (2001) for *Z. officinale*. The results of the current study will surely contribute for further research and conservation of wild and endemic varieties of Zingiberaceae that are available in limited quantities.

The establishment of genetic stability of *in vitro* regenerated plants will be an essential requisite for large scale multiplication. Despite the advantages of the *in vitro* propagation, genetic instability has been observed in micropropagated species hence it is necessary to establish a system that produces genetically stable and identical plants, especially in the case of commercial plant species such as *C. amada* and *C. longa*. Several strategies can be used to assess the genetic stability of *in vitro* derived plants such as karyological analysis and isozyme markers, but they have their own limitations (Isabel et al. 1993). However, karyological assessment stands effective in species like *Zingiber* where polyploidy is a common phenomenon. Bimolecular analytical techniques like polypeptide and DNA polymorphisms profiling facilitates direct and reliable measurements to detect culture-induced variation at the DNA and protein level (Cloutier and Landry 1994). Rout and Das (2002) have reported the use of RAPD markers for genetic fidelity testing of the micropropagated plants of *Plumbago zeylanica* and conservation of genetic richness as well. RAPD analysis along with cytophotometry was successfully used by Panda et al. (2007) for assessing the genetic stability in the tissue culture raised plants of *Curcuma longa.* Of several molecular markers used RAPD marker have been proven as the most useful, powerful and simplest tool for the detection of genetic fidelity of *in vitro* propagated plants in many plant species (Williams et al. 1990; Salvi et al. 2001; Panda et al. 2007). RAPD analysis as a genetic markers to determine the extent of genetic variation or fiedility of regenerated plants has been used in plant and crops such as *Allium* (Al Zahim et al. 1991), *Lolium* (Wang et al. 1993), *Triticum* (Brown et al. 1993), *Beta* (Munthali et al. 1996), *Pongamia* (Kesari 2010), rice sugarcane (Jain et al. 2005), where RAPD was used to detect somaclonal variation or purity (Yang et al., 1999). Polypeptide profiling (SDS-PAGE) is another useful biochemical marker system with functional/expressed gene diversity and has been more popularly applied to seed

analysis for diagnostics and estimating out-crossing rates (Ferreira et al. 2000). Earlier SDS-PAGE has been reported to use as a tool for testing genetic fidelity in regenerated plantlets in *Pongamia pinnata* (Kesari 2010).

2.3 Material and Methods

2.3.1. Plant material and preparation of explants

Mature rhizomes of *C. amada*, *C. longa*, *Z. moran* and *Z. zerumbet* were collected from different parts of NE region of India (Kamrup, Barpeta, Nagaland and Sivasagar) having axillary buds. Collected rhizomes were placed in sand and soil mixture (1:3) in a pot and kept for sprouting. After 8-10 days, axillary buds of the rhizomes sprouted and were used as starting material. Outer scales of the buds were removed and were cut into small pieces (1-2 cm long) and washed under running tap water to remove dust and adhering soil particles. The explants were treated with 0.1-0.2% Bavistin solution, a systemic fungicide (BASF, India) for 30 min under continuous agitation followed by rinse with sterile distilled water. After that, explants were surface sterilized with 0.1% Na-hypochlorite solution along with (2% m/v) Tween 20 for 5 min and rinsed with sterile distilled water until all traces of Tween 20 were removed. Finally explants were pretreated with 70% ethanol for 2 min before the second surface sterilization with 0.1% (m/v) mercuric chloride for 10 min followed by five rinses with sterile distilled water.

2.3.2 Inoculation and establishment of explants

Both ends of the explants exposed to sterilants were trimmed and were incubated in culture tubes (Borosil, India Ltd.) containing culture media. The standard MS medium (Murashige and Skoog 1962) and modified MS medium (MSR) containing additional supplements like yeast extract (300mg/L) and casein hydrolysate (100mg/L) with 0.8% agar (Hi-media, UK) and 3% sucrose were used as basal media for initiation and multiplication. The pH of the media was adjusted to 5.8 and autoclaved at 121 ^{o}C for 15 min. Cultures were incubated at 25±2 °C under 16 h/daylight. A light intensity of 40 µmol m^{-2} s^{-1} was provided by cool white fluorescent light. It took 3-6 weeks to get the first organogenetic response of the explants. The steps involved in tissue culture are summarized as flow diagram Fig. 2.1.

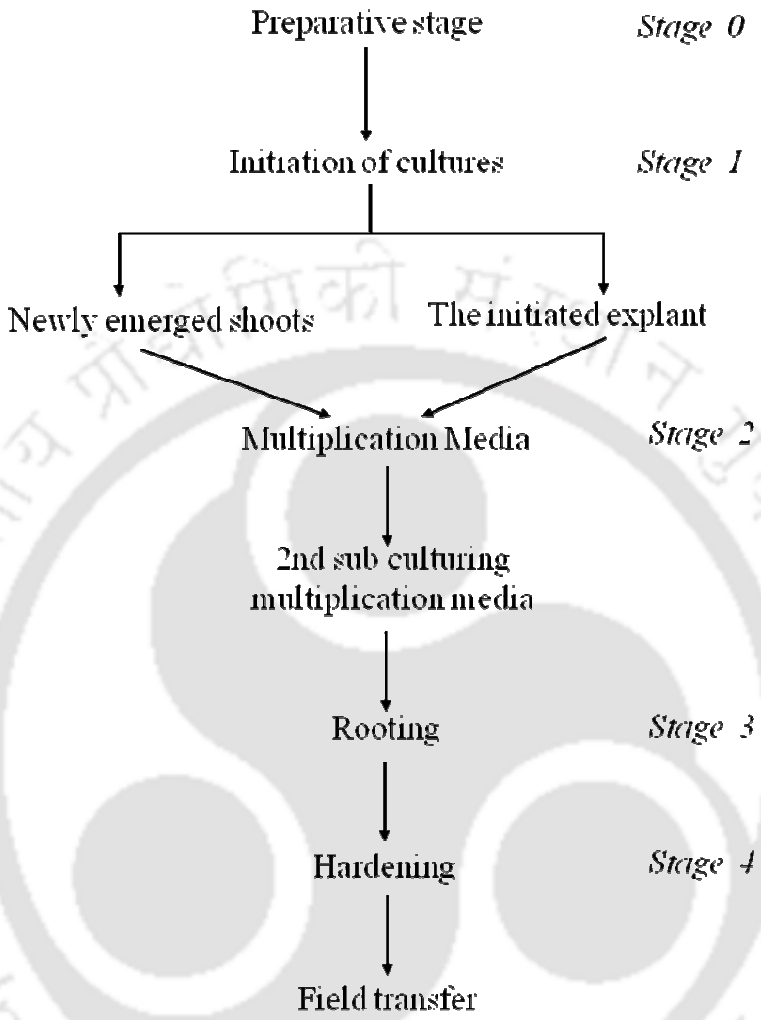


Figure 2.1: Flow scheme for the *in vitro* regeneration studies

2.3.3 Media and culture conditions

Basal MS medium and modified MSR medium supplemented with different combinations and concentrations of plant growth regulators (PGR) and varying sucrose concentrations was used in the study (Table 2.1 and 2.2). Standard procedures were followed for media preparation and maintenance (Vincent et al. 1992). Different concentrations of BA and Kn were tried individually and in combination with NAA and IBA for shoot multiplication and maintenance (MM). However, in case of *Zingiber* cytokinins were tried singly and with combinations to see the synergistic effect of the regulators for regeneration and multiplication. For rooting response, various concentrations of auxins NAA, IAA and IBA were tested with half strength of MM medium. To study the effect of gelling concentration on shoot multiplication, shoot tips were cultured on MM medium solidified with 0.6, 0.7 and 0.8% agar (Hi-media, UK). The effect of carbohydrate at varying concentrations on *in vitro* shoot multiplication was also carried out by supplementing MM medium with sucrose at 1, 2 and 3% respectively. Of several media combinations tried, those inducing the greatest response were recorded. Sub-culturing was carried out after 3 weeks of culture. After 8 weeks, the number of shoots per explant, shoots length, number of roots per shoot and root length were recorded.

2.3.4 Acclimatization of plantlet

Healthy and rooted shoot clumps were removed from culture tubes and washed thoroughly with tap water to remove the adhering medium and subsequently were planted in poly-bags containing sand and clay at the ratio of 1:4, and kept in the mist chamber. Plantlets were maintained in the greenhouse under a semi-shade and a high humid (RH-80%) conditions with 16-h day, 8-h night cycle at 28±2ºC for hardening. Intermittent mist was supplied for 30 seconds at 15 minutes intervals. The percentage survival was determined after two months. Later, the plantlets were isolated carefully and transferred to the garden for further establishment.

Table 2.1: Nutrient composition of the media used in current study

Nutrient Composition	MS (1962) (mg/L)	Nutrient composition	MSR (mg/L)
Major nutrients (20x)		**MS A (100x)**	
NH_4NO_3	1650	$CaCl_2.2H_2O$	440
$(NH_4)_2SO_4$	0	**MS B (10x)**	
KNO_3	1900	NH_4NO_3	1650
$Ca(NO_3)_2.4H_2O$	0	KNO_3	1900
$MgSO_4.7H_2O$	370	**MS C (100x)**	
$CaCl_2.2H_2O$	440	KI	83
KH_2PO_4	170	$CoCl_2.6H_2O$	2.5
K_2SO_4	0	**MS D (100x)**	
Iron Stock (200x)		KH_2PO_4	170
$FeSO_4.7H_2O$	27.8	H_3BO_3	620
$Na_2EDTA.2H_2O$	37.3	Na_2MoO_4	25
Minor nutrients (200x)		**MS E (100x)**	
$MnSO_4.4H_2O$	22.3	$MgSO_4.7H_2O$	3700
$ZnSO_4.7H_2O$	8.60	$MnSO_4.4H_2O$	223
H_3BO_3	6.3	$CuSO_4.5H_2O$	2.5
KI	0.83	$ZnSO_4.7H_2O$	860
Na_2MoO_4	0.25	**MS F (100x)**	
$CuSO_4.5H_2O$	0.025	$FeSO_4.7H_2O$	278.5
$CoCl_2.6H_2O$	0.025	$Na_2EDTA.2H_2O$	372.5
Vitamins (200x)		**Others**	
Inositol	100	Thiamine	0.001
Glycine	10.0	Myoinositol	100
Thiamine	1.0	Caesin hydrolysate	300
Nicotinic acid	1.0	Yeast extract	300
Pyridoxin HCl	1.0		

Table 2.2: PGR concentrations and combinations used for the genera *Curcuma* and *Zingiber* in the study

For shoot initiation			For multiplication and maintenance		For rooting		
PGR	**mg/L**	**µM/L**	**mg/L**	**µM/L**	**PGR**	**mg/L**	**µM/L**
BA	1.0	4.43			**IAA**	0.5	2.85
	2.0	8.86	2.0	8.86		1.0	5.7
	3.0	13.29	3.0	13.29	**IBA**	0.5	2.46
	4.0	17.72				1.0	4.92
	5.0	22.15			**NAA**	0.5	2.68
Kn	1.0	4.64	1.0	4.64		1.0	5.37
	2.0	9.38	2.0	9.38			
	3.0	13.92					
	4.0	18.56					
	5.0	23.20					
BA + Kn	1.0 + 1.0	4.43 + 4.64	1.0 + 1.0	4.43 + 4.64			
	2.0 + 1.0	8.86 + 4.64					
	3.0 + 1.0	13.29 + 4.64					
	1.0 + 2.0	4.43 + 9.38					
	2.0 + 2.0	8.86 + 9.38	2.0 + 2.0	8.86 + 9.38			
	3.0 + 2.0	13.29 + 9.38					
	1.0 + 3.0	4.43 + 13.92					
	2.0 + 3.0	8.86 + 13.92					
	3.0 + 3.0	13.29+13.92					
BA + NAA	3.0 + 0.5	13.29 + 2.68	3.0 + 0.5	13.29 + 2.68			
	3.0 + 1.0	13.29 + 5.37					
BA + IBA	3.0 + 0.5	13.29 + 2.46	3.0 + 0.5	13.29 + 2.46			
	3.0 + 1.0	13.29 + 4.92					
Kn + NAA	1.0 + 0.5	4.64 + 2.68					
	1.0 + 1.0	4.64 + 5.37					
Kn + IBA	1.0 + 0.5	4.64 + 2.46					
	1.0 + 1.0	4.64 + 4.92					

2.3.5 Statistical analysis

Three replicates (10 explants/ replicate) were inoculated per treatment to test the effects of medium consistency on multiplication rates. After three weeks of culture period, records were made on percentage of explant initiating shoot buds, mean number of multiple shoot buds and mean length of the longest shoot per explant. For rooting, percentage rooting, mean root number and mean root length per explant were measured after three weeks. All experiments were carried out independently and repeated thrice. The data were analyzed using one-way ANOVA (SPSS 16.0 version, 2008) and significant differences between treatment means were assessed using Duncan's multiple range test (DMRT) at a 5% probability level ($P<0.05$).

2.3.6 Testing cyto- genetic fidelity of regenerated plants

2.3.6.1 RAPD analysis in Curcuma

For RAPD experiments, 10 regenerated plants of *C. amada* and *C. domestica* collected from 60-day-old plantlets selected randomly and mother plant were analyzed. Total genomic DNA of the mother plants and the regenerated plants was extracted from fresh tender leaves using SDS protocol (Kesari et al. 2009; Das et al. 2010). One gram of leaf tissue was ground in liquid nitrogen and suspended in 10ml of extraction buffer (100 mM Tris, 0.5M NaCl, 50 mM EDTA) containing 1% β-marcaptoethanol. The suspension was incubated at 65 ^{o}C in water bath for 30 min, extracted with 5M potassium acetate and centrifuged at 2795 *g* at 4 ^{o}C for 30 min. The aqueous phase was precipitated with isopropanol and again centrifuged at 2795 g at 4 ^{o}C for 20 min. The pellet was dissolved in TE buffer (10mM Tris HCl, 1mM EDTA pH 8.0) and treated with RNAse. DNA was purified by ethanol precipitation. The quality and quantity of the extracted DNA was confirmed to be consistent both spectro-photometrically and by running the extracted DNA on 1.0% agarose gels containing 0.5 µg/ml of EtBr.

PCR amplification of the genomic DNA was carried out using 10 arbitrary decamer oligonucleotide primers (Operon Tech, USA). Each reaction mixture of 20µl contained 50ng/µl of template DNA, 1× assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin), 0.2 mM each dNTPs (Banglore Genei, India), 5 pmol of each primer and 0.5U of *Taq* polymerase (Banglore Genei, India). The

reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems, USA) programmed for 40 cycles. The first amplification cycle consisted of initial denaturation step of 5 min at 94 ^{o}C. This was followed by 40 cycles of 45 sec at 94 ^{o}C, annealing for 1 min at 32 ^{o}C, and extension at 72 ^{o}C for 2 min. An additional cycle of 10 min at 72 ^{o}C was used for primer extension. The amplification products were electrophoresed in 1.3% agarose gels in 1x TAE (50× stock contained 2M Tris, 0.5M EDTA and glacial acetic acid). The gels were visualized and photographed under UV light by a gel documentation system (Bio Rad, USA). The size of amplification products was estimated using λ DNA marker (Banglore Genei, India). PCR amplification was repeated twice and only primers producing reproducible bands were considered for analysis. Amplified DNA and polypeptide fragments were scored as present or absent both in the regenerated and in mother plant. Electrophoretic bands of low visual intensity that could not be readily distinguished as present or absent were considered as ambiguous markers and were not scored.

2.3.6.2 Protein profiling in Curcuma

Genetic fidelity was also tested by studying protein profiles of *Curcuma* regenerated plantlets and comparing those with the mother plants. Protein extraction was carried out for the same regenerated plants from which DNA was extracted. Young leaves were homogenized in 10 mM Tris-EDTA buffer (pH 8.0) containing 10% SDS, 5 mM β-mercaptoethanol and 0.1 mg cm-3 phenylmethanesulphonyl fluoride (PMSF). After centrifugation at 10,000 g for 10 min (4 ºC), the supernatant was boiled for 10 min. Protein content was measured by Bradford method (1976) using bovine serum albumin as a standard. Proteins were separated using discontinuous SDS-PAGE (10% running gel, pH 8.8 and 5% stacking gel, pH 6.8) at 4 ºC according to Laemmli (1970). After electrophoresis, gels were stained overnight with 0.25% Coomassie Brilliant Blue R250, destained, fixed and photographed. Molecular mass of polypeptides was determined according to the mobility of the standard proteins.

2.3.6.3 RAPD analysis in Zingiber

Two months old *in vitro* raised plants, 10 from each of *Z. moran* and *Z. zerumbet* were considered for RAPD analysis. The genomic DNA was isolated from tender leaves in a similar way described for *Curcuma*. The quality and quantity of the extracted DNA was checked both with spectrophotometric method and by running on 1.0% agarose gels pre-stained with 0.5 µg /ml of EtBr.

For PCR amplification of the *Zingiber* genomic DNA, 10 arbitrary decamer oligonucleotide primers were used from Operon Technologies, Almeda, USA. Reaction mixture and PCR compositions were same as those used for *Curcuma* species. PCR amplification was carried out in a mini thermal cycler (Applied Biosystems, Foster City, CA, USA) programmed for 35 cycles. The amplification cycles were as follows: first denaturation step of 5 min at 95 ºC, followed by 35 cycles of 30 s at 95 ºC, annealing for 1 min at 32-34 ºC, extension at 72 ºC for 2 min and a final extension cycle of 10 min at 72 ºC. The PCR products were electrophoresed in 1.3% agarose gels in 1× TAE (50× stock contained 2 M Tris, 0.5 M EDTA and glacial acetic acid). The gels were visualized and photographed under UV radiation by a gel documentation system (BioRad, Hercules, USA). The size of amplified products was estimated using λ DNA marker (Banglore Genei). To avoid any ambiguity, only the bands with higher intensity were considered.

2.3.6.4 Cytogenetic stability testing in Zingiber

Further, ploidy levels of the cloned plants in *Zingiber* were checked by chromosome counting after acclimatization in order to confirm the absence of cytogenetic instability. Root tips of the mother plant and 3 months old regenerated plants in both the species were fixed during somatic cell division in the morning hours from 9.00 am -10.30 am. Fixed root tips were treated with 1 N HCl for 5 min and then stained with Acetocarmine (2 %) for 2 h. Slides were prepared and mounted to count the number of chromosomes in metaphase stage for each species under study under high power (45× magnification). Regenerated plants were studied individually to see if there was any variation.

2.4 Results and Discussion

2.4.1 Culture initiation and multiplication

Establishment of the aseptic cultures in *Curcuma* and *Zingiber* species posed considerable difficulties due to high rate of contamination in primary cultures, which reappeared even after repeated sub-culturing. The problem was overcome by two fold surface sterilization of the explants with 0.1% Na-hypochlorite solution along with (2% m/v) Tween 20 for 5 min and mercuric chloride 0.1% (m/v) for 10 min before inoculation. In rhizomatous plants, contamination is a major problem during initiation and further successful establishment of aseptic cultures (Borthakur et al. 1999; Balachandran et al. 1990). It was observed that for Zingiberacean species, percentage response and number of explants showing contamination was highly dependent on the season during which the sampling was made. Explants collected during rainy season did not survive due to heavy fungal and bacterial contamination. Spring (April-June) season known as an actively growing season, was found to be the most favorable for initiation of culture, for almost 80% of culture developed adventitious buds and rate of contamination was also less. *In vitro* seasonal effect on bud growth has been reported in rhizomatous species such as *Z. zerumbet* Smith (Stanly and Keng 2007) and *C. orchioides* Gaertn (Wala and Jasrai 2003). The axillary buds of rhizome used as explants were available throughout the year for explantation for initiating tissue culture. In most of the earlier reports on micropropagation of *C. longa* direct shoot multiplication and plant regeneration were achieved using active sprouted shoot buds of rhizome, which were available during planting season (Salvi et al. 2002; Nayak and Nayak 2006). The axillary bud explants responded to induced multiple shoots in different degrees in two different media tried *viz.* MS and MSR media with varying concentrations and combinations of cytokinin and auxins as given in Tables 2.3-2.6. Subsequent subculturing on the optimal multiplication medium repeatedly for two or three cultures increased the multiplication rate in all the four species under study.

2.4.1.1 C. amada and C. longa

There was no organogenic response observed till 4 weeks of inoculation in the excised meristem of both the species of *Curcuma* cultured *in vitro* on MS basal medium devoid of growth regulators. The addition of BA (13.31 µM) and Kn (4.64µM) to the basal medium showed the first visible sign of growth with axillary buds sprouting (Figure 2.2A and B). *In vitro* raised explants responded to induce multiple shoots in different degrees in two different media tried viz., MS and MSR media with varying concentrations and combinations of cytokinins and auxins. Cytokinins were not effective for the sprouting of axillary buds; hence combinations of cytokinin and auxins were used. BA was far more effective than Kn for inducing proliferation of axillary buds. The greatest response for enhanced induction of rhizome buds in *C. amada* supplemented with (13.31µM) BA + (2.46 µM) IBA were recorded in MS (93.33%) and MSR medium (100%) respectively after 2 weeks (Table 2.3).

Rhizome buds of *C. amada* when cultured on MSR based medium containing BA (13.31µM) + IBA (2.46 µM) started to proliferate producing new shoots within 10 days where as in MS basal media with same hormone combination, buds started to proliferate only after 3 weeks of culturing. The highest mean number of buds per explants was also recorded in the same hormone combination which was 4.3 in MS and 8.9 in MSR medium (Figure 2.2C). This medium also showed the highest mean length of the longest shoot 5.5 cm (Table 2.3). The buds of *C. longa* showed a regeneration frequency of 80 and 100% in MS and MSR medium respectively. Response was 100% in MSR medium with BA 13.31µM + IBA 2.46 µM, but with a lesser average number of shoots per explant. The highest mean number of shoots per culture recorded was 8.2 in MSR medium (Figure 2.2D) supplemented with BA (13.31µM) + NAA (2.68 µM), showing greater response than in MS medium having same hormone concentration (4.2). The highest mean length of the longest shoot in MSR medium was also recorded (4.5 cm) in the same medium (Figure 2.2D). Similar results were reported for *C. longa* where the shoot multiplication was found maximum in MS media containing BA (3 mg/L) after 4 weeks of culture (Panda et al. 2007). Earlier, methods of effective culture initiation and proliferation have been standardized for *Curcuma* species by Tyagi et al. (2004). BA was found to be more effective growth hormone than Kn for multiple shoot regeneration

when used alone in both media tried but combination of BA with lower concentration of auxin (NAA or IBA) was found to be ideal for shoot multiplication.

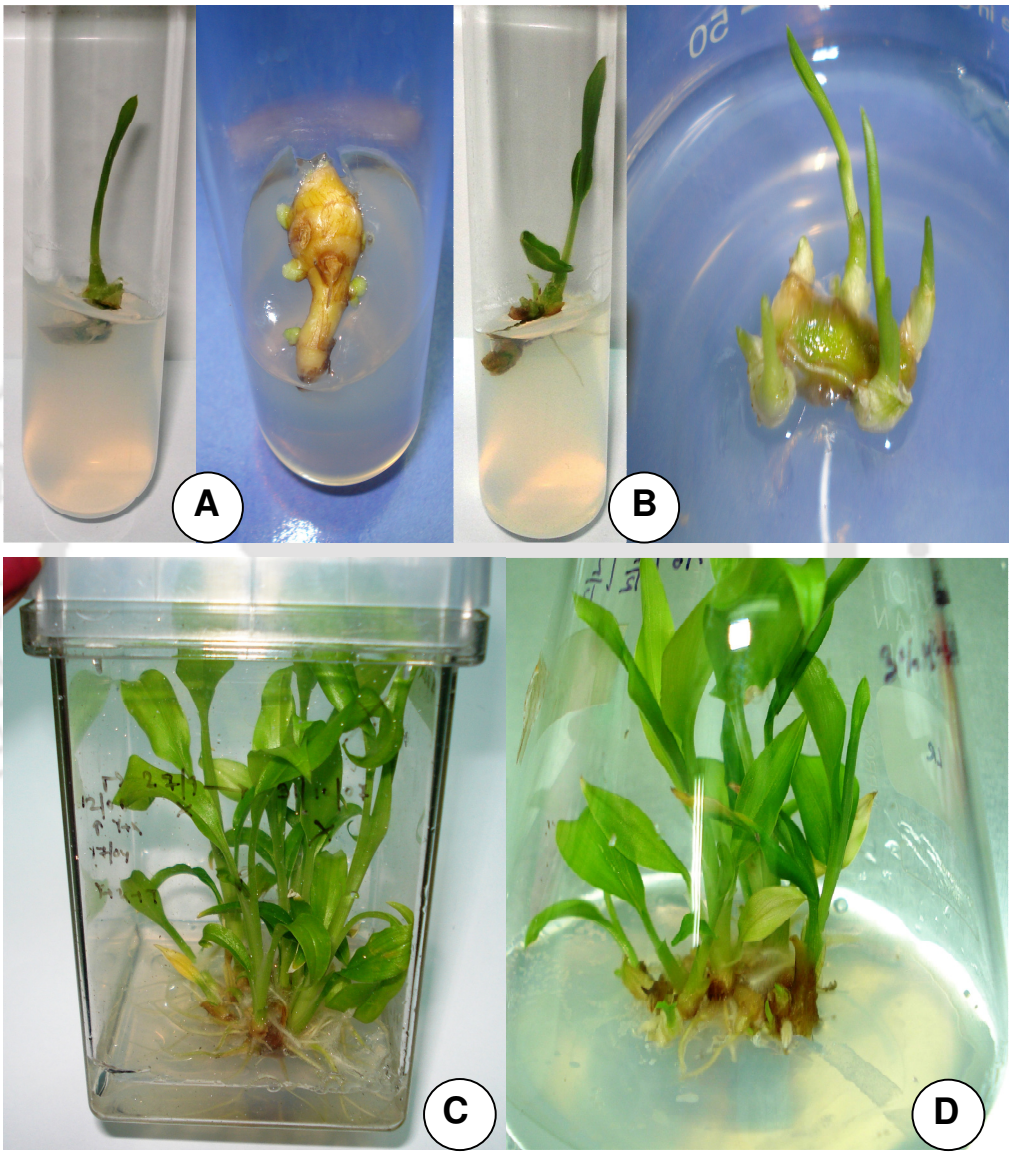


Figure 2.2: Sprouting and multiple bud formation of axillary buds after 4 weeks of culture in MSR medium supplemented with 13.31 µM BA + 2.46µM IBA
A- *C. amada*, **B**- *C. longa*
Multiple shoots produced in MSR medium supplemented with 13.31 BA + 2.46 IBA in *C. amada* (**C**), and with 13.31µM BA + 2.68 µM NAA in *C. longa* (**D**) after 10 weeks of culture

Presence of IBA in small amount (2.46 µM) supplemented with BA had significant positive effect on multiple shoot production in *C. amada* (Figure 2.2A). When the aseptic shoots of *C. amada* were cultured on MSR medium containing BA (13.31 µM) with 2.46 µM of IBA, an average of 8.9 shoots were produced from each explants and was chosen as the optimum shoot multiplication medium for the species (Table 2.3). The aseptic shoots of *C. longa* cultured on MSR medium supplemented with 13.31 µM BA and 2.68µM NAA produced 8.2 shoot per explants (Figure 2.2D). Thus the greatest response for enhanced multiplication was recorded on MSR basal medium supplemented with BA and NAA (Table 2.4). Balachandarn et al. (1990) also reported that higher concentration of BA was not suitable for *Curcuma* species. Subsequent sub-culturing on the optimal multiplication medium repeatedly for two or three cultures did not increase the multiplication rate.

The aseptic shoots of *C. amada* and *C. longa* cultured on MS medium supplemented with different concentration of BA in combination with and NAA and IBA produced shoots and roots and were also reported in other Zingiberaceae species (Chan and Thong 2004; Bharalee et al. 2005). Loc et al. (2005) reported that MS medium supplemented with 20% (v/v) coconut water, 3 mg/L BA and 0.5 mg/L IBA could induce the formation of 6 shoots per explants for *C. zedoaria*. Bharalee et al. (2005) found that MS medium supplemented with 4 mg/L BAP and 1.5 mg/L NAA was the best medium for shoot multiplication of *C. caesia* (3.5 shoots per explants) and MS with 1 mg/L BAP+0.5 mg/L NAA for *C. zedoaria* (4.5 shoots per explants). Balachandaran et al. (1990) reported that *C. domestica* could produce 3.4 shoots per explants, *C. caesia* produced 2.8 shoots per explants using MS medium supplemented with 3mg/L BAP. Nayak (2000) reported MS medium supplemented with 5 mg/L BAP was most effective for shoot multiplication of *C. aromatica* producing an average of 3.3 shoots per explants. The results indicated that BA (13.39µM) and NAA as low as (2.68µM) supplemented in the MSR medium was sufficient for the induction of multiple shoots from the buds and shoots of *C. amada* and *C. longa.* Even, the dormant axillary bud of rhizome became active and sprouted on this medium with subsequent production of multiple shoots.

Table 2.3: Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma amada* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Conc. [µM]	MS medium response [%]	Shoots /explant	shoot length [cm]	MSR medium response [%]	shoots /explant	shoot length [cm]
BA	4.43	0	-	-	30.0 ± 10.0	1.80 ± 0.11b	1.47 ± 0.05a
	13.31	43.3 ± 6.67	1.33 ± 0.13a	2.70 ± 0.85c	56.6 ± 6.67	2.40 ± 0.15**?**	2.68 ± 0.07c
	22.19	16.6 ± 3.33	1.00 ± 0.00a	2.10 ± 0.17b	63.3 ± 3.33	1.50 ± 0.11ab	2.01 ± 0.06b
Kn	2.32	0	-	-	53.3 ± 3.33	1.50 ± 0.12ab	1.66 ± 0.08e
	4.64	46.6 ± 6.67	1.50 ± 0.13abc	2.60 ± 0.08c	70.0 ± 0.00	2.00 ± 0.16abc	2.90 ± 0.06d
	9.29	56.6 ± 3.33	1.16 ± 0.34cd	1.60 ± 0.08a	56.6 ± 6.67	1.30 ± 0.11a	2.60 ± 0.12b
BA + NAA	13.31 + 2.68	73.3 ± 6.67	3.40 ± 0.19f	4.00 ± 0.10e	93.3 ± 3.33	7.60 ± 0.25g	4.96 ± 0.07f
	13.31 + 5.37	60.0 ± 0	2.40 ± 0.14de	3.10 ± 0.09d	56.6 ± 6.67	2.10 ± 0.12bc	3.23 ± 0.08e
Kn + NAA	4.64 + 2.68	53.3 ± 3.33	2.30 ± 0.13de	2.50 ± 0.09c	90.0 ± 5.78	3.20 0.15e	3.18 ± 0.07**?**
	4.64 + 5.37	50.0 ± 0	1.90 ± 0.18bcd	1.70 ± 0.08a	66.6 ± 3.33	2.40 ± 0.13cd	2.03 ± 0.11b
BA + IBA	13.31 + 2.46	93.3 ± 6.67	4.30 ± 0.29g	4.90 ± 0.09 f	100.0 ± 0.00	8.96 ± 0.43h	5.56 ± 0.10g
	13.31 + 4.92	53.3 ± 3.33	2.30 ± 0.12de	2.64 ± 0.81c	83.3 ± 8.82	3.90 ± 0.16f	2.50 ± 0.05c
Kn + IBA	4.64 + 2.46	90.0 ± 10.0	2.70 ± 0.18ef	2.60 ± 0.07c	93.3 ± 6.67	2.70 ± 0.13cde	2.13 ± 0.07b
	4.64 + 4.92	63.3 ± 3.33	2.40 ± 0.14de	2.03 ± 0.07b	66.6 ± 3.33	2.90 ± 0.22de	2.11 ± 0.08b

Table 2.4: Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma longa* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Conc. [µM]	MS medium response [%]	shoots /explant	shoot length [cm]	MSR medium response [%]	shoots /explant	shoot length [cm]
BA	4.43	NR	--	--	56.67 ± 3.33	1.10 ± 0.09a	1.57 ± 0.04b
	13.31	46.67 ± 8.82	1.20 ± 0.20a	1.37 ± 0.12abc	90.00 ± 5.77	2.71 ± 0.11e	2.02 ± 0.11cd
	22.19	40.00 ± 5.77	1.57 ± 0.03abc	1.62 ± 0.02bcd	66.67 ± 3.33	2.00 ± 0.15cd	1.57 ± 0.18c
Kn	2.32	13.33 ± 3.33	1.00 ± 0.00a	1.08 ± 0.06a	56.67 ± 6.67	1.29 ± 0.06a,b	1.49 ± 0.20a
	4.64	46.67± 3.33	1.85 ± 0.08bc	1.89 ± 0.05de	86.67 ± 8.82	2.63 ± 0.26e	2.22 ± 0.06de
	9.29	50.00 ± 5.77	1.08 ± 0.08a	1.27 ± 0.07ab	60.00 ± 5.77	1.78 ± 0.04bc	1.82 ± 0.24bc
BA + NAA	13.31 + 2.68	80.00 ± 10.0	4.27 ± 0.30f	4.25 ± 0.07h	100.00 ± 0.00	8.17 ± 0.17h	4.54 ± 0.13i
	13.31 + 5.37	60.00 ± 5.77	1.71 ± 0.27bc	1.68 ± 0.11c,d	60.00 ± 5.77	2.57 ± 0.14e	2.91 ± 0.08g
Kn + NAA	4.64 + 2.68	70.00 ± 5.77	2.25 ± 0.39cd	2.24 ± 0.02ef	66.67 ± 3.33	3.17 ± 0.12f	3.10 ± 0.05g
	4.64 + 5.37	56.67 ± 3.33	1.37 ± 0.19ab	1.37 ± 0.01abc	63.33 ± 3.33	1.74 ± 0.30bc	2.53 ± 0.01f
BA + IBA	13.31 + 2.46	73.33 ± 3.33	3.61 ± 0.22e	3.58 ± 0.12g	100.00 ± 0.00	7.03 ± 0.15g	3.96 ± 0.10h
	13.31 + 4.92	53.33 ± 3.33	1.87 ± 0.13bc	1.93 ± 0.04de	56.67 ± 3.33	2.42 ± 0.21de	2.42 ± 0.04ef
Kn + IBA	4.64 + 2.46	56.67 ± 3.33	2.49 ± 0.16d	2.50 ± 0.07f	80.00 ± 0.00	3.33 ± 0.11f	3.72 ± 0.06h
	4.64 + 4.92	53.33 ± 3.33	1.81 ± 0.12bc	1.82 ± 0.04d	60.00 ± 5.77	1.58 ± 0.21bc	1.76 ± 0.13bc

2.4.1.2 Z. moran and Z. zerumbet

Four to six weeks after inoculation, new shoots were found to be emerged from the rhizome buds of both *Z. moran* and *Z. zerumbet* explants (Figure 2.3A, B). Various combinations of plant growth regulators were used with MS and MSR media for culture initiation. The explants on different combinations and concentrations of PGRs showed a large variability in culture response. *Z. zerumbet* showed response to a total of 14 PGR combinations tried, where as *Z. moran* responded to all the 15 combinations used in the study. However, MS medium with BA (4.43 µM) showed poor response in *Z. zerumbet* and no response at all in case of *Z. moran.* The PGR combination of BA (13.29 µM) + Kn (13.92 µM) showed no response at all in *Z. zerumbet*. The greatest response for enhanced induction of rhizome buds in *Z. zerumbet* supplemented with (8.86µM) BA + (9.38 µM) Kn were recorded in MS (96.66%) and MSR medium (100%) respectively after 2 weeks; where as that of *Z. moran* found to be 83.33% (MS) and 100% (MSR) with the same combinations of PGR used (Table 2.5). Methods of culture initiation and multiple shoot regeneration are well established in *Z. officinale* (Balachandran et al. 1990, Tyagi et al. 1998, Khatun et al. 2003, Sultana et al. 2009).

The MSR medium containing BA (8.86 µM) + Kn (9.38 µM) proved to be best medium for shoot proliferation in case of both the *Zingiber* species studied (Figure 2.3A, B). Rhizome buds started to proliferate soon after 10 days of inoculation in MSR medium compared to the same combination tried in MS medium which responded after about 2 weeks. Though shoot multiplication was observed in single treatment of cytokinins, the rate of multiplication was poor. Among them BA (8.86 µM) offered better provision to develop an average of 3.7 and 4 shoots per explant in *Z. moran* and *Z. zerumbet* respectively (Table 2.5, 2.6). The presence of BA along with Kn in the medium markedly increased the number of shoots produced per explant. The highest shoot induction was found in MSR medium supplemented with 8.86 µM BA + 9.38 µM Kn, which produced highest number of 9 shoots in *Z. zerumbet* and 12 shoots in *Z. moran* explants (Figure 2.3C, D). Higher concentration of BA was found to be inhibitory in shoot multiplication irrespective of the Kn concentrations used. Although explants showed a fair response to individual cytokinins used, the combinations of two regular cytokinins (BA and Kn) was found to be an ideal medium for shoot multiplication in *Zingiber* (Tables 2.5, 2.6).

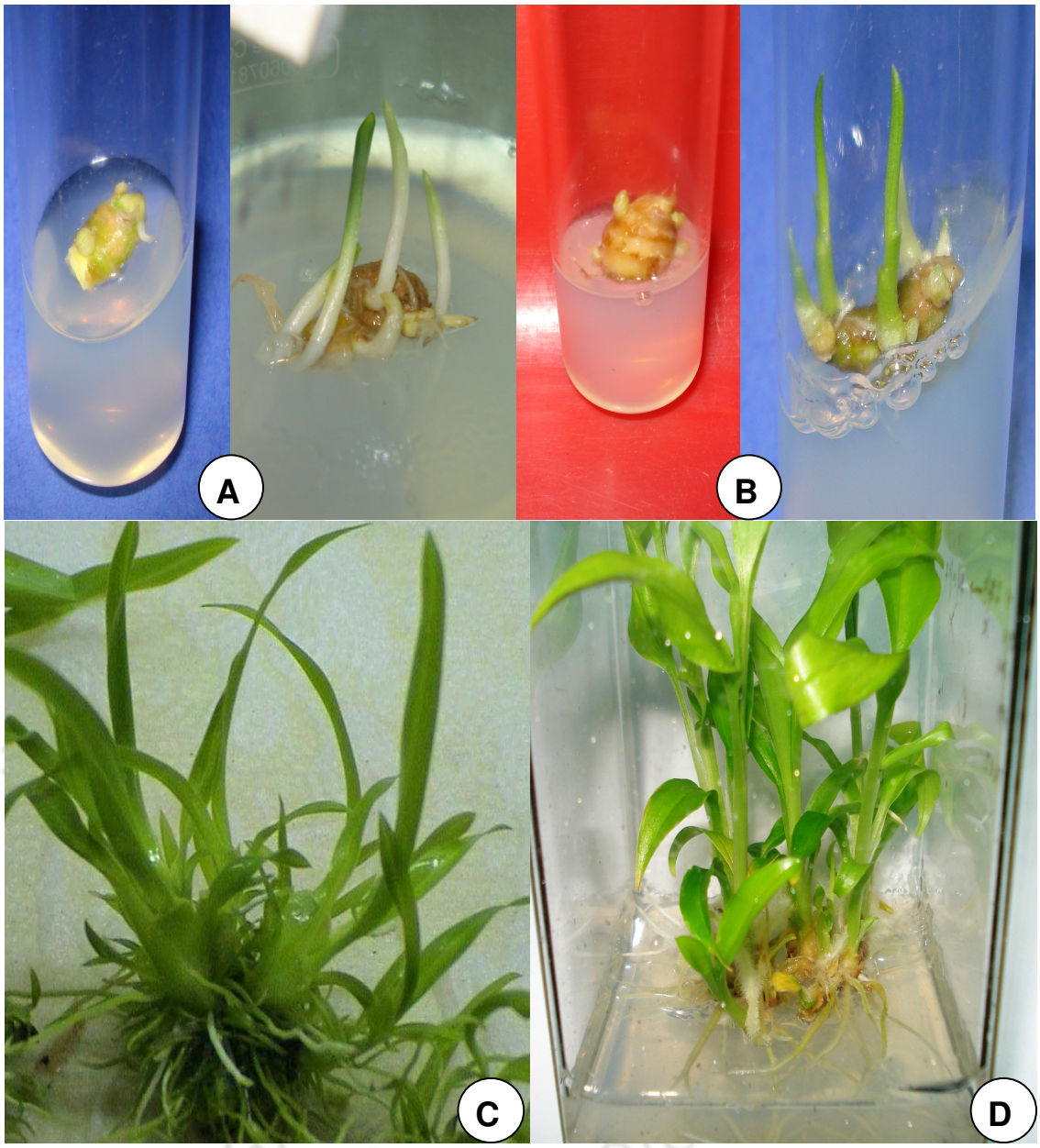


Figure 2.3: Sprouting and multiple bud formation of axillary buds after 4 weeks of culture in MSR medium supplemented with 8.86 µM BA + 9.38 µM Kn
A- *Z. moran*, **B**- *Z. zerumbet*
Multiple shoots produced in MSR medium supplemented with 8.86 µM BA + 9.38 µM Kn after 10 weeks of culture; **C**- *Z. moran*, **D**-*Z. zerumbet*

Table 2.5: Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Z. moran* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth Regulators	Conc. (µM)	MS medium response (%)	Mean number of shoots / explant	Mean length of the longest shoot (cm)	MSR medium response (%)	Mean number of shoots / explant	Mean Length of the longest shoot (cm)
BA	4.43	NR	--	NR	36.66±0.57	2.55±0.05ef	1.62±0.06a
BA	8.86	46.66±0.57	3.69±0.07a	2.50±0.04ab	73.33±0.57	3.70±0.09ac	2.70±0.03bd
BA	13.29	33.33±0.57	1.80±0.04ab	2.00±0.05ab	56.66±0.57	1.84±0.06bcd	2.02±0.02f
Kn	4.64	NR	--	--	56.66±0.57	1.81±0.06af	1.70±0.02ae
Kn	9.38	46.60±0.57	2.21±0.06bc	2.6±0.03ce	76.66±1.15	2.80±0.07ab	2.94±0.03c
Kn	13.92	36.60±1.15	2.17±0.4c	1.84±0.03ae	60.0±1.0	1.52±0.05cd	2.30±0.03d
BA + Kn	4.43+4.64	63.33±0.57	4.77±0.09ab	4.22±0.04cd	86.66±0.57	7.42±0.13cf	4.92±0.06fg
BA + Kn	8.86+4.64	53.33±0.57	2.44±0.06cd	2.97±0.04ab	60.0±1.0	3.00±0.08ab	3.30±0.03g
BA + Kn	13.29+4.64	50.0±1.0	2.56±0.05ab	2.53±0.03ae	63.33±0.57	3.18±0.07c	3.12±0.04bd
BA + Kn	4.43+9.38	56.66±0.57	3.00±0.07a	1.90±0.02af	60.0±1.0	2.45±0.06f	2.10±0.05d
BA + Kn	8.86+9.38	83.33±0.57	6.17±0.12cd	4.98±0.05de	100±0	9.16±0.17de	5.81±0.06bc
BA + Kn	13.29+9.38	53.30±0.57	3.06±0.08ce	2.43±0.03ef	56.66±0.57	3.8±0.07d	2.50±0.02ae
BA + Kn	4.43+13.92	56.66±0.57	3.00±0.09	1.93±0.03be	76.66±0.57	2.71±0.06fg	2.19±0.03f
BA + Kn	8.86+13.92	53.33±0.57	2.42±0.06	1.92±0.03eg	60.0±1.0	2.55±0.06f	2.01±0.05g
BA + Kn	13.29+13.92	NR	--	--	33.33±0.57	1.2±0.05e	1.50±0.05g

Similar results were found by Anish et al. (2008) in *Bosenbergia pulcherrima*, a threatened ginger.

The highest mean number of buds per explants was also recorded in the same hormone combination which was 6.2 in MS and 9.2 in MSR medium for *Z. moran* (Table 2.5). MSR medium along with BA and Kn with concentrations (8.86 µM + 9.38 µM) showed the highest regeneration frequency followed by BA (4.43 µM) + Kn (4.64 µM) in both the *Zingiber* species (Tables 2.5, 2.6). However, in *Z. zerumbet,* highest average number of shoots per explant (7.23) was observed in MSR media supplemented with BA (8.86 µM) and Kn (9.38 µM) which was followed by 6.8 with the hormone combination BA 4.43 µM + Kn 13.92 µM in the same media (Table 2.6). This was much higher than that obtained with the same growth regulators used with MS medium. Beneficial effect of Kn when treated along with BA attributed to the improvement of multiplication in the present study. The application of BA at 8.86 µM seems to be suitable for optimum multiplication as concentrations above and below this level reduced shoot production. The highest mean number of shoots per culture for *Z. moran* was recorded 9.2 in MSR medium supplemented with BA (8.86µM) + Kn (9.38 µM), showing greater response than in MS medium having same hormone concentration (6.2). The highest mean length of the longest shoot in MSR medium was also recorded (5.8 cm) in the same medium (Table 2.5). On the other hand, the highest mean length of the longest shoot in *Z. zerumbet* was obtained as 4.4 cm with MSR medium supplemented with BA 8.86 µM + Kn 4.64 µM which was however similar to that found in MS medium associated with the same growth regulators (Table 2.6).

Moreover, BA (8.86µM) in combination with Kn (9.38 µM) was found to be most effective in shoot induction and multiplication in the two *Zingiber* species studied. The combination yielded 9 and 12 shoots per explant in *Z. zerumbet* and *Z. moran* respectively which is much higher than the earlier report of 4.7 shoots per explant for *Z. zerumbet* by Hsu et al. (1991) who used 4 mg/L BA in MS basal medium.

Table 2.6: Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Z. zerumbet* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth Regulators	Conc. ((μM)	MS medium response (%)	Mean no of shoots / explant	Mean shoot length (cm)	MSR medium response (%)	Mean no of shoots / explant	Mean shoot length (cm)
BA	4.43	3.33±0.57	3.00±0.06a	1.39±0.03ab	26.66±0.57	2.37±0.05bc	1.58±0.03d
BA	8.86	46.66±1.52	4.00±0.08ac	1.81±0.02b	70.00±1.0	3.42±0.06e	1.90±0.03ab
BA	13.29	40.00±1.0	2.75±0.06abc	1.45±0.03b	63.33±0.57	2.42±0.08ac	1.94±0.04a
Kn	4.64	13.30±0.63	2.50±0.05bc	1.75±0.03cd	43.33±0.57	2.30±0.06ab	1.44±0.03abc
Kn	9.38	40.00±1.0	2.66±0.07c	1.8±0.05de	86.66±0.57	2.50±0.05a	1.41±0.05def
Kn	13.92	40.00±1.0	1.58±0.05b	1.75±0.04d	43.33±0.57	2.58±0.06b	2.21±0.04d
BA+Kn	4.43+4.64	86.66±1.52	4.50±0.10ab	1.88±0.09a	76.66±0.57	4.17±0.12f	1.86±0.05de
BA+Kn	8.86+4.64	60.00±1.0	3.38±0.06c	4.40±0.07f	60.00±1.0	3.27±0.10f	4.36±0.10cd
BA+Kn	13.29+4.64	56.66±0.57	3.05±0.07ad	1.92±0.08ae	66.66±0.57	3.60±0.09ef	3.98±0.09ab
BA+Kn	4.43+9.38	63.33±0.57	3.26±0.08cd	2.18±0.04ef	53.33±0.57	3.31±0.10ae	2.93±0.03bc
BA+Kn	8.86+9.38	96.66±0.57	5.65±0.08cbd	3.60±0.10e	100.00±0.0	7.23±0.14bc	2.88±0.04b
BA+Kn	13.29+9.38	70.00±2.64	3.47±0.10cb	3.09±0.09c	6.00±1.0	5.50±0.25cd	3.43±0.07d
BA+Kn	4.43+13.92	56.66±0.57	2.70±0.08c	2.15±0.04d	63.33±1.52	6.78±0.15d	3.91±0.05e
BA+Kn	8.86+13.92	43.33±0.57	2.53±0.07ab	2.1±0.03bc	46.66±0.57	2.42±0.06d	2.36±0.04f
BA+Kn	13.29+13.92	NR	--	--	NR	--	--

2.4.2 Effect of agar and carbon source

2.4.2.1 C. amada and C. longa

When different concentrations of agar were tested, 0.7% was found to be better than the others for shoot multiplication, while 0.6% agar produced the highest shoot length (Table 2.7). Shoots produced on medium with 0.8% agar were long, slender, elongated with some shoots showing signs of drying and browning. Carbon source in culture medium had a significant effect on the growth of *Curcuma* species. MSR medium containing maltose gave best result as far as shoot induction and multiplication was concerned in both the *Curcuma* species studied, compared to MS medium containing sucrose as the carbon source. A significant increase in shoot length and number was observed when maltose was added to the medium suggesting that non reducing sugars such as maltose could be best carbon source for *in vitro* multiplication of *Curcuma* species. Maltose has been reported to be a superior source of carbohydrate than sucrose in several species, including cereals (Last and Brettel 1990, Pande and Bhojwani 1999). Sugars (sucrose or maltose) at 2% were found to be most effective carbon source for shoot multiplication. Further the concentration of sugar in the culture medium had a positive significant effect on the growth of *Curcuma* species. A significant increase in shoot length, number and length of roots were observed when the concentration of sugar was 2% suggesting that low concentration of sugar could be best for *in vitro* multiplication of *Curcuma* species (Figure 2.4). At concentration above than 3% *Curcuma* plantlets were etiolated and died. Similar effect was noticed by Yildiz et al. (2007) in case of sugar beet where sugar concentration more than 1% resulted in necrosis of the plantlets. Sucrose as a carbon source supports growth of plant cells in culture. A sucrose concentration of 1-5% is generally used for *in vitro* tissue culture, since it is also synthesized naturally by the tissue (Pierik, 1987). The photosynthetic activity of the plantlets grown *in vitro* depends on the amount of carbon in the medium. Langford and Wainwright (1987) reported that the highest net photosynthetic rate was obtained in rose plantlets grown in media containing less than 3% sucrose. In rhizomatous plants like Zingiberaceae, the surplus of sucrose is usually stored in the rhizomes. As there were no roots in our *in vitro* cultures, it

is possible that synthesized sucrose accumulated in the leaves of the plantlets as well as in the culture

Table2.7: Effect of agar and sugar concentration on response and multiple bud formation as observed in MSR media supplemented with BA (13.31µM) + IBA (2.46µM) for *C. amada*; with BA (13.31µM) + NAA (2.68µM) for *C. longa*; with BA (8.86µM) + Kn (9.39µM) for *Z. moran* and *Z. zerumbet.*

		Percentage of response (%)				**No of multiple buds per explant**			
Agar g/L	**Sugar g/L**	***C. amada***	***C. longa***	***Z. moran***	***Z. zerumbet***	***C. amada***	***C. longa***	***Z. moran***	***Z. zerumbet***
	1.0	35	33	38	41	3.8	4.0	3.5	4.0
0.6	2.0	45	47	52	48	3.2	4.5	3.8	4.1
	3.0	41	42	40	44	3.1	4.4	3.1	3.3
	1.0	46	51	50	40	3.8	4.3	3.5	3.9
0.7	2.0	100	100	100	100	8.9	8.2	9.2	7.23
	3.0	44	50	54	52	5.0	4.8	4.5	4.3
	1.0	36	30	30	34	3.8	4.5	3.8	4.1
0.8	2.0	45	47	52	46	4.5	4.0	5.0	4.7
	3.0	45	42	50	47	4.2	3.6	3.7	3.2

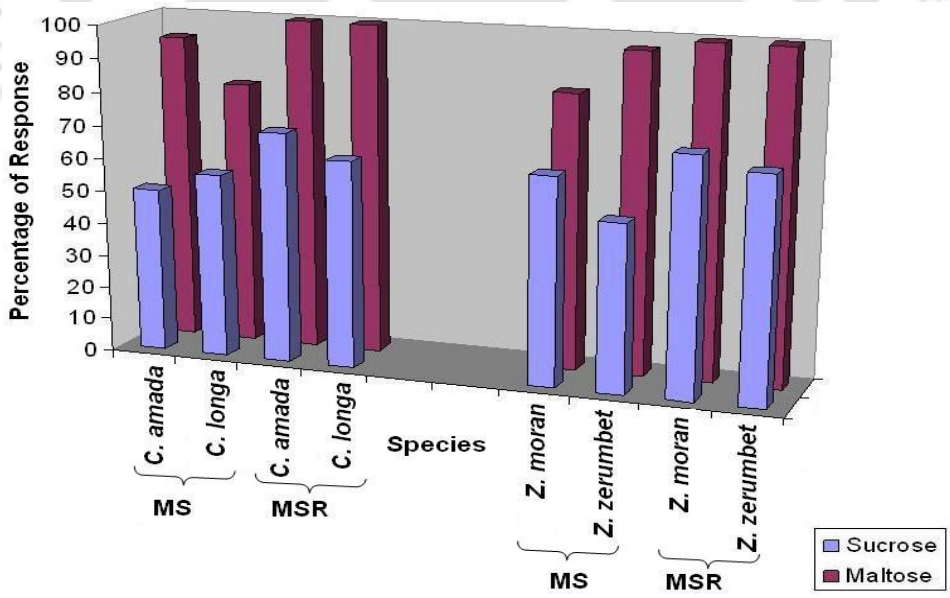


Figure 2.4: Effect of C-source on response percentage of the four species studied with two different media MS and MSR

media. Thus increasing the overall sucrose concentration leading to cell death. Similar observations were recorded in ginger species at higher concentration range of sucrose (Barthakur and Bordoloi 1992). However, a very high sucrose concentration at a later stage of culture has been reported to be useful in the formation of microrhizomes in many gingers (Sharma and Singh 1995; Shirgurkar et al. 2001; Nayak 2000; Rout et al. 2001; Islam et al. 2004; Tyagi et al. 2006; Zheng et al. 2008).

2.4.2.2 Z. moran and Z. zerumbet

In case of *Zingiber* species, the agar at a concentration of 0.7% was found to be optimum for both regeneration and multiplication. Sucrose concentration at 2% showed greater number of multiple shoots when used with MSR medium compared to that used at same concentration of sucrose with MS medium. However 3% sucrose with MS medium has been used invariably by many workers for members of Zingiberaceae with better results (Tyagi 2004; Stanly and Keng 2007). Maltose did not serve as a good carbon source for the genus *Zingiber*. A significant increase in shoot length and number was observed when sucrose was added to the medium at a lower percentage (2%). Sucrose at concentration 2% was found to be most suitable for shoot multiplication for both *Z. moran* and *Z. zerumbet* species (Table 2.7; Figure 2.4). Concentrations higher than 3% caused a defoliating effect on the *Zingiber* plantlets. However higher concentration of sugar source has been found to be ideal for *in vitro* micro rhizome production in *Z. officinale* (Zheng et al. 2008). Sucrose in culture medium functions both as a carbon source and osmotic regulator. Both the functions are critical for regeneration and callus formation (Last and Brettell 1990). Sucrose is rapidly hydrolyzed to glucose and fructose, nearly doubling the osmolality of the medium. In many plants it has been reported that although glucose seems to help initiate or promote the early stage of development in tissue culture but the excess of sugar could be detrimental to both regeneration and embryoid production (Hakim et al. 1991; Navarro-Alvareg et al. 1994). This can be explained as the higher sugar concentration is beneficial for rhizome formation and callus induction but not for regeneration and proliferation for *in vitro* cultures.

2.4.3 Rooting response and acclimatization

Multiplied shoots showed simultaneous formation of roots in the multiplication medium itself which contained both BA and NAA or IBA. However, the number of roots produced in this medium was limited, and the proliferation was also slow. When the multiplied shoots were put on optimal rooting media, the rooting and proliferation response was greater. Shoots started to root after 15 days of culture in the rooting medium, but an additional two weeks of growth were necessary for sufficient development to acclimatize plants in the greenhouse. The type and concentration of auxins influenced the average number of roots produced per explant, as well as the mean length of the roots in all the four species studied

2.4.3.1 C. amada and C. longa

Rooting was observed in a combination of BA with IBA and NAA in *C. amada* and *C. longa*. Root initiation in presence of both cytokinins and auxins observed in present study indicates the inherent root inducing tendency of explants of rhizome origin rather than the influence of hormones applied exogenously. Such response has been reported earlier in *C. haritha* (Bejoy et al. 2006). Well developed shoots (5-6 cm) when excised and transferred further to ½ strength MS medium supplemented with NAA (2.68 µM) 83.3% of the shoots produced well developed roots (Table 2.8, Figure 2.5). All regenerated plantlets with well developed roots were transferred to sterilized soil in plastic cups, covered with polythene bags to prevent desiccation and later transferred to the green house.

Plantlets developed *in vitro* could be hardened in the green house for two months suggesting the suitability of the *in vitro* protocol to restore the plantlets in heterotrophic condition (Figure 2.5). Hardened plants of *C. amada* and *C. longa* recorded about 80% and 100% survivability and were reared to maturity. The results coincides with the findings of Panda et al. (2007) who obtained about ninety percent of successful survival of the regenerated *C. longa* plantlets after being transferred to the field.

Table 2.8: Effect of auxins on *in vitro* rooting in shoots of *C. amada* and *C. longa* cultured in half-strength MS medium (after 10weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Conc. (µM)	*C. amada* Response (%)	Root number /shoot	Root length (cm)	*C. longa* Response (%)	Root number /shoot	Root length (cm)
NAA	2.68	76.6 ± 3.33	5.2 ± 0.18e	4.4 ± 0.12d	83.3 ± 3.33	7.1 ± 0.24d	5.2 ± 0.12e
	5.37	56.6 ± 6.67	1.6 ± 0.12ab	1.7 ± 0.11a	66.6 ± 6.67	3.0 ± 0.13b	3.3 ± 0.09d
IAA	2.85	60.0 ± 5.78	3.8 ± 0.15d	3.1 ± 0.09c	70.0 ± 5.78	3.8 ± 0.20c	2.5 ± 0.10b
	5.70	50.0 ± 5.78	1.2 ± 0.12a	2.1 ± 0.06b	46.6 ± 3.33	2.0 ± 0.15a	1.8 ± 0.12a
IBA	2.46	73.3 ± 3.33	3.0 ± 0.15c	3.0 ± 0.10c	56.6 ± 6.67	3.5 ± 0.15bc	3.0 ± 0.14c
	4.92	56.6 ± 8.29	1.8 ± 0.13b	2.2 ± 0.08b	43.3 ± 3.33	2.1 ± 0.14a	1.5 ± 0.10a

Table 2.9: Effect of auxins on *in vitro* rooting in shoots of *Z. moran* and *Z. zerumbet* cultured in half-strength MS medium (after 10 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Conc (µM)	*Z. moran* Response (%)	Root number/ shoot	Root length (cm)	*Z. zerumbet* response (%)	Root number/ shoot	Root length (cm)
NAA	2.68	90.0±1.0	5.21±0.08a	5.41±0.09ab	86.6±0.57	7.12±0.12ab	5.51±0.70ab
	5.37	56.6±1.15	1.88±0.06ab	2.05±0.05b	60.0±0.0	3.05±0.06b	3.55±0.08a
IAA	2.85	66.6±0.57	3.77±0.06b	3.45±0.05c	66.6±0.57	4.14±0.12c	3.8±0.11a
	5.70	50.0±1.0	1.73±0.07c	2.11±0.02a	43.3±0.57	2.0±0.05c	2.02±0.04c
IBA	2.46	73.3±0.57	3.59±0.07d	3.25±0.05c	56.6±1.15	3.94±0.08a	3.13±0.05d
	4.92	53.3±1.15	1.82±0.05a	2.11±0.02a	36.6±0.57	2.07±0.04d	1.9±0.04d

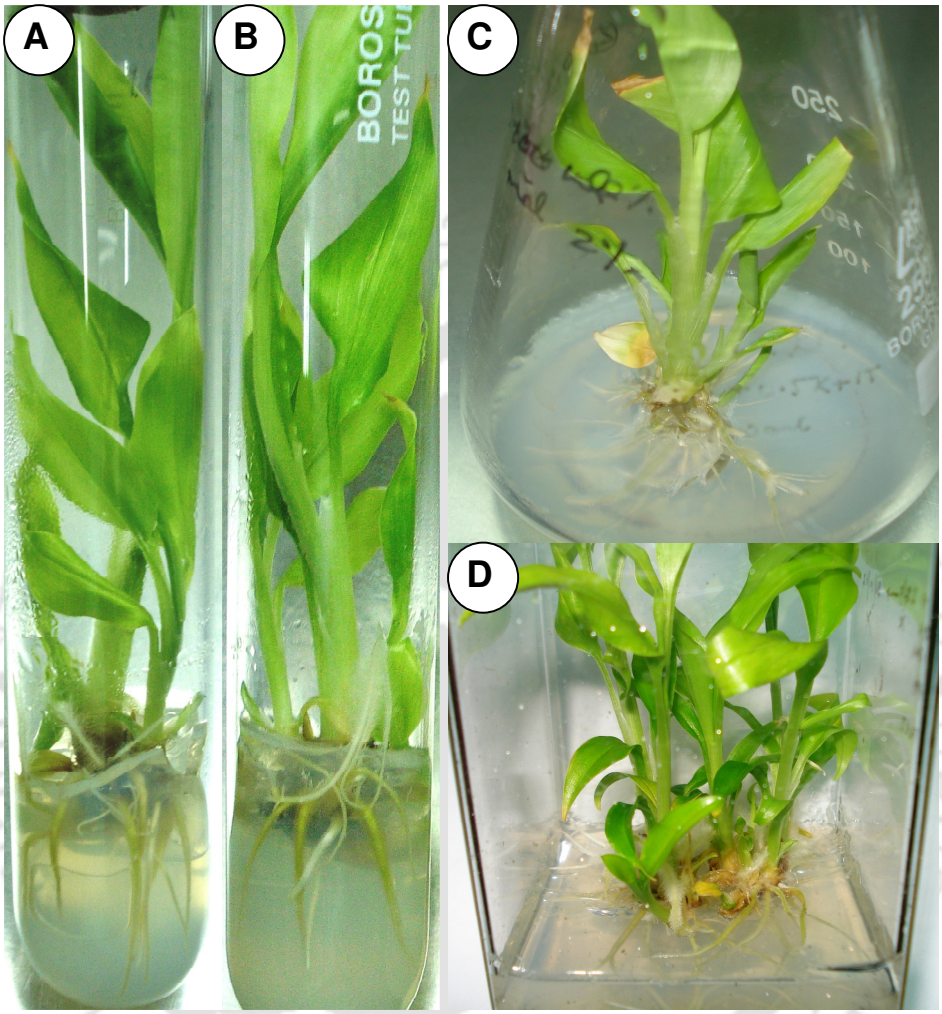


Figure 2.5: Rooting response shown by explants in the half-strength of MS medium supplemented with NAA at 2.68 µM after 8 weeks of culture;
A- *C. amada*; **B**- *C. longa*, **C**- *Z. zerumbet*, **D**- *Z. moran*

2.4.3.2 Z. moran and Z. zerumbet

The regenerated plants were subcultured in rooting media after six-eight weeks of culture. Half strength of both MS and MSR media along with three different auxins were studied for *in vitro* rooting response in *Z. zerumbet* and *Z. moran*. All the three auxins IAA (2.85-5.7 µM), IBA (2.46-4.92 µM) and NAA (2.68-5.37 µM) revealed higher rooting ability at lower concentrations. However, the higher concentrations used also produced roots at a low rate and lengths of the roots were also shorter. NAA at 2.68 µM showed the highest, above 90% and 86.6% rooting in *Z. moran* and *Z. zerumbet* respectively (Table 2.9). This was followed by IBA (2.46 µM) for *Z. moran* (73.3%) and IAA (2.85 µM) for *Z. zerumbet* (66.6%) respectively. Actively growing plantlets with profuse root system were transferred to greenhouse after 30-50 days of culture and acclimatized similarly as described in case of *Curcuma.* About 90% shoots survived in non-sterile potting mixture.

Hardened plantlets of both the species were found to grow healthily with 80-100% survival after 4 weeks of repotting. After 35-45 days of transfer, new leaves were also developed in the *in vitro* shoots (Figure 2.6).

2.4.4 Cyto-genetic fidelity assessment

Testing the genetic purity of the *in vitro* raised plantlets in tissue culture studies confirms success of the process. RAPD based genetic assessment of genetic stability of *in vitro* grown micropropagated plants has been reported in many other plant species (Rout and Das 2002). The source of the explants and mode of regeneration (somatic embryogenesis/ organogenesis/ axillary bud multiplication) are known to play a major role in determining the presence or absence of variation (Salvi et al. 2001). Use of rhizomatous buds as explants for micropropagation lowers the risk of genetic instability as the organized meristem is generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Salvi et al. 2002). Bimolecular analysis using RAPD and SDS-PAGE of micropropagated plantlets of *Curcuma* showed a profile similar to the mother plant indicating that no variation had occurred *in vitro* at DNA and protein level. Results depicted here concur with findings of Suri et al. (1999) who found better genetic stability in regenerated plantlets obtained from rhizomes

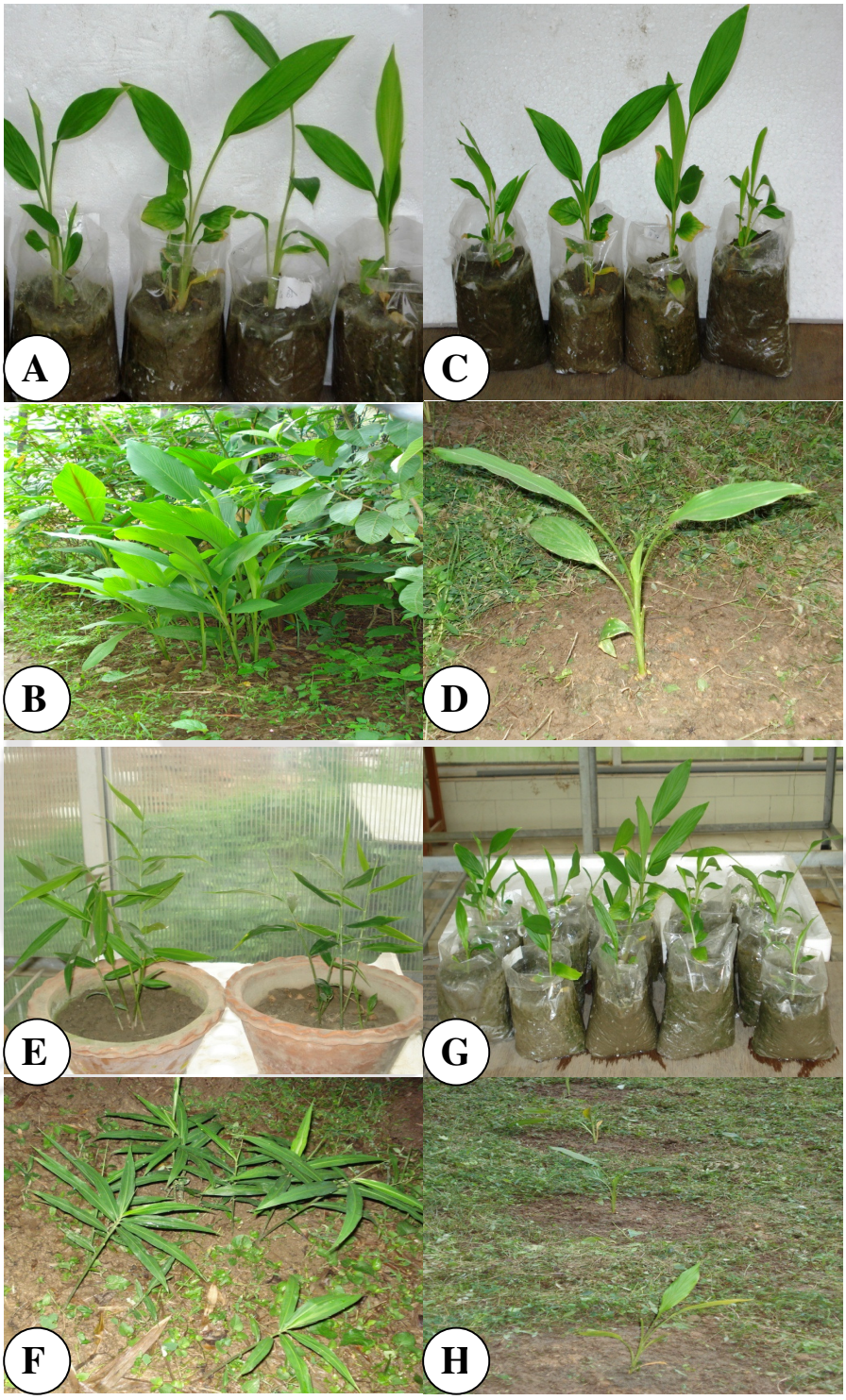


Figure 2.6: Hardened and field transferred regenerated plants of the two genera:
C. amada -(**A**) hardened plants in green house, (**B**) field transferred plants
C. longa -(**C**) hardened plants in green house, (**D**) field transferred plants
Z. moran -(**E**) hardened plants in green house, (**F**) field transferred plants
Z. zerumbet -(**G**) hardened plants in green house, (**H**) field transferred plants

compared to leaf explants. Rhizomatous buds were used as explants for micropropagation of *Curcuma* and *Zingiber* species in the current study as it lowers the risk of genetic stability.

2.4.4.1 RAPD profiling in Curcuma

To confirm the genetic stability of regenerated plants maintained in culture for a period of 2 months, RAPD analysis was carried out. Out of the 10 different RAPD primers tested, 9 and 7 primers produced clear and scorable bands in *C. amada* and *C. longa* respectively. Nine selected RAPD primers utilized in this study for *C. amada* gave rise to a total of 46 scorable bands, ranging from 300-1800 bp in size. The number of bands for each primer varied from 1-8, with an average of 5.1 bands per primer. Similarly, the 7 selected RAPD primers utilized in this study for *C. longa* gave rise to a total of 36 scorable bands, ranging from 500-2000 bp in size and the number of bands for each primer varied from 1-5, with an average of 5 bands per primer. Each primer generated a unique set of amplification products that were monomorphic across all the micropropagated plants. Number of monomorphic bands was highest, six in case of primer OPA 03 for *C. amada* and was lowest in case of the primer OPA 10 in *C. longa*. Overall, no changes in the amplified fragments were detected among all micropropagated plantlets with reference to donor plant, which confirmed the genetic stability of these plantlets derived *in vitro*. Figure 2.7 shows representative examples of amplified banding patterns produced by RAPD primers.

2.4.4.2 Protein profiling in Curcuma

In SDS-PAGE analysis, 12 unique bands were observed and the protein patterns of both micropropagated plants and the mother plant exhibited relatively high degree of identity (Figure 2.8). The difference among polypeptides is in the relative intensity of the stained bands rather than in their numbers. It was observed that the low molecular mass proteins produced high intensity bands while the high molecular mass proteins were present in lower concentrations. The consistency in the polypeptide profiles of all the regenerated plants along with the mother plant suggested the absence of genetic variability of the cloned plants.

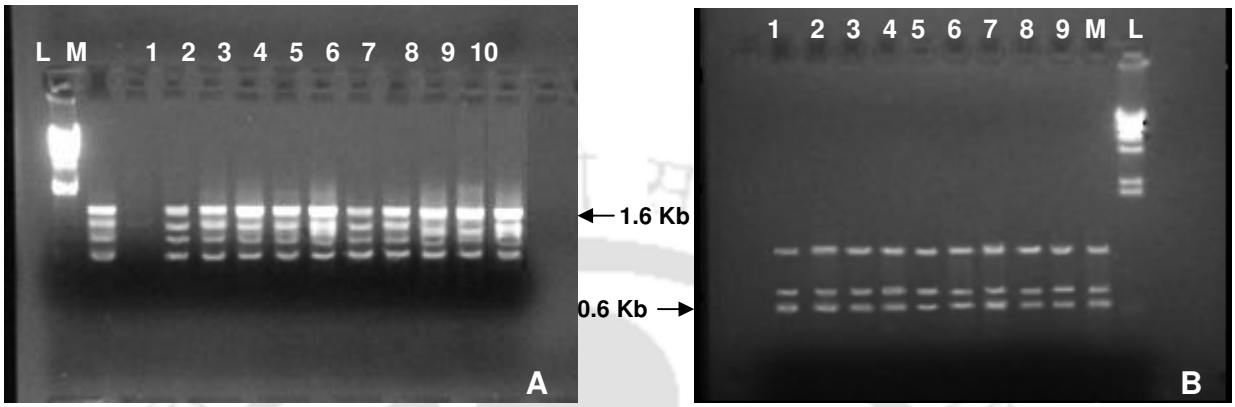


Figure 2.7: RAPD profiling of regenerated plants of *C. amada* (**A**) with OPA 04 and *C. longa* with OPA 11 (**B**)

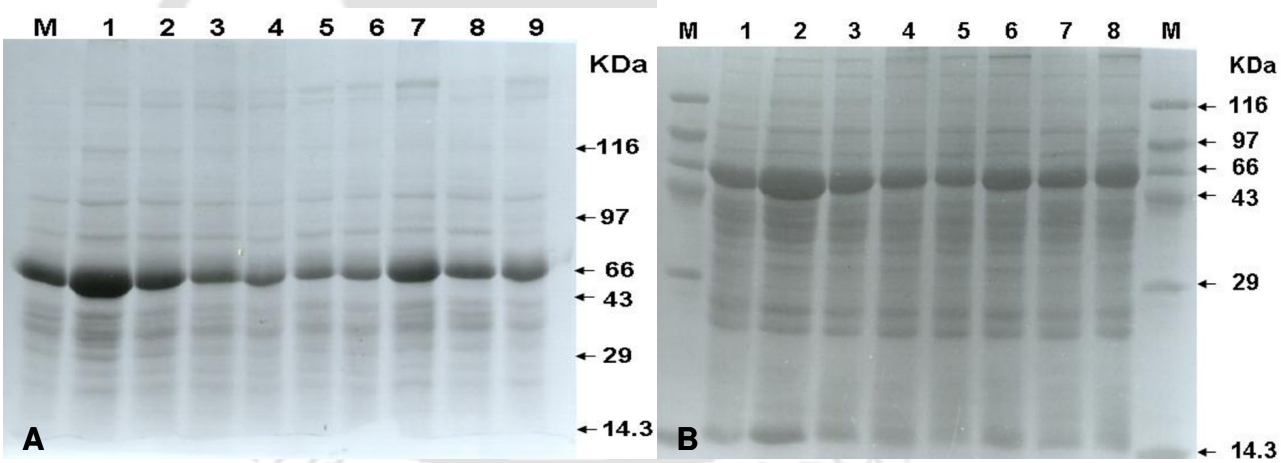


Figure 2.8: SDS-PAGE profiling of regenerated plants of *C. amada* (**A**) and *C. longa* (**B**)

2.4.4.3 RAPD profiling in Zingiber

The genetic purity of regenerated plants of *Zingiber* species were also confirmed by RAPD profiling using 10 primers of which 8 and 6 primers produced clear reproducible bands for *Z. moran* and *Z. zerumbet* respectively. In case of *Z. moran* a total of 45 scorable bands were produced by the 8 selected RAPD primers which ranged from 500-3000 bp in size. An average of 5.6 bands per primer was produced whereas the number of bands for each primer varied from 3-8. Similarly the 6 selected RAPD primers on the basis of clarity of banding patterns used for *Z. zerumbet* revealed a total of 36 scorable bands, ranging from 200-800 bp in size and the number of bands for each primer varied from 2-5, with an average of 6 bands per primer. The amplification products were monomorphic across all the micropropagated plants. No variation was detected among all the regenerated plants with reference to the mother plant. A typical RAPD profile of *Z. zerumbet* and *Z. moran* is shown in the figure 2.9 depicting all regenerated plants were clones of its mother plant.

2.4.4.4 Ploidy check in Zingiber

The ploidy levels of the regenerated plants were checked by chromosome study and counting the number of chromosomes and compared with that of the mother plants. The number of chromosome was found 2n=22 in *Z. moran* (Figure 2.10 A, B) and *Z. zerumbet* (Figure 2.10 C, D) respectively both in donor and *in vitro* raised plants. The stable nature of the regenerants was thus ascertained by consistency of chromosome numbers.

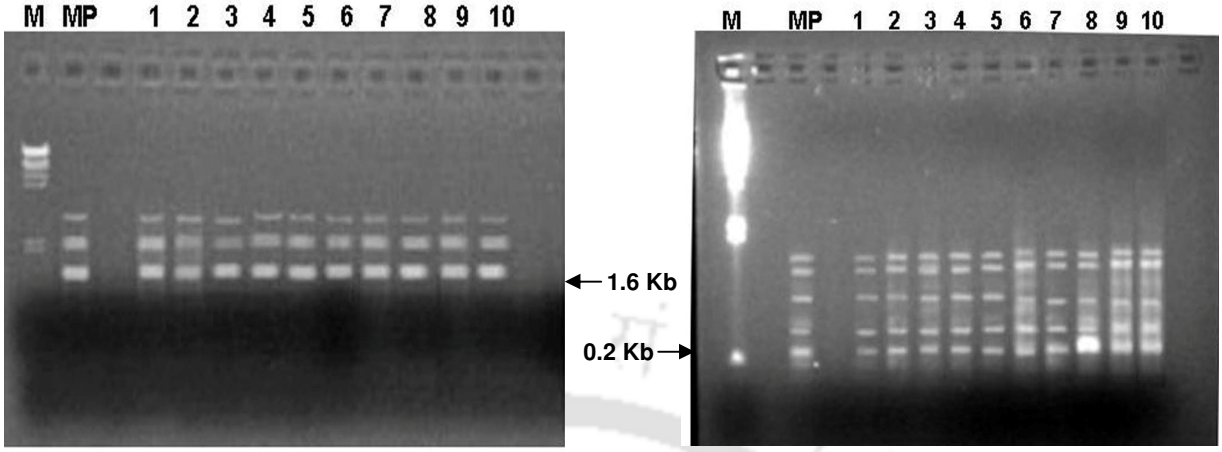


Figure 2.9: RAPD profiling of regenerated plants of *Z. moran* (**A**) with OPA 04 and *Z. zerumbet* with OPA 10 (**B**)

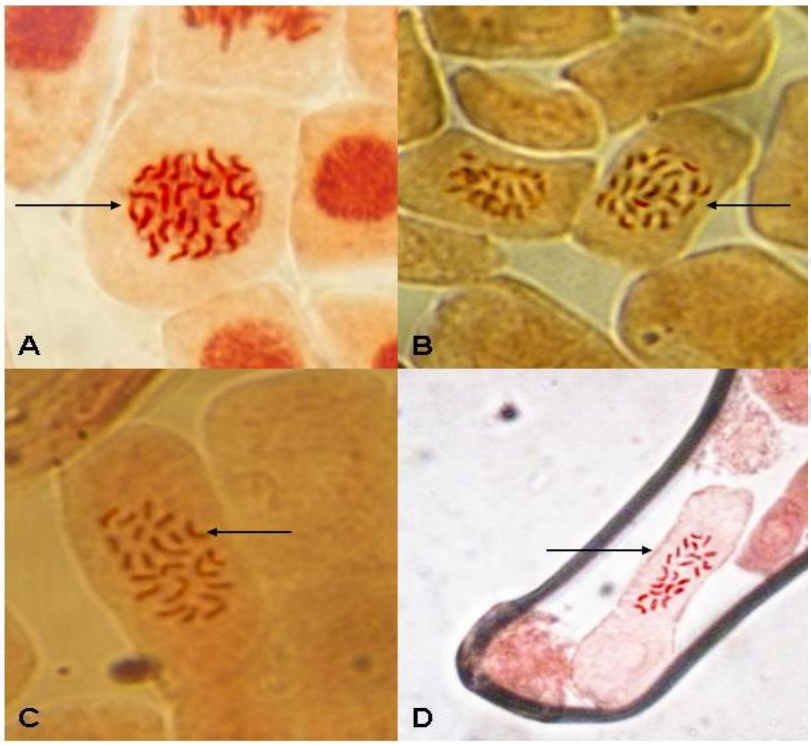


Figure 2.10: Light microscopic images showing somatic chromosomes during metaphase division with exactly equal chromosome counts depicting absence of cytological variation in *in vitro* raised plants; **A**- *Z. moran* mother plant; **B**- *Z. moran* regenerated plant; **C**- *Z. zerumbet* mother plant; **B**- *Z. zerumbet* regenerated plant (Magnification = 400x)

2.5 Conclusion

The production of whole plant by *in vitro* technique is an efficient, reliable and rapid strategy. This provides a faster method of large scale propagation. *In vitro* propagation in some wild and domestic *Curcuma* and *Zingiber* species of NE India was attempted with the aim of developing a general and reproducible protocol that could be used for further improvement and large scale plantation. In the present investigation, multiplication was found to occur by development of axillary buds which is ideal for maintaining genetic stability. However the rate of bud multiplication was significantly different according to the various concentrations and combinations of growth regulators used. Rate of multiplication was found to be dependent mainly on plant growth regulators, its concentration and nutrient media. The results demonstrated that for all the tissues used in the present study, MSR is more effective than MS media for obtaining a large number of plantlets in a short time.

The effect of different growth regulators and culture conditions on *in vitro* multiplication and rhizome formation was studied by Rout et al. (2001) for *Z. officinale*. The importance of cytokinins has also been demonstrated for *in vitro* shoot production of other plants including Zingiberaceae (Chithra et al. 2005; Kesari et al. 2008). Cytokinins promote axillary shoot formation by inhibiting apical dominance. But the wild species with therapeutic value are investigated for the first time. In the current investigation, cytokinins along with lower concentrations of auxins revealed an increased proliferation and multiplication rate in *Curcuma* species. Explant type, growth medium, and endogenous hormone levels are known to influence shoot induction and these factors may contribute toward the variation observed in the present study. BA has been found to be more favourable than Kn in multiple shoot induction. However, persistence of explants in culture media containing higher concentration of cytokinins suppressed shoot elongation in present study which is contrary to what has been reported by other researchers who used rather high concentrations of plant growth regulators for the multiple shoot formation for some of the Zingiberaceae species (Khatun et al. 2003; Chan and Thong 2004, Bharalee et al. 2005, Sultana et al. 2009). In case of *Zingiber* species, the synergistic effect of the cytokinins was found fruitful. This was earlier found by many workers in other species of Zingiberaceae (Vincent et al. 1992; Nayak 2000; Khatun et al.

2003). However, Stanley and Keng (2007) used the combination of BA and IBA for *Z. zerumbet* with much less number (2.3) of shoots per explant. But this was optimum for micropropagation when cultured in liquid medium. Fortunately the present study documents a better and convenient mode of *in vitro* propagation in the modified MS media with low percentage of agar. The semi-solid gel of agar proved to be beneficial for the regeneration and multiple bud formation in explants for all of the four species studied.

Sucrose is widely used as a standard carbon source for plant tissue culture, and different concentrations and different osmotic environments have been used. To the best of understanding till date, there are no reports on use of maltose as a carbon source in any members of Zingiberaceae. What is clear is that different carbon source is effective in supporting shoot multiplication probably due to the ability of this species to metabolize a wide variety of carbohydrates. Sugar at 2% concentration was also found to be satisfactory in micropropagation of *Z. officinale* earlier by Sharma and Singh (1995). However higher concentration of sugar source has been found to be ideal for *in vitro* microrhizome production in *Z. officinale* (Sharma and Singh 1995; Zheng et al. 2008). Increase in sugar concentration in the culture medium decreased the height of *Curcuma* plantlets significantly in current study. Hence, from the results of the study it can be concluded that maltose and sucrose are better carbon sources for *in vitro* micropropagation of the Zingiberaceae species in as low as 2% concentration.

The results obtained from RAPD profiling suggest that, *in vitro* regeneration and multiplication of *C. longa, C. amada*, *Z. moran* and *Z. zerumbet* using rhizome with axillary buds could be successfully used for rapid clonal propagation of these valuable medicinal plants with the least possibility of genetic variations. These can also be used as a source of disease free planting material for the farmers. The RAPD technique has been proved powerful in analyzing the genetic stability of regenerated plants in many other plant species (Rout and Das 2002; Lakshmanan et al. 2007). This is preferred over the other markers for cost effectiveness and simplicity and can be applied to rapid evaluation of genetic fidelity of micropropagated plants for the conservation of genetic richness.

Tissue culture techniques have been applied typically when traditional methods of propagation have either failed or proved inadequate. Plant tissue culture techniques have been increasingly applied to many medicinal plants in particular for mass propagation,

conservation of germplasm, study and production of bioactive compounds, and for genetic improvement. Medicinal plants have vast genetic diversity, which is a valuable source of agronomic gene/s of interest for the future. Large-scale plant tissue culture is found to be an attractive alternative approach to the traditional methods of plantations, as it offers a controlled supply of bio-chemicals independent of plant availability and more consistent product quality (Kesari et al. 2010). Application of *in vitro* propagation techniques may help in the conservation of biodiversity of locally used medicinal plants. Therefore, as a principal output of this chapter, the protocols have been standardized for mass propagation of the valuable medicinal herbs, *C. longa, C. amada, Z. moran* and *Z. zerumbet* through shoot morphogenesis, as well as cyto-genetic purity was established for the *in vitro* raised plants by RAPD, SDS-PAGE profiling and chromosome counting.

Present study is a successful effort to optimize the *in vitro* protocols for direct plantlet regeneration, rapid multiplication and effective conservation of wild and domestic species of *Curcuma* and *Zingiber*. It is interesting to note that till date, there is no any report on the endemic plant species *Z. moran*. The results will surely contribute for further research and conservation of wild and endemic varieties of Zingiberaceae that are available in limited quantities. The improved *in vitro* propagation protocol for the conservation of wild but medicinally sound species of *Zingiber* from NE India reported here could be helpful for the endemic species of *Z. moran* in terms of conservation, utilization and pharmaceutical application.

Chapter 3

Chapter 3

Systematic characterization for genetic diversity studies in members of the genus *Curcuma* and *Zingiber moran* ecotypes

3.1 Introduction

Genetic diversity is a fundamental component of biodiversity, forming the basis of species and ecosystem diversity. It represents all of the genetically determined differences that occur between individuals of a species in the expression of a particular trait or set of traits. There are three fundamental levels of genetic diversity: genetic variation within individuals (heterozygosity), genetic variation among individuals within a population, and genetic differences among populations. An understanding of the extent and distribution of genetic variation within and among plant populations is essential for determining appropriate genetic management strategies for utilization and conservation purposes (Bawa and Dayanandan 1998). Genetic variation plays a critical role in the ability of populations to respond to changing environments. Hence the extent and causes of genetic variation provide the basis of any evolutionary studies (Weir 1996). Moreover, continual analysis identifies new, previously undescribed species and many groups with more than one species together or divide species into more than one variety. Among nature's valuable biodiversities, plant and plant diversity contribute directly or indirectly to enrichment of life experiences of humans. A key drive for support of nature conservation is the human perception that diversity of life forms has a value beyond that associated with the importance of diversity for environmental sustainability and economic reasons. Plant diversity is a key contributor to environment sustainability on a global scale. Studies of species richness demonstrate the greater productivity of more diverse plant communities. Evolutionary relationships are more important in plant conservation and in plant improvement as well (Muthusamy et al. 2009).

Biodiversity Conservation Act (1999) emphasizes the conservation of biodiversity rich areas and their sustainable use, especially in the developing countries. For a country like India which is diverse with all variety of flora and fauna, conservation of natural

wealth becomes a priority in the urban sprawl. India's Biological Diversity Act, 2002 also aims to promote conservation, sustainable use and equitable sharing of benefits of India's biodiversity resources. India has one of world's richest medicinal plant heritages. In the present study Northeastern biodiversity hotspot was chosen for its enormous amount of naturally occurring medicinal wealth the value of which is more or less to a large extent restricted to experts in the field and the traditional folks. All eight states of the NE region are with varying climatic and forest vegetation zones which encompasses different types of medicinal plants that either do not occur in other parts of the country or have less potential. This chapter primly tries to emphasize on the species that are endangered (due to their over exploitation) and calls for immediate conservation strategies for their sustenance. The investigation was carried out on two important genera of the family Zingiberaceae which would surely add to the conservation endeavor to ensure the sustainability of nature's wealth for future.

The tropical monocotyledonous family Zingiberaceae is well known for their medicinal and commercial value and is found to prevail in entire Northeastern region of India in wild state. The climate and geographic conditions of NE India makes the region a natural reservoir for the members of Zingiberaceae. *Curcuma* and *Zingiber* are two major genera of this family with immense commercial and medicinal significance and earliest of spices to be known in the east (Singh et al. 2008). The present study was carried out on different domestic and wild species of *Curcuma* and an endemic species *Zingiber moran* from NE India for accessing the genetic variances existing both at inter and intraspecific level. Any scientific work on this family is largely hurdled by many difficulties like species identification, inaccessible habitat, short vegetative growth etc. (Sasikumar 2005; Das et al. 2010). Some of the species descriptions are without Latin diagnosis or type specimen, therefore the legitimate status of many species is suspicious and remains unclear (Per. Comm. G.C. Sarma, GU). Due to over-exploitation of such species for their extreme medicinal properties, the germplasm are getting in the verge of extinction. Therefore, characterization of such species will open a ground for their conservation, utilization and further improvement programs. It is here, molecular systematic approach will be significant especially towards protection and utilization of bioresources from NE India (Rangan et al. 2008). The knowledge of genetic variability is a pre requisite to

study the evolutionary history of a species, as well as for other studies like intraspecific variations, genetic resources conservation etc (Islam et al. 2007). One of the scientific methods to elucidate a genetic relationship is to use molecular markers, which is now possible with the advent of several molecular techniques. New technological developments (Rafalski et al. 1996) have expanded the range of DNA polymorphisms assays for genetic mapping, marker assisted breeding, genome finger printing and investigating genetic relatedness. Current study was emphasized on interspecific variations among 9 different species of *Curcuma* and intraspecific variations prevailing in *Z. moran* from NE India using different molecular markers.

Thus the objectives of this chapter encompasses (i) to evaluate the degree of genetic variability and analyze genetic proximity among 9 species of *Curcuma* at the inter and intra-specific level, (ii) to quantify the level of genetic diversity within 10 ecotypes of *Z. moran* using different markers system (RAPD, ISSR and AFLP) and, (iii) to compare the informativeness of RAPD, ISSR and AFLP assays employed for analyzing the genomes of both the genera.

3.2 Literature Review

For any future analysis using a species it is useful to have information regarding its genetic diversity and relationship with cultivated and wild species. The effectiveness of any scientific improvement programme depends upon the nature and magnitude of existing genetic variability. The knowledge of genetic variability hence, is the prime requisite for studies like phylogeny, intraspecific variations and genetic resources conservation of a species (Islam et al. 2007). Moreover, knowledge on the evolutionary relationship is important in ensuring the management of plant populations that is conducted to allow continuation of effective plant evolution, allowing long-term plant diversity and survival. Traditionally, morphological and agronomic traits have been used to measure genetic diversity but both these approaches have their limitations when it comes to precise positioning of the species in a dendrogram, since they are not always complete representative of the genetic structure (Noli et al. 1997). Conventional taxonomic techniques along with molecular biology tools may go a long way in

providing accurate and powerful way of analyzing genetic relationship in the genera *Curcuma and Zingiber.* Recently, these techniques have been used in some plants including certain members of Zingiberaceae (Palombi and Damiano 2002; Jatoi et al. 2006). Till now, little reference is available on genetic diversity analysis of the members of genus *Curcuma* (Syamkumar and Sasikumar 2007; Arunyawat 1997; Jatoi et al. 2006; Das et al. 2011) and *Zingiber* (Kavitha et al. 2010). But there has been no effort to analyze the rich source of *Curcuma* and *Zingiber* species from NE India to study genetic variability. However biochemical and pharmacological studies are available in many members of these two genera (Singh et al. 2002; Purakayashtha et al. 2006; Sabulala et al. 2006; Bos et al. 2007; Habib et al. 2008). Molecular markers may contribute to analyze taxonomic relationships and intraspecific diversity of the genera.

Traditionally, phenotype and geographic regions form the basis of conservation of biological resources; however more detailed information is desired by the conservation biologists. Such additional and advanced information could be obtained by using molecular techniques which provide answer to several diversity questions, such as the amount of diversity present at intra- and inter-specific levels, the structure of diversity (i.e. how the diversity is partitioned among and within populations in space and over time), the population history, genetic lineages, gene genealogies, distinguishing between differentiation and divergence, genetic distance, relatedness (i.e. kinship or degree of relatedness among relatives), to ascertain whether two individuals are genetically identical, gene flow, linkage disequilibria, and inter-locus correlation of allelic variation. The molecular markers because of their ability to elucidate diversity in their genome can also help to define the taxonomic boundaries where phenotypic differences are ambiguous. Evolutionary biologists generally assume the alleles to be evolutionary units and the individuals as the means of distributing alleles, the molecular markers can be used to delimit the taxa (Sharma and Jana 2002; Sharma 2009). Molecular markers that can address questions on biodiversity or systematic should be heritable, discriminate between the individuals, populations, taxa, easy to evaluate and provide results that can be compared with that of similar studies (Hills and Mortiz 1990).

Use of molecular markers in biodiversity studies started with the advent of isozyme polymorphism and has proliferated since then. Nucleic acid markers were

developed later on with the advent of restriction fragment length polymorphism (RFLP) in 1970s (Jeffreys 1979; Botstein et al. 1980) followed by sequencing (Sanger et al. 1977) and polymerase chain reaction (PCR) in 1983 (Mullis and Faloona 1987). Unlike protein and isozymes, the nucleic acid markers are able to reveal many loci, are not influenced by environmental conditions and large information can be obtained from tiny amount of plant material at any stage of development. (Sharma 2009). With the advent of newer and efficient sets of molecular markers like Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR), and Inter Simple Sequence Repeats (ISSR) genetic relationship analyses are being attempted to ascertain details and to arrive at better relationship patterns (Vos et al. 1995; Bartish et al. 2000). The usefulness of molecular markers in genetic diversity studies has been convincingly established in many plants (Belaj et al. 2003; Awasthi et al. 2004; Sharma et al. 2009; Rangan et al. 2008; Kesari et al. 2010; Das et al. 2011). DNA markers portray genome sequence composition, thus enabling to detect differences in the genetic information carried by the different individuals. A wide variety of DNA-based markers have been developed in past few years.

RAPD analysis is the simplest, inexpensive, user-friendly and least laborious method and has been successfully used to estimate the genetic distances and diversity in a wide range of plant species especially at the sub-species and cultivar level (Shasany et al. 2002; Fico et al. 2003; Nayak et al. 2005; Saritnum et al. 2005). The essential feature RAPD is the identification of polymorphism by the detection of differences in DNA occurring between the individual plants. The most important aspect of this marker is that it can be mapped as any other standard genetic marker. RAPD markers are dominant in nature and can assay a greater number of potential polymorphic loci. The greatest advantage of the RAPD approach lies in its technical simplicity paired with the independence of any prior DNA sequence information. However, the technique lacks robustness primarily because of short length primers and amplification process is sensitive to fluctuations in laboratory conditions, change in chemicals and equipments etc. Despite of such drawbacks, RAPD have been used extensively in description of inter- and intra-specific variability or differentiation at the species and genus level (Sharma et al. 2001; Petros et al. 2007). RAPD technology has been used in diverse applications in

plants, and lower organisms (Lavanya et al. 2009) which include studies in genetic diversity, phylogeny, systematic and population genetics, development of genetic linkage maps, targeting genetic markers and few other applications such as germplasm screening, evaluating genetic introgression and inbreeding.

Inter simple sequence repeats (ISSR) marker is a variant of polymerase chain reaction that uses simple sequence repeat (SSR) primers to amplify regions between their target sequences (Kahl 2001). This fingerprinting technique exploits the abundant and random distribution of simple sequence repeats in plant genome by amplifying the DNA sequences between closely linked SSRs. The technique is quicker and straight forward and does not require any high developmental cost. ISSR markers are highly polymorphic, robust in usage and can be automated. These are mostly dominant markers, though occasionally few of them exhibit co-dominance (Banu 2009). ISSR markers are reported to have advantage over other markers like RAPD and RFLP (Parsons et al. 1997). The ISSR amplification can easily differentiate between closely related individuals (Zietkiewicz et al. 1994) and reveal a much larger number of fragments per primer than RAPD analysis. These are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Reddy et al. 2002). ISSR markers can be used in population genetics studies of a plant as they can detect very low levels of genetic variation (Zietkiewicz et al. 1994). They have the potential for analyzing biogeographic patterns among populations of a single plant species and have been successfully used to understand the genetic structure and differentiation among various plant species across their natural populations (Wolfe et al. 1998).

Amplified fragment length polymorphism (AFLP) is another promising technique for characterization of genetic diversity because it possesses a high degree of reproducibility and discriminating power. The high marker density sites that can be obtained with AFLP are the key feature of this marker system. It is a highly sensitive method for detecting polymorphism throughout the genome and is becoming increasingly popular (Lavanya et al. 2009). This marker is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and ligation of oligonucleotide bases to the restriction fragments (Vos et al. 1995). The technique generates huge number of restriction fragments for polymorphism. Moreover, it possesses

the capacity for screening of many different DNA regions distributed across the genome. The number of restriction fragments to be amplified can be controlled by selecting the different base number at the end and the composition of nucleotides in the adapters. Although like RAPD, AFLP are dominantly inherited, they can survey up to 100-200 loci and are reproducible. AFLP is a novel DNA fingerprinting technique and involves the display of a set of DNA fragments from a specific DNA sample. It is a fast and reliable method to generate hundreds of informative genetic markers (Vos et al. 1995) and requires no sequence information or probe collection prior to the generation of the AFLP fingerprints. This is of particular benefit when studying organisms with very little or no DNA marker information is available. The AFLP technique has been widely applied in mapping, DNA fingerprinting, analyses of genetic relationships and genetic diversity (Powell et al. 1996; Russel et al. 1997).

The diversity analysis of forest plants in India using AFLP is still in a nascent state (Banu 2009). AFLP is considered more powerful and reliable than RAPD in discriminating genetic diversity (Powell et al. 1996; Vos et al. 1995) but also more laborious and time consuming. On the other hand, ISSR markers can identify allele with high reliability and reproducibility, abundant throughout the genome and show a higher level of polymorphism than any other genetic markers (Gutierrez et al. 2005). The sequences flanking specific microsatellite loci in a genome are considered to be conserved within species, across species in a genus and perhaps even across the related genera (Varshney et al. 2002). In contrast, ISSR amplifications can easily differentiate between closely related individuals. For inter and intra-population assessment with limited genetic variability, the molecular markers of choice must be very informative. Although newer techniques like AFLPs, microsatellites (SSR and ISSR) are preferred due to their informativeness, RAPD analysis is still used because of its simplicity, low cost and lower infrastructure requirement (Williams et al. 1990; Caetano-Anollés et al. 1991). RAPD supports the simultaneous detection of polymorphism at many loci in the entire genome. Advantages of ISSR markers lie in that these are accurate, highly polymorphic and can be automated (Medhi et al. 2009). Dominant markers like RAPD, ISSR and AFLP, are able to detect multiple loci distributed throughout the genome. However, the greatest advantage of AFLP technique is its ability to reveal polymorphism at the DNA

sequence level within individuals and between individuals due to its potential to generate higher number of loci per assay and hence higher polymorphism rate (Surekha and steve 2005). These methods are adequate for describing genetic diversity and effects of fragmentation, and are easy to implement when little or no molecular genetic research has been conducted for the species (Bartish et al. 2000; Nybom 2004).

Studies are available on the genetic diversity analysis using RAPD, SSR and ISSR markers on the various members of family Zingiberaceae (Jatoi et al. 2006; Syamkumar and Sasikumar 2007; Hussain et al. 2008; Das et al. 2011), but there has been no report on any of the endemic *Zingiber* species of NE India. Hence the objective of the chapter was aimed to clarify genetic relationship of nine *Curcuma* species and ten different ecotypes of *Z. moran* and to access the degree of genetic diversity and analyze genetic proximity prevailing among the indigenous and endemic species from NE India using three different sets of molecular markers viz. RAPD, ISSR and AFLP respectively. The use of molecular markers to study the genetic diversity and relationships of *Z. moran* species will be helpful to resolve the identity of the ecotypes and analyze the intraspecific diversity existing among them. The study was also intended to discuss about the usefulness of the three compared methods for assessment of genetic diversity. Further, the study would surely exhilarate the process of planning strategies for the conservation and optimal utilization of such important species.

3.3 Materials and methods

3.3.1 Genetic diversity studies in Curcuma and Z. moran

3.3.1.1 Plant material

The plant material used for the present study consisted of 9 different species of *Curcuma*, both wild and cultivated and ten ecotypes of *Z. moran* collected from various regions of NE India. Various collection sites of the nine different *Curcuma* species and ten ecotypes of *Z. moran* are shown in figures 3.1 and 3.2. The details of the collected species are summarized in Table 3.1(a, b). The species were collected during the monsoon season and maintained in the departmental green house of IIT Guwahati and Botanical Garden, Gauhati University. The vegetative and reproductive features of the specimen under study

are depicted in figure 3.3. Fresh, healthy green leaves of these samples were used for DNA extraction and subsequent fingerprinting analysis.

Table 3.1(a): *Curcuma* species used in the study

Species	Collection Site	Latitude and Altitude	Rhizome character	Habitat
C. amada SK 130	Amingaon	26° 11'N, 56 m	Light yellow, creeping, soft, strong smell like raw mango	Hilly slopes and moist grasslands
C. angustifolia SK 261	Shillong	25°34'N, 1540 m	White, tuber is hard with characteristic odor.	Hilly moist slopes
C. caesia SK270	Goalpara	26° 10'N, 63.3 m	Whitish, soft tuber with mild smell, bluish inside	Moist, shady lands
C. zedoaria SK160	Darrang	20° 9'N, 104 m	Cone shaped tuber towards the end, Yellowish, with strong smell	Shady, humid places
C. aromatica SK 335	Nagaon	26° 11'N, 55 m	Hard, whitish in color with strong smell	Both shady and hot-humid places
C. longa SK145	Barpeta	26° 10'N, 56 m	Yellowish, smaller tuber, with characteristic smell	Shady, moist places
C. domestica GS 205	Kahikuchi	26° 9'N, 55.5 m	Reddish in color, larger tubers with strong smell	Shady, hot and humid places
C. domestica GS 466	Nagaland (Tizu)	27° 4'N, 900 m	Dark yellow in color, tuber has strong odor	Shady, moist, hilly places
C. spp (wild) SK435	Kokrajhar	26° 24'N, 65 m	White and hard tuber with mild smell	Hilly moist slopes

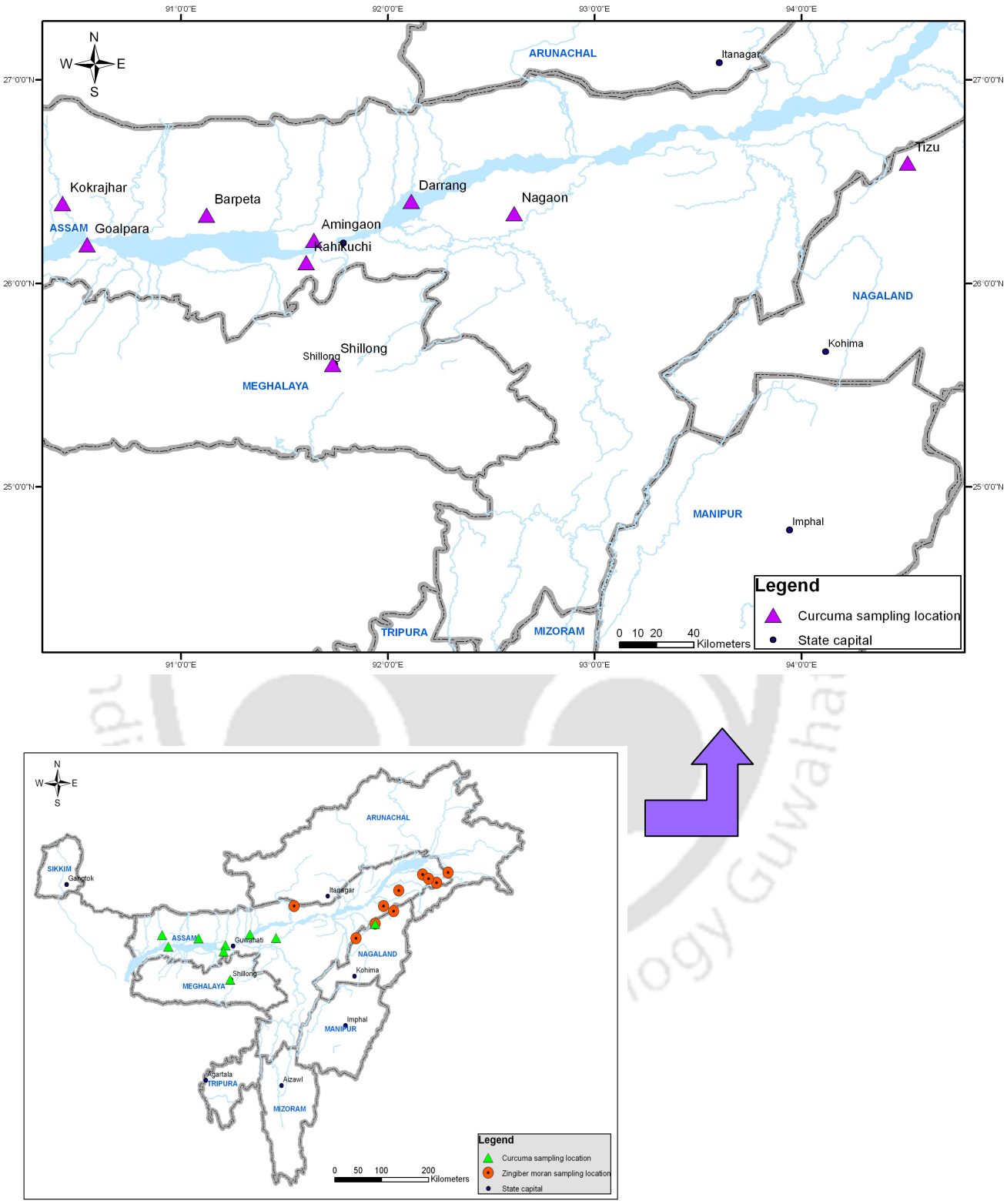
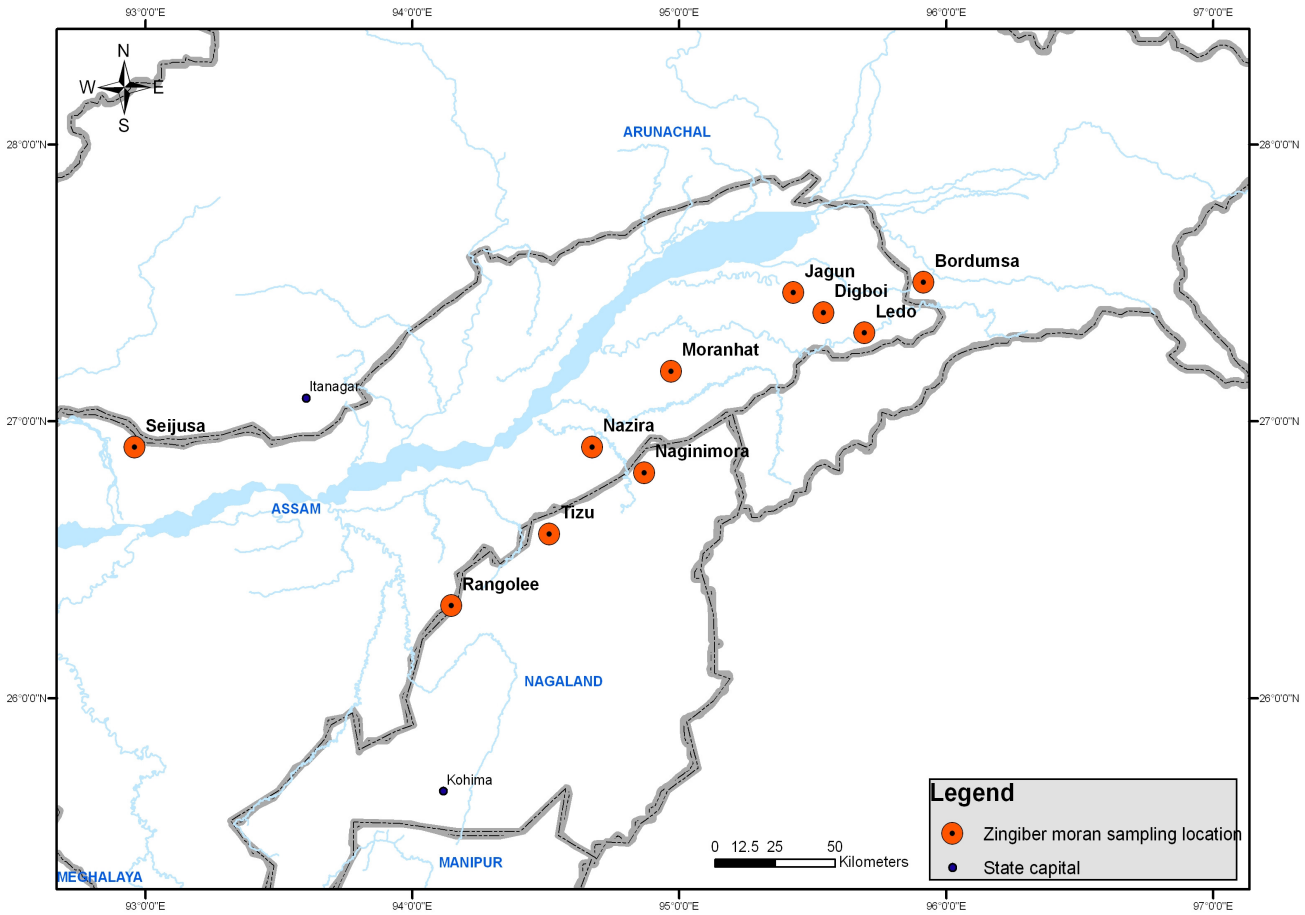


Figure 3.1: Map showing the collection sites of *Curcuma* species used in the study

Table 3.1(b): Source information of *Z. moran* ecotypes used in the study

Species tag	Collection site	State	Latitude and Altitude	Rhizome character	Habitat
ZM01	Moran	Assam	26° 3'N, 96 m	Off white in color, with strong pungent scent, small in size	Shady Humid places
ZM02	Golai	Assam	27°54'N, 100 m	Off white in color, with strong pungent scent, small in size	Shady Humid places
ZM03	Digboi	Assam	27° 48'N, 101 m	Off white in color, with strong pungent scent, small in size	Shady Humid places
ZM04	Jagun	Assam	29° 3'N, 104 m	Off white in color, with strong pungent scent, small in size	Shady Humid places
ZM05	Paanbari	Assam	27° 5'N, 86 m	Off white in color, with strong pungent scent, small in size	Shady Humid places
ZM06	Bordumsa	Arunachal Pradesh	26° 42'N, 530 m	Off white in color, with strong pungent scent, small in size	Shady Humid, hilly places
ZM07	Seijusha	Arunachal Pradesh	25°15'N, 535 m	Off white in color, with strong pungent scent, small in size	Shady Humid, hilly places
ZM08	Tizu	Nagaland	27° 4'N, 900 m	Off white in color, with strong pungent scent, small in size	Shady Humid, hilly places
ZM09	Rangolee	Nagaland	26° 68'N, 550 m	Off white in color, with strong pungent scent, small in size	Shady Humid, hilly places
ZM10	Naginimora	Nagaland	25° 55'N, 552 m	Off white in color, with strong pungent scent, small in size	Shady Humid, hilly places



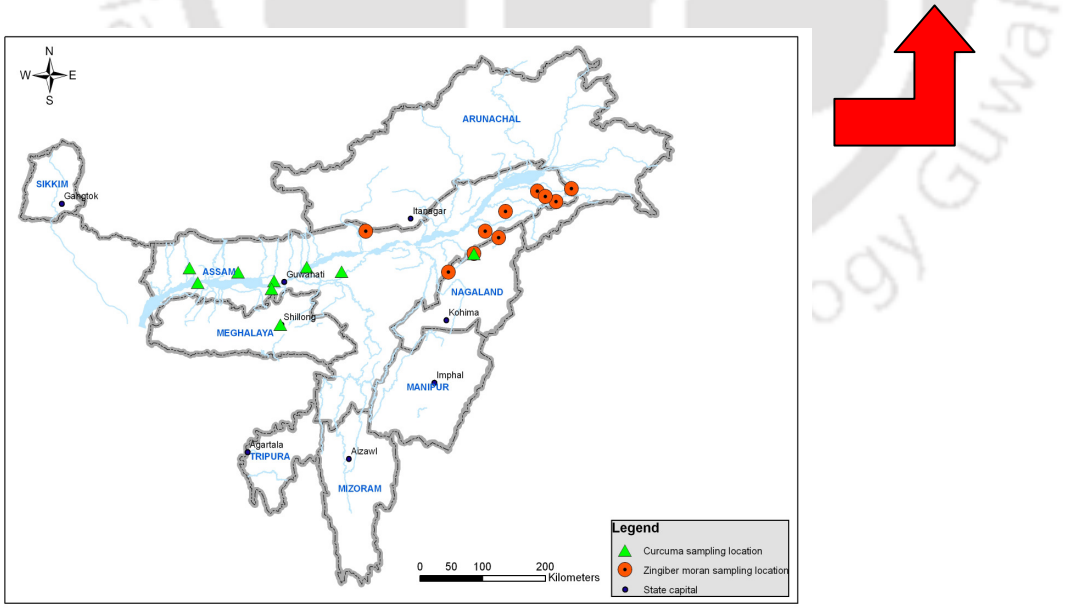


Figure 3.2: Map showing the collection sites of *Z. moran* ecotypes used in the study

Figures 3.3: Plant material along with reproductive parts in their natural habitats used in the study; *C. amada* (**A**), *C. angustifolia* (**B**), *C. caesia* (**C**), *C. aromatica* (**D**), *C. zedoaria* (**E**), *C. longa* I (**F**), *C. longa* II (**G**), *C. longa* III (**H**), *C. wild* (**I**), *Z. moran* (**J**)

3.3.1.2 Genomic DNA isolation

Genomic DNA was isolated from tender leaves (~5 g fresh weight) of the test plants using modified SDS method of McCouch et al. 1988 (Kesari et al. 2009). The quality and quantity of the extracted DNA was confirmed to be consistent both spectro-photometrically and by running the extracted DNA on 1.0% agarose gels stained with ethidium bromide.

3.3.1.3 RAPD analysis

For RAPD fingerprinting, PCR amplification of the genomic DNA was carried out using 20 arbitrary decamer oligonucleotide primers for *Curcuma* and 10 primers for *Z. moran* ecotypes (Table 3.2) (Operon Tech, USA). Each reaction mixture of 20µl contained 50ng/µl of template DNA, 1 x assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1 % gelatin), 0.2 mM each dNTPs (B'LGenei, India), 5 pM of each primer and 0.5U of *Taq* polymerase (B'LGenei, India). The reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems 9700). Thermal cycling conditions were as follows: pre-denaturing step of 5 min at 94 ^{o}C, followed by 35 cycles each of 45 sec at 94 ^{o}C, annealing for 1 min at 32 ^{o}C, extension for 1 min 30 sec at 72 ^{o}C and followed by one final extension cycle of 5 min at 72 ^{o}C. The amplification products were electrophoresed in 1.5 % agarose gels in 0.5x TBE (10x stock contained 0.8 M Tris, 0.8 M boric acid, 0.5 M EDTA). The gels were photographed under a UV transilluminator.

3.3.1.4 ISSR analysis

For ISSR amplification, 20 and 10 selected primers were considered for *Curcuma* species and *Z. moran* ecotypes respectively. Table 3.3 lists each marker, repeat type and length, primer sequence and annealing temperature. The PCR composition was same as that used for RAPD analysis with a final volume of the reaction mixture being 20µl. The PCR conditions followed were: denaturation at 94 ^{o}C for 4 min, 35 cycles at 94 ^{o}C for 45 sec, annealing time was 1 min with varied temperatures as per the melting temperature of the ISSR primer used (Table 3.3), extension at 72 ^{o}C for 1min 30 sec and final extension at

72 ^{o}C for 5 min. The amplified products were visualized in a 1.5% agarose gel containing ethidium bromide and photographed for further analysis.

Table 3.2: Sequence information of RAPD and ISSR oligonucleotide primers used for amplification and polymorphism study amongst nine *Curcuma* species and ten *Z. moran* ecotypes from North East India.

RAPD primers used for 9 species of *Curcuma*					
S.No.	**Primer**	**Sequence (5'-3')**	**S.No.**	**Primer**	**Sequence (5'-3')**
1	**OPC 07**	GTCCCGACGA	11	**OPAA 01**	AGACGGCTCC
2	**OPL 11**	ACGATGAGCC	12	**OPAB 01**	CCGTCGGTAG
3	**OPO 08**	GCTCCAGTGT	13	**OPAB 05**	CCCGAAGCGA
4	**OPAH 15**	CTACAGCGAG	14	**OPAB 14**	AAGTGCGACC
5	**OPAM 20**	ACCAACCAGG	15	**OPAH 13**	TGAGTCCGCA
6	**OPAN 01**	ACTCCAGGTC	16	**OPAF 02**	CAGCCGAGAA
7	**OPAO 01**	AAGACGACGG	17	**OPAJ 19**	ACAGTGGCCT
8	**OPAP 20**	CCCGGATACA	18	**OPX 20**	CCCAGCTAGA
9	**OPAN 05**	GGGTGCAGTT	19	**OPA 08**	GTGACGTAGG
10	**OPAP 10**	TGGGTGATCC	20	**OPA 12**	TCGGCGATAG
RAPD primers used for 10 ecotypes of *Z. moran*.					
1	**OPA 01**	CAGGCCCTTC	6	**OPA 12**	TCGGCGATAG
2	**OPA 02**	TGCCGAGCTG	7	**OPA 14**	TCTGTGCTGG
3	**OPA 04**	AATCGGGCTG	8	**OPA 16**	AGCCAGCGAA
4	**OPA 05**	AGGGGTCTTG	9	**OPA 17**	GACCGCTTGT
5	**OPA 11**	CAATCGCCGT	10	**OPA 18**	AGGTGACCGT

Table 3.3: ISSR oligonucleotide primers selected for amplification and polymorphism of *Curcuma* and *Z. moran*

S.No.	Primer	Sequence (5'-3')	S.No	Primer	Sequence (5'-3')
ISSR primers used for 9 species of *Curcuma*					
1	**HB 12**	CACCACCACGC	11	**816**	CACACACACACACACAT
2	**HB 13**	GAGGAGGAGGC	12	**817**	CACACACACACACACAA
3	**HB 14**	CTCCTCCTCGC	13	**818**	CACACACACACACACAG
4	**HB 15**	GTGGTGGTGGC	14	**824**	TCTCTCTCTCTCTCTCG
5	**P 3**	AGAGAGAGAGAGAGAGTG	15	**825**	ACACACACACACACACT
6	**P 6**	CCACCACCACCACCA	16	**826**	ACACACACACACACACC
7	**P 8**	CACCACCACCACCAC	17	**844**	CTCTCTCTCTCTCTCTAC
8	**807**	AGAGAGAGAGAGAGAGT	18	**872**	GATAGATAGATAGATA
9	**809**	AGAGAGAGAGAGAGAGG	19	**17898A**	CACACACACACAAC
10	**811**	GAGAGAGAGAGAGAGAC	20	**17898B**	CACACACACACAGT
ISSR primers used for 10 ecotypes of *Z. moran*					
1	**809**	AGAGAGAGAGAGAGAGG	6	**17898A**	CACACACACACAAC
2	**824**	TCTCTCTCTCTCTCTCG	7	**17898B**	CACACACACACAGT
3	**825**	ACACACACACACACACT	8	**17899A**	CACACACACACAAG
4	**826**	ACACACACACACACACC	9	**17899B**	CACACACACACAGG
5	**HB 14**	CTCCTCCTCGC	10	**HB 15**	GTGGTGGTGGC

3.3.1.5 AFLP analysis

AFLP markers were obtained with the Plant Mapping Kit (Applied Biosystems) and assay was performed as per the manufacturer's protocol followed by Vos et al. (1995) with minor modifications. DNA (200 ng) was double digested with two restriction endonucleases *Eco*R1/*Mse*I for 2 h at 37 °C and heated at 70 °C for 15 min to inactivate the enzymes. The DNA fragments were then ligated to their respective adapters using ligation solution (*Eco*R1/*Mse*I adapters, 0.4mM ATP, 10mM Tris-HCl at pH 7.5, 10mM Mg-acetate, 50mM K-acetate) and 1µl T4 DNA ligase at 20 °C for 2 hrs. The diluted RE-ligation mixture (10 fold) was used for preselective amplification with preselective primers to reduce the overall complexity of the mixture by increasing concentration of the target sequences so that the double digested fragments become predominant. The pre-selective amplification cycles contained 20 cycles of denaturation (at 94 °C for 60 s), primer annealing (at 56 °C for 60 s) and primer extension (at 72 °C for 2 min). The

preselective PCR product was diluted in a ratio of 1:50 with TE buffer (pH 8.0) and then used as a template for the selective amplification.

The selective amplification of specific target sequences was performed using selective primers available in the AFLP kit. Four primer combinations were used (Table 3.4). The *Eco*RI primers are labeled with different fluorescent dyes (6-FAM, JOE and NED), which make possible multiplexing of three primer pairs in one reaction. The selective amplification involved PCR amplifications with following thermal cycling conditions: denaturation at 94 °C for 2 min followed by primer annealing at 65 °C for 30 s and primer extension at 72 °C for 2 min. The annealing temperature was reduced by 1 °C every cycle till it reached 56 °C. At this annealing temperature, 20 more cycles of PCR amplifications were performed. This was followed by a final cycle of primer extension step at 60 °C for 30 min. Preselective and selective amplifications were carried out in a Gen Amp polymerase chain reaction (PCR) system 9700 thermocycler (Applied Biosystems) following the manufacturer's protocol.

Table 3.4: AFLP primers combinations used for genetic diversity studies for 9 species of *Curcuma* and 10 ecotypes of *Z. moran*

Purpose	Oligonucleotide sequence
Adapters	
*Eco*RI- Adap seq 1	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI- Adap seq 2	3'-CATCTGACGCATGGTTAA-5'
*MSe*I- Adap seq 1	5'-GACGATGAGTCCTGAG-3'
*MSe*I- Adap seq 2	3'-TACTCAGGACTCAT-5'
Primers	
Preamplification	
*Eco*RI- Primer	5'-GACTGCGTACCAATTCA-3'
*MSe*I- Primer	5'-GATGAGTCCTGAGTAAG-3'
Selective amplification	*MSe*I - CAA / *Eco*RI - ACT *MSe*I - CAC / *Eco*RI - ACA *MSe*I - CAG / *Eco*RI - AAC *MSe*I - CAT / *Eco*RI - ACC

One microlitre of the selective amplification product was mixed with 0.5 µl of the GeneScan 500 ROX internal size standard (Applied Biosystems P/N 402985) to accurately size the amplified fragments and 8.5 µl of Hi-Di Formamide (Applied Biosystems P/N 4311320). The PCR products of selective amplifications were denatured prior to separation by capillary gel electrophoresis on an automated DNA sequencer (ABI Prism 310, PE Applied Biosystems). The electropherograms generated by the sequencer were interpreted with Gene scan software. Genotyper software was then used to create a list of fragments detected in each lane by fragment size. Fragments sized from 50 to 500 base pairs (bp) with a peak height >50 in the electropherogram were retained for subsequent analysis. Peakmatcher software (DeHaan et al. 2002) was used to convert the list of fragments detected in each sample into a binary table (1/0) for the presence or absence of each fragment in each sample. Peak matcher was set to retain markers with repeatability >90%.

3.3.1.6 Data analysis

For all the three types of marker systems, duplicate samples from each individual were tested and only clear, unambiguous, and reproducible bands amplified in both were considered for the scoring and data. Only the presence of band was considered irrespective of the intensity of bands just to ignore any taxonomic weighing. The numbers of polymorphic and monomorphic amplification products were determined for each primer for 9 genotypes of *Curcuma* and 10 ecotypes of *Z. moran*. To compare the efficiency of primers, polymorphic information content (PIC) as a marker discrimination power was computed using the formula PIC = 1- $\sum p_i^2$, where p_i is the frequency of i^{th} allele at a given locus (Anderson et al. 1993) and also marker index (MI) was calculated calculated as given in Sorkheh et al. (2007). The basic parameters for genetic diversity were calculated in the POPGENE application (Yeh et al. 1999). The polymorphism of amplification products (P), the number of observed alleles (n_a), the mean number of effective alleles (n_e), the mean Nei's gene diversity index (h), the Shannon index (I) were calculated within the species and within ecotypes using the POPGENE software.

Level of similarity among species/ ecotypes was established as percentage of polymorphic bands, and a matrix of genetic similarity has been obtained by using the Dice's coefficient (1945) using the SIMQUAL program of NTSYS. Applying the UPGMA (Sneath and Sokal 1973) method on this matrix using the SHAN subroutine through the NTSYS- pc (Numerical taxonomy system, 2.2 version) (Numerical taxonomy system, Applied Biostatistics, N.Y.) (Rohlf 2002) dendrograms generated representing the genetic relationships among 9 species of *Curcuma* and 10 ecotypes of *Z. moran*. The correlation between the original similarity indices and cophenetic values was calculated and the Mantel's test (Mantel 1967) was performed using 250 permutations to check the goodness of fit for *Curcuma* species and *Z. moran* ecotypes respectively, to a specific cluster in the UPGMA cluster analysis to the similarity matrix. Further, principal component analysis (PCA) was undertaken for the families with modules STAND, CORR and EIGEN of NTSYS-pc (Rohlf 2002) using the Euclidean distances derived from the standardized values using the software package NTSYS-pc-2.2.

3.4 Results and discussion

3.4.1 Genetic diversity studies in Curcuma and Zingiber

The differential rate of polymorphism in the taxa at inter and intraspecific level is generally dependant on various factors like breeding system, habitat specialization, impact of human communities, preferential selection and the type of molecular marker used. Habitat heterogeneity plays a prominent role in maintaining diversity by diversifying selection, especially in clonal species, in contrast to habitat homogeneity which results in a gradual decrease in diversity through directional selection (Hangelbroek et al. 2002). Although vegetatively propagated species are supposed to display low genetic variance due to lack of genetic recombination, still a fairly wide range of genetic divergence is found to exist in the genera *Curcuma* and *Zingiber* (Kavitha et al. 2010; Jatoi et al. 2008). This is an advanced criterion in terms of adaptability and survival of any species to avoid the risk of extinction (Hangelbroek et al. 2002). However there has been a loss of genetic variation among these two genera due to factors like habitat destruction, deforestation, uncontrolled and extensive harvesting of the plants for their medicinal significance at the rural level etc.

The wide range of polymorphism detected in the current study is of considerable significance and displayed the resolving power of the marker systems used. Morphological markers for identifying the individuals are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith and Smith 1989). In contrast, molecular markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy et al. 2002). In the present study, three DNA based marker systems were utilized for the first time to trace the genetic relationships among the nine *Curcuma* species and ten *Z. moran* ecotypes from NE India.

3.4.2 Polymorphism detection by RAPD, ISSR and AFLP marker systems

3.4.2.1 RAPD analysis

The genetic diversity among the nine different *Curcuma* species was evaluated by 12 selected primers which yielded species specific DNA profiles and proved to be informative. A total of 55 mappable RAPD markers were generated by 12 primers. The amplicons ranged between 0.3-1.8 kb in size. Amplicon number per primer ranged from 2 (OPAM 20) to 8 (OPC 07) with an average of 4.92. Polymorphism also varied in different species of *Curcuma* with a maximum of 8 bands for the primer OPC 07 and a minimum of 1 band in the primer OPAM 20 with a mean of 4.58 (Table 3.5). RAPD profile of nine different *Curcuma* species analyzed showed the polymorphic index value of 93.22 % across all the species examined in the current study. The details of amplification products, polymorphic fragments generated, polymorphism information content (PIC) and marker index (MI) values for each primer are showed in Table 3.5. The RAPD profile generated by OPAN 01 for the 9 different *Curcuma* species is shown in the figure 3.4A. PIC values for RAPD primers varied from 0.17 (OPAM 20) to 0.48 (OPAN 05) whereas marker indices ranged between 8.64 (OPAM 20) to 48.14 (OPAN 05).

Table 3.5: Degree of polymorphism and polymorphic information content for RAPD and ISSR primers in 9 species of *Curcuma*

Markers	Primer code	Total no of bands	Total no of polymorphic bands	POL %	PIC	MI
RAPD	OPAN 01	6	5	83.33	0.33	27.43
	OPAO 01	5	5	100	0.39	39.50
	OPC 07	8	8	100	0.44	44.44
	OPL 11	3	3	100	0.41	41.15
	OPAM 20	2	1	50	0.17	8.64
	OPAN 05	4	4	100	0.48	48.14
	OPAB 10	5	4	80	0.38	30.61
	OPO 08	6	6	100	0.36	36.21
	OPA 8	3	3	100	0.42	42.79
	OPA 12	7	7	100	0.45	45.14
	OPAH 15	5	4	80	0.34	26.86
	OPAP 20	5	5	100	0.40	40.43
	Total	**59**	**55**	**93.22**	**-**	**-**
	Mean	**4.92**	**4.58**	**-**	**-**	**-**
	Range	**2- 8**	**1- 8**	**50-100**	**0.17- 0.48**	**8.64 - 48.1**
ISSR	HB12	4	3	75	0.33	24.99
	HB 13	4	4	100	0.40	40.74
	HB 14	5	5	100	0.45	45.43
	HB 15	2	2	100	0.41	41.97
	P3	5	5	100	0.43	43.45
	P6	3	3	100	0.32	32.92
	P8	4	4	100	0.46	46.91
	807	1	1	100	0.44	44.44
	809	3	3	100	0.34	34.56
	811	4	4	100	0.25	25.92
	816	4	4	100	0.37	37.04
	817	4	4	100	0.37	37.04
	818	1	1	100	0.19	19.75
	824	5	5	100	0.35	35.55
	825	4	4	100	0.48	48.14
	826	4	4	100	0.32	32.09
	844	4	4	100	0.38	38.27
	17898 A	2	2	100	0.46	46.91
	17898 B	6	6	100	0.38	38.68
	Total	**69**	**68**	**98.55**	**-**	**-**
	Mean	**3.63**	**3.58**	-	-	-
	Range	**1- 6**	**1- 6**	**75-100**	**0.197-0.481**	**19.75- 48.14**

POL – Polymorphism;
PIC - Average polymorphic information content for polymorphic bands;
MI - Marker Index = POL (%) x PIC

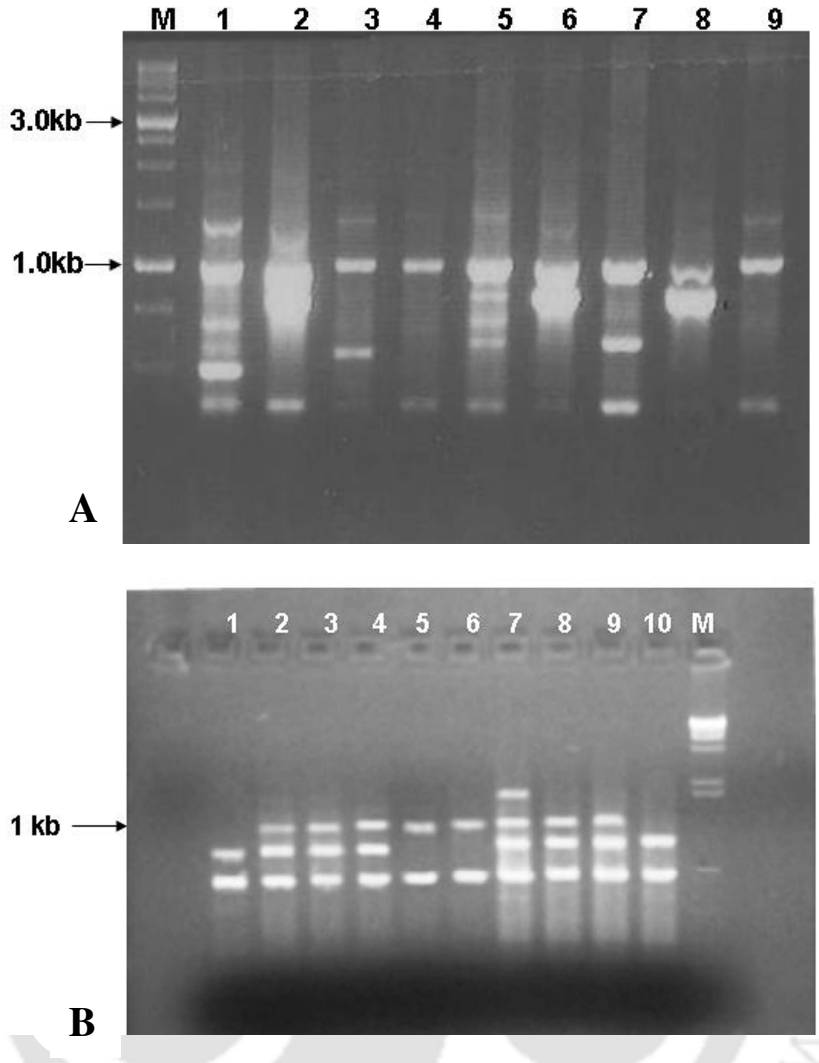


Figure 3.4: (**A**) RAPD profiles generated by the primer OPAN 01 in nine *Curcuma* species from NE India. Lane M, 1 kb DNA ladder; Lane 1-9, *Curcuma* species 1-9
(**B**) RAPD profiles generated by the primer OPA 01 in *Z. moran* ecotypes from NE India. Lane 1-10, ZM 01-10; Lane M, λ-DNA marker

Ten selected RAPD primers were used to execute the genetic diversity prevailing among ten different ecotypes of *Zingiber moran*. From 10 initially applied RAPD primers for evaluation of genetic diversity, all the primers showed reproducible fragments with easily recordable fingerprints. Nine out of 10 primers produced polymorphic bands with 63.16% of polymorphism among the ten *Z. moran* ecotypes studied (Table 3.6). A total of 38 RAPD fragments were consistently resolved by the 10 primers in the ecotypes that ranged between 0.3 - 2.0 kb in size. Primers varied in their ability to detect variation within the ecotypes under study. Amplified fragments per primer ranged between 2-7 where as polymorphic bands varied from 1-4 per primer with a mean of 2.4 (Table 3.6). A wide range of polymorphic percentage (20-100%) in the RAPD profile was observed with an average of 63.16 % across all the ecotypes studied. The RAPD banding pattern generated by OPA 01 is shown in the Figure 3.4B. Maximum PIC value was recorded for OPA 05 (0.39) and a minimum value showed by primer OPA 12 (0.03). Marker indices for RAPD primers ranged between 0.72 (OPA 12) to 22.72 (OPA 11).

3.4.2.2. ISSR analysis

Twenty ISSR primers were used to characterize the genetic diversity present among the nine species of *Curcuma.* Nineteen of these primers showed a total of 69 reproducible fragments that ranged from 0.2-0.85 kb in size. High percentage of polymorphism with all the 19 primers (98.55 %) was displayed among the nine species of *Curcuma* with 68 polymorphic bands. It was observed that the number of visible bands ranged from 1 (807, 818) to 6 (17898 B) with an average of 3.63 whereas the average number of polymorphic bands per primer obtained was 3.58 (Table 3.5). The percentage of ISSR polymorphism for 9 different *Curcuma* species studied ranged from 75 to 100 %. Figure 3.5A displays the ISSR fingerprints using the primer HB 12. The PIC values for ISSR primers ranged from 0.19 (818) to 0.48 (825) with an average of 0.38, whereas MI ranged between 19.75 (818) to 48.14 (825).

For studying the genetic diversity present among the ten ecotypes of *Z. moran* 10 ISSR primers were used of which, all the primers showed a total of 52 well resolved and reproducible fragments that ranged from 0.2-0.85 kb in size. Out of these 52 bands, only 18 bands were polymorphic among the 10 ecotypes as given by eight primers. Other two

Table 3.6: Degree of polymorphism and polymorphic information content for RAPD and ISSR primers in 10 ecotypes of *Z. moran*.

Markers	Primer code	Total no of bands	Total no of polymorphic bands	POL %	PIC	MI
RAPD	OPA 01	4	3	75	0.20	15.38
	OPA 02	2	1	50	0.09	4.5
	OPA 04	2	2	100	0.25	25
	OPA 05	3	3	100	0.39	38.6
	OPA 11	5	4	80	0.284	22.72
	OPA 12	5	1	20	0.036	0.72
	OPA 14	4	3	75	0.28	21
	OPA 16	3	0	-	-	-
	OPA 17	7	4	57.14	0.211	12.06
	OPA 18	3	3	100	0.23	22.6
	Total	**38**	**24**	**63.16**	**-**	
	Mean	**3.8**	**2.4**	-	**-**	
	Range	**2-7**	**0-4**	**0-100**	**0.03-0.39**	**0.72-22.72**
ISSR	809	6	2	33.33	0.16	5.33
	824	3	1	33.33	0.1	3.33
	825	4	0	-	-	-
	826	6	1	16.7	0.07	1.17
	17899A	6	2	33.33	0.07	2.33
	17899B	7	5	71.43	0.27	19. 3
	17898A	6	1	16.7	0.03	0.5
	17898B	7	3	42.86	0.16	6.86
	HB 14	4	3	75	0.21	15.75
	HB 15	3	0	-	-	-
	Total	**52**	**18**	**34.61**	**-**	
	Mean	**5.2**	**1.8**	**-**	**-**	
	Range	**3-7**	**0-5**	**16.7-75**	**0.03-0.27**	**0.5-19.3**

POL – Polymorphism;
PIC - Average polymorphic information content for polymorphic bands;
MI - Marker Index = POL (%) x PIC

ISSR primers (HB 15 and 825) produce monomorphic fragments for 10 ecotypes. The average number of bands per primer was 5.2 and the average number of polymorphic bands per primer was 1.8. A detailed study of amplification pattern of the ISSR markers revealed that for each primer the number of visible bands ranged from 3 (824, HB 15) -7 (17899B) with an average of 5.2 bands per primer; whereas the average number of polymorphism obtained was 1.8 (Table 3.6). The percentage of ISSR polymorphism varied greatly from 16.7 to 75% for the ten ecotypes studied with a fair level of total polymorphism 34.61% for all the 10 ISSR primers. Figure 3.5B displays the ISSR fingerprints using the primer HB 12. The PIC values for ISSR primers ranged from 0.03 (17898 A) to 0.27 (17899 B), whereas MI ranged between 0.5 (17898 A) to 19.3 (17899 B) respectively.

3.4.2.3. AFLP analysis

A total of 147 AFLP bands were recorded with four primer pair combinations and 143 of these bands were polymorphic. The size of amplified products ranged from 50-500 bp. Total number of bands per primer ranged from 3 (*Msel*- CAA /*Eco*RI - ACT) to 81 (*Msel*- CAT /*Eco*RI – ACC) with an average of 36.75 whereas polymorphic bands per primer ranged from 3 (*Msel*- CAA /*Eco*RI - ACT) to 78 (*Msel*- CAT /*Eco*RI – ACC) with an average of 35.75 (Table 3.7). The polymorphic information content (PIC) of each primer was evaluated which was found highest (0.61) for the primer combination *Msel*- CAG / *Eco*RI - AAC and the lowest (0.25) for the primer combination *Msel*- CAC / *Eco*RI - ACA (Table 3.7) with an average of 0.35 per primer combination. The highest MI of 59.8 obtained for the primer pair *Msel*- CAG / *Eco*RI – AAC and the primer combination *Msel*- CAC / *Eco*RI – ACA showed the lowest MI of 25 with an average of 35.18.

In current analysis for assessing genetic variance among the ten *Z. moran* ecotypes, using four AFLP primer combinations resulted in a total of 81 scorable bands among those 77 were clearly polymorphic (95.06%). All the 4 sets of AFLP primers yielded an average of 20.25 bands per primer whereas the average number of polymorphic fragments per primer combination was 19.25 (Table 3.7). The size of amplicons varied from 50-500 bp. Polymorphic bands per primer ranged from 8 (*Msel*-

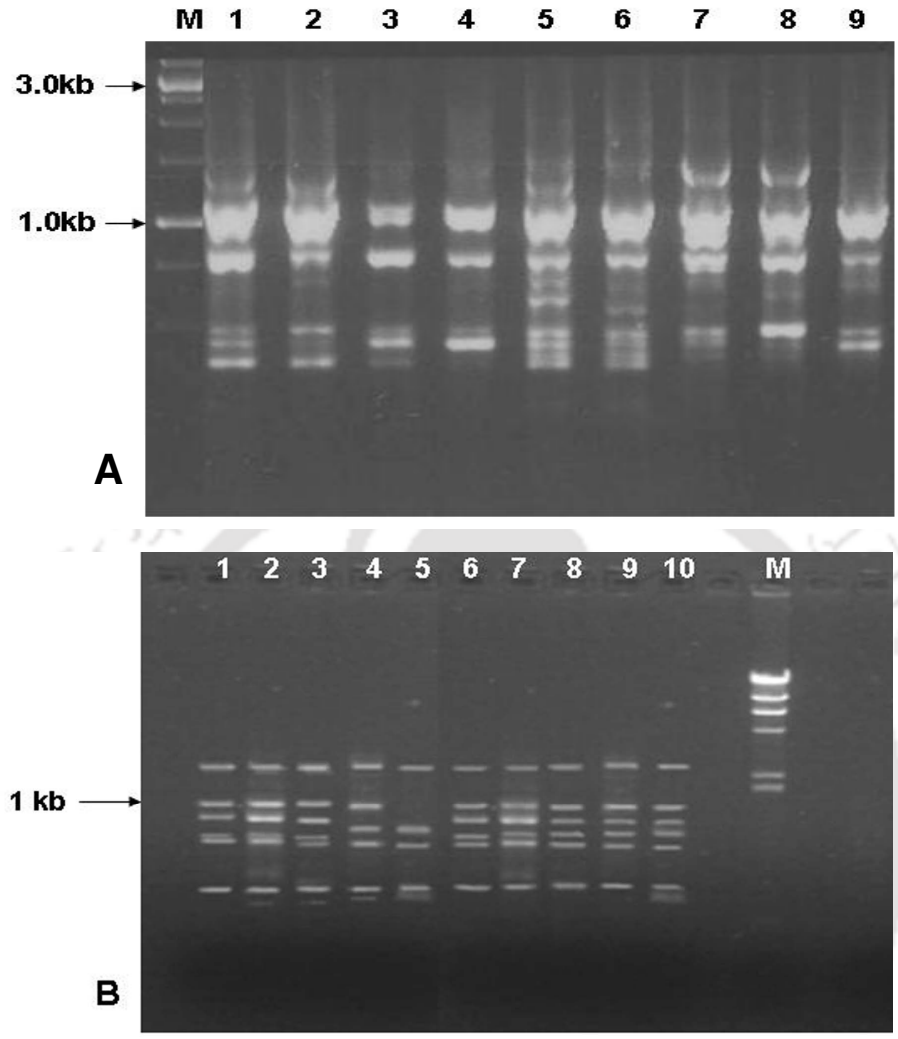


Figure 3.5: (**A**) ISSR profiles generated by primer HB12 for *Curcuma* species from NE India. Lane M, 1 kb DNA ladder; Lane 1-9, *Curcuma* species 1-9
(**B**) ISSR profiles generated by primer HB12 for *Z. moran* ecotypes from NE India. Lane 1-10, ZM 01-10; Lane M, λ-DNA marker

Table 3.7: Degree of polymorphism and polymorphic information content for AFLP primers applied to 9 species of *Curcuma* and 10 ecotypes of *Z. moran*

Primer combinations	Total number of bands	POL		PIC	MI
		Number	%		
I. Curcuma					
*Mse*I - CAA / *Eco*RI -ACT	3	3	100	0.28	28
*Mse*I - CAC / *Eco*RI -ACA	12	12	100	0.25	25
*Mse*I - CAG / *Eco*RI -AAC	51	50	98.0	0.61	59.8
*Mse*I - CAT / *Eco*RI -ACC	81	78	96.3	0.29	27.9
Total	**147**	**143**	**97.27**	**-**	**-**
Average	**36.75**	**35.75**	**-**	**-**	**35.18**
Range	**3-81**	**3-78**	**96.3-00**	**0.25-.61**	**25-59.8**
II. Z. moran					
*Mse*I - CAA / *Eco*RI -ACT	19	18	94.74	0.38	36
*Mse*I - CAC / *Eco*RI -ACA	8	8	100	0.39	39
*Mse*I - CAG / *Eco*RI -AAC	20	19	95	0.319	30.30
*Mse*I - CAT / *Eco*RI -ACC	34	32	94.12	0.3	28.23
Total	**81**	**77**	**95.06**	**-**	**-**
Average	**20.25**	**19.25**	**-**	**-**	**-**
Range	**8-34**	**8-32**	**94.1-100**	**0.3-0.39**	**28.2-39**

POL - Polymorphism
PIC - Average polymorphic information content for polymorphic bands
MI - Marker Index = POL (%) x PIC

CAC /*Eco*RI - ACA) to 32 (*Msel*- CAT /*Eco*RI – ACC). The PIC values were found to be maximum of 0.39 and a minimum of 0.3 with the primer combinations *Msel*- CAC / *Eco*RI - ACA and *Msel*- CAT / *Eco*RI - ACC respectively. Comparatively higher MI values were obtained for AFLP primers ranging from 39 (*Msel*- CAC / *Eco*RI – ACA) to 28.23 (*Msel*- CAT / *Eco*RI – ACC).

The three marker systems utilized to trace the genetic relationships and diversity among the nine *Curcuma* species and ten *Z. moran* ecotypes revealed a considerably higher level of polymorphism. All the three markers yielded considerable number of polymorphic bands at their discriminating pace and thereby were efficient enough to

resolve the species and ecotypes genetically. The details of amplification products, polymorphic fragments generated, polymorphism information content (PIC) and marker index (MI) values for each of the three primer sets are showed in Table 3.5, 3.6 and 3.7.

3.4.3 Gene Diversity

In this study, relatively higher level of polymorphism and genetic diversity among the nine *Curcuma* species was revealed by RAPD, ISSR and AFLP markers. Screening genetic diversity at the interspecific level, the average values of n_a, n_e, h were ranged from 1.93-1.99, 1.37-1.62 and 0.24-0.36 respectively (Table 3.8). The mean Shannon's indexes (I) for *Curcuma* species were 0.53, 0.50 and 0.38 based on RAPD, ISSR and AFLP respectively. Considerable amount of polymorphism and genetic variance were detected among the ten *Z. moran* ecotypes at the intraspecific level. The ecotypes of different sources were screened using RAPD, ISSR and AFLP markers which revealed the average values of observed alleles (n_a), effective alleles (n_e), Nei's gene diversity index (h) and Shannon's indexes (I) from 1.34-1.95, 1.25-1.60, 0.26-1.44 and 0.20-0.49 respectively (Table 3.8).

3.4.4 UPGMA clustering and PCA analysis

Based on the RAPD, ISSR and AFLP analyses, the similarity coefficient among all the species of *Curcuma* were calculated. The genetic similarity value derived from the RAPD data ranged from 0.098 between *C. longa* and *C. zedoaria* to 0.806 between *C. domestica I* and *C. domestica II.* The genetic similarity coefficients for all the nine species of *Curcuma* using ISSR markers showed a wide range from 0.278 (between *C. angustifolia* and *C. caesia)* to 0.756 (between *C. aromatica* and *C. longa).* Similarly, for AFLP, the lowest genetic similarity coefficient was for the pair of *C. angustifolia* and *C. longa* (0.04) while the highest value (0.648) was calculated for the pair of *Curcuma* species (*C. amada* and *C. zedoaria*), indicating a broad genetic basis.

In the present study, the genetic relationships among 9 species of *Curcuma* were analyzed by 12 RAPD, 19 ISSR and 4 AFLP primer combinations on the basis of Dice genetic distance (Dice 1945). Resulting clusters were expressed as UPGMA dendrograms constructed using SHAN neighbor-joining tree separately for each molecular marker used

Table 3.8: Genetic diversity parameters in 9 *Curcuma* species and 10 ecotypes of *Z. moran*

Parameters	Values					
	Curcuma			*Z. moran*		
	RAPD	**ISSR**	**AFLP**	**RAPD**	**ISSR**	**AFLP**
Number of observed alleles, n_a	1.93±0.24	1.99±0.12	1.97±0.16	1.63±0.48	1.34±0.48	1.95±0.21
Mean number of effective alleles, n_e	1.62±0.31	1.5701±0.30	1.37±0.30	1.46±0.43	1.25±0.36	1.60±0.37
Mean Nei's gene diversity index, h	0.36±0.14	0.34±0.13	0.24±0.15	0.26±0.22	1.44±0.20	0.33±0.17
Shannon index, I	0.53±0.19	0.51±0.17	0.38±0.19	0.37±0.31	0.20±0.29	0.49±0.22

Each value= mean ± SD

The coefficients on the x-axis represent the similarity indices (DICE) of the different species chosen for the study. Based on UPGMA clustering algorithm from RAPD, the genotypes were grouped into two major clusters at a similarity index value of 0.20 (Figure 3.6A). *C. amada* and *C. zedoaria* were the two extremes in the dendrogram. Cluster I consisted of only one group having *C. caesia* and *C. zedoaria*. Cluster II consists of individuals belonging to domestic species along with *C. angustifolia* and *C. aromatica.* Within cluster II, three subgroups were evident, one containing *C. amada* and *C. spp*, while subgroup 2 included *C. angustifolia* and *C. longa.* In subgroup 3, *C. aromatica* was placed with the domestic varieties *C. domestica I* and *II.* Similarly, the dendrogram obtained from ISSR profiles showed two distinct groups for nine species of *Curcuma* studied at a similarity index value of 0.34 (Figure 3.6B), placing *C. caesia* in one and the rest in the second cluster. Cluster II again formed three distinct subgroups where *C. amada* and *C. zedoaria* are found to form subgroup 1 separately with *C. angustifolia.* The cultivated species are grouped with *C. aromatica* and *C. spp.* in subgroups 2 and 3 respectively. The dendrogram prepared based on AFLP analysis for studied *Curcuma* species also formed two clusters showing higher level of diversity. The AFLP discriminated most genotypes and grouped individuals together though belonging

to two different species (such as *C. amada* and *C. caesia*) (Figure 3.6C). *C. angustifolia* alone formed cluster I. In cluster II, cultivated species are grouped together along with other closer species. *C. amada, C. zedoaria* and *C. spp* were placed in subgroup 1, where as *C. longa* and *C. domestica I* was placed in subgroup 2. In subgroup 3, *C. domestica II*, *C. aromatica* and *C. caesia* grouped separately.

The dendrograms based on three markers data were basically same with minor changes showing inter-specific differences compared to intra-varietal ones. The adjoined group of cultivated varieties with wild ones suggests that they have been evolved in course of time. *C. domestica* I, II and *C. aromatica* have physiological similarity of strong aroma and were also sub-grouped together in the RAPD dendrogram having least genetic distance. Coinciding with the results of RAPD, the clusters based on ISSR analysis divides the *Curcuma* species at their genetic distances segregating them more precisely. Different hierarchical positions of the nine *Curcuma* species in the dendrograms elucidated that genomes of each species are not exactly the same. AFLP markers separated the three varieties of a single species (*C. longa, C. domestica* I & II) in two subgroups depicting that intraspecific variations also exists (Figure 3.6). Dendrogram revealed that the species that are the derivatives of genetically similar type clustered more together. Similar findings were reported by Vanijajiva et al. (2005) in a genetic relationship study among *Boesenbergia* and related genera.

The separation approach as revealed by the Mantel test comparing the results of RAPD, ISSR and AFLP, indicated a significant correlation within the nine different *Curcuma* species. The cophenetic correlation coefficient between dendrogram and the original similarity matrix were also significant for RAPD (r = 0.96), ISSR (r = 0.94) and AFLP (r = 0.97) supporting a good degree of confidence in the association obtained for the nine species of *Curcuma*. Principal Coordinate Analysis (PCA) derived on the basis of RAPD data illustrated that the first three principal coordinate components accounted for 24.99 %, 21.45 % and 13.78 % variation respectively among the *Curcuma* species. ISSR and AFLP marker based PCA maps showed the three coordinates of 25.77 %, 17.36 % and 11.91 % (for ISSR) and 32.17 %, 14.25 % and 11.64 % (for AFLP) of the total

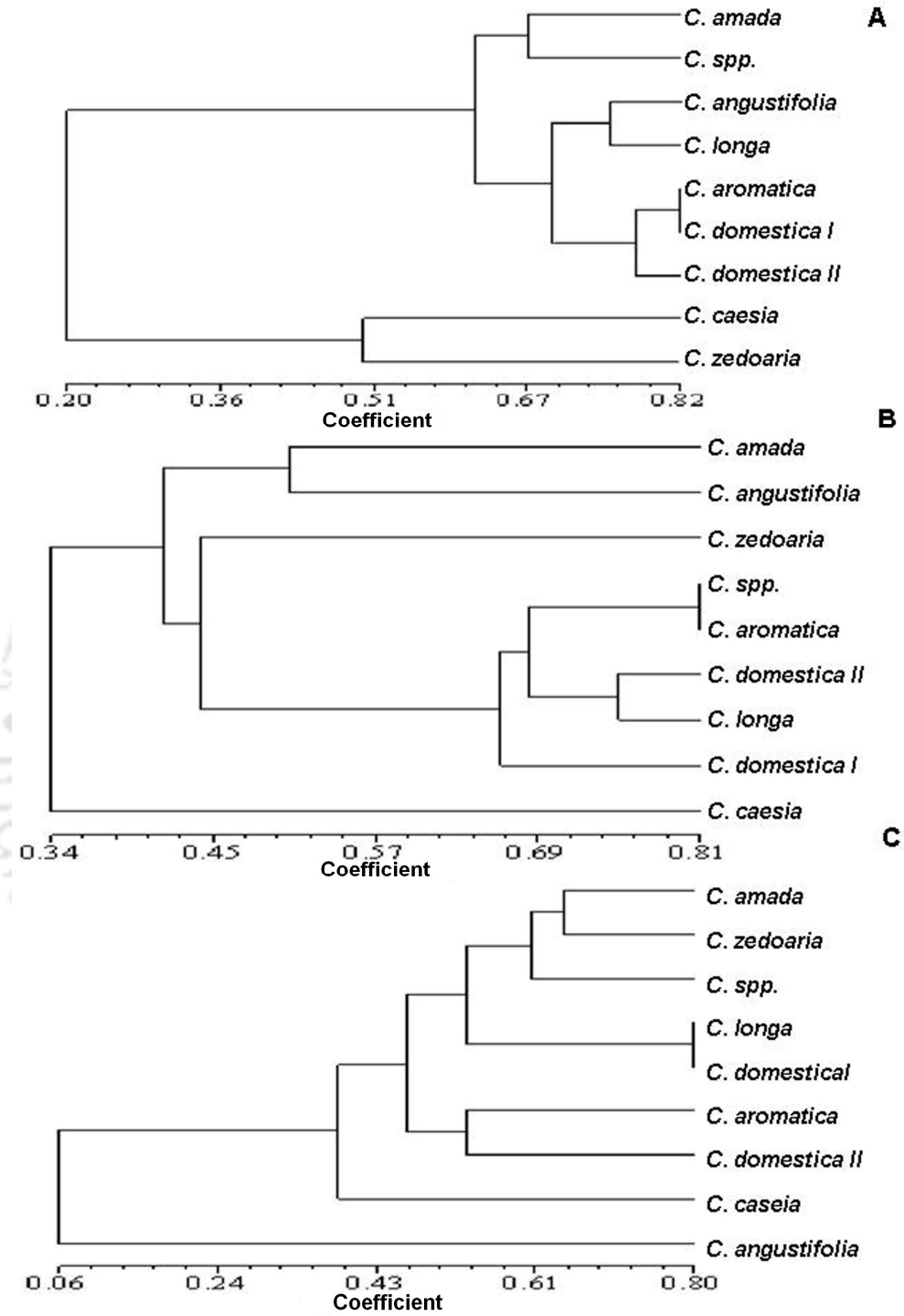


Figure 3.6: Dendrograms indicating the phylogenetic relationship of 9 *Curcuma* species from NE India, as revealed by UPGMA cluster analysis using SHAN neighbor-joining tree based on polymorphic markers. The genetic distances were from Dice similarity coefficient. (**A**)RAPD; (**B**) ISSR; (**C**) AFLP

variance respectively. Thus the first three most informative coordinates accounted for 60.22, 55.04, and 58.06 % of the genetic similarity variance based on RAPD, ISSR and AFLP markers. PCA showed the multidimensional relationships that describe portions of the genetic variance in a data set for *Curcuma* species (Figure 3.7). It was evident from the 3D plot that the species from hilly areas fall close to each other genetically. This analysis allowed understanding the spatial distribution of studied species and broadly classifies them. Similar result was reported by Islam et al. (2005) in intraspecific variation study of *C. zedoaria*. From PCA analysis it is evident that both the methods, phenogram and three dimensional plots of PCA were effective in studying genetic relationships and the groups found were comparable.

Dendrograms based on the UPGMA clustering of the three marker data, showed similarity among all the ten *Z. moran* ecotypes. In case of RAPD, the genetic similarity value showed a wide range from 0.46 (between ZM 08 and ZM 07) to 0.93 (between ZM 02 and ZM 03). In contrast, the genetic similarity coefficients derived from the ISSR data ranged from 0.61 (between ZM 06, 07 and ZM 08*)* to 0.92 (between ZM 09 and ZM 10). Whereas for AFLP, the genetic similarity coefficient varied significantly from 0.25 to 0.88 (ZM 08-ZM 10 and ZM 04-ZM05) respectively.

Dice genetic distance (Dice 1945) was used to analyze the genetic relationships among 10 different ecotypes of *Z. moran.* The three marker profiles broadly divided the ten ecotypes into two major clusters based on UPGMA clustering algorithm (Figure 3.8). In the RAPD based dendrogram, cluster I consisted the only ecotype ZM 08. Cluster II consists of individuals belonging to hilly habitats along with the ones belonging to the plains. Cluster II further formed three subgroups, one with ZM 04 and ZM 05, second included ZM 01, 02 and ZM 03. The subgroup 3 comprised of individuals collected from plains ZM 06, 07, 09 and ZM 10. The ISSR based dendrogram also divided ecotypes into two distinct groups at a similarity index of 0.61, placing ZM 06 and ZM 07 in one and the rest in the second cluster. Within the second cluster three distinct subgroups were formed with ZM 08 alone forming subgroup 1. The hilly ecotypes were grouped together in subgroups 2 and 3 respectively. Dendrogram generated from AFLP profiles formed the two principal clusters at a similarity index of 0.25. The ecotypes were discriminated at a higher level of diversity and most were grouped together belonging to similar geographic

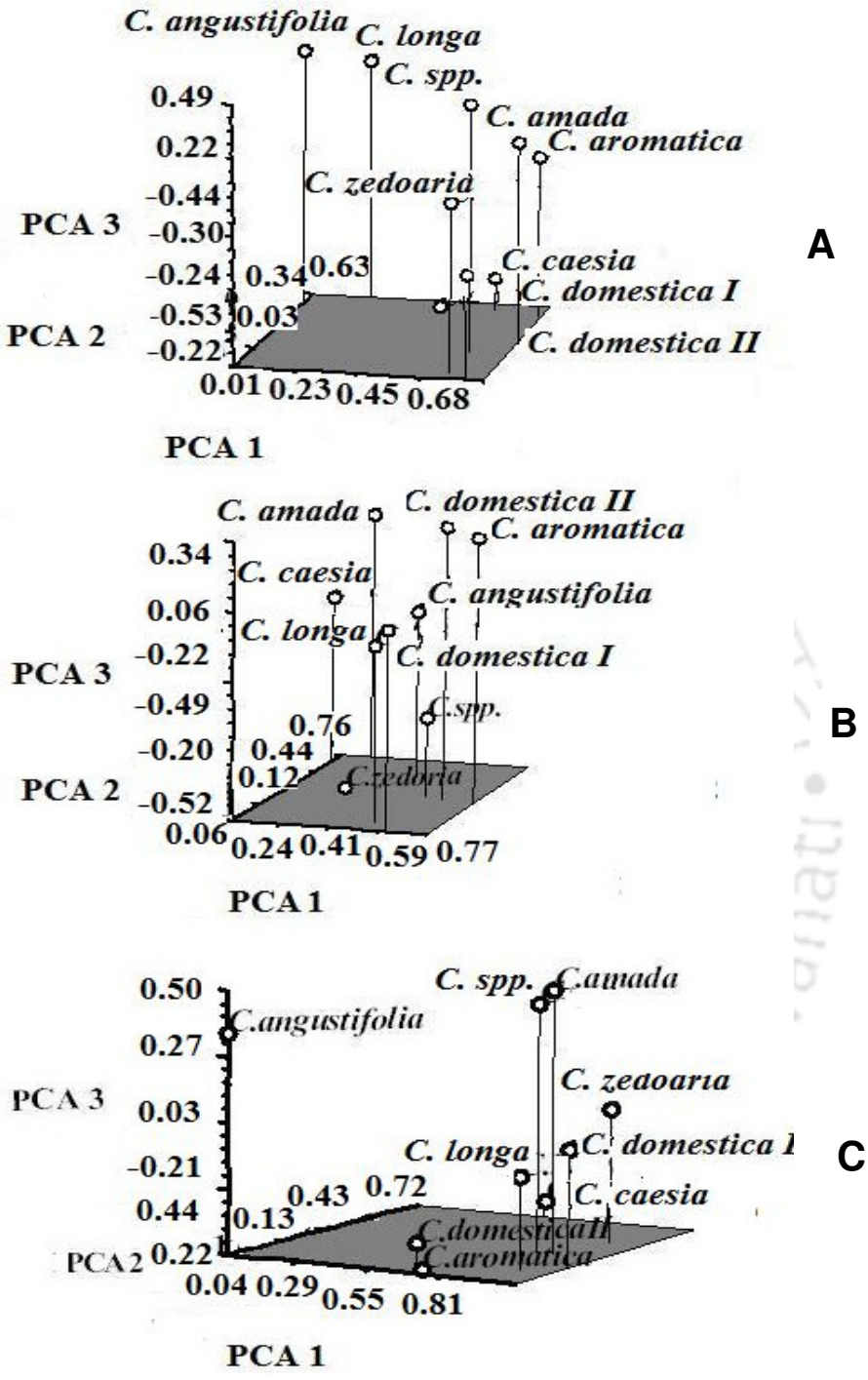


Figure 3.7: Principle coordinate analysis showing grouping of 9 *Curcuma* species for the first, second and third principle coordinate estimated for RAPD (**A**), ISSR (**B**), and AFLP (**C**) markers

background. Like in RAPD, ZM 08 alone formed cluster I. In cluster II, ecotypes from elevation topography were grouped together along with other closer ecotypes. In subgroup 1, ZM 02 and ZM 03 were placed where as ZM 04, 05 and ZM 06 was placed in subgroup 2. Whereas ZM 01, ZM 07, ZM 09 and ZM 10 were grouped separately in the third subgroup. Dendrogram constructed on AFLP data, depicted a high level of genetic polymorphism among the ecotypes. It was clear that AFLP technique was able to differentiate *Z. moran* ecotypes under study by a higher number of unique markers compared to RAPD and ISSR techniques. This supports the findings of Kavitha et al. (2010) where intraspecific genetic diversity is studied for four *Zingiber* species using AFLP markers and found striking differences among the four species from Western Ghats. The two distinct clusters formed by AFLP analysis, grouped the ecotypes in accordance with their genetic relatedness. The first cluster formed of ZM 08 alone depicting its isolated geographic status. The second cluster contained the other ecotypes where three subgroups were formed. Species sharing a common ecological niche were found to be in close proximity genetically. Ecotypes from hills of Arunachal ZM 04 and ZM 05 showed genetic similarity with ZM 06 which was collected from Jagun, a site near Assam-Arunachal border, which might have moved to the plains by ecological drift.

With the three sets of markers used in the present study the relatively higher level of polymorphism (63-95 %) between the *Z. moran* ecotypes was revealed by the three markers of which AFLP markers showed highest polymorphic ability per primer (94-100 %) indicating its potential in detecting intraspecific variations. In contrast, ISSR markers were less informative in characterizing the *Z. moran* ecotypes. However it was not significant because only one system of marker is not enough to reflect the complete diversity profile of any plant species, it does yield some estimate of the diversity that can provide the basis for future in-depth studies using different molecular approaches. Out of 10 RAPD markers used, 3 produced 100% polymorphism while 5 of them produced more than 50% polymorphism among *Z. moran* ecotypes. Similar results were reported by Nayak et al. (2005) in *Curcuma longa* and Saowaluck and Paisooksantivatana (2010) in *Zingiber montenum* ecotypes. The total informative bands amplified by AFLP (19.25 /primer) was considerably higher than those by RAPD (2.4) and ISSR (1.8) respectively (Tables 3A and 3B). There was a close correspondence between the similarity matrices of

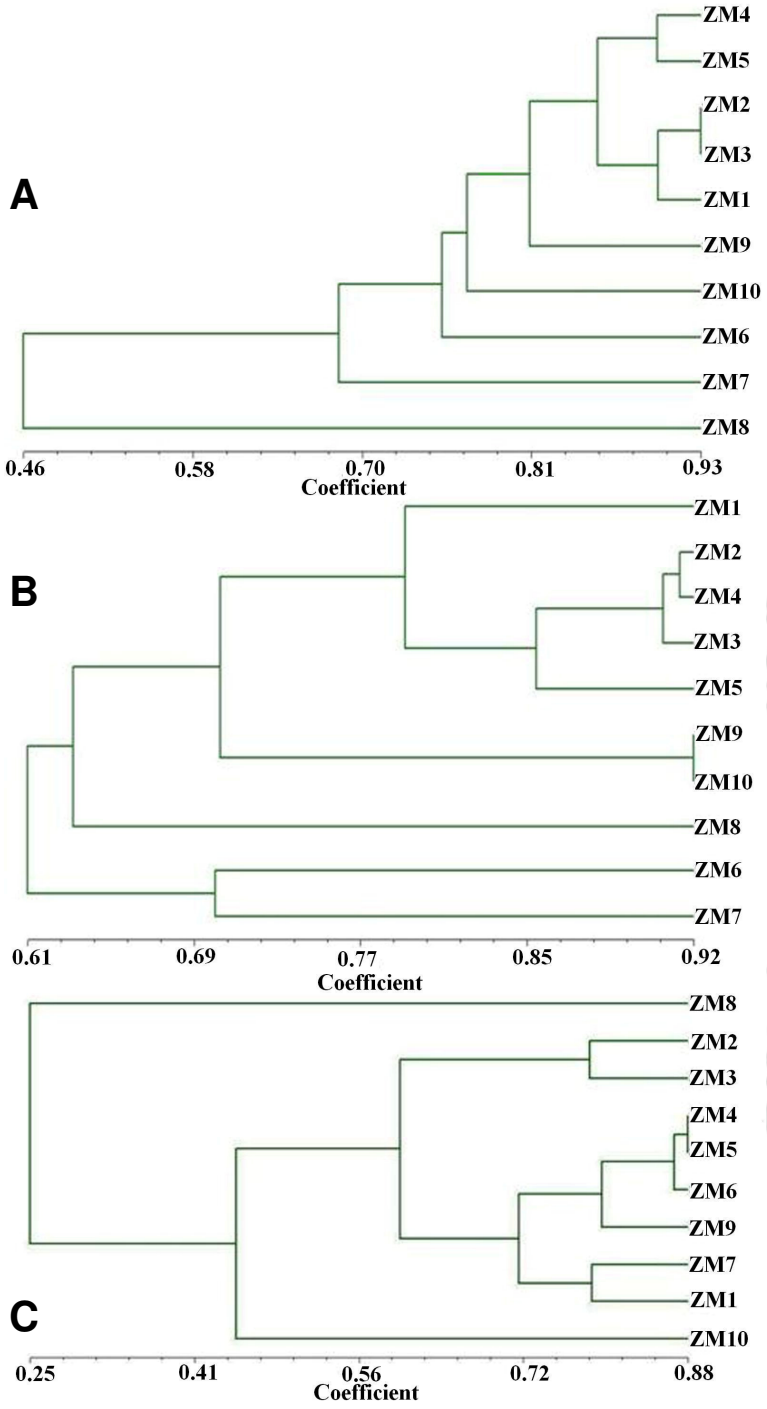


Figure 3.8: Dendrograms indicating the phylogenetic relationship among 10 *Z. moran* ecotypes from NE India, as revealed by UPGMA cluster analysis using SHAN neighbor-joining tree based on polymorphic markers. The genetic distances were from Dice similarity coefficient. (**A**) RAPD; (**B**) ISSR; (**C**) AFLP

RAPD, ISSR and AFLP established by means of high matrix correlation value of 0.901, 0.856 and 0.914 respectively. The average MI values (Tables 3.6 and 3.7) derived from the three marker sets also differed greatly indicating that they vary in their discriminating power (AFLP>RAPD>ISSR). Comparatively high MI (33.38) derived from AFLP system was mainly due to the higher number of polymorphic bands (77) in comparison with RAPD (24) and ISSR (18). This indicated that AFLP markers can probe a large number of informative loci which have been reported in many other plants (Albert et al. 2003; Martin et al. 2006; Kunkeaw et al. 2009; Kesari et al. 2010; Das et al. 2011).

Thus, the 3 marker-systems divided the *Z. moran* ecotypes into two distinct clusters. The ecotypes belonging to the different states show relatively low similarity. The ecotypes ZM 02 and ZM 03 showed higher similarity in their molecular data which were collected from the similar geographic location. Similarly, ZM 04 and ZM 05 possess maximum genetic similarity in all three dendrograms. These two ecotypes from Arunachal Pradesh also share a morphological resemblance in rhizome traits. ZM 08 was found to form a single group in all three dendrograms, which supports the geographic isolation of the ecotype collected from the hills of Nagaland. The lack of gene flow and the effect of genetic drift due to small population size might have caused the ecotype to differentiate genetically from other mainland ecotypes of *Z. moran.*

The discrimination pattern revealed by the three marker systems, based on Mantel test showed a significant correlation within the ten *Z. moran* ecotypes. The ecotypes of hilly areas produced a mixed distribution pattern that did not allow discrimination among them whereas the ecotypes of plain land were very distinctly discriminated in the 3D plot (Figure 3.9). Also significant cophenetic correlation coefficients were found between dendrograms and the original similarity matrices as r = 0.90 (RAPD), r = 0.85 (ISSR) and r = 0.91 (AFLP). This illustrated a good degree of confidence in the association obtained for the ten ecotypes under study. Principal Coordinate Analysis (PCA) derived on the basis of RAPD, ISSR and AFLP data illustrated the multidimensional relationships describing the genetic diversity among the *Z. moran* ecotypes (Figure 3.9 A, B & C). The results of Mantel tests performed are also in good agreement with the cluster analysis. PCA established the association among the ten ecotypes of *Z. moran* placing them in distinct groups which were congruent with that of cluster analysis. Ecotype ZM 08 that

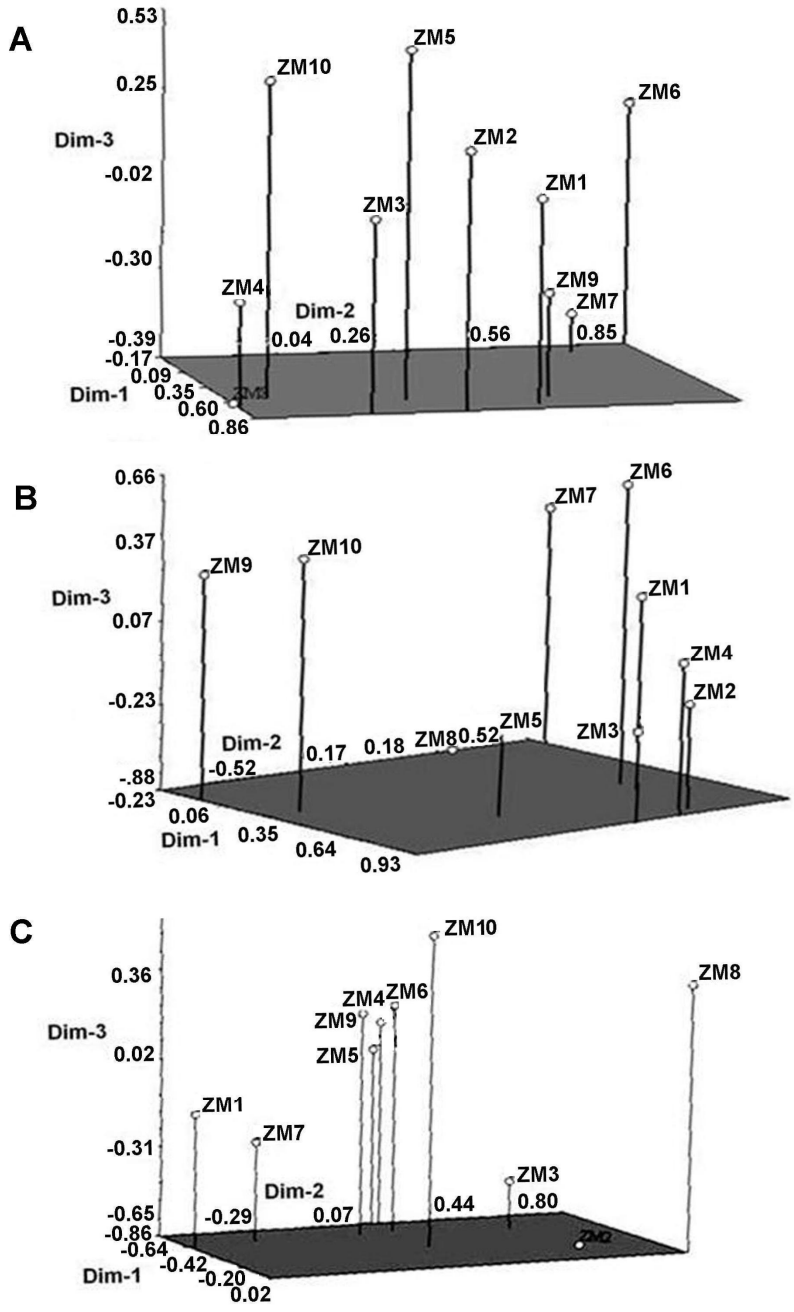


Figure 3.9: Principle coordinate analysis showing grouping of 10 ecotypes of *Z. moran* for the first, second and third principle coordinate estimated for RAPD (**A**), ISSR (**B**), and AFLP (**C**) markers

was clearly distinct from the other ecotypes in the PCA, confirmed its genetic distinctness.

Different criteria and statistical approaches in diversity studies are generally used to obtain a reliable and true picture of genetic diversity (Jatoi et al. 2008). Estimates of genetic diversity, similarity coefficients and allele amplification are good indicators that reflect a similar diversity pattern of the germplasm under investigation. The results obtained from the current study, clearly reveal that genetic variance exists among species and within the ecotypes that appear to be the same species from morphological point of view. Similar observations were noted by some workers where considerable genetic variance occurred within ecotypes (Hangelbroek et al. 2002; Haldimann et al. 2003; Jatoi et al. 2008). It signifies the importance of collection sources for capturing genetic variability. This is the first attempt to study *Z. moran* endemic species from NE India that are extremely valuable source of medicinal aspects. Molecular marker based genetic variability studies are reported in a few members of Zingiberaceae by earlier workers (Shyamkumar and Sasikumar 2007; Hussain et al. 2008; Saowaluck and Paisooksantivatana 2010). But use of three sets of marker system to study the genetic variances existing in the members of Zingiberaceae is scarce (Das et al. 2011). *Z. moran* is a promising species and requires extensive study which would help to devise effective strategies for *in situ* conservation and utilization of the species.

3.4 Conclusion

Finally, a conclusion can be drawn that, employing molecular marker based study of genetic variations facilitates in the delineation of *Curcuma* and *Zingiber* species in dendrograms which are suggestive of an evolutionary pattern among these wild species. The results provided an insight into the phylogenetic relationship between cultivated and wild relatives of *Curcuma* as well as among the *Z. moran* ecotypes. This information has a pivotal role in successful utilization of such wild genetic resources since each of them has significant value in traditional system of medicine and other uses. On the other hand, the degree of genetic diversity found within the species *Z. moran* may open possibilities for conservation, utilization and scientific research of this elite germplasm. Unfortunately, this species is already endangered and is limited to only a few districts of

Assam, Arunachal Pradesh and Nagaland. These approaches may definitely contribute to the application of sustainable management practices for these neglected species of NE India.

DNA markers have been valuable tools for the preservation and analysis of germplasm diversity (Hill et al. 1996; Yang et al. 2007). The present study clearly state the feasibility of the PCR based marker systems in detecting genetic variation at both inter and intraspecific level. Moreover, the results also demonstrate the usefulness, limitations and resolution power of the three types of molecular markers used for analyzing the *Curcuma* and *Z. moran* genomes. ISSR markers are more discriminating than RAPD and AFLP to evaluate the genetic relationship among *Curcuma* species from the rich flora of NE India. Although RAPD markers are very quick and easy to develop but reproducibility is less than ISSR which detect at predetermined sites, such as DNA repetitive regions of the genome which are known as fast evolving sequences. ISSR fingerprinting opens new and interesting possibilities in the characterization of the C*urcuma* plants especially from NE region of India which still awaits proper systematic identification. The results obtained from the current study reveal that 10 ecotypes of *Z. moran*, inhabiting in the few parts of NE India, exhibited variations in their DNA fingerprinting profiles for each of the three different DNA markers, although these have been considered as the one variety of the same species and found to share the same ecological niche. The diversity assessment conducted herein provides additional markers for the establishment of a broad based descriptive marker array (RAPD, ISSR and AFLP) for improved germplasm curation and the identification of germplasm for genetic mapping and other improvement strategies of these genera under study. The interspecific and intraspecific genetic diversity found in *Curcuma* species and *Z. moran* is of utmost importance as the two genera have immense pharmacological importance, yet genetically uncharacterized. Among the three marker systems tested, AFLP proved to be advantageous in predicting intraspecific variance as it samples a large number of informative fragments with random genomic origin.

According to Strauss et al. (1992), molecular markers are usually considered selectively neutral and thus do not necessarily reflect the diversity in functional characters (Karhu et al. 1996; Hintum van and Treuren van 2002). The genetic variability

of plants emerges from interaction of mutation, selection, random genetic drift and differential migration, geographical and reproductive isolation, topographic conditions etc. (Kumar et al. 2009; Tripathi et al. 2007). RAPD markers proved to be useful in discriminating among closely related taxa (Kumar et al. 2009) which was again established in this study. RAPD markers can be used successfully for estimation of genetic relationships and eventually in characterization of *Curcuma* and *Zingiber* germplasm. Polymorphic information content (PIC) analysis is used to measure the informativeness of genetic marker which could be useful to evaluate markers and can be used for genetic linkage studies, mapping, phylogenetic analysis etc. (Anderson et al. 1993). The advantages of using three different marker systems are that they exhibit no plasticity, unlikely to be similar because of convergent evolution and they can generate information at many different loci (Kesari 2010). New technological developments have expanded the range of DNA polymorphisms assays for genetic mapping, marker assisted breeding, genome finger printing and investigating genetic relatedness (Rafalski et al. 1996). The dominant markers are able to detect multiple loci distributed throughout the genome. AFLP markers are advantageous as it can produce information about genetic relationship by using less primer combinations and also powerful in detecting genetic variation between individuals at intraspecific level (Thomas and Scoot 1993; Rongwen et al. 1995). An added advantage of AFLP-based DNA fingerprinting is its potential in revealing large genetic polymorphisms with a nearly complete coverage of the whole genome (Badr et al. 2000; Heun et al. 1997). In the current study, four sets of AFLP primers were sufficient to reproduce adequate number of polymorphic DNA fragments with regard to comparative and statistical evaluations. Moreover, polymorphism detection was also much more efficient due to amplification with greater number of loci. Therefore, genotype identification could be possible even with only one or two AFLP primer combinations, a situation which is not so easily achieved with other molecular marker techniques (Ozkan et al. 2005; Karudapuram and Larson 2005).

Current study is the first effort of molecular characterization with multiple molecular markers in species of *Curcuma* and ecotypes of *Z. moran* from NE India. Exploration and evaluation of diversity would be of great significance for *in situ* conservation of important *Curcuma* species as well as the endemic *Z. moran* especially

for their scientific and commercial programmes. Furthermore, the scientific data presented here indicates that the application of PCR based fingerprinting using whole DNA and arbitrary primers would provide a rapid and sensitive method for detection of genetic variations among the different species of these genera and also other genera of Zingiberaceae. Results of the study hold great possibilities for further research on *Z. moran* and other species from NE India bearing therapeutic significance leading to their conservation and optimum utilization for human benefit.

Chapter 4

Chapter 4

Antimicrobial spectrum of different solvent based extraction from rhizomes of species of the two genera *Curcuma* and *Zingiber*

4.1 Introduction

Traditional medicine based on herbal remedies has always played a key role in the health systems of many countries including India where the native folk have been exploiting a variety of herbals for effective curing of various ailments. The plant parts used, preparation, and administration of drugs vary from one place to other. Although the knowledge of herbal medicines is gradually perishing, however, some of the traditional tribal men are still practicing the art of herbal healing effectively. These plants are frequently used by the local inhabitants of that area for treatment of various diseases. Ethno-medicinal studies have offered immense scope and opportunities for the development of new drugs. Some modern drugs have been deducted from folklore and traditional medicines. Living close to nature, traditional societies have acquired unique knowledge about the use of wild flora and fauna, most of which are unknown to the people who live away from such natural ecosystem as forests. After years of observations and analysis, trials, error, experimentation or even use of intuitive methods the innovative member of human communities have selected and identified useful and harmful members of the flora and fauna. Such knowledge and practices/experience were subjected to further modification or enriched with new knowledge of practice by succeeding generations and become a part of the tradition, culture, art, belief, folklore and knowledge base of these traditional communities. The traditional knowledge, skill and practices thus developed are freely exchanged, cared for and nourished as a common property of the communities (Pushpangadan and Kumar 2005). The value and importance of traditional knowledge are now being increasingly acknowledged all over the world. The pharmaceutical industry continues to investigate and confirm the efficacy of many medicines and toxins used by traditional communities (Posey and Dutfield 1996). The forests have been the source of invaluable medicinal plants since the time man realized the preventive and curative properties of plants and started using them for human heath care.

India has one of world's richest medicinal plant heritages. The wealth is not only in terms of the number of unique species (6160) documented but also in terms of the tremendous depth of traditional knowledge on their use for human and livestock health (Muthusamy et al. 2009). The old traditional Indian Systems of Medicine (ISM), is one of the most ancient medicine practices known to the world, and derives maximum formulations from plants and plant extracts that exist in the forests. About 400 plants are used in regular production of Ayurvedic, Unani, Siddha and tribal medicine. About 75% are from tropical and 25% from temperate forests, 30% of preparations are derived from roots, 14 % bark, 16% whole plants, 5% flowers, 10% fruits, 6% leaves, 7% seeds, 3% wood, 4% rhizomes 6% stems and only less than 20% of the species used are cultivated (Anonymous 1997). Forest degradation processes adversely affect the resource base of medicinal plants. The rural poor, whose dependence on these products is very heavy, are the worst sufferers. The problems are compounded by market-demand driven harvesting without any concern for representation and conservation. In the process essential regenerative components of a plant like roots, tubers, fruits, seeds flowers and bark are indiscriminately collected, leading to degradation and depletion and even extinction of particular species. Due to this ruthless exploitation, many important medicinal plants species are becoming rare and some of them are critically endangered. It is estimated that 10% of all plant species are currently endangered in India (Pandey et al. 2005). The practice of ethnomedicine is an important vehicle for understanding indigenous societies and their relationships with nature (Anyinam 1995). According to the World Health Organization (WHO) as many as 80% of world's population depends today on traditional medicine for their primary health care needs (Azaizeh et al. 2003). Recent decades have seen significant changes occurring within several aspects of ethnomedicine as a result of environmental degradation and tremendous changes in modern, social, and economic systems (Anyinam, 1995). These factors in totality resulted in disappearance of ethnomedicinal plants at regional as well as global scale (Baillie et al. 2004). Ethnomedicines are of particular relevance in developing countries like India (Ali 1999; Jamir et al. 1999; Sharma et al. 2001; Buragohain 2008; Ignacimuthu et al. 2008), where modern health service is limited.

The emergence of multiple drug resistant bacteria (MDR) has become a major cause of failure of the treatment of infectious diseases (Mathias et al. 2000; Gibbons 2005). As a result, society is facing one of the most serious public health dilemmas over the emergence

of infectious bacteria displaying resistance against many and in some cases all, effective antibiotics (Kapil 2005). Much like the situation in human medicine, the use of antibiotics in agriculture, livestock and poultry has accelerated the development of antibiotic resistant strains of microbial pathogens, potentially complicating treatment for plants, animals and humans (Sorum and Abee-Lund 2002; White et al. 2002). Furthermore changing patterns of susceptibility and the availability of new antimicrobial agents demands continuous updating of knowledge concerning treatment of disease caused by such pathogens. Thus there comes the need of alternative strategies for the management of disease resistant bacteria. One of the possible strategies towards this objective involves the rational localization of bioactive phytochemicals which have antibacterial activity, may be one of the important approaches for the containment of antibiotic resistance (Gottlieb et al. 2002; Newman et al. 2000). Even today plants are the almost exclusive source of drugs for the majority of the world population. People in developing countries utilize traditional medicine for their primary health care needs (Palombo et al. 2001; Cowan 1999). The potential of higher plants as a source for new drugs is thus still largely unexplored (Dubey et al. 2004). This is also true in India and only a small percentage of plants of this region have been evaluated for antibacterial activity against human pathogens (Patwardhan et al. 2004; Kumar 2004). Thus considering the vast potentiality of plant as a source of new therapeutic agents, in this chapter a detailed investigation was conducted to test the efficacy of crude rhizome extracts of selected members of Zingiberaceae (wild and cultivated) collected from NE India against important human pathogenic bacteria and fungi.

4.2 Literature Review

Plants have been used medicinally in different countries and are a rich source of many potent and powerful drugs (Gislene et al. 2000; Srinivasan 2001). Medicinal herbs have always been used as traditional primary health care agents, especially in Asian countries, and over the last 20 years, there have been rapid changes in the popularity of the use of natural systems to maintain health and for alternative therapy in Western countries (Kirana et al. 2007). However, scientific studies on the use of most traditional medicinal plants have not been carried out to assure their efficacy and non-toxicity. Researchers from divergent fields are developing a keen interest in investigating plants with a new eye for their

antimicrobial usefulness and as an alternative source to existing drugs. Plants with their wide variety of chemical constituents offer a promising source of new antimicrobial agents with general as well as specific activity (Evans 1996). There are several reports on the presence of antimicrobial compounds in various plants (Ravindra et al. 2005; Prusti et al. 2008; Nair et al. 2005; Tushar et al. 2010). Although large number of plant species has been tested for antimicrobial properties, the vast majority of them have not been adequately evaluated (Srinivasan 2001; Jantan et al. 2003). Recently, there has been a renewed interest in plant essential oils and their antimicrobial activity due to the spread of antibiotic resistance (Gislene et al. 2000; Cavanagh 2007).

In a biodiversity rich country like India, over 4686 ecosystem specific species of plants are used by ethnic communalities for human and veterinary healthcare, across the various ecosystems from Ladakh from trans-Himalayas to the southern coastal tip of Kanyakumari and from the deserts of Rajasthan and Kachch to the hills of Northeast (Muthusamy et al. 2009). Across the codified medicinal systems of Ayurveda, Siddha, Unani and Sowa-rigpa, a recent study enumerates around 2400 unique species of plants that are fully documented in terms of their biological properties, actions and drug formulations for a range of health conditions (Muthusamy et al. 2009). Among those, plants belonging to the family Zingiberaceae occupy a prime position in virtue of their rich medicinal properties. All of the members of Zingiberaceae possess a rich amount of essential oils in their rhizomes, leaves and flowers or seeds as well. The family is distributed widely throughout the tropics, particularly in Southeast Asia. It is an important natural resource that provides many useful products for food, spices, medicines, dyes, perfume and aesthetics (Bhavnani and Ballow 2000; Jantan et al. 2003). The aromatic plants are known to be used traditionally by most of the Asian countries including India for various health aliments. Around 200 species of Zingiberaceae belonging to 20 different genera have been reported alone in India of which NE India houses a vast reservoir of these plants. Most of the members of Zingiberaceae are found here at wild states which are yet to be explored. In recent years, several reports have been published concerning the biological properties (antimicrobial, antioxidant, anticancer and a stimulated effect on the immune system) of Zingiberaceae plant extracts (Ekwenye and Elegalam 2005; Cavanagh 2007) containing many essential oils like terpenes, alcohols, ketones, flavanoids, carotenoids, gingeroles and phytoestrogens (Habash et al. 2000; Sacchetti et al. 2005). Less polar compounds like curcuminoids,

gingeroles etc. have been reported to have great antifungal, antioxidant, insecticidal and anti-inflammatory activities (Bhavnani and Ballow 2000).

The genus *Curcuma* is a perennial herb with simple and large leaves with oblong, ovate or cylindrical rhizomes and has about 70 species, of which *C. xanthorrhiza*, *C. zedoaria*, *C. aromatica*, *C. caesia* and *C. amada* are important members. The most common species of the genus is *C. longa* popularly known as haldi, turmeric or Indian saffron. The rhizome of the species is yellowish red in color with characteristic odor and slightly pungent bitter taste and is used as a household remedy as anti inflammatory (antiseptic and irritant). The genus *Curcuma* has been extensively worked on regarding antimicrobial and other biological properties (Negi et al. 1999; Yano et al. 2000; Kim et al. 2003; Matsuda et al. 2004; Sacchetti et al. 2005; Cousins et al. 2007; Policegoudra 2007). Curcumin (diferuloyl methane), the yellow pigment from the rhizomes of turmeric is getting much attention of cancer investigations because of its chemopreventive properties against human malignancies. Besides its daily dietary use, turmeric is used in Asian herbal medicines (Ayurveda) for skin and gut-diseases (Ramachandran and You 1999). Medical use of turmeric as a wound healing agent with anti-inflammatory, antitumor and anti-proliferative effects has been well recognized (Rao et al. 1995; Ramachandran and You 1999). *C. aromatica* and *C. zedoaria* rhizome essential oils were found to possess fair antifungal and insecticidal properties (Singh et al. 2002). *C. amada* also has a record of traditional use in medicine in diverse ethnic groups and is an ingredient in culinary preparations in the Indian subcontinent (Jatoi et al. 2007). The *C. amada* rhizome is large and branched, with a buff colored external surface, fresh color of rhizome is pale yellow with a brilliant fragrance of green mango. The rhizome is considered good as a stomachic because of its bitter, aromatic, cooling, astringent and carminative qualities (Anonymous 1997). Combined with other medicines, rhizomes are also used to improve blood qualities (Kapoor 1990). Mango ginger is used therapeutically as a carminative, stomachic, in treatment of piles, and topically for contusions and sprains (Rao et al. 1989;). Traditionally the mango ginger rhizome has been extensively used as appetizer, alexteric, antipyretic, aphrodisiac, laxative and in the ancient Indian system of medicine Ayurveda, to cure biliousness, itching, skin diseases, bronchitis, asthma hiccough and inflammation (Kiritikar and Basu 1984; Warrier et al. 1994). The antimicrobial activity of *C. amada* alone or in combination with ginger and turmeric was demonstrated earlier against some pathogenic bacteria by Dorman and Deans (2000). In

2007, a new antimicrobial compound was isolated and identified by Policegoudra et al. from *C. amada*.

Zingiber is another common genus which comprises the common ginger, *Z. officinale*. The rhizomes of the genus are larger, oblong, fibrous and whitish in color with strong pungent smell. Other members of this genus are *Z. zerumbet*, *Z. casuamonar*, *Z. wrayi* etc. with immense medicinal values. Almost all the plants in this genus contain active phenolic compounds in their rhizomes which possess numerous biological properties. Most studied species across the globe is the common ginger (*Z. officinale*). The underground rhizome of the plant has been used as a medicine in Asian, Indian and Arabic herbal traditions since ancient times (Altman and Marcussen 2001). Next to *Curcuma*, the genus *Zingiber* has been studied for its various medicinal values. It has been used extensively for thousands of years in Asian countries for headaches, nausea and common cold (Grant and Lutz 2000) and in Mediterranean and Western parts in herbal medicine practice for the treatment of arthritis, rheumatological conditions and muscular discomfort (Bordia et al. 1997; Langner et al. 1998). Reports on biological properties are available in *Z. officinale*, *Z. zerumbet, Z. casuamonar, Z. wrayi, Z. nimmonii* etc. (Jantan et al. 2003; Chairgulprasert et al. 2004; Sabulal et al. 2006; Ali et al. 2008; Akihiro et al. 2008). *Z. zerumbet* commonly known as shampoo ginger has also been known for it various therapeutic uses. It is a perennial herb with large rhizomes and characteristic odor. The rhizomes are widely used as a folk medicine for sprains, indigestion, cure of swelling, sores and loss of appetite, toothache and other aliments. The juice of the boiled rhizome is has also been used as a medicine for worm infestation in children (Bhuiyan et al. 2009). Recently, *Z. zerumbet* has received considerable attention because of the pharmacological significance of 'Zerumbone', the most abundant component of the rhizome oil. Zerumbone possesses striking anti-inflammatory and anti-HIV activities (Das et al.1997; Chien et al. 2008). *Z. zerumbet* is used to treat stomach-aches in Indonesian traditional medicine under the name of '*Jamu*' (Burkill 1966).

Like ginger and turmeric, other members of Zingiberaceae also have tremendous medicinal values which are not evaluated properly till date. In fact, many members are still not recognized taxonomically and scientifically but have been used traditionally for their medicinal virtue. Literature is available on many members of the family Zingiberaceae all over the world on their antiallergic, antioxidant, anti-scavenging and antimicrobial activities

(Sekiwa et al. 2000; Srinivasan 2001; Kumar et al. 2001; Hseih et al. 2001; Jirovetz et al. 2003; Burt 2004; Konning et al. 2004). In 2005, Khattak and coworkers have reported on antifungal effects of *Alpinia galanga* and *Curcuma longa* from Thailand. Tewtrakul and Subhandhirasakul (2007) studied antiallergic property of different Zingiberaceae members and found excellent results. *Alpinia* is another significant genus comprising many members with economic and medicinal value. Plants are mostly wild and some of them produce seeds. *A. nigra, A. galanga, A. bracteata* are some of the key species and biochemical studies are reportd on their medicinal use of rhizome essential oils (Qiao et al. 2007; Yang and Eilerman 1999). Ibrahim et al. (2009) investigated antimicrobial properties of *A. conchigera* rhizome oil against bacterial and fungal pathogens. *Kaempferia* stands a unique status in Indian system of traditional medicine since long back. Common species like *K. galanga, K. rotunda, K. pulchera, K. indica* etc. are with immense use in healthcare and treatments of various aliments (Sarma 2005; Tushar et al. 2010). In India investigations are reported on the antimicrobial and other biological activities of different Zingiberaceae members; viz. *Ammomum* (Sabulal et al. 2006), *Curcuma* (Srinivasan 2001; Kim et al. 2003), *Hedychium* (Gopanraj 2005; Parekh et al. 2010), *Kaempferia* (Jantan et al. 2003; Sukari 2008), *Zingiber* (Indu et al. 2006; Pushpangadan and Kumar 2005; Daswani et al. 2010; Indu and Menon 2010) etc. However no attempt has been made till date to study the wild and endemic members from NE India which holds immense hidden medicinal properties. This might be due to the inaccessible nature of habitats of these plants and also very short reproductive and vegetative phase during the monsoons which pose great difficulties in collection and identification as well.

Therefore, in this chapter the objectives were aimed to investigate the antimicrobial activity of the rhizome essential oil of selected Zingiberaceae members including the endemic species *Zingiber moran*, from NE India against common pathogenic bacteria and fungi. Also to evaluate the minimal inhibitory concentrations (MIC) of the rhizome essential oils in an attempt to contribute to the use of these plants as an alternative for microbial control and food preservation. The second objective was to compare the amount of oil yield and antimicrobial activity of the rhizome extracts derived using various polar and non polar solvents. This study is the first report on these important species of Zingiberaceae from NE India using different organic solvents, wide range of microbial agents and different techniques of evaluation.

Bacterial pathogens transmitted commonly through foods are responsible for a significant portion of food-related illnesses (Mead et al. 1999) and pose a high risk to public health. Pathogenic strains from *Campylobacter*, *Salmonella*, *Listeria* and *Escherichia coli* are well recognized as important food borne pathogens. Bacterial food borne illnesses are still a global health concern and present a continuous challenge for food safety. Assurance of food safety, and prevention and control of bacterial food borne diseases necessarily rely on the ability to detect the pathogens especially in a low number in foods. Increasing investigations on rapid, sensitive and specific detection of bacterial food borne pathogens have advanced the development of detection methods from conventional culture plating techniques to newer techniques. Raman spectroscopy is such a technique based on molecular vibrations and is emerging as an important nondestructive, noninvasive, analytical tool for biologic materials including whole bacteria (Naumann 2000) because of the high specificity and high resolution of vibrational spectra and weak background signal from the aqueous environment (Maquelin et al. 2002). A number of studies employing the Raman spectroscopic technique for the microbiologic analysis (detection, identification and characterization) of bacteria in forms of single cells, colonies or aqueous culture (after drying) have been reported (Kirschner et al. 2001; Grow et al. 2003; Zeiri et al. 2004). Researchers have examined the bactericidal activity of some metal nanoparticle using Raman spectroscopy and surface enhanced Raman spectroscopy (SERS) (Lindsay et al. 2007; Peter et al. 2002; Raffi et al. 2010). However, reports are scanty in evaluating antibacterial potential of biological samples like essential oils till date. Hence, another aim of the study was to characterize the rhizome essential oils of the selected gingers against Gram-positive and Gram-negative bacteria with the help of micro-Raman spectroscopy.

4.3 Materials and Methods

4.3.1 Plant material

The plant material used in this study was healthy and mature rhizomes from four different species of Zingiberaceae viz. *Z. moran*, *Z. zerumbet*, *C. amada*, and *C. longa* collected from different places of NE India during the month of May-July (Table 4.1). These plants were maintained at the departmental green house of the Indian Institute of Technology Guwahati (IITG) and botanical garden of Gauhati University (GU). The mature rhizomes of the

collected plants (around 1 kg each) were dried at 65 ^{o}C in an oven for approximately 4-5 days till the dry weight was constant, manually crushed and stored at 4 ^{o}C until all further use. All experiments were performed with the same dried material.

Table 4.1: Four selected Zingiberaceae species collected from NE India and their characteristics used in the study

Species	Tag no.	Collection Site	Latitude & altitude	Habitat	Rhizome character
C. amada	ZSD 01	Kamrup (Assam)	26° 11'N, 56 m	Hilly slopes and moist grasslands	Light yellow, creeping, soft, strong smell like raw mango
C. longa	ZSD 07	Kahikuchi (Assam)	26° 9'N, 55.5 m	Shady , moist places	Dark Yellow, small tuber, with characteristic smell
Z. moran	ZSH 02	Tizu (Nagaland)	27° 4'N, 900 m	Shady , moist, hilly places	Blackish white in color, much smaller in size, tuber has strong pungent odor
Z. zerumbet	ZSH 04	Kokrajhar (Assam)	26° 24'N, 65 m	Shady , hot and humid places	Yellowish in color, larger tubers with strong characteristic smell

4.3.2 Extraction of crude oil

The rhizome crude oil was extracted from 50 g of each of the oven-dried and coarsely powdered plant material following the Soxhlet extraction method as per the standards AOAC (American Oil Chemical Society) procedure (Arlington 1995) using different solvents viz, n-Hexane, petroleum ether, acetone, isopropanol and methanol at their boiling points. Extracted oils were subjected to simple distillation and evaporation under reduced pressure to remove the solvent. The crude oil was expressed as % vol wt^{-1} and was stored at $4^{o}C$ until further use (max. 2 months) for all chemical analysis and bioactivity studies. Moreover, essential oil of the rhizomes was also extracted by the process of hydro distillation using Clevenger apparatus. This part of oil was washed with anhydrous sodium-sulfate and stored at $4^{o}C$.

4.3.3 Antibacterial assay

4.3.3.1 Disc diffusion method

Bacterial cultures viz. *Listeria monocytogenes*, *Salmonella paratyphi*, *Yersinia enterocolitica* (MTCC 859), and *E. coli* ETEC (enterotoxic) were grown in nutrient agar media (stored slants maintained at 4-5 ^{o}C) were transferred to 10 ml nutrient broth and incubated overnight at 37 ^{o}C. A preculture was prepared by transferring 1 ml of this culture to 9 ml nutrient broth (Hi media) and incubated for 48 h. The cells were harvested by centrifugation (2795 *g* for 5 min), washed and suspended in saline. Inoculum (100 µl) was then spread onto the solidified agar plate. The assay was carried out by disc diffusion technique (Bauer et al. 1966). Observations for growth inhibition zone were recorded after 24 h.

4.3.3.2 Growth kinetics

The effect of the rhizome oil fractions were also examined by determining the growth kinetics of bacteria. Bacterial cultures under study were grown in liquid NB media for 12 h. After two subcultures, 100 µl of 6 h grown fresh culture was inoculated in 2 ml fresh liquid NB and treated with rhizome oil fractions at concentrations 10, 20, 30, 40 and 50 µg /ml. Cultures were incubated at 28 ^{o}C and 180 rpm in orbital shaking incubator for 24 hrs. Optical density (OD) measurements were taken at 600 nm using a UV-Vis

spectrophotometer (Carry 100) to monitor the bacterial concentration after 8h of growth and every two hours thereafter.

4.3.3.3 Viability assay

To investigate the effect of rhizome oils on viability of bacteria the three bacterial strains viz. *Listeria monocytogenes*, *Salmonella paratyphi*, and *Escherchia coli* ETEC (enterotoxic) were grown in liquid nutrient broth (NB) for 12 h. These were subcultured twice and 6 h grown fresh cultures were used for viability test. Aliquots of 100 µl of the cultures were subcultured in fresh NB media (2 ml) and treated with rhizome oils at various concentrations (10, 20, 30, 40, 50 µg /ml). Cultures were kept for incubation at 28 ^{o}C and 180 rpm in orbital shaking incubator for 4 hrs. After incubation, 100 µl of the growth mixtures (bacteria + rhizome oil + media) were inoculated on solid NB agar (2 %) plates and kept at 37 ^{o}C for 24 hrs. Control plate contained only inoculum without the test oil fractions. Bacterial growth was observed and numbers of colonies were counted after 12 h and every 2 h thereafter.

4.3.3.4 Raman spectroscopy

Antibacterial efficacy was also characterized by examining the characteristic Raman spectra of test bacteria both gram positive (*L. monocytogenes*) and gram negative (*E. coli*) after treating with the rhizome oil. Bacterial cultures raised from single colony were grown in nutrient broth for 12 h. After one subculture, the 6 h grown fresh culture were taken for experiment. The cultures were treated with the rhizome oil fractions (10 µg each) and kept for incubation at 28 ^{o}C and 180 rpm in an orbital shaker. The excitation wavelength for recording Raman spectra was 488 nm Argon ion laser. Readings were taken after 12 h of incubation from inoculation. Changes in the Raman shift of the bacteria were observed.

4.3.4 Antifungal assay

Fungal slant cultures viz. *Aspergillus niger* (NRRL 3), *A. niger* (NRRL 326) and *A. terreus* were maintained in potato dextrose agar (PDA) medium. Inoculum (300 µl, 10^6 spores ml^{-1}) from the spore suspension cultures of the different fungal strains was then spread onto the Czapek Dox Agar (CDA) plates. The assay was done out in the same way as described for bacterial cultures and observations were recorded after 48 h. Ampicillin (USB Amersham 50 mg ml^{-1}) and Co-trimoxazole (Hi-media, 100 µg $disc^{-1}$) were used as positive controls.

The results were represented as the diameter of zone of inhibition (ZOI) (mm) ± SD, excluding the disc diameter (4 mm). Minimal inhibitory concentration (MIC) of all the fractions was determined according to (Lennette et al. 1974). The different concentrations (25-100%) of rhizome oil fractions were obtained by diluting it in pure dimethyl sulphoxide (DMSO). The minimum inhibitory concentration (MIC) was recorded as the lowest dilution of the tested sample inhibiting the visible growth of the organism after 24-48 h for the bacterial and fungal cultures respectively on the agar plate. The response of the positive control (antibiotic) was treated as 100% inhibition and with respect to that the percentage of inhibition were calculated for each fraction.

4.3.5 Statistical Analysis

Each assay was performed three times and the results were expressed as their mean ± standard error. The data were analyzed by one way analysis of variance (ANOVA) using Instat 3.036 version (http://www.ssc.rdg.ac.uk/software/download.html). Differences between means were tested by LSD tests and were considered significance at $p < 0.05$.

4.4 Results and Discussion

4.4.1 Plant material

In view of the medicinal and economic values of the family Zingiberaceae, four different plants from two genera were selected for the study. The plants were collected during early monsoon (May-June) which was the only time for their true vegetative and reproductive growth. During collection, the rhizomes were immature and it was difficult to collect the mature rhizomes due to the long hibernation of the plants (September-May). Therefore, healthy mature rhizomes were collected during the late monsoon period (July-September) when the vegetative and reproductive stages are at its best. The rhizomes of *Z. moran* were darker than the common ginger (*Z. officinale*), smaller in size and had a much stronger ginger-like pungent aroma whereas that of *Z. zerumbet* were yellowish in color, large and soft compared to *Z. officinale* and *Z. moran*. Rhizomes of *C. longa* were orange-yellow and that of *C. amada* were light yellow in color. *C. longa* rhizomes had its characteristic turmeric smell while *C. amada* possessed a brilliant aroma of raw mango which persisted even after drying and grinding. After collection, rhizomes were washed in running tap-water for 2-4 hrs and then kept for drying. Dried rhizomes were crushed manually and the

powdered form was preserved at 4 ^{o}C. Vegetative plant body and rhizomes of the species under study are shown in the figure 4.1.

4.4.2 Oil extraction and yield

The highest importance of the studied genotypes lies in the fact that they have essential oils with potential medicinal values in rhizomes. Essential oils from many members of Zingiberaceae have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Burt 2004; Ali et al. 2008). Many of them are used in cancer treatment, food preservation, aromatherapy and fragrance industries (Afzal et al. 2001; Shukla and Singh 2007). Therefore, it is important to exploit the right oil extraction technique for the recovery of maximum oil from the mature rhizomes for optimum utilization. Essential oils are aromatic liquids obtained from plant materials. To obtain the essential oils from rhizomes, Soxhlet extraction process was used here with different organic solvents. In this extraction technique, some organic solvent of low boiling point is added to the plant material to extract oil and the solvent can be recovered during the process. It is costlier than the conventional method but is very easy in implementation.

For all the extractions, three rhizomes to solvent ratios viz. 1:5, 1:6 and 1:7 were tried of which, 1:6 (wt vol^{-1}) has been found to be as optimal ratio for getting higher yield in less time with good quality of oil. It was found that minimum time was required for extraction of oil using non polar solvents (n-Hexane and petroleum ether) whereas for polar solvents (acetone, isopropanol and methanol) the time taken for extraction was almost double for the same amount of the rhizome material (Table 4.2). The oils of all the four species were dark yellow in color with strong characteristic aroma of their own. The polar solvents yielded less viscous and more amount (2.8-8 ml) of oil than the non polar ones (1.2-2.8 ml). Similar results were also reported by earlier too (Sabulal et al. 2006; Norajit et al. 2007). However, the advantage of using organic solvents in extraction technique is that they can be recovered during the process. Whereas for extracting oil with hydrodistillation Clevenger apparatus was used which yielded lesser amount of oil compared to the solvent

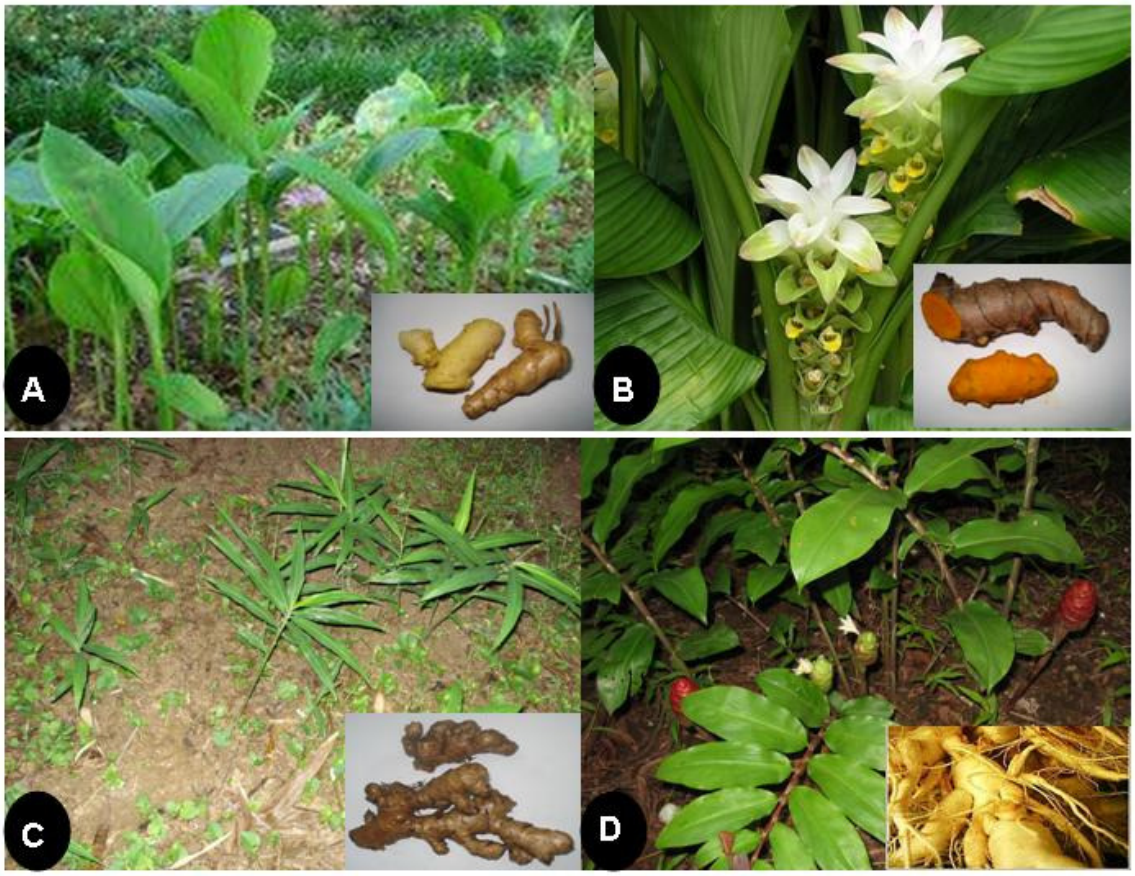


Figure 4.1: The Zingiberaceae species collected from Northeast India depicting their reproductive parts and rhizome.
A. *C. amada*; **B.** *C. longa*; **C.** *Z. moran;* **D.** *Z. zerumbet.*

Table 4.2: Effect of solvent in oil yield from rhizomes of *Curcuma* and *Zingiber*

Solvent	Initial volume (ml)	Average yield (ml)	Solvent recovery (ml)	Average yield (ml)	Solvent recovery (ml)
		Curcuma		*Zingiber*	
n-Hexane	300	2.8	260	1.6	240
Petroleum ether	300	2.5	275	1.2	220
Acetone	300	4.5	180	4.5	210
Isopropanol	300	5.0	230	3.2	180
Methanol	300	8.0	220	2.8	170
Water	-	2.0	-	3.0	-

extractions (2-3 ml per 50 g of rhizomes), however the oil was least viscous and colorless. The average yield from hydrodistillation was 2.5 ml for all four plants in the study. Further, this process is more advantageous as it is a continuous process, took lesser time and effort than Soxhlet extraction and also is cost effective as it requires only water.

4.4.3 Antibacterial and antifungal assay

Antimicrobial screening was carried out against various pathogenic bacteria and fungi with different fractions isolated from rhizome oils of four selected plants of Zingiberaceae at different concentrations. Interestingly, all the fractions were found to exhibit antimicrobial effect. The results of the antimicrobial activity of the extracts were qualitatively and quantitatively assessed by evaluating the presence of inhibition zones and zone diameter measurements. The inhibition rate was solely dependent on rate of diffusion. Diluted samples were noted to be more effective than the concentrated ones. Oil at (75%) in DMSO gave more inhibition than 100% rhizome oil. Hence, the antimicrobial activity was a linear

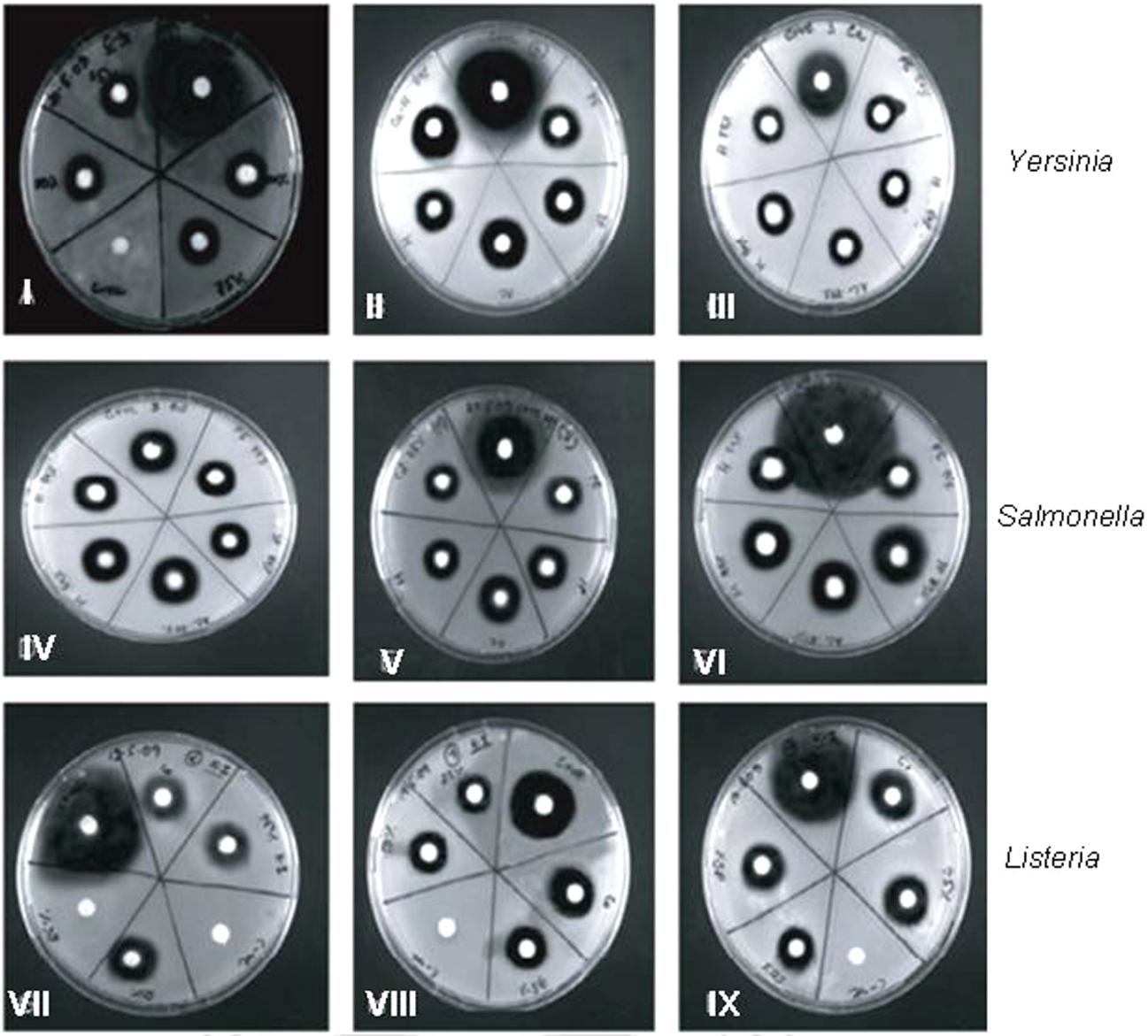


Figure 4.2: Zone of inhibitions as depicted by the antibacterial assay of rhizome oils of the selected species of the genera *Curcuma* and *Zingiber*.
Lane 1: Antibacterial effect of *C. amada* against *Yersinia* (I, II, III);
Lane 2: *Z. moran* against *Salmonella* (IV, V, VI) and
Lane 3: *Z. zerumbet* against *Listeria* (VII, VIII, IX).

function of concentration in all cases except the crude extract because of high viscosity. All of the four Zingiberaceae species studied showed antimicrobial activity against all test pathogens. *Z. moran* showed excellent antimicrobial activity at different concentrations tested against all the strains of bacteria and fungi used in present study. Among all the dilutions, 50% (10µg/ml) concentrated oils of *Z. moran*, *Z. zerumbet* and *C. amada* were found to be most effective against all the pathogens. None of the polar solvents showed the best response in 100% concentration against any of the pathogens tested, however the polar solvents (methanol, acetone and isopropanol) showed better inhibition than the non polar solvent extracts against all bacterial and fungal pathogens studied. Zone of inhibition by the action of crude rhizome oil against tested pathogenic bacteria is depicted in figure 4.2.

The results showed that the crude rhizome extracts from all the species of Zingiberaceae studied inhibited the growth of all the tested bacteria at a minimal concentration limit of 25% or 10µg/ml and maximum inhibition at 75% or 30µg/ml (Table 4.3A). All the extracts showed maximum inhibitory effect over the bacterial strain *Listeria monocytogenes*. This agrees with the observation found by Thongson et al. (2004) where Gram-positive *Listeria* were found more sensitive than the Gram-negative *Salmonella* to rhizome extracts of *Z. officinale* and *C. longa*. Polar (methanol and acetone) extracts of *Z. moran* showed the maximum zone of inhibition. Isopropanol and acetone extract of all the oil samples showed similar and comparatively greater inhibitory effect over the bacterial strains. The highest MIC value was observed in *Listeria* for petroleum ether extracts of *Z. zerumbet* and *C. amada* (100%), whereas it was lowest (25%) for all other strains.

The growth kinetics of the bacterial strains revealed a prominent decrease in optical densities of the bacterial cultures after treatment with the rhizome extracts. Figure 4.3 depicts the growth curves of the bacterial pathogens studied with the methanol and hydrodistilled fraction of rhizome oils. Similar results were obtained in the viability test carried out using bacteria with 10^6 CFU/ml. A drastic decrease in number of colonies was observed in all the four bacterial strains after treatment with the rhizome extracts (Figure 4.4) at their minimal inhibitory concentrations (10µg/ml) obtained from the growth kinetics assay. Hence, the rhizome oils of the four studied Zingiberaceae members are effective against the common pathogenic bacteria at a very low concentration of 10µg/ml. From the figures it is evident that the hydrodistilled fraction of rhizome oil of *Z. moran* has a much greater efficacy than the other fractions and species studied.

Table 4.3A: Antibacterial activity of four species of *Zingiberaceae* using different solvent extracts at varied concentrations

	Z. zerumbet			*Z. moran*			*C. amada*			*C. longa*		
Bacteria	**Conc. %**	**MIC %**	**Mean [a] ±SE**	**Conc. %**	**MIC %**	**Mean [a] ±SE**	**Conc. %**	**MIC %**	**Mean [a] ±SE**	**Conc. %**	**MIC %**	**Mean [a] ±SE**
Listeria	100	50	12.17± 0.3	50	25	15.3± 0.53	100	25	12.2± 0.51	75	25	14.3± 0.52
	25	25	12.4± 0.3	50	25	14.83± 0.53	25	25	12.4± 0.51	50	25	15.3± 0.52
	50	25	16± 0.3	50	25	21.2± 0.53	50	25	16± 0.51	50	25	12.5± 0.52
	50	25	12.5± 0.3	75	25	18.43± 0.53	50	25	14.5± 0.51	50	25	17.6± 0.52
	50	25	13.65± 0.3	50	25	10.33± 0.53	50	25	18.6± 0.51	50	25	16.5± 0.52
Yersinia	25	25	11.9± 0.36	25	25	14.07± 0.46	25	25	11.9± 0.36	75	25	11.2± 0.43
	25	25	11.8± 0.36	25	25	13± 0.46	25	25	11.8± 0.36	25	25	13.5± 0.43
	50	25	19.2± 0.36	50	25	11.93± 0.46	50	25	19.2± 0.36	50	25	14.2± 0.43
	50	25	13.2± 0.36	50	25	14.17± 0.46	50	25	21.1± 0.36	75	25	18± 0.43
	50	25	16.8± 0.36	50	25	18.13± 0.46	50	25	22.4± 0.36	50	25	19.3± 0.43
S. paratyphi	25	25	11.9± 0.33	75	25	18.57± 0.63	25	25	10± 0.32	50	25	13.33± 0.62
	50	25	12.27± 0.33	25	25	12.27± 0.63	50	25	13± 0.32	25	25	16.1± 0.62
	50	25	16.3± 0.33	50	25	13.33± 0.63	50	25	20.2± 0.32	50	25	17.3± 0.62
	50	25	14.5± 0.33	50	25	14.5± 0.63	50	25	14.5± 0.32	50	25	20.1± 0.62
	75	25	15.57± 0.33	50	25	18.23± 0.63	75	25	15.57± 0.32	25	25	19.4± 0.62
E. coli ETEC	50	25	12.07± 0.39	50	25	19.57± 0.57	50	25	12.07± 0.35	50	25	15.2± 0.33
	50	25	11.1± 0.39	50	25	10.9± 0.57	50	25	10.1± 0.35	50	25	10.02± 0.33
	50	25	11.87± 0.39	50	25	23.43± 0.57	50	25	11.87± 0.35	50	25	20.5± 0.33
	75	25	13± 0.39	75	50	18.9± 0.57	75	25	13± 0.35	50	25	17.9± 0.33
	50	25	11.7± 0.39	50	25	23.9± 0.57	50	25	15.87± 0.35	50	25	18.23± 0.33

[a]Zone of inhibition from oil and reference antibiotics are given as mean ± SE of three replicates.

Reference antibiotic Ampicillin (50 mg ml^{-1}) was taken for bacteria

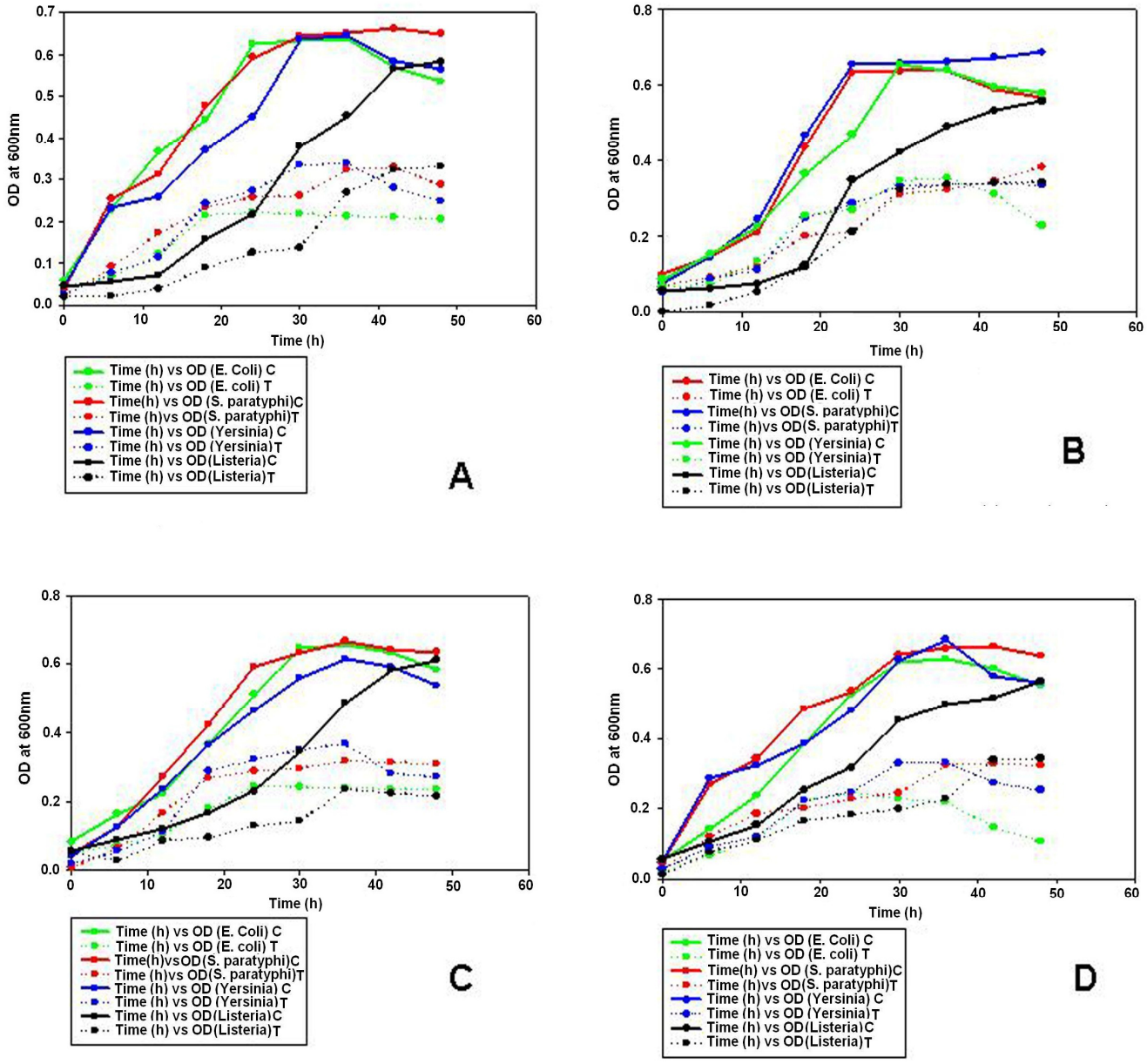


Figure 4.3: Effect of rhizome essential oils on Growth kinetics of the test bacteria as measured by Optical Density before and after treatment.
A. Antibacterial effect of *C. longa* acetone extract; **B.** Antibacterial effect of *C. amada* methanol extract; **C.** Antibacterial effect of *Z. zerumbet* isopropanol extract;
D. Antibacterial effect of *Z. moran* water extract.

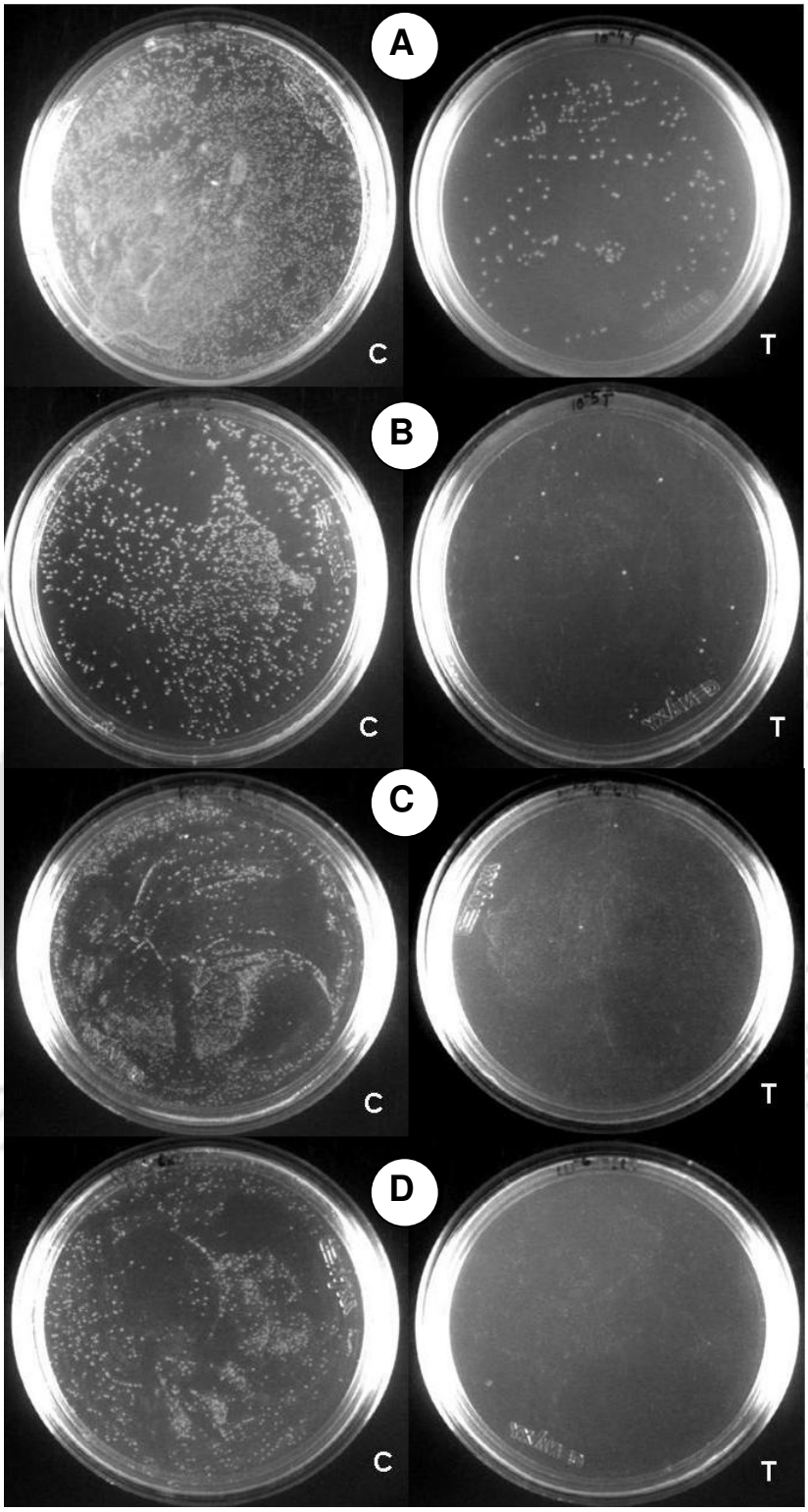


Figure 4.4: Viability assay of the test bacteria evaluating antibacterial potential of the selected rhizome extracts of *Curcuma* and *Zingiber* from NE India
Lane A. *C. amada*, **Lane B.** *C. longa*, **Lane C.** *Z. moran* **Lane D.** *Z. Zerumbet*

There are many reports on diverse use of Zingiberaceae members in traditional systems of medicine in treatment of various skin diseases, stomach disorders, cold and cough etc, (Indu et al. 2006; Sabulal et al. 2006; Tewtrakul and Subhadhirasakul 2007). Some of the members are also reported to possess antimicrobial property (Sabulal et al. 2006; Norajit et al. 2007). However many wild members of the family are still to work out which are equally important for their medicinal values and have been used extensively in traditional medicines by folk people. *Z. moran* and *Z. zerumbet* in current study are such wild relatives of ginger with great therapeutic significance. *C. amada* is another wild species found in NE India and are hence included in the current pharmacological study along with the domesticated variety *C. longa*.

Antifungal activities exhibited by essential oils of many Zingiberaceae against plant and human pathogenic indicators have been reported earlier (Norajit et al. 2007, Tewtrakul and Subhadhirasakul 2007). In this study, acetone and methanol extracts showed better results and petroleum ether extract showed minimum inhibitory activity against the pathogenic indicators (Table 4.3B). Similar findings were observed by Parekh and Chanda (2007) in *Trapa natans* L. Antifungal assay showed the maximum inhibition of 22 mm by *Z. moran* at 75% and a minimum of 6 mm by *Z. zerumbet* at 25% against all tested fungi (Figure 4.5). Rhizome oil of all species showed maximum antifungal activity against *A. niger* 326 followed by *A. niger* 3 and *A. terreus*.

The outcomes of the present research agree with the observations of earlier findings on biocidal effects of *Z. officinale* and *C. longa* rhizome extracts (Singh et al. 2002; Ali et al. 2008). Antioxidant and antimicrobial activity were tested on other gingers by various workers in different countries of Asia (Chien et al. 2008; Akihiro et al. 2008). An important characteristic of the essential oils and their compounds is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structure and rendering them more permeable (Sikkema et al. 1994). Extensive leakage from the bacterial cells or exit of critical molecules and ions lead to death (Danyer and Hugo 1991). Current findings support the fact showing excellent antibacterial effect from all the four rhizome essential oils. Many of the earlier studies against fungal pathogens were limiting as those covered only some important and domestic varieties of Zingiberaceae. Kim et al. (2003) reported on antifungal properties of *C. longa* against broad range of phytopathogenic fungi. Whereas Yin and Cheng (1998) showed that ginger had no

Table 4.3B: Antifungal activity of four species of *Zingiberaceae* using different solvent extracts at varied concentrations

	Z. zerumbet			*Z. moran*			*C. amada*			*C. longa*		
Fungi	**Conc. %**	**MIC %**	**Mean[a] ±SE**	**Conc. %**	**MIC %**	**Mean[a] ±SE**	**Conc. %**	**MIC %**	**Mean[a] ±SE**	**Conc. %**	**MIC %**	**Mean[a] ±SE**
A. niger NRRL 3	50	25	5.3± 0.46	50	25	6.6± 0.51	50	25	7.2± 0.87	100	50	9.2± 0.31
	50	50	6.5± 0.46	50	25	9.5± 0.51	50	25	8.2± 0.87	75	25	8± 0.31
	75	25	5.3± 0.46	50	50	9± 0.51	50	50	6.56± 0.87	75	50	5.5± 0.31
	50	25	6.6± 0.46	75	25	7.5± 0.51	50	25	8.56± 0.87	100	50	5.8± 0.31
	50	25	8.89± 0.46	75	25	10.3± 0.51	50	25	11.9± 0.87	75	25	12 ± 0.31
A. niger NRRL 326	50	50	4± 0.46	75	50	6.1± 0.51	50	50	7.2± 0.87	100	50	9.2± 0.31
	50	25	00± 0.46	50	50	9.5± 0.51	50	50	8.2± 0.87	75	25	8± 0.31
	75	50	5.3± 0.46	50	50	9± 0.51	50	25	6.56± 0.87	75	50	5.5± 0.31
	50	25	6.6± 0.46	75	25	7.5± 0.51	50	25	8.56± 0.87	100	25	5.8± 0.31
	50	25	8.89± 0.46	75	25	10.3± 0.51	50	25	11.9± 0.87	75	50	12 ± 0.31
A. terreus	-	-	00± 0.46	50	25	7± 0.51	50	50	7.2± 0.87	100	50	9.2± 0.31
	-	-	00± 0.46	50	50	9.5± 0.51	50	25	8.2± 0.87	75	50	8± 0.31
	75	50	5.3± 0.46	50	25	9± 0.51	50	50	6.56± 0.87	75	25	5.5± 0.31
	50	25	6.6± 0.46	75	25	7.5± 0.51	50	25	8.56± 0.87	100	25	5.8± 0.31
	50	25	8.89± 0.46	75	25	10.3± 0.51	50	25	11.9± 0.87	75	25	12 ± 0.31

[a]Zone of inhibition from oil and reference antibiotics are given as mean ± SE of three replicates.

Reference antibiotic Co-trimoxazole (100 µg $disc^{-1}$) was taken for fungus

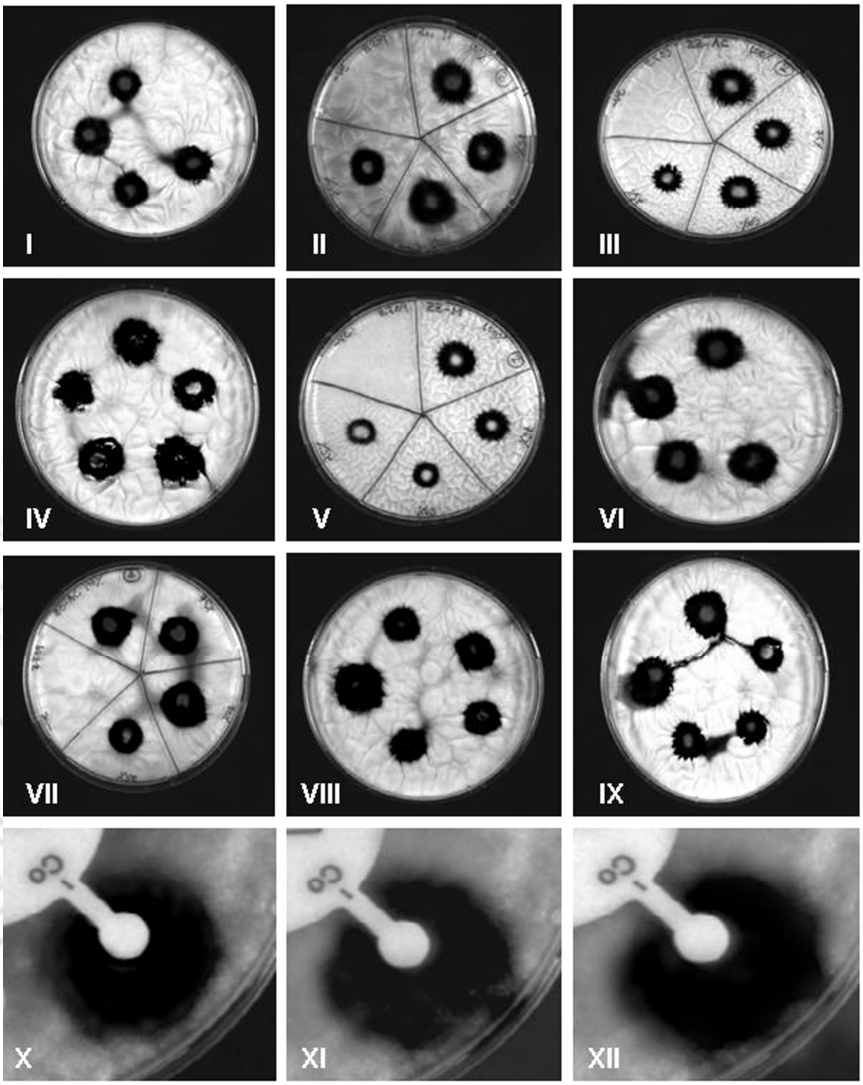


Figure 4.5: Zone of inhibitions as depicted by the antifungal assay of rhizome oils of the selected species of the genera *Curcuma* and *Zingiber*.
Lane 1: Effect of Z. *zerumbet* against *A. niger* 326 (I, II, III);
Lane 2: *Z. moran* against *A. niger* 3 (IV, V, VI);
Lane 3: *C. amada* against *A. niger* 326 (VII, VIII, IX) and
Lane 4: Reference antibiotic Co-trimoxazole (100 µg $disc^{-1}$) for *Z. zerumbet* (X), *Z. moran* (XI) and *C. amada* (XII).

significant action against some fungi (*A. niger* and *A. flavus*) *in vitro*. However, Ficker et al. (2003b) found that, out of 29 plant extracts, ginger extract had the broadest range of anti-fungal activity measured either by the fungi inhibited or as the average diameter of the zones of inhibition. A far better antibacterial effect was obtained in the current study against *E. coli*, *S. paratyphi* and *L. monocytogenes* from ginger (*Z. officinale*) extracts using solvent unlike reported by Indu et al. (2006) where raw extract was used. It is unclear exactly how the essential oils are inhibiting fungal growth and why some essential oils have better activity against fungi than bacteria as found in early reports (Cavanagh 2007). It could be the fact that the anti microbial activity of volatile compounds results from the combined effect of direct vapor absorption on microorganisms and indirect effect through the medium that absorbed the vapor (Inouye et al. 2000). Fungi grow mainly on the surface of the medium and might be more susceptible to direct vapor contact while the antimicrobial effect against bacteria might be more dependant on the vapor accumulation into agar. This could probably explain why no clear zone of inhibition was observed after 24 h of inoculation with essential oil against the fungal pathogens tested.

4.4.4 Raman spectroscopy

The antibacterial efficacy of the hydrodistilled fractions of the four plant species were found to be the best effective among all the solvents used. Therefore, these fractions were further characterized using the micro-Raman spectroscopy against both Gram-positive and Gram-negative strains of bacteria. Confocal micro-Raman spectroscopy is a promising tool for detection and identification of molecules contained in a tiny space based on measuring the molecular vibration frequencies from the scattered light (Xie and Li 2003). All the biological molecules show their characteristic micro-Raman spectra and peaks at specific wavelength. This technique has been used to study single living cells and useful information about the composition, secondary structure and interactions of DNA-protein complexes inside the living cells can be yielded from the positions, intensities and the line widths of the Raman peaks in the spectra (Xie and Li 2003). Bacterial cells too have their specific absorption wavelength with the confocal micro-Raman spectrum and can be detected easily by the peaks shown by them. Raman spectroscopy has been successfully used in pathogen detection to identify and quantify microorganisms without drying (Lindsay et al. 2007). In the current study, all the four rhizome oil fractions revealed excellent antibacterial effect by

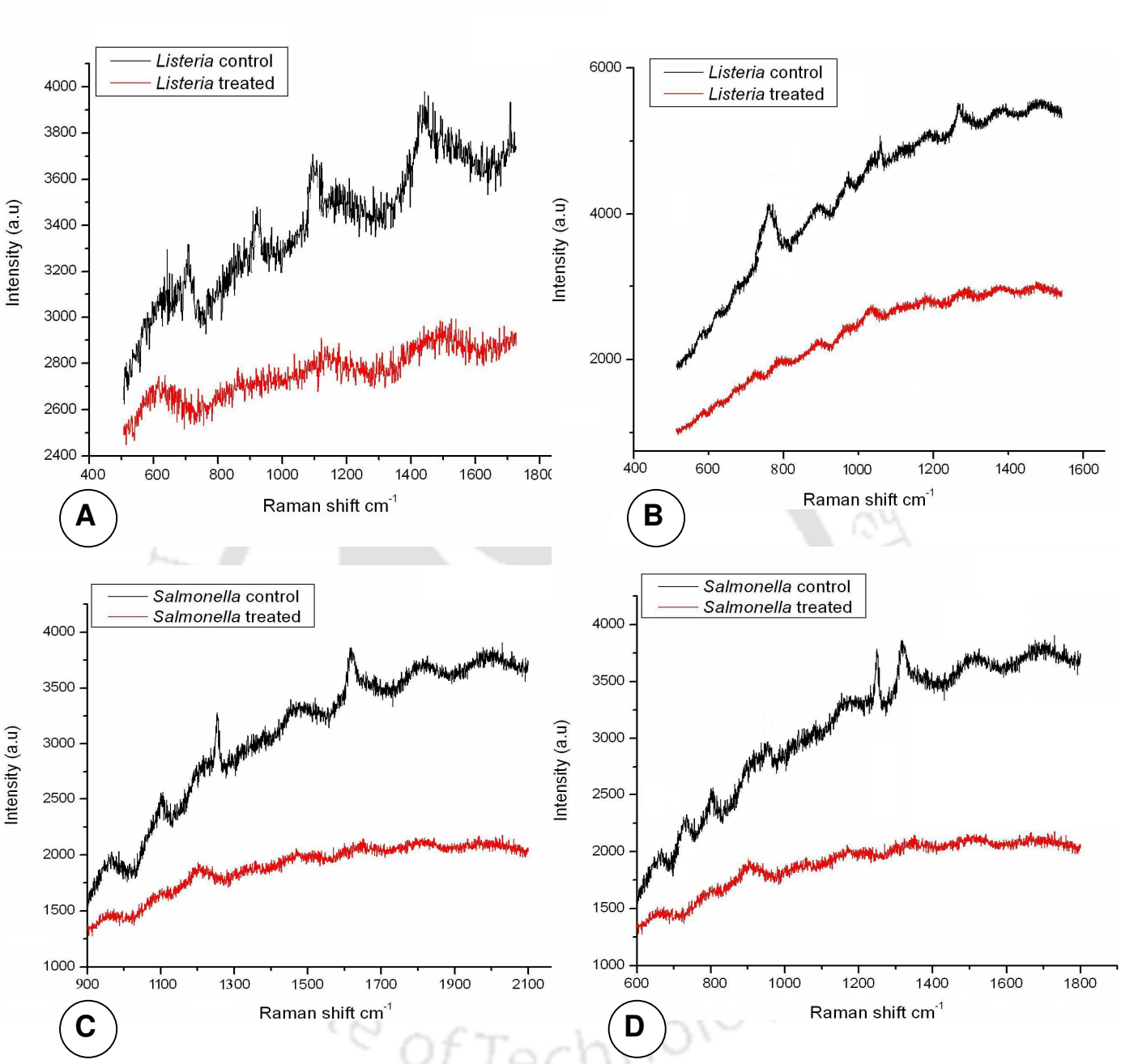


Figure 4.6: Micro-Raman spectra of the studied bacteria before and after treatment with the rhizome essential oils. **A.** *Z. moran*; **B**. *Z. zerumbet*; **C**. *C. amada* and **D.** *C. longa*

showing the characteristic Raman shifts for *S. paratyphi* and *L. monocytogenes*. The Raman shift thus depicts the fall in bacterial cell number by inhibiting the growth after treatment (Figure 4.6). The characteristic Raman peaks of *Salmonella* was observed at about 1242nm and 1637 nm (Sengupta et al. 2004) were found to fall significantly after treatment with *Z. moran* essential oil. Similarly, two specific Raman peaks shown by *Listeria* at 750, 1100, 1350 and 1440 nm of wavelengths (Luo and Lin 2008; Green et al. 2008) were decreased in the treated samples with rhizome essential oils at their MICs (Figure 4.7). The results suggested that the concentrations of the rhizome essential oils were sufficient enough to kill or inhibit the growth of the pathogen. Interestingly, no literature is available in evaluating antibacterial potential of biological samples like essential oils till date. Hence, a mere advanced characterization in evaluating the antibacterial potential of the rhizome essential oils using micro-Raman spectroscopy is presented here. The characteristic Raman spectra of both *S. paratyphi* and *L. monocytogenes* studied were found to decrease significantly after treatment with the rhizome essential oils. Reports are available on utilization of Raman spectroscopy for depicting antibacterial efficacy of various metal nanoparticles (Peter et al. 2002; Raffi et al. 2010). However, Raman spectroscopy still awaits wide use and application in the biological field. This is a clear evidence of the strong antibacterial property of the essential oil of *Z. moran* and thus can be used as a broad spectrum antibacterial agent effective against both Gram-positive and Gram-negative bacteria.

4.5 Conclusion

In view of various ethno medicinal properties, Zingiberaceae of NE India is undoubtedly very important plant family housing key source-species that are potential source of various products. The study reported here has an applied significance. *In vitro* evaluation of plants for antimicrobial property is the first step towards achieving the goal for developing eco-friendly management of infectious diseases of human by search for new bio-molecules of plant origin. Moreover, the ability to detect the presence of and identify the type of bacterial pathogen is a key requirement for ultimately reducing the incidence of disease. In the current study all the four plants of Zingiberaceae including *Z. moran* were selected on the basis of traditional medicinal knowledge. The tests of the essential rhizome oil from *Z. moran* for various biological properties demonstrated that the essential oils from the wild plant appears

to be the potential source of bioactive compounds which have tremendous medicinal, economic and therapeutic value. The fractionated oil of the species also expressed significant bactericidal and fungicidal activity against selected strains of pathogenic microorganisms. The membrane disruption could be one of the likely mechanisms of action of the rhizome oil, inactivating the microbial adhesions, between the chemical content of oils and the antimicrobial activities. With the advantage of bacterial zone of inhibition and MIC determination, combining optic characterization light scattering signal, we conclude that the rhizome oils have strong antibacterial effect. This study is a positive demonstration of the utility of screening NE India's endemic Zingiberaceae plants for their ethnomedical use and thus can be used to discover bioactive natural products that will lead to the development of new pharmaceuticals. The oils of *Z. moran* is reported to possess herbal-spicy-odor, and hence can be used as an alternative in the perfume industry for aftershaves, skin waters, soaps, insect repellants, room fresheners etc. It is obvious that such indigenous species from NE India possess very important properties of cosmetics and medicinal importance. Further, characterization of members of the family Zingiberaceae for its added attributes thus also has important implications for germplasm utilization and may establish a case study of genetic enhancement of *Zingiber* species (Rangan, personal communication).

The results of the study provide further evidences of antimicrobial activities of some native NE Indian Zingiberaceae plants. *C. longa* has been studied by earlier workers and is perhaps the highest studied species in the genus. *C. amada* and *Z. zerumbet* have also been attempted by a few researchers still they need further extensive study on the biological properties as revealed in the study. A scientific and systematic investigation with regard to the various biological activities of these plants is lacking. Present study indicates that the wild species *Z. moran* is particularly worthy further study due to the range of microbes it is capable of inhibiting. Further evaluation of the antibacterial properties of these extracts against a more extensive panel of microbial agents is warranted. Likewise, purification and identification of the bioactive components is needed to examine the mechanisms of action of these agents. Whilst the extracts examined in this present study appear promising as antimicrobial agents. As an added advantage, there needs no preliminary cautions before using these compounds as medicines or drugs preparation because all these plant-products are edible and have been proven traditionally.

Chapter 5a

Chapter 5a

Isolation and characterization of potential bioactive compounds from rhizome essential oil of *Zingiber moran*

5a.1 Introduction

Plants have formed the basis for traditional medicinal systems for thousands of years, with the first records dating from about 2600 BC in Mesopotamia (Khine 2006). Ancient Egyptian, Chinese, and Indian documents show that medicine in these societies included numerous plant-based remedies and preventives. The Greeks and Arabs both contributed substantially to the assimilation, codification, and development of plant-based medicines. The isolation of the active principles from the plants and herbs such as strychnine, morphine, and colchicines began in the early 1800s (Newman et al. 2000). Peoples used oils from various plants (Cedar, Cypress, Ginger, Licorice, Poppy, Turmeric etc.), among other things, substances that are still in use today for the treatment of a variety of illnesses and infections (Khine 2006). Recently, there is a growing interest and inclination towards the use of herbal products in the field of medicine and cosmetics across the world. Despite the great success already achieved in natural products chemistry and drug development, people have barely begun to tap the potential of the molecular diversity. Only 5 to 15% of the estimated 250,000 species of higher terrestrial plants in existence on earth have been chemically and pharmacologically investigated in a systematic fashion (http://www.aaas.org/international/africa/gbdi/mod1b.html; Nnadozi et al. 2000). In this regard, India is blessed with biodiversity and habitat diversity as well. However, most of the wild plants found in this subcontinent are still to be identified and explored for their better use and conservation *in situ*. Plants have an advantage in this area based on their long-term use by the local folk (over hundreds or thousands of years) for traditional system of medicine. Many bioactive compounds obtained from such plants have been established to have low human toxicity (Fabricant and Farnsworth 2001). The traditional medicine or ethnomedicine may be defined broadly as the use of plants by humans as medicines (Farnsworth 1990; 1994). Traditional medicine is a broad term used

to define any non-Western medical practice (Bannerman 1983). Ethno-pharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biologic activities. It is based on botany, chemistry, biochemistry, pharmacology, and many other disciplines (anthropology, archaeology, history, and linguistics) that contribute to the discovery of natural products with biologic activity (Fabricant and Farnsworth 2001). The current study deals with such an endeavor to investigate one of the indigenous plant species of the family Zingiberaceae collected from NE India. The medicinal family Zingiberaceae is found to grow naturally in this region of India which has been a vital part of the ethno medicinal practices. Many members of this family are also cultivated as spice crops viz, ginger, turmeric, cardamom, galangal etc. in this region. Spices are high value and export oriented commodity crops, which play an important role in agricultural economy of the country. India is the principal source for supply of spices in the global market and the share of spices in the export earnings from agricultural and allied products is 8.5% (Yadav et al. 2004). The NE region has tremendous potential for production of spice crops as the climatic condition of the region is highly suitable for cultivation of a large number of spices including ginger and turmeric. Among all, ginger is the main cash crop supporting the livelihood and improving the economic level of many ginger growers of NE region. Ginger is grown in almost all states of the region (Govind et al. 1998). Apart from improved varieties like Nadia, China, Varada etc. a number of local varieties exist in this region. These are the high yielder of rhizomes as compared to the standard cultivars like Nadia and Rio-De-Janeiro but have more fiber content. *Z. moran* or locally known as 'Moran adaa' is such a local ginger variety endemic to this NE Indian region which was selected as the study material in the present investigation.

In the previous chapter, the antimicrobial assay of some selected wild and domestic Zingiberaceae species was conducted which showed their potentiality to be used as drugs. Among these, *Z. moran* was found to have an excellent antimicrobial spectrum. This species is a wild relative of common ginger (*Z. officinale*) and is found to grow in few particular places of NE India. Till date, there is no any report regarding any kind of investigation of this variety. Yadav et al. (2004) reported on this plant as an endemic wild ginger variety with high pungency and smaller size commonly found in

tribal villages of Nagaland. However, literatures are available on some other wild members of the genus *Zingiber*, viz. *Z. zerumbet*, *Z. waryi*, *Z. casuamonar, Z. nimmonii* etc. (Chairgilprasert et al. 2005; Sabulal et al. 2006; Yang et al. 2009). After testing the antimicrobial property of the essential oil in previous chapter, the results were motivating enough to investigate the bioactive compounds present in hydrodistilled fraction of the *Z. moran* oil which was the most effective oil fraction against all pathogens tested. Hence, the objective of the present chapter was set to isolate and characterize at least three potential natural antimicrobial compounds from the wild ginger (*Z. moran*) rhizome oil. The chapter is divided into two sections, where, first section (5a) deals with the chemical characterization of the isolated compounds; whereas the second section (5b) meets the objective to examine the anticancerous property of the isolated rhizome oil fractions against human cervical adenocarcinoma cell lines (HeLa) so as to establish the hidden medicinal significance of this plant in the scientific world. To the conscious circle, this is the first scientific study reported on this important species of Zingiberaceae from NE India.

5a.2 Literature Review

In the previous chapter, status and significance of traditional medicines were discussed in the world and Indian contexts. These are used by about 60% of the world's population not only for primary health care and not just in rural areas in developing countries, but also in developed countries as well where modern medicines are predominantly used (Kamboj 2000). Use of plants as a source of medicine has been inherited and is an important component of the health care system in India. In the Indian systems of medicine, most practitioners formulate and dispense their own recipes; hence this requires proper documentation and research. In western world also, the use of herbal medicines is steadily growing with approximately 40% of population reporting the use of herb to treat medical illnesses within the past years (Bent and Ko 2004). Public, academic and government interest in traditional medicines is growing exponentially due to the increased incidence of the adverse drug reactions and economic burden of the modern system of medicine (Dubey et al. 2004).

Plants, besides their immense biodiversity are also very rich in metabolites with a huge chemical diversity of which only a small fraction have been characterized. An estimate showed that about 100,000 plant secondary metabolites or natural products have been identified (Seth and Sharma 2004). For the vast majority of natural products, their function for the plants as well as their potential role in human health care is still to be elucidated. The area of pharmacognosy has been evolved from a mere descriptive botanical science to a discipline integrating biochemistry and molecular genetics (Phillipson 2003). This has led to the identification and biochemical characterization of several natural products, which in turn have led to the development of important novel drugs. As a strong support to this fact, over one quarter of new drugs that have been approved in last 30 years are based on a lead from a molecule of plant origin (Muthusamy et al. 2009). Moreover, 9 of the top 20 selling drugs are derived from the knowledge of plant secondary metabolites (Tulp and Bohlin 2002). Due to the limited success of the combinatorial-biochemistry approach to develop novel drugs, there is renewed interest in plants as well as in other lower organisms to look for active natural products with a putative pharmaceutical importance (Muller-Kuhrt 2003). Indeed, it is clear that the search for active products will be far more successful using the enormous biodiverse library of molecules from plants and other living beings compared to a random chemical library (Muthusamy et al. 2009).

Since plants and their natural enemies such as, bacteria, viruses, nematodes, and insects have coevolved, plants produce a wide range of natural products that are involved in their defence against these arrays of natural enemies (Wink and Schimmer 1999). Moreover, plants have sedentary life which makes them to adapt to different environments and to develop systems of defence against various stresses. Some plant secondary metabolites serve as attractants for pollinators or as protectors of UV radiation. In principle, the molecules involved in these actions are observed to possess highly specific structures designed through evolution to interact with certain protein folds, but not with the majority of them. Thus, they form ideal candidate molecules with pharmaceutical potential (Heimann and Bauer 1999). Natural products play an important role in pharmaceuticals, agrochemicals, food-supplements/ additives, herbal medicines and in cosmetics. Plants offer a rich source of novel and diverse compounds for new

drugs. Almost one-third of the world's prescribed drugs originate from compounds derived from plants. About 40% of all medicines are either natural products or derivatives thereof (Muthusamy et al. 2009). Herbal medicine has been a tradition of healthcare since ancient times and one of the roots of pharma-research. Hence there is a need to integrate traditional medicine into the modern medicine practices. This requires clinical validation by conducting controlled clinical trials (Fang 2003). The methods used for clinical validation for modern medicines must be applied to prove the safety and efficacy of the finished herbal products. The design and the scope of the studies should be in accordance with traditional use and in consultation with the traditional medical practitioners.

The major hindrance in the amalgamation of herbal medicines into modern medical practices is the lack of scientific and clinical data, and better understanding of efficacy and safety of the herbal products. To ensure the quality and safety of its products and practices standardization is of vital importance (Gogtay et al. 2002). Most of the herbal products do not have drug regulatory approval to demonstrate their safety and efficacy. The traditional use can provide valuable clues for the selection, preparation and indications for use of herbal formulation, as efficacy has been established by the common use. The historical use provides the source to study the specific plant species with potential to be used in a particular disease. A systematic approach through experimental and clinical validation of efficacy is required for a plant identified for traditional medicine, as is done in modern medicine; animal toxicity studies are also required to establish the potential adverse effects. Efficacy testing of the traditional and new herbal products in experimental screening method is important to establish the active component and appropriate extract of the plant (Chakravarty 1993). However, there should be adequate data from *in vivo* and *in vitro* studies to validate the therapeutic potential claimed. There is a need to establish the pharmacological studies for identifying and comparing the various preparations for potency.

The plant species (*Z. moran*) under study in this chapter is an unexplored member of the medicinal family Zingiberaceae which has tremendous therapeutic values proven by the traditional practitioners of NE India since long back. The species lacks any sort of scientific investigation for which it has been a hidden plant and as is restricted to only a few part of NE Indian states, is gradually facing the danger of extinction. The crude

rhizome extracts of *Z. moran* was tested for antimicrobial activities in the last chapter along with other members of the family. Considering the best antimicrobial spectra shown by the *Z. moran* hydrodistilled oil, it was further characterized by biochemical means. Literatures are available where a wide number of Zingiberaceae members were investigated chemically for the therapeutic phytochemicals present in rhizome essential oil (Yang and Eilerman 1999; Singh et al. 2002; Purkayastha et al. 2006; Qiao et al. 2007; Natta et al. 2008). In fact the genus *Zingiber*, after *Curcuma* is the best known for the members with enormous medicinal importance. Apart from the common ginger (*Z. officinale*), many other wild and cultivated species of this genus have been studied across the world and characterized for the beneficial phytochemicals present in the rhizome oil (Chairgilprasert et al. 2005; Sabulal et al. 2006; Cheien et al. 2008; Yang et al. 2009). Characteristically the rhizomes of *Zingiber* are strongly aromatic and possess mainly terpenoids and flavanoids. Common secondary metabolites found in the genus *Zingiber* are zingiberene, gingeroles, zingiberol, geraniol, phellandrene, camphene etc. Essential oils are complex mixtures of several tens of components, mainly mono- and sesquiterpenes, more scarcely diterpenes, phenylpropanoids, or acyclic nonterpenic compounds. The essential oil of some important members of Zingiberaceae are investigated by many workers (Mao et al. 2003; Purakayashtha et al. 2006). However, the wild and medicinally significant members are least reported with regard to its biochemical study.

Analytical chemistry techniques like column chromatography (CC), thin layer chromatography (TLC), UV-visible spectroscopy, Fourier transform infrared (FTIR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy etc. have extensively been used for characterizing plant based natural products and in elucidating the structures (Sabulal et al. 2006; Policegoudra et al. 2007; Singh et al. 2008).

5a.3 Materials and Method

5a.3.1 Plant material and rhizome essential oil extraction

The study material used in the experiment was the essential oil of *Z. moran* extracted from the mature rhizomes by hydrodistillation. Mature rhizomes of the plant *Z. moran* were collected in bulk (about 10 kg) from the natural habitats at the time of harvesting

(October to December). Collected rhizomes were washed with running water to remove soil and dust and were shade dried till the dry weight became constant. The essential oil from these rhizomes was isolated by hydrodistillation process using Clevenger apparatus. The extracted oil was stored in 4 ^{o}C and used for further experiments.

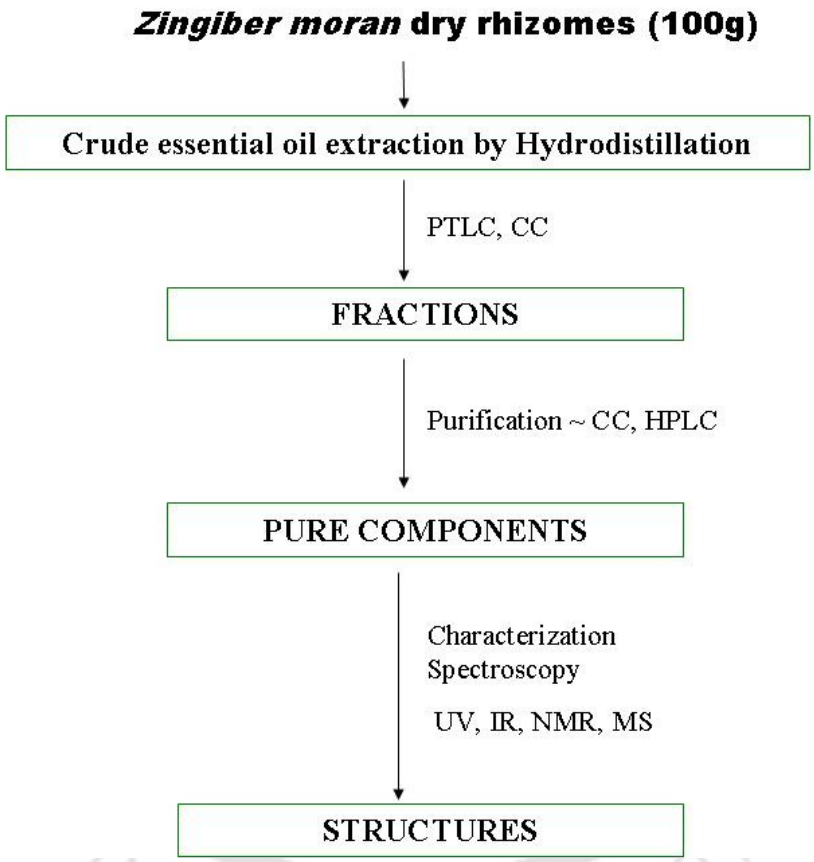


Figure 5a.1: Schematic representation of steps involved for isolation and identification of the bioactive compounds from *Z. moran* rhizome

5a.3.2 Fractionation of the essential oil

The rhizome essential oil of *Z. moran* was fractionated using the analytical chemistry techniques mentioned below. These fractions were identified and characterized further. The entire procedure of isolation and purification is showed in figure 5a.1.

5a.3.2.1 Thin-layer chromatography (TLC)

An uniform slurry of adsorbent (silica gel with little $CaSO_4$) neither too thick nor too thin in consistency was prepared by ethyl acetate (1:1, w/v), spread on a glass plate with an applicator and dried at 120 ^oC in an oven. An aliquot of the hydrodistilled oil fraction diluted in ethyl acetate was loaded on the activated silica gel TLC plates (2x20 cm) with a capillary tube. The plates were developed using n-Hexane: chloroform (80:20), chloroform: ethyl acetate (90:10) and ethyl acetate: methanol (90:10) solvents. The spots were located by exposing the plate to iodine fumes. Fractions having the same number of spots with similar R_f values on the TLC plate were pooled. The pooled fractions were numbered (Fr.1–Fr.3). After optimization of the solvent system polarity for TLC, the crude oil was subjected to column chromatography.

5a.3.2.2 Column chromatography (CC)

Activated silica gel (60–120 mesh) was packed onto a glass column (450 x 40 mm) using n-Hexane solvent. The glass tube is clumped vertically keeping glass wool at the constricted end. Half of the tube is filled with eluting solvent and mobile slurry of adsorbent prepared in eluting solvent is added in portions to it. About 50g of activated alumina (Al_2O_3) was used to prepare the slurry in n-Hexane (400ml). Stopper of the column is opened to run the solvent slowly while tapping the column gently with a glass plunger when the adsorbent settles down. Another portion of slurry was added before complete settling of the slurry added previously. The process was repeated until whole of the slurry has been added. Top portion of the surface was protected by placing a filter paper on it. For large-scale isolation of compound, 20 g of crude water extract was loaded on the top of silica gel. The column was eluted stepwise at a flow rate of 1 ml/min with 300 ml of n-Hexane, 2000 ml of n-Hexane : ethyl acetate (75 : 25 to 0 : 100 v/v), 2000 ml of chloroform : ethyl acetate (75 : 25 to 0:100 v/v), 2000 ml of ethyl acetate : acetone (75

: 25 to 0:100 v/v) and 1500 ml of acetone : methanol (75 : 25 to 0 : 100 v/v). About 18 fractions measuring 100 ml each were collected and concentrated by using the rotary evaporator. An aliquot of all these fractions were loaded on TLC plate, fractions having similar R_f values were pooled and numbered. Among these, the three fractions showing single spots in TLC were selected for further study. The whole process was repeated for 10 times to get the optimum amount of the desired fractions and all the three fractions were collected in gross amount (~ 1.5g). After isolation of the fractions, they were subjected to further purification and characterization.

5a.3.3 Further purification of the bioactive fractions

The three fractions depending on their R_f values obtained in pure form from the first round of column chromatographic separation were further subjected to CC for further purification. About 500 mg of bioactive fractions Fr.1, Fr.2 and Fr.3 were further purified using silica gel (60–120 mesh) column (450x20 mm). Each of the fractions was run separately. For the first fraction (Fr.1) the column was eluted stepwise at a flow rate of 1 ml/min with 100 ml of hexane, 200 ml of hexane : chloroform (90 : 10 to 0 : 100 v/v), 800 ml of chloroform : ethyl acetate (90 : 10 to 0 : 100 v/v), 600 ml of ethyl acetate : acetone (90 : 10 to 0 : 100 v/v) and 400 ml of acetone : methanol (90 : 10 to 0 : 100 v/v). For Fr.2, the column was eluted stepwise at a flow rate of 1.5 ml/min with 100 ml of hexane : chloroform (90 : 10 to 0 : 100 v/v), 400 ml of chloroform : ethyl acetate (95 : 05 to 0 : 100 v/v) and 200 ml of ethyl acetate : acetone (95 : 05 to 0 : 100 v/v). And for Fr.3,the stepwise column elution parameters were, flow rate of 1 ml/min with 100 ml of hexane : chloroform (90 : 10 to 0 : 100 v/v), 400 ml of chloroform : ethyl acetate (95 : 05 to 0 : 100 v/v) and 400 ml of ethyl acetate : acetone (95 : 05 to 0 : 100 v/v) and 200 ml of acetone : methanol (90 : 10 to 0 : 100 v/v). All fractions measuring 100 ml each were collected and concentrated in a rotary evaporator. An aliquot of the fractions was loaded on the TLC plate, which showed a single spot in the TLC profile there by confirming the purity of the compounds. These pure compounds thus obtained were subjected to various spectroscopic techniques for elucidation of the structures.

5a.3.4 High-performance liquid chromatography

The purified compounds were tested for its purity using HPLC, using LC-10AT liquid chromatograph (LC; Shimadzu, Singapore) equipped with C-18 column (300x4.6 mm 5l µ Thermo Hypersil) and Varian HPLC software. Methanol and Acetonotrile of HPLC grade were used (60 : 40) as a mobile phase for the chromatographic separation. Elution was carried out in an isocratic solvent system at a temperature of 25 ^{o}C, a flow rate of 0.75 ml/min and a run time of 30 min. The concentration of the compound was 100 ppm and injection volume was 25 µl. Ultraviolet (UV) detection was carried out with a diode array detector (Shimadzu) and the detection wavelength was 425 nm.

5a.3.5 Characterization of bioactive compounds

5a.3.5.1 Fourier Transform Infrared spectrometry

Fourier Transform Infrared (FTIR) spectrum of the isolated compounds were recorded on a Perkin-Elmer Spectrum-One FT-IR spectrometer (USA), KBr disc in the range 4000-250 cm^{-1}, at room temperature. About 0.5 mg of each of the isolated compounds was mixed with dried potassium bromide (KBr) to make the pellet and then was used to record the spectrum.

5a.3.5.2 High Resolution Mass spectrometry (HRMS)

Mass spectrum of the isolated compounds was recorded on instrument Water Q-TOF premier mass spectrometer (USA) by electro-spray ionization (ESI) technique with a flow rate of 0.2 ml min) 1 on the C-18 column and at a total run time of 30 min. Diode array was used as a detector and the ESI probe served as the positive ion mode in analysis. About 1 mg of each of the isolated and purified compounds was dissolved in 5 ml of methanol was used for recording the spectrum. An aliquot of formic acid was used as an ionizing agent.

5a.3.5.3 Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance (NMR) spectra for both ^{1}H and ^{13}C were recorded for each of the isolated compounds using $CDCl_3$ with Mercury Plus 400 MHz NMR Spectrometer (Varian, USA), operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature.

Chemical shifts were recorded in parts per million (ppm) on the scale. A region from 0 to 12 ppm for ^{1}H and 0 to 200 ppm for ^{13}C was employed. Signals were referred to internal standard tetramethylsilane (TMS). About 45 mg of the isolated compound dissolved in 0.75 ml of $CDCl_3$ was used for recording the spectra.

5.4 Result and Discussion

5a.4.1 Plant material and rhizome essential oil extraction

The essential oil of *Z. moran* rhizomes was extracted by hydrodistillation using Clevenger apparatus. One whole round of extraction process took about 3-4 h to yield 3-4 ml oil from 50 g of crushed rhizomes. As the process was a continuous one it demanded less labor and it took almost one month to yield sufficient amount of essential oil required for the study. The oil was colorless and possessed strong characteristic pungent aroma. Extracted oils were allowed to settle in glass vials, later washed with anhydrous Sodium sulfate (Na_2SO_4) to remove the remaining water particles and kept at 4 ^{o}C for further use.

5.4.2 Fractionation and purification of the bioactive compounds

The rhizome essential oil of *Z. moran* was fractionated using the chromatographic separation techniques like TLC and CC. As the crude essential oil contained many components, TLC could not serve as an efficient mean of separation and hence column chromatography was followed to isolate at least three major components of the oil. Once the polarity of the solvent systems was optimized, the essential oil was subjected to CC from which three major components were selected on the basis of their R_f values and separation ability. The initial separation of the three selected components by CC is as shown in the figure 5a.2. The purity of the fractions/compounds was further confirmed by HPLC. The spectra showed single peaks for each of the three isolated compounds confirming the purity of the same as shown in figure 5a.3 (A, B & C) for compound 1, 2 and 3 respectively.

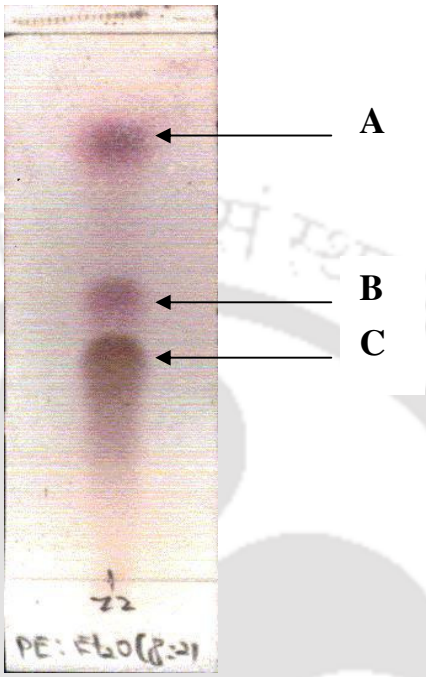


Figure 5a.2: TLC profiles of the three isolated fractions of *Z. moran* essential oil. **A**- Fraction 1, **B**-Fraction 2, **C**-Fraction 3

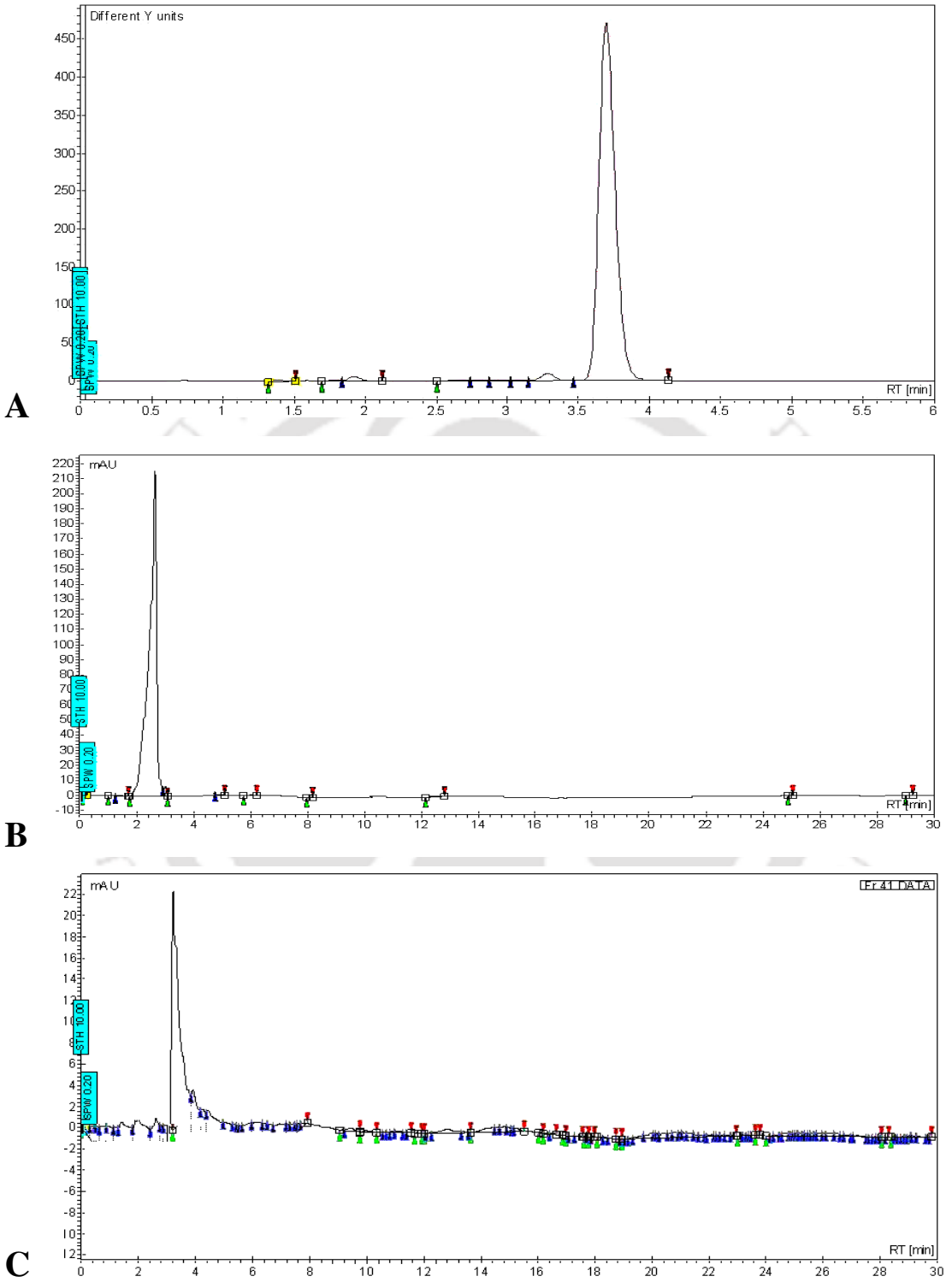


Figure 5a.3: HPLC profiles of the three isolated fractions of *Z. moran* essential oil.
A- Fraction 1, **B**-Fraction 2, **C**-Fraction 3

5a.4.3 Identification of the bioactive fraction

The pure compounds were then subjected to various spectroscopic analyses, i.e. Fourier transform infrared spectroscopy (FTIR), high resolution mass spectroscopy (HRMS) and nuclear magnetic resonance spectroscopy (NMR) to deduce their respective structures.

Compound 1

NMR spectrum showed as many as two CH_3 groups as singlets. Three CH_3 signals were observed as doublets and indicated that they were attached to CH carbons. The corresponding ^{13}C signals for the CH_3 groups were also observed. The region between 20.0 to 40.0 ppm indicated quite a lot of CH and CH_2 signals with complex splittings. The region between 1.5 and 4.88 ppm in ^1H spectrum showed CH signals along with the corresponding ^{13}C signal. A C=C signal at 165.0 ppm was also observed. Some quaternary carbons and aromatic carbons in the region 97.2–116.2 ppm were observed. Aliphatic carbon signals were observed at 24.8 ppm.

^1H NMR (400 MHz, $CDCl_3$)

δ 1.00 (s, 3H, -CH_3), 1.03 (S, 3H, -CH_3), 1.17-1.22 (m, 2H), 1.30-1.40 (m, 1H), 1.57-1.69 (m, 3H), 1.85-1.90 (m, 1H), 2.61-2.65 (m, 1H, -CH-), 4.47 (s, 1H, -CH=), 4.70 (s, 1H, -CH=) (Annexure 1).

^{13}C NMR ($CDCl_3$)

δ 24.0 (C-1), 24.3 (C-9), 26.0 (C-6), 26.4 (C-7), 29.4 (C-4), 29.9 (C-2), 38.0 (C-5), 47.4 (C-3), 48.6 (C-10), 99.5 (C-8) (Annexure 2).

FTIR spectrum

IR spectral data of the compound 1 showed C–C stretching at 1628 cm^{-1}, alkyl stretching at 2925 cm^{-1} indicating the presence of olefinic and aliphatic methyl groups (Annexure 3).

FTIR: νmax cm^{-1}: 1628 (C=C stretching frequency.), 1456 and 1377 (*gem*-dimethyl). Peaks were observed at 2925, 2857, 1628, 1456, 1377, 1121, 801.

HRMS spectrum

MS data showed parent molecular ion peak at 137.0148 (Annexure 4).

Theoritical value $(M+H)^+$ =137.1330

Observed Value $(M+H)^+$ = 137.0148

Based on all these spectral data, the structure was deduced to be a probable monoterpene and was designated as a 2,2 dimethyl 3- methylene bicycle (2.2.1) heptane or Camphene (Figure 5a.4).

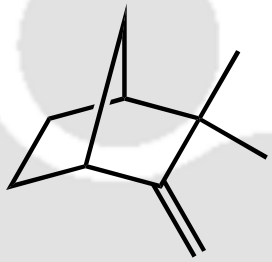


Figure 5a.4: Structure of compound 1 (Camphene, $C_{10}H_{16}$)

Compound 2

NMR spectrum showed as many as three CH_3 groups as singlets. Other CH_3 signals were observed as doublets and indicated that they were attached to CH carbons. The aldehydic HC=O signal was observed in 9.68 ppm. The corresponding ^{13}C signals for all the three CH_3 groups were also observed. The region between 1.5 and 2.6 ppm indicated quite a lot of CH and CH_2 signals with complex multiple splittings. The region between 5 and 10 ppm in ^1H spectrum showed CH signals attached to OH groups along with the corresponding ^{13}C signal. A carbonyl signal at 191.5 ppm was also observed. Aliphatic carbon signals were observed at 17.0 to 40.5 ppm. A signal at 132.1 and 163.6 ppm was also observed for the C=C.

The NMR, spectra were analyzed as below:

^{1}H NMR (400 MHz, $CDCl_3$)

δ 1.59 (s, 1.5H, -CH_3), 1.61 (s, 1.5H, -CH_3), 1.68 (s, 3H, 2×-CH_3), 1.99 (s, 1.5H, CH_3), 2.17 (s, 1.5H, -CH_3), 2.19-2.27 (m, 2H, -CH_2-), 2.59 (t, *J* = 7.6 Hz, 2H, -CH_2-), 5.09 (dd, *J* = 7.2 and 5.2 Hz, 1H, -CH-), 5.87 (d, *J* = 8.0 Hz, 1H, -CH-), 9.89 (d, *J* = 8 Hz, 0.5H, -CHO), 9.99 (d, *J* = 8.0 Hz, 0.5H, -CHO)

Two isomers cis and trans of the compound are present (Annexure 5).

^{13}C NMR (100 MHz, $CDCl_3$)

δ 17.4, 17.6, 24.9, 25.5, 25.7, 27.0, 32.5 (2C), 40.5 (2C), 122.3, 122.6, 127.3, 128.5, 132.7, 133.5, 163.6 (2C), 190.5, 191.0. (Annexure 6).

FTIR spectrum

IR spectral data of the compound 2 showed aldehydic O=CH stretching at 1673 cm^{-1}, alkyl stretching at 2920 cm^{-1} and C=C stretching at 1633 cm^{-1} indicating the presence of OH and aldehyde groups (Annexure 7).

FTIR: νmax cm^{-1}: 1673 (Conjugated C=O), 1444 and 1378 (*gem*-dimethyl). Peaks were observed at 2920, 2857, 1673, 1633, 1378, 1153, 1121, 1044, 842.

1675, aldehydic C=O starching frequency; 1633, C=C stretching frequency

HRMS spectrum

MS data showed parent molecular ion peak at 153.0400 (Annexure 8)

Theoritical value $(M+H)^+$ =153.1279

Observed Value $(M+H)^+$ = 153.0400

Based on all these spectral data, the structure was deduced to be a probable monoterpene and was designated as a 3,7 dimethyl octa- 2, 6 dienal; (mixture of a pair of terpenoids) or Citral (Figure 5a.5).

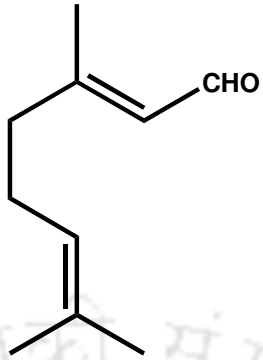


Figure 5a.5: Structure of compound 2 (Citral, $C_{10}H_{16}O$)

Compound 3

IR spectral data of the compound 1 showed O–H stretching at 3409 cm^{-1}, alkyl stretching at 2926 cm^{-1} and C=C stretching at 1640 cm^{-1} indicating the presence of OH and olefenic group. Mass spectrum showed a HRMS peak at 155.0525. NMR spectrum showed as many as three CH_3 groups as singlets. Other three CH_3 signals were observed as doublets and indicated that they were attached to CH carbons. The corresponding ^{13}C signals for the remaining six CH_3 groups were also observed. The region between 1.1 and 2.4 ppm indicated quite a lot of CH and CH_2 signals with complex multiple splittings. At about 2.0 ppm in ^{1}H spectrum showed CH signals attached to OH groups along with the corresponding ^{13}C signal. Aliphatic carbon signals were observed at 17.5 to 42.1 ppm. The FTIR and NMR spectra were analyzed as below.

^{1}H NMR (400 MHz, $CDCl_3$)

δ 1.25 (d, *J*= 3.2 Hz, 3H, -CH3), 1.48-1.55 (m, 2H, -CH2-), 1.59 (s, 3H, -CH3), 1.66 (s, 3H, -CH3), 1.9-2.08 (m, 2H, -CH2-), 2.47 (brs, 1H, -OH), 4.99-5.04 (m, 1H, -CH-), 5.06-5.12 (m, 1H, -CH-), 5.16-5.22 (m, 1H, -CH-), 5.48-5.93 (m, 1H, -CH-) (Annexure 9).

^{13}C NMR (100 MHz, $CDCl_3$)

δ 17.6, 22.8, 25.6, 27.6 42.1, 73.2, 111.6, 124.5, 131.4, 145.1 (Annexure 10).

FTIR spectrum

IR: νmax cm^{-1}: 1640 (Conjugated C=C), 1452, 1376 and 1412 (*gem*-trimethyl).

3403 for OH; Peaks were observed at 2970, 2927, 2857, 1640, 1452, 1376, 1113 (>CO), 996, 920. (Annexure 11)

HRMS spectrum

MS data showed parent molecular ion peak at 155.0525 (Annexure 12).

Theoritical value $(M+H)^+$ = 155.1436

Observed value $(M+H)^+$ = 155.0525

Based on all these spectral data, the structure was deduced to be a probable monoterpene and was designated as a 3, 7 dimethyl octa- 1, 6 dien-3-ol or Linalool (Figure 5a.6).

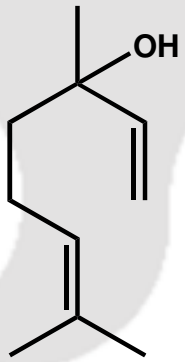


Figure 5a.6: Structure of compound 3 (Linalool, $C_{10}H_{18}O$)

5.5 Conclusion

The repeated fractionation of the hydrodistilled extract of *Z. moran* by silica gel column chromatography yielded pure compounds. The structures of the isolated compounds were deduced as Camphene, Citral and Linalool after extensive analysis of spectroscopic data. These three are the first bioactive compounds isolated and characterized from the wild

ginger *Z. moran* rhizome essential oil extracted by hydrodistillation. Camphene or 2,2-dimethyl-3-methylene-bicyclo[2.2.1] heptane was the compound identified from the fraction 1. It is a volatile monoterpene with a pungent characteristic smell. In industrial purpose, Camphene is produced by catalytic isomerization of the more common alpha-pinene. Citral is the second fraction of the essential oil of rhizome of *Z. moran*. Citral, or 3,7-dimethyl-2,6-octadienal or lemonal, is either of, or a mixture of, a pair of terpenoids with the molecular formula $C_{10}H_{16}O$. The two compounds are double bond isomers. The *E*-isomer is known as geranial or citral A. The *Z*-isomer is known as neral or citral B. In the fraction isolated from the *Z. moran oil*, the mixture of both the isomers was found to be present. Linalool or 3,7-dimethyl octa-1,6-dien-3-ol was identified as the third compound from the fraction 3 of the *Z. moran* oil which is characterized by strong pleasant smell.

The identification and isolation of chemical constituents is an important step and provides a basis for further in depth studies. However, the characterization of the mechanism and the biological activity of chemical constituents have special significance. In case of ginger (*Z. officinale*) a wide spectrum of chemical constituents has been identified. Most of the phytochemicals found in the genus *Zingiber* also are characterized pharmacologically. Still there are lot more endemic and wild members of this genus and also the family Zingiberaceae which needs chemical characterization. Special efforts are needed to explore their biological activity and the quantity required for action. For essential oil extraction from various plant-parts including the rhizomes of ginger, hydrodistillation or steam-distillation has been proved to be the pre-dominated and effective one (Chattoopadhyay 2004; Jatoi et al. 2007). In bio-chemical investigation gas-chromatography and gas-chromatography-mass spectroscopy was the dominant tool used for the separation and detection of volatile compounds from the ginger rhizomes (Jatoi et al. 2007). However, in the current study the crude essential oil was first subjected to chromatographic separations (TLC and CC) where from three different fractions were isolated in pure form and were selected for their characterization. Generally, there occurs large quantitative variation in both major and minor chemical constituents in the rhizome essential oils. The aromas of different spices including the gingers are caused by volatile odorous compounds that differ widely in yield and compositions in different individuals

and even among different parts of the same plant (Chattopadhyay 2004). Hence a comprehensive study is needed to address this issue systematically using new technology and new approaches. In the present study, only three of the chemical constituents from this 'new' ginger variety were isolated and characterized. Investigation is in process for isolating other bioactive compounds and testing of the compounds their mode and site of action of these in the same laboratory. Further research is a crying need to examine the effect of extraction and analysis methods, plant parts and forms used, and techniques on the quality, quantity and the reproducibility of chemical constituent measurements. Moreover chemotypic variation of the essential oil of this significant plant species using various ecotypes and different plant-parts could also be interesting.

Chapter 5b

Chapter 5b

Cytotoxic assay of bioactive compounds extracted from *Z. moran* rhizome essential oils by hydrodistillation against HeLa cancer cell lines

5b.1 Introduction

Cancer is a growing health problem around the world, particularly with the steady rise in life expectancy, increasing urbanization and the subsequent changes in environmental conditions, including lifestyle. According to a recent report by the World Health Organization (WHO) in 2003, there are now more than 10 million cases of cancer per year worldwide. The interest in biodynamic phytotherapy has been increased many fold all over the world because of reasonably safe and affordable remedies for many diseased conditions. In developing countries like India, the indigenous system of medicine, together with folklore medicine continue to play a significant role in healthcare system of the population. During the past few decades, there have been renewed attention and interest in the use of traditional medicine globally (Sheldon 2000). The apex body like WHO has recognized the traditional medicines as an important contributor to achieve its health goals set so for. According to the WHO estimates, 80% of the world's population is still dependent on traditional medicines. About 65% of the population in the rural area in developing countries uses forms of traditional medicine to meet their primary healthcare needs (Anonymous 1992). Moreover, traditional medicine practices conserved over decades from old civilizations can serve as an effective basis for the discovery and development of modern therapeutic drugs.

Although cancers are characterized by the deregulation of cell signaling pathways at multiple steps, most current anticancer therapies involve the modulation of a single target. The ineffectiveness, lack of safety, and high cost of mono-targeted therapies have led to a lack of faith in these approaches. As a result, many pharmaceutical companies are increasingly interested in developing multi-targeted therapies. Many plant based products however, accomplish multi-targeting naturally and, in addition, are inexpensive and safe compared to synthetic agents. However, because pharmaceutical companies are not

usually able to secure intellectual property rights to plant based products, the development of plant based anticancer therapies has not been prioritized. Nonetheless, plant-based products like curcumin, has shown significant promise against cancer and other inflammatory diseases which is derived from *C. longa*, a promising medicinal plant from the family Zingiberaceae (Sa and Das 2008).

Plants have been one of the important sources of medicines even since the dawn of human civilization. In spite of tremendous development in the field of allopathy during the 20th century, plants still remain one of the major sources of drug in the modern as well as traditional system of medicine throughout the world. Over 60% of the total pharmaceuticals are plant-based. Phytochemicals are non-nutritive components in the plant-based diet ('phyto' is from the Greek word meaning plant) that possess substantial anticarcinogenic and antimutagenic properties. Among the medicinal plants used to derive phytochemicals for drug manufacturing, Zingiberaceae stands in a prime position with its significant members. *C. longa* (turmeric) and *Z. officinale* (ginger) of this family are two important cultivated medicinal crops of India. Turmeric is a holistic gift of nature. Its use in medication, culinary and cosmetics is well known since centuries. Its constituents, especially curcumin has been found to possess tremendous therapeutic potency to the extent of incorporating the curcumin nucleus in many other compounds. Ginger is another crop with versatile use in food, medication, culinary and traditional aspects. Indian Vaidyas, Hakims as well as tribal people have developed and recorded the knowledge of the various uses of these plants. Various scientists and research workers reported a lot of scientific work on these two plant species. Anticancerous property of bioactive compounds from the essential oils of turmeric and ginger has been well established in the past decade (Dorai et al. 2000; Pillai et al. 2004; Shukla and Singh 2007; Wang et al. 2003; Manju and Nalini 2005). However, there are far more in nature's lap than one can imagine. Apart from the common ginger and turmeric, many other Zingiberaceae species are found to possess a fair range of antimicrobial and anticancerous properties (Sakinah et al. 2007; Wahab et al. 2009). Some wild varieties of Zingiberaceae family which are found to grow in the rich vegetation of NE Indian flora possess enormous medicinal and therapeutic values. These plants and plant-derived products have been used by the local people in the indigenous system of medicine and

health care for centuries. *Zingiber moran*, a wild relative of ginger, is such a species known only for its immense medicinal properties. The species is a native of Assam and its habitat is restricted to a few districts of Assam, Nagaland and Arunachal. The highly scented rhizomes possess essential oils and are used for various home-made therapies amidst the local folk of this region.

The objective of this chapter was therefore to evaluate the *in vitro* cytotoxic effect of the three isolated and purified fractions of rhizome essential oil of *Z. moran* on HeLa cell line. The cell morphology and anti-proliferative activity of all the fractions were aimed to be accessed by qualitative methods. The experiments also intended to examine the dose dependency of the compounds in inducing cellular apoptosis on the HeLa cancer cell lines.

5b.2 Literature Review

Cancer cells are characterized by loss of growth control, invasiveness and metastasis. This can be caused by chemical and biological agents called carcinogens which damage or alter the DNA and lead to the transformation of genes controlling cell proliferation, differentiation and apoptosis. In normal tissue the rate of normal cell growth and death are kept in balance whereas in cancer this balance is disrupted leading to either cellular overgrowth and or lack of apoptosis of damaged cells later becomes malignant (Chathoth et al. 2008). Apoptosis or programmed cell death is the mechanism by which old or damaged cells are normally self destroyed, so the induction of apoptosis can be considered as a promising approach towards cancer therapy. Indeed, some chemotherapy relies on apoptosis of tumor cells (Chathoth et al. 2008). The limited progress achieved by cancer therapy in the last three decades (Parkin et al. 2005; Jemal et al. 2007) has increased the interest of researchers in cancer chemoprevention. It is becoming accepted that cancer chemoprevention (use of chemicals to prevent, stop or reverse the process of carcinogenesis) is an essential approach to control cancer. Moreover, since the process of carcinogenesis can take several decades to complete, it makes more sense to prevent cancer at its earliest stages by using low toxic chemicals (chemoprevention) than to wait until the disease has reached its final stages, where it becomes necessary to use more toxic chemicals (chemotherapy). Chemoprevention was described as the use of natural or

synthetic chemicals allowing suppression, retardation or inversion of carcinogenesis (Duvoix et al. 2005). Chemopreventive products presents low side effects and toxicity, neutralization of carcinogens as well as their effects on cells. Most chemopreventive agents known until today are plant extracts, subdivided into two classes; (i) blocking agents, which inhibit the initiation step by preventing carcinogen activation and (ii) suppressing agents, which inhibit malignant cell proliferation during promotion and progression steps of carcinogenesis (Duvoix et al. 2005). Chemoprevention and chemotherapy are one of the methods used in cancer treatment, refers to the approach of treating cancer cells with anticancer drugs that can destroy cancer cells and stop uncontrolled cell growth. Research over the last decade has shown that phytochemicals, chemical agents obtained from plant (fruits, vegetables, spices etc.) exert their inhibitory effect on carcinogenesis and tumor progression. Extensive research in the last few years has demonstrated that some dietary compounds such as curcumin, capsaicin, 6-gingerol, ajoene etc. which are present in common spices have inhibitory effect on human cancers (Chathoth et al. 2008). This suggests that these phytochemicals may serve as chemotherapeutic agents. *In vitro* and *in vivo* experimental studies indicate that these phytochemicals interfere with several cell signaling pathways and lead to apoptosis and cell cycle arrest (Chathoth et al. 2008). A population based study of cancer incidence showed a lower percentage of cancer victims in Southeast Asian countries as they consume large amounts of these phytochemicals through their diets (Dorai and Aggarwal 2004). Since these phytochemicals are consumed in daily life as dietary components, they have received much attention among the public, medical and research community. Furthermore, as these agents are obtained from natural sources, and have been consumed by people for centuries, they can be considered as safe chemotherapeutic agents (Chathoth et al. 2008).

Cancer causes significant morbidity and mortality and is a major public health problem worldwide. An effective cancer preventive program, diet and exercise may decrease the incidence of cancer. Plant-derived compounds are known to have curative potential. Till date, several hundred scientific studies focused on the activity of non-nutritional compounds present in the diet, preventing the occurrence of degenerative diseases, such as cancer. This heterogeneous class of molecules, generally known as

phytochemicals includes vitamins (carotenoids) and food polyphenols, such as flavonoids, phytoalexins, phenolic acids indoles and sulfur rich compounds (Russo 2007; Sporn and Suh 2002; Surh 2003). More than 10,000 phytochemicals have been described, and among them more than 6,000 compounds are included in the class of flavonoids (Hairborne 1993). They are widely present in plant derived foods and beverages (fruits, vegetables and beverage such as tea, wine beer and chocolate), and in many dietary supplements or herbal remedies. Due to the variety of their physiological roles in plant tissues in regulating enzymes involved in cell metabolism and in mechanisms of defence against foreign agents (radiations, viruses, parasites), phytochemicals have been associated to pleiotropic effects in animal cells. Phytochemicals attracted scientists' interests since the demonstration that their biological targets in mammalian cells were the same involved in inflammatory processes and oncogenic transformation, such alterations of cell cycle control, apoptosis evasion, angiogenesis and metastases. In addition, a large number of epidemiological studies suggest that a daily intake of phytochemicals can reduce the incidence of several types of cancers (Sporn and Suh 2002; Surh 2003; Hairborne 1993; Russo et al. 2005; D'Incalci et al. 2005).

Epidemiological, biological and clinical studies have provided various lines of evidences that dietary factors have a profound impact on etiology and prevention of human cancers (Surh 2003). It has been estimated that 10–70% (average 35%) of human cancer mortality is attributable to diet factors (Doll and Peto 1981). Therefore, chemoprevention of cancers by nutraceuticals and phytochemicals has become a flourishing research field in the past decade (Manson 2003; Surh and Ferguson 2003; Gosslao and Chen 2004). Researchers have worked on many medicinal plants and plant-products and successfully established the anticancerous property of these across the world (Katiyar et al. 1996; Bode et al. 2001; Morrison 2003). Among these, the members medicinal family Zingiberaceae acquire a prominent position. The chief plants like *C. longa, C. amada, C. caesia, C. zedoaria, Z. officinale, Z. casuamonar, Z. zerumbet* etc. have been extensively investigated for various therapeutic properties. Moreover, anticancerous properties of the golden spices *C. longa* and *Z. officinale* have been established by a good number of studies all over the world (Manju and Nalini 2005; Miyoshi et al. 2003; Wang et al. 2003). The bioactive compounds found in the rhizome

essential oil of these species possess a variety of health supporting virtues. The chief phenolic compounds viz; curcumin, gingerol, zerumbone etc. are effective against tumor formation tested against many cancer cell lines (Ramachandran and You 1999; Dorai et al. 2000; Pillai 2004). Such natural compounds are effectively confirmed to trigger apoptosis in the malignant cells. Ginger is one of the widely used spices and has been used in traditional oriental medicines to ameliorate such symptoms as inflammation, rheumatic disorders and gastrointestinal discomforts for long time (Peng 1992). Its extract and major pungent principles, such as [6]-gingerol and [6]-paradol have recently been shown to exhibit a variety of biological activities including anticancer activities (Habsah et al. 2000; Leal et al. 2003; Wang et al. 2003; Ficker et al. 2003).

Cervical cancer remains a critical public health problem that is second only to breast cancer in overall disease burden for women throughout the world (Monsonego 2006). This cancer has been targeted by researchers to discover new anticancer drugs that can replace the current unsafe regimens for such disease (Liu et al. 2008). Although readily detectable in its pre-malignant stage, cervical cancer remains the second most common women's cancer worldwide. Cervical cancer is important not only because it is the most prevalent cancer in women in several developing countries, but also because it is often diagnosed in young patients as well as at the age of 48 years, giving the treatment of this disease a degree of societal importance (Ries et al. 2006). The understanding that infection with human papillomaviruses (HPVs) leads to the development of cervical cancer, predominantly through the action of viral oncogenes, may lead to effective treatment strategies. Natural products are suitable alternatives that can be used instead of platinum-based drugs in control of cervical cancer, which show some harmful side effects (Wang et al. 2008); however, studies have demonstrated that natural products are significantly less harmful when tested on human peripheral blood lymphocytes compared to cisplatin (Zubairi et al. 2007).

Ginger rhizome extracts are found to inhibit the proliferation of several cancer cell lines, while the growth of Chang normal liver cells, normal human dermal (2F0-C25), and colon (CCD-18 Co) fibroblasts was less affected (Sakinah 2007; Murakami et al. 2002). With regards to the cyto-selective toxicity and the versatile biological activities of the bioactive compounds isolated from *Z. moran*, the present study was suggested to

explore the antiproliferative effects and the apoptogenic ontogeny on human cervical cancer cell (HeLa). A HeLa cell is an immortal cell line used in medical research particularly in cancer research. The cell line was derived from cervical cancer cells taken from Henrietta Lacks, who died from her cancer in 1951. Initially, the cell line was said to be named after a "Helen Lane" in order to preserve Lacks's anonymity (Terry 2006). The current study is the first attempt to check the effects of *Z. moran* rhizome essential oil on biological system.

5b.3 Materials and methods

5b.3.1 Materials

The material used in the study are the three different rhizome oil fractions of *Z. moran,* isolated by hydrodistillation subsequently purified and identified by analytical chemistry methods described in the previous chapter. These compounds were identified as Linalool, Citral and Camphene. The *in vitro* cytotoxic effect of these three compounds was evaluated on Human cervical adenocarcinoma cell line (HeLa). The cell culture reagents and plastic-wares were purchased from Sigma-Aldrich (Bangalore, India). All other chemicals, unless otherwise stated, were obtained from Merck (Mumbai, India) and were used without further purification.

5b.3.2 Cell culture and maintenance

Human cervical adenocarcinoma cell line (HeLa) was obtained from National Centre for Cell Science (NCSS) Pune, India. Cell line was grown in Dulbecco's modified essential medium (DMEM), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 1% antibiotic - antimycotic solution (1000 U ml/l penicillin G, 10 mg ml/l streptomycin sulphate, 5 mg ml/l gentamycin and 25 µg ml/l amphotericin B). Cells were maintained at 37 ^{o}C in a humidified atmosphere supplied with 5% CO_2 (HF 160W, Heal Force). Cells were frequently examined under an inverted microscope for confluency and viability.

5b3.3 Cytotoxicity assay

Initially, each compound was dissolved in DMSO to make a stock solution of 10 mg/mL, and then appropriate concentrations of Camphene Citral and Linalool fractions (ranging

from 10 µg/ml to 100 µg/ml), were prepared with cell culture medium (DMEM without serum) before each experiment. The final concentration of DMSO was maintained around 1% in all cases. The cytotoxic effects of these fractions on HeLa cells were determined by MTT (methyl-thiazolyldiphenyl-tetrazolium bromide) assay (Mosmann 1983). Cells grown in T-25 culture flasks were harvested by trypsinization and were then seeded in a 96-well culture plate (Cell Bind, Corning®), at a rate of approximately $1x10^4$ cells/well. The plate was incubated for 24 h to allow adequate cell growth in wells. Subsequently, the medium from each well was removed and the cells were washed twice with Dulbecco's phosphate buffered saline without Ca^{+2} and Mg^{+2}. The cells were then exposed to different concentrations of test samples. After 24 h of treatment, the contents of each well were replaced with equal volume of MTT solution (0.5 mg/mL dissolved in serum free DMEM) and the plates were further incubated for a period of 4 h. Finally, the MTT solution was replaced with equal amount of DMSO and the absorbance (Abs.) was read at 570 nm in a Multi-mode multi-well plate reader (infinite m200, Tecan). The cell viability (%) was determined by following equation. The concentration of each compound was plotted against corresponding viability %, and the IC_{50} value was calculated as the concentration of the compound at which 50% cell death was achieved.

$$\text{Viability (\%)} = \frac{\text{Abs. of treated}}{\text{Abs. of untreated}} \times 100$$

5b.3.4 Morphological studies with light microscopy

The effect of each compound on cell morphology was assessed by microscopic examination. Initially, HeLa cells were cultured in a 24-well culture plate (Cell Bind, Corning®) for 24 h till a sub-confluent monolayer was obtained. Then, they were exposed to various concentrations of extracts ranging from 10 µg/ml-100 µg/ml. Cells under treatment were observed periodically through inverted light microscope. After 24 h of treatment, cells exposed to different concentrations were imaged with the help of an inverted light microscope (OXDY-1, ProSciTech) equipped with CCD camera (ODCM 900, ProSciTech).

5b.3.5 Fluorescence microscopic assay (Live / dead staining)

The effect of each of the three compounds on cell morphology and viability was also assessed qualitatively by fluorescence based microscopic examination. Once the cancer cells were treated with the tested compounds for 24 h, cells were subjected to staining with the florescent dyes Acridine orange (AO) and Ethidium bromide (EB). Stained cells were visualized and images were captured with the help of an inverted light microscope equipped with fluorescence unit (ProSciTech) and CCD camera.

5b.4 Result and Discussion

Natural products provide a great chemical structural diversity. In this respect, sesquiterpines have been demonstrated to stimulate cytotoxicity and apoptosis in several cancer cell lines at low micromolar concentrations with an acceptable clinical range of new anticancer drugs (Song et al. 2005; Tabopoda et al. 2007). Sesquiterpene have been found abundantly in the genus *Zingiber*, a group of plants which are commonly found in Southeast Asia (Sakinah 2007). Apoptosis is biological phenomena that involved in process ranging from embryogenesis to ageing, from normal tissue homoeostasis to many human diseases. Apoptotic cells share a number of common features such as cell shrinkage, nuclear condensation, membrane blebbing, chromatin cleavage, and formation of pyknotic bodies of condensed chromatin (Lin et al. 2007; Doonan and Cotter 2008). These distinctive morphological features form the basis of some of the most widely used techniques for the validation of apoptosis, and thus morphologic description using light or electron microscopy remains one of the best ways to identify apoptosis (Doonan and Cotter 2008). The programmed cell death (apoptosis) compared to necrosis is a desired somatic defense mechanism against cancer cells (Mandoky 2008). Moreover, apoptosis is reported earlier to be triggered by natural products (Zhou et al. 2008; Lee et al. 2008). Camphene, Citral and Linalool were found to be the promising natural products that possess potentiality to induce apoptosis on cervical cancer cell lines (HeLa) in the current study which was intended to provide an evidence of apoptosis on HeLa cells induced by these products.

5b.4.1 Cytotoxicity assay

The effect of the three purified rhizome essential oil fractions of *Z. moran* namely Camphene, Citral, and Linalool on HeLa cell viability was assessed by the MTT (methyl thiazole tetrazolium) colorimetric assay which is based on the reduction of MTT by the mitochondrial succinate dehydrogenase of intact cells to a purple formazan product (Hussain et al. 1993). The yellow tetrazolium salt-MTT is converted to purple formazan crystals by metabolically active cells and thus provides a quantitative estimate of the viable cells (Singh and Bhat 2004). In the initial screening experiments, all the three fractions showed significant effect on HeLa cell proliferation at a concentration less than 50µg/ml.

To study the effect of the test compounds on cell growth and proliferative potential of HeLa cell line, these were dissolved in DMSO and used at a concentration range between 10-100µg/ml. The cancer cell lines were exposed to the compounds one day after seeding and their viability was assessed using the MTT assay. Results are presented as percentage of viability and each value was measured in triplicates. All of the three compounds, showed significant effect on HeLa cell viability at a concentration as low as10µg/ml. Figure 5b.1 shows the effect of Camphene, Citral and Linalool fractions on the viability of HeLa cells as estimated by MTT assay. It is clear from the graph that exposure to the test compounds at a range between 10-30 µg/ml strongly inhibited the growth of the cell line by 60-80% indicating that the compounds affect cell viability in a dose dependant manner. There was no growth inhibition of the cell line by DMSO vehicle (used at the same final concentration of 1%). The results clearly suggested a significant loss of cell viability upon increasing concentration of the fractions. IC_{50} values were calculated to be 38.54µg/ml for Camphene, 39.65µg/ml for Citral and 55.44µg/ml for Linalool fraction. Similar results were reported by Li et al. (2008) for Selenium-enriched green tea extract where they found an IC_{50} value of 278.6µg/ml and 431.6µg/ml.

Cancer cell death phenomenon could be induced through different pathways (Humphreys and Halpern 2008). Enzyme MTT-dehydrogenase present in mitochondria of living cells could serve as the indicator of apoptosis (Lin et al. 2005). This enzyme could be measured *in vitro* using colorimetric MTT assay which results showed an obvious decrease of living cells in *Z. moran* essential oil compounds treated group. From

the graphs, Camphene and Citral were found to exert better cytotoxic effects on HeLa cancer cells at lower concentrations compared to Linalool which needed a little higher concentration to induce similar effects. Quantification of apoptosis with differential scoring of treated and control HeLa cells revealed a significant ($P < .05$) difference in the number of apoptotic cells in a time-dependent manner but with insignificant difference between numbers of necrosis positive cells in control and treated cells, concluding that none of the three compounds did not induce necrotic effects at a concentration below 50 µg/ml. Fluorescence microscopy examination confirmed the onset of apoptosis features. The morphological criteria that implicate apoptotic cell death were further confirmed by live-dead staining assay by fluorescence microscopy. There has been substantial interest in finding potential chemopreventive constituents from ginger in the past 5 years, but the compounds that have been dealt with were limited (Katiyar et al. 1996; Lee and Surh 1998; Surh et al. 1998; Bode et al. 2001; Keum et al. 2002; Leal et al. 2003; Miyoshi et al. 2003). Surh and coworkers have studied the inhibitory effects of 6-[paradol] and [6]-gingerol on the cell proliferation and DNA synthesis of HL-60 cells and demonstrated that their cytotoxicity was associated with induction of apoptosis and/or inhibition of activator protein 1 (AP-1) (Lee and Surh 1998; Surh et al. 1998; Bode et al. 2001). They also reported that [6]-paradol and its analogs [10]-paradol, [3]-dehydroparadol, [6]-dehydroparadol and [10]-dehydroparadol could induce apoptosis of an oral squamous carcinoma KB cell lines (Keum et al. 2002). Miyoshi et al. (2003) reported that galanals A and B isolated from ginger could induce apoptosis of human T lymphoma Jurkat cells. In addition, supercritical CO_2 extract of ginger was reported to exhibit selective cytotoxicity against nine human cancer cell lines (Leal et al. 2003) and ethanol extract of ginger was reported to be able to inhibit tumor promotion in SENKAR mouse skin (Katiyar et al. 1996). The present work studied the cytotoxicity against HeLa cell lines of 3 diarylheptanoids isolated from the wild ginger *Z. moran* that enables comparison and evaluation of their anti cytotoxic activity. It indicates that all three of the compounds (Camphene, Citral and Linalool) exhibit significant effect (IC_{50} <40µg/ml for Camphene and Citral) and (IC_{50} <56µg/ml for Linalool) on the cell proliferation of HeLa cells.

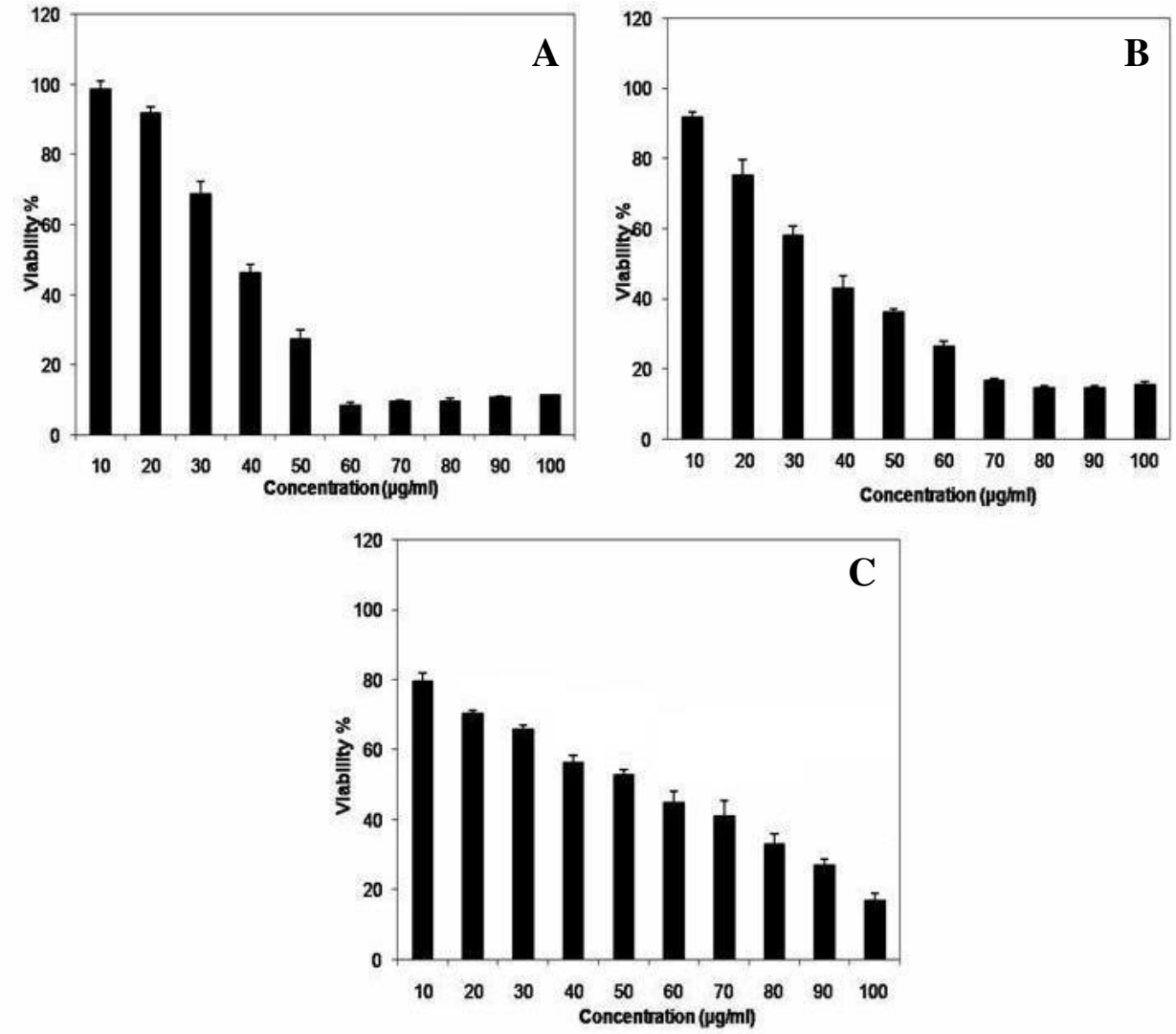


Figure 5b.1: Effect of Camphene (**A**), Citral (**B**) and Linalool (**C**) isolated from *Z. moran* rhizome oil on viability of HeLa cells at various concentrations after 24 h of treatment showing IC_{50} values for the respective compounds.

5b.4.2 Morphological studies

Apoptosis possesses distinctive morphological features which form the basis of some of the most widely used techniques for the identification and quantification of apoptosis. Thus, morphologic description of apoptosis using microscopy still remains one of the best ways to define apoptosis and to contrast it with necrosis (Doonan and Cotter 2008). In the current investigation, inverted light microscopy was employed to obtain detailed information about the cell morphology of HeLa upon treatment with the test compounds at IC_{50} in a time-dependent manner (0, 24, and 48 h). HeLa cells treated with Camphene, Citral and Linalool stimulation had typical morphological changes that implicate apoptosis such as DNA fragmentation, membrane blebbing, and apoptotic body formation, which could be evidenced by the visual bright light microscopy. There were significant morphological changes observed to the HeLa cancer cells during treatment with the test compounds after 24, and 48 h post addition. Prominent growth reduction was identified to the HeLa cells, after treatment with the test compounds as compared to untreated cells. Control cells were observed to have rapid cell growth. At 24 h post treatment, few rounded cancer cells (cell shrinkage) were observed after Camphene and Citral treatment. Whereas, the cancer cells treated with Linalool showed more rounded cells with budding cell membrane, an effect called blabbing occurred due to cell apoptosis. An increased number of rounded cells were observed at 48 h post treatment with all the three compounds. Representative inverted light microscopic images of treated cells are shown in figure 5b.2. A progressive nuclear shrinkage with increased rounded cancer cells was noticeable to the HeLa cells treated with Linalool at 48 h post treatment as compared to Camphene and Citral, which resulted in higher number of rounded cells. There was gradual increase in number of dead cells with an increase in the dose of each fraction. Also, it was noticed that cell death caused by lower doses (<50µg/ml) exhibited loss of cell-cell contacts, detachment from the substratum, membrane blabbing, and formation of apoptotic bodies which were distinctive morphological features of apoptosis. While, cell death caused by higher doses (>50µg/ml) exhibited characteristic features of necrosis, such as increased volume of nucleus and cytoplasm, vacuolization of the cytoplasm, chromatin flocculation, dissolution of nuclear membrane, and dissolution of cytoplasmic membrane leading to cell lysis. The micrographs showed distinct

morphological changes that correspond to a typical cellular surface morphology implicating apoptosis, including cell membrane blebbing, microvilli disappearance or reduction (blunt microvillus), and separated apoptotic bodies after treatment. These findings were similar that confirms to previous reported cytological features of HeLa cells undergoing apoptosis (Majumdar et al. 2001). Untreated HeLa cells were found to restore the typical morphological features of cervical cancer cells, (viz; numerous microvilli) on the cell surface with membrane connections. These results strongly provide evidence that the test compounds has promising anticancer activity toward human cervical cancer based on the observation of distinctive morphological features of HeLa cells upon treatment with the three test compounds.

5b.4.3 Fluorescence microscopic assay (Live-dead staining)

The effect of the three test compounds of HeLa cell morphology and viability were tracked by inverted fluorescence microscope staining with AO/EB (100µg/ml). This is a qualitative assessment of cell viability and apoptotic cell death where AO stains live cells and emits green fluorescence upon excitation with blue filter, where as EB stains dead cells and emits red fluorescence upon excitation with green filter. The results of live-dead staining assay are shown in figure 5b.3, 5b.4 and 5b.5 for the three compound Camphene, Citral and Linalool (at their IC_{50} values) respectively. Live cells exhibit bright-green nucleus showing condensation of chromatin as dense-green areas. Apoptotic, necrotic, and viable HeLa cells were scored under fluorescence microscope. The study revealed that all three of the test compounds triggered morphological features that relates to apoptosis in a time-dependent manner (Figures 5b.3, 5b.4, 5b.5) whereby, early apoptosis is obvious by intercalated AO within the fragmented DNA.

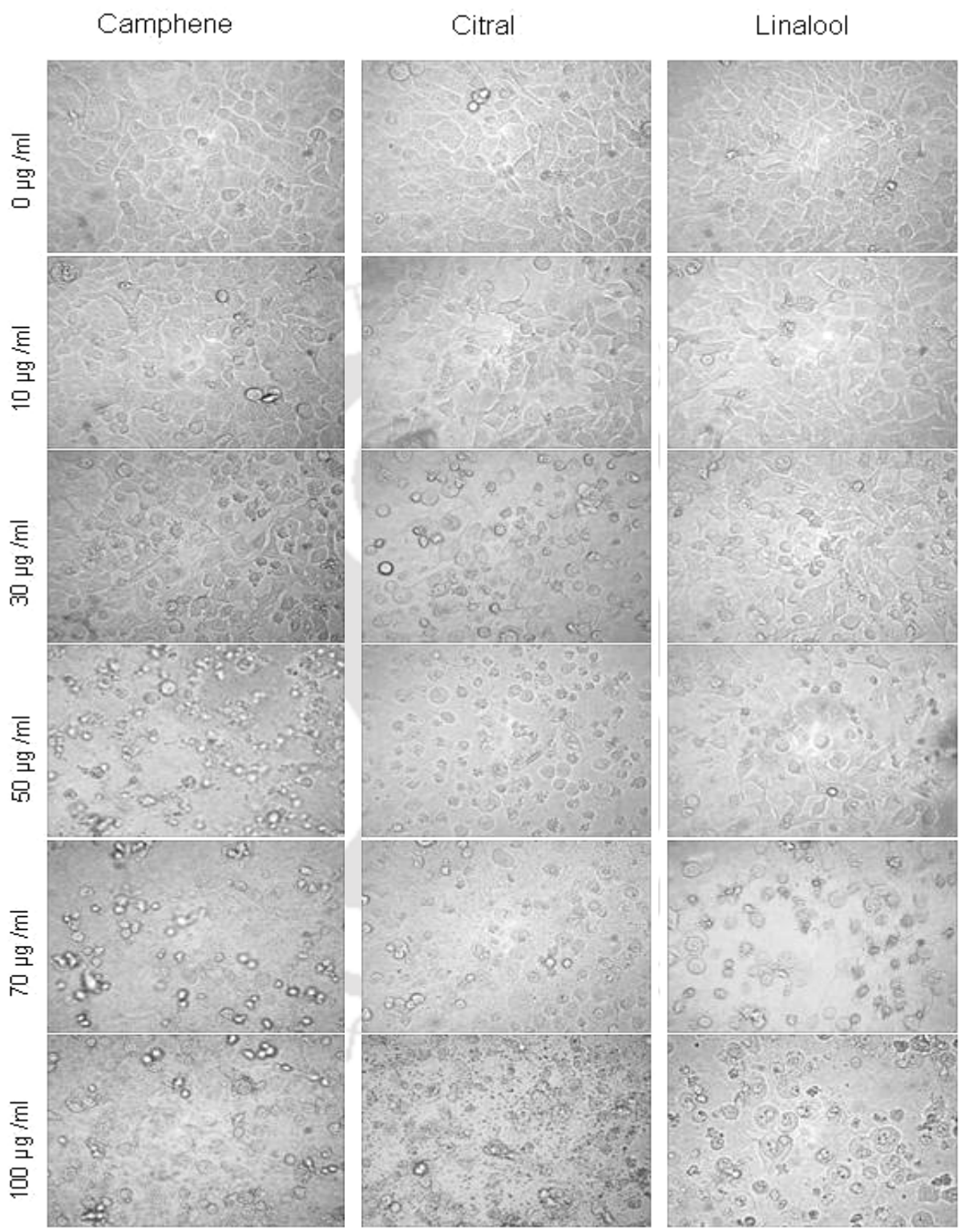


Figure 5b.2: Inverted light microscopy images of HeLa cells exposed to various doses of Camphene, Citral and Linalool isolated from *Z. moran* rhizome oil showing distinctive morphological features of apoptosis. (400 X)

In several of such cases, the fluorescent bright-green color could be seen in treated HeLa cells only. In contrast, untreated cells were observed with a green intact nuclear structure. At 48 h treatment with the test compounds, blebbing and nuclear margination were noticed (moderate apoptosis). In addition, late stages of apoptosis, such as apoptotic body separation and presence of reddish-orange color due to the binding of EB to denatured DNA, were observed after 72-hour treatment with Camphene, Citral and Linalool. Differential scoring of treated HeLa cells also showed a significant difference in apoptosis positive cells, which indicates clearly that the test compounds had a time-dependent apoptogenic effect.

The number of dead cells was found to increase after the post treatment of cells with the two stains. It is quite clear from the figure that intensity of red cells stained by EB, were increased after the treatment. In contrast, the green cells were observed to decrease which gave the live cell count. The results revealed a decrease in the viability of cancerous HeLa cells after treatment with three compounds Camphene, Citral, and Linalool in comparison to untreated cells. The observations of qualitative analysis of viability (%) as determined by live-dead staining assay were in good agreement with the quantitative analysis data as estimated by MTT assay.

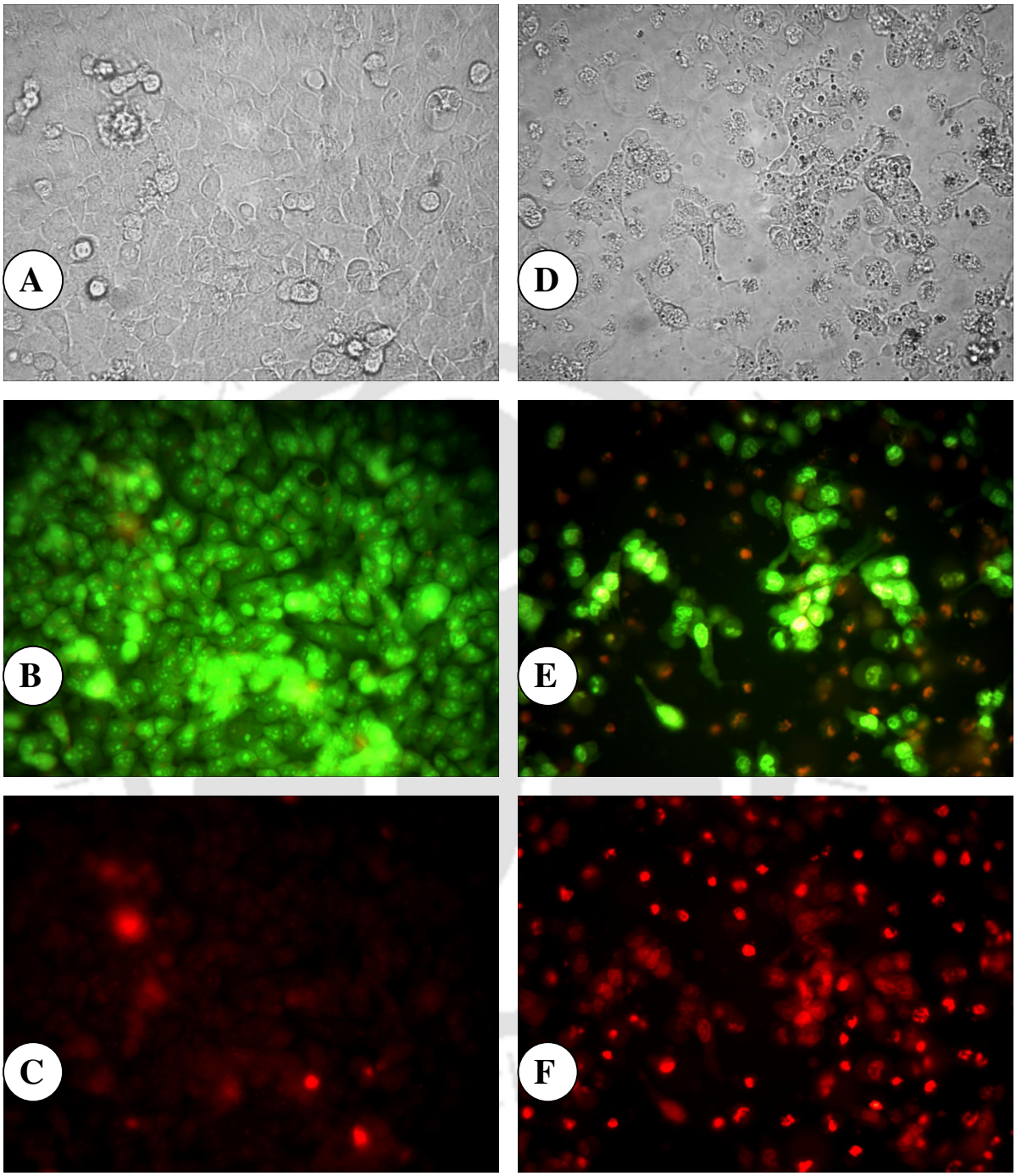


Figure 5b.3: Inverted fluorescence micrographs of HeLa cells treated with Camphene at concentrations 38µg/ml as observed after staining with AO/EB.
Green fluorescence was due to AO, gives the live cell-count
Red fluorescence was due to EB, gives the dead cell-count (400 X)
A,**B**,**C**= control cells; **D**,**E**,**F**= treated cells after 48h

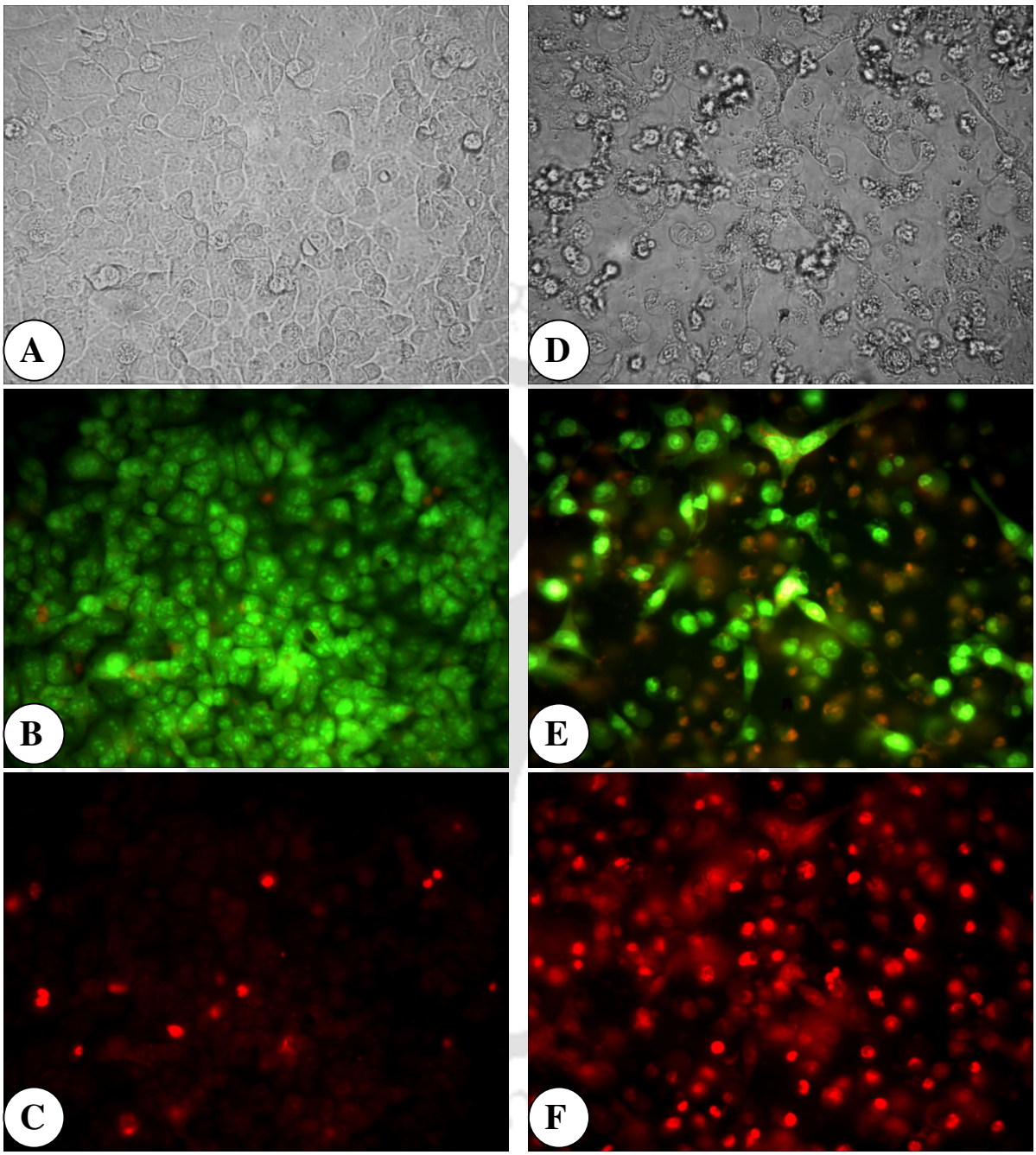


Figure 5b.4: Inverted fluorescence micrographs of HeLa cells treated with Citral at concentrations 39µg/ml as observed after staining with AO/EB.
Green fluorescence was due to AO, gives the live cell-count
Red fluorescence was due to EB, gives the dead cell-count (400 X)
A,**B**,**C**= control cells; **D**,**E**,**F**= treated cells after 48h

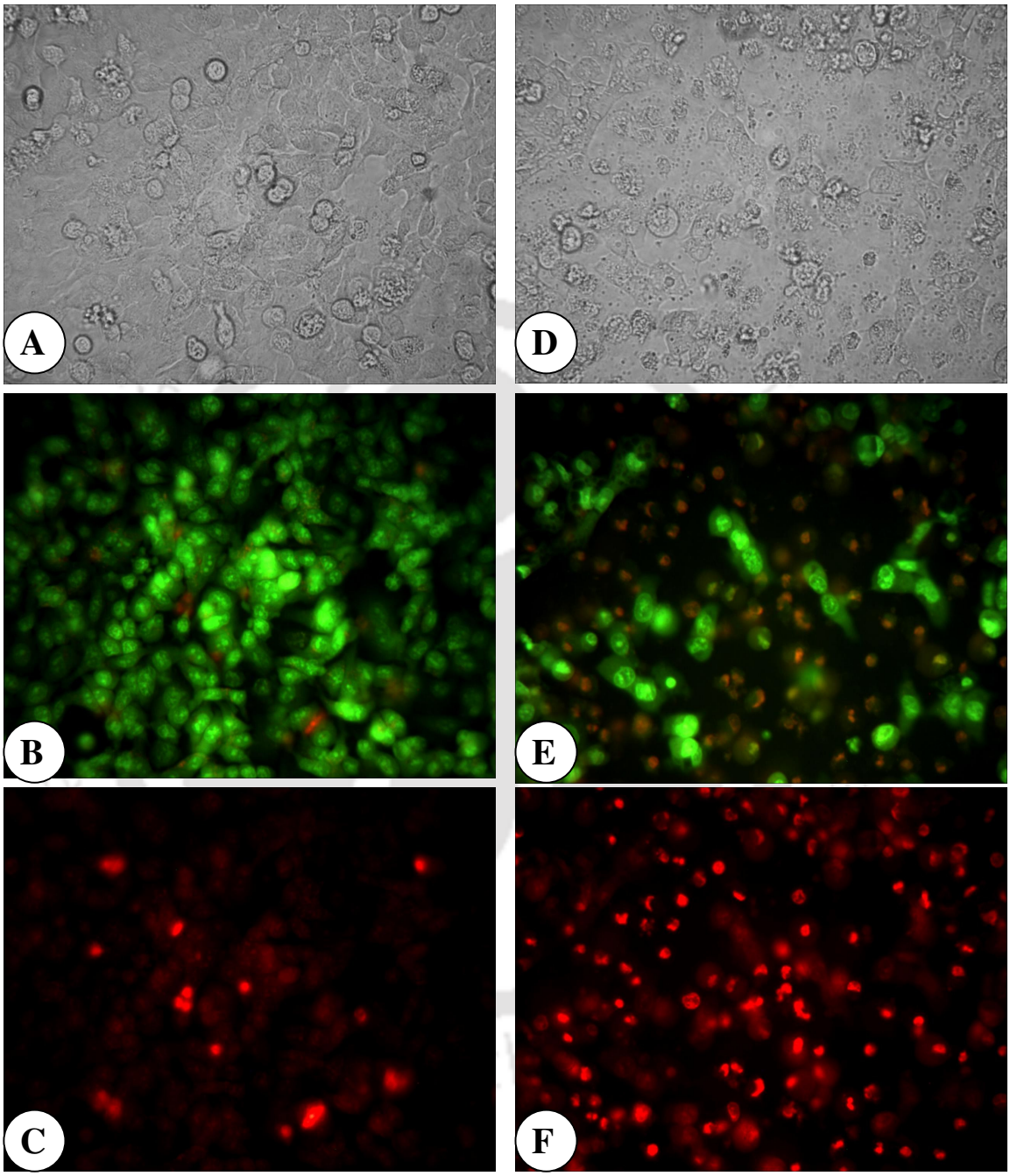


Figure 5b.5: Inverted fluorescence micrographs of HeLa cells treated with Linaool at concentrations 55µg/ml as observed after staining with AO/EB.
Green fluorescence was due to AO, gives the live cell-count
Red fluorescence was due to EB, gives the dead cell-count (400 X)
A,**B**,**C**= control cells; **D**,**E**,**F**= treated cells after 48h

Apoptosis as a desired biodefense cell death can be induced in cancer tissues (Kang et al. 2007). This induction is made possible using natural products from a variety of plants derived compounds (Matito et al. 2003). One of these natural compounds of plant derived is terpenoids (Tian et al. 2003), which included mono-, sesqui-, di-, and triterpenoids. These bio-compounds are synthesized by tandem reactions of the phosphorylated isoprene unit (Rasulav et al. 2007). Some of these dietary compounds possess chemopreventive properties that have been proven *in vivo* and *in vitro*. Clinical trials results also demonstrated that some terpenoids have the potentiality for treating cancers without major toxicity (Liu et al. 2005). Thus, further understanding of their biological and physiological mechanisms may lead to the identification of more effective compounds in this category for the prevention and treatment of targeted cancer types. Many mono-sesquiterpene from other *Zingiber* species has been reported previously to induce apoptosis in several cancer cell lines such as liver cancer (Sakinah et al. 2007) and leukemia cell lines (Xian et al. 2007). The current results of this study matches with previous studies in terms of the potential anticancer property of bioactive compounds isolated from genus *Zingiber* and the typical features of apoptosis of HeLa (Sakinah et al. 2007; Liu et al. 2008; Lin et al. 2007).

5b.5 Conclusion

Chemoprevention by edible phytochemicals is now considered to be an inexpensive, readily applicable, acceptable and accessible approach to cancer control and management. With healthcare costs being a key issue today, it would be cost-effective to promote the awareness and consumption of phytochemicals as a cancer-preventive strategy for the general public. Several nutrients and non-nutritive phytochemicals are being evaluated in intervention trials for their potential as cancer chemopreventive agents. Despite significant advances in our understanding of multistage carcinogenesis, little is known about the mechanism of action of most chemopreventive agents. The chemopreventive effects that most dietary phytochemicals exert are likely to be the sum of several distinct mechanisms. Disruption or deregulation of intracellular-signaling cascades often leads to malignant transformation of cells, and it is therefore important to identify the molecules in the signaling network that can be affected by individual chemo

preventive phytochemicals to allow for better assessment of their underlying mechanisms.

Programmed cell death or apoptosis, is now recognized as an important phenomenon that plays a key role in most of the developmental processes right from embryogenesis to senescence or aging. Apoptosis is a highly regulated mechanism by which cells undergo cell death in an active way and is also necessary to eliminate pathogen invaded cells or activated or auto-aggressive immune cells (Hengartner 2000). Programmed cell death is an active process that describes the orchestrated collapse of a cell, resulting in membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation, and DNA fragmentation (Hengartner 2000). Anti-cancer agents along with various extra cellular stresses are reported to induce apoptosis in many cell systems (Mathiasen and Jaattela 2002; Cutin et al. 2002).

As one of the most challenging tasks concerning cancer is to induce apoptosis in malignant cells, researchers increasingly focus on natural products to modulate apoptotic signaling pathways. The natural compounds isolated from the plant *Z. moran*, has chemopreventive properties, which are mainly due to its ability to arrest cell cycle and to induce apoptosis. Naturally occurring substances that block or suppress the proliferation of tumor cells are potentially potent antitumor agents. Anticancer agents from edible plants have an added advantage in their clinical application on account of their low toxicities (Vimala et al. 1999). This study suggests that the compounds Camphene, Citral and Linalool obtained from *Z. moran*, are effective as anticancer agent and have a chemotherapeutic potential on cervical cancer cells. Hence the report can be concluded that there are immense possibilities in using these natural compounds as a new drug for treatment of cervical cancer.

Our results, for the first time demonstrate that these three bioactive compounds from *Z. moran* essential oil induce apoptosis in human cervical adenocarcinoma cancer cells. The *in vitro* cytotoxicity studies revealed the potential cytotoxic activity of Camphene, Citral and Linalool fractions against HeLa cell line. Three of the compounds, being hydrophobic, pass easily through the plasma membrane into the cytosol (Oetari et al. 1996). Inverted light microscopic observations of treated cells revealed the dose dependent effect of compounds on the type of cell death; cells were prone to apoptosis at

lower effective dose and necrosis at higher dose. Besides, qualitative assessment of cell death and viability (%) was performed by inverted fluorescence microscopy through live AO/EB dual staining; the results were comparable to that of the MTT assay. Fragmented apoptotic nuclei (stained green) were found to be progressively stained with EB (red). A significant population of apoptotic cells was noted at 24 h after treating with Camphene, Citral and Linalool. The investigation reveals that the three compounds isolated from *Zingiber moran* plant has a significant therapeutic value and the cytotoxic activity of these compounds via apoptotic pathway has been established. However further detailed research is needed to exploit its potential as a drug.

In the present study, three natural compounds from the rhizome essential oil of the edible plant *Z. moran* were extracted, isolated purified and identified and the effect of these three compounds was also investigated against cervical cancer cell line (HeLa). It was established that the three compounds (Camphene, Citral and Linalool) have stronger cytotoxic effects toward cancer cells compared to normal cells and can stimulate apoptosis of HeLa cells. Therefore, *Z. moran* would have a bright future in the treatment of tumors and further work may lead to relative antitumor agents to be used in clinical settings.

Summary and Future Scope

Summary

The importance of plants as valuable sources for chemical compounds of medicinal value is well known from time immemorial. But presently due to the depletion of forest areas and other wild lands particularly in the tropical countries, medicinal plant materials are becoming scarce. Unless there is immediate action to salvage the remaining unprotected hotspot areas, the indigenous germplasm will soon be lost for ever. Apart from habitat degradation and loss, in medicinal plants in particular, injudicious collection is yet another important reason for genetic depletion and endangerment of species. Northeastern India is often called India's forgotten corner and it was perceived that the remoteness of the place has helped preserve its biodiversity. Comprehensive environmental impact assessments reveal the possible danger posed by a series of proposed dams across NE region to the biodiversity of the region. Besides, other reasons like tribal life style, deforestation, natural calamities, climatic changes etc. play a major role in the process of loss of biodiversity in this region. In such a situation, scientific approaches for collection, identification and conservation of wild, elite germplasm of medicinally important plants could be a promising step regarding protecting and conserving the biodiversity. Therefore, to meet the dual objective of conservation and proper utilization of the rich medicinal wealth of NE India present work was based on a series of scientific studies on two significant genera *Curcuma* and *Zingiber* from the medicinal family Zingiberaceae.

The very **first phase** of the research work was carried out by initiating the scientific and systematic collection of germplasm of *Curcuma* and *Zingiber* from NE region of India. The collected species were identified with taxonomic tools and properly maintained both as type and herbarium specimen. This study aimed to give comprehensive information about the indigenous germplasm present unnoticed till date. In addition, an endemic, traditionally significant species *Zingiber moran* was also identified for the first time and its chromosome count was also established.

Although, India is the largest producer of *Curcuma longa*, still there is a scope to improve its cultivation and export potential because the climatic condition of India is

much favorable to maximize its production. Besides *C. longa*, other commercial and medicinal species like *C. amada, Z. moran* and *Z. zerumbet* also hold the utmost application possibilities as cash crops. Application of tissue culture techniques can definitely aid a role to increase the percentage of its therapeutically active phyto-constituents. Owing to the market potential of the two selected genera *Curcuma* and *Zingiber*, both as spice, vegetable and a raw source of many medications, enormous planting material is required to raise the economically significant members. With this objective, in the **second phase**, an efficient micropropagation protocol was devised and cyto-genetic stability of the regenerated plants was assessed through RAPD markers, protein profiling and chromosome counting for the production of elite clones having higher survival rate.

Preservation of biological diversity at the most basal level entails maintenance of genetic variation. Differing patterns in magnitude and distribution of genetic variation among taxa demand different conservation measures. The basic principle governing the conservation of any species is the inclusion and maintenance of maximum genetic diversity (Ananthakrishnan, 2001). Therefore the **third phase** of the study dealt with teasing out the intra- and interspecific genetic diversity among 9 *Curcuma* species and 10 ecotypes of *Z. moran* using molecular markers (RAPD, ISSR and AFLP). In spite of being vegetatively propagated, species under study revealed higher range of inter and intraspecific variations. Intraspecific genetic variations can also be utilised as a possible solution to the vulnerability of many monocultured plants to disease and pests. Moreover, greater genetic diversity leads to greater productivity in plant communities, greater nutrient retention in ecosystems as well as greater ecosystem stability.

Fourth phase involved screening of the antimicrobial spectra of rhizome oils of selected *Curcuma* and *Zingiber* species against various pathogenic indicators. The results confirmed the broad antimicrobial potential of all the species under study. The investigation throws light into the biological activity and therapeutic value of the most commonly used medicinal plants (and plant constituents) in traditional system of medicine. This knowledge can be used to further develop the traditional medicines in India in a more rational and scientific way.

In the **fifth phase**, best reporting rhizome essential oil of *Z. moran* obtained from hydrodistillation was progressed further for chemical characterization using different analytical techniques like TLC, CC, NMR, FTIR, HRMS etc. Finally three major bioactive components were isolated and identified which were Camphene, Citral and Linalool respectively. These compounds are reported for the first time in this wild variety of ginger. The biological applicability of these components was further tested by investigating the cytotoxic activity against human cervical adenocarcinoma cell line (HeLa). This resulted in excellent antiproliferative activity of the isolated compounds indicating the hidden potentiality for developing as anticancer agents in near future.

For the biodiversity rich developing countries in the tropics, chemical and genetic prospecting of their plant genetic resources is a priority area not only to fish out genotypes of potential economic importance but also to add value to them (Sabu 1992). The present study essentially directed towards this objective, involves scientific collection, identification, maintenance, micropropagation, analysis of genetic variations, and biochemical characterization in *Curcuma* and *Zingiber* of Zingiberaceae, an important medicinal family of India, predominantly found in NE India. Such studies could be extended also for the other Zingiberaceae members with pharmacological importance. The scientific knowledge documented so far in the present study will surely help in preparation of action plan for the development of herbal drug industry and to boost up tribal and rural economy in this region. Owing to the worldwide increasing demands of herbal products, in long term, such studies form the basis for sustainable development in terms of economics, environmental as well as socio-cultural considerations. Although these studies can exploit therapeutic potential of the plants under study, *Z. moran* in particular as drug but further studies are required to get its maximum utility.

Future Scope

Theses are the potential works which can be explored on the basis of present work:

- Development of extensive karyological studies for various members of Zingiberaceae from NE Indian flora.

- The novel approach with systematic collection and maintenance to study the unexplored germplasm of Zingiberaceae from NE India could be extended for other indigenous medicinal plants of the region.

- Expedition of propagation rate and generation of superior clones of other members of *Zingiber* and *Curcuma* through *in vitro* regeneration studies. Mass multiplication with high genetic stability particularly for elite clones could be helpful in encouraging rapid propagation and commercialization of the important genera.

- Investigation of the greater genetic diversity prevailing in the genera *Curcuma* and *Zingiber* within Northeast India and thereby signifying the key issues for crop improvement, genetic enhancement, conservation and utilization of germplasm.

- Exploring the broad antimicrobial spectra of the species under *Curcuma* and *Zingiber* and establishing their pharmacological significance in a more scientific way.

- Investigation of traditionally and locally popular elite plant species with respect to their anticancerous and other biological properties steps need to be announced regarding integrated cultivation and processing of *Curcuma* and *Zingiber* involving rural people in different districts.

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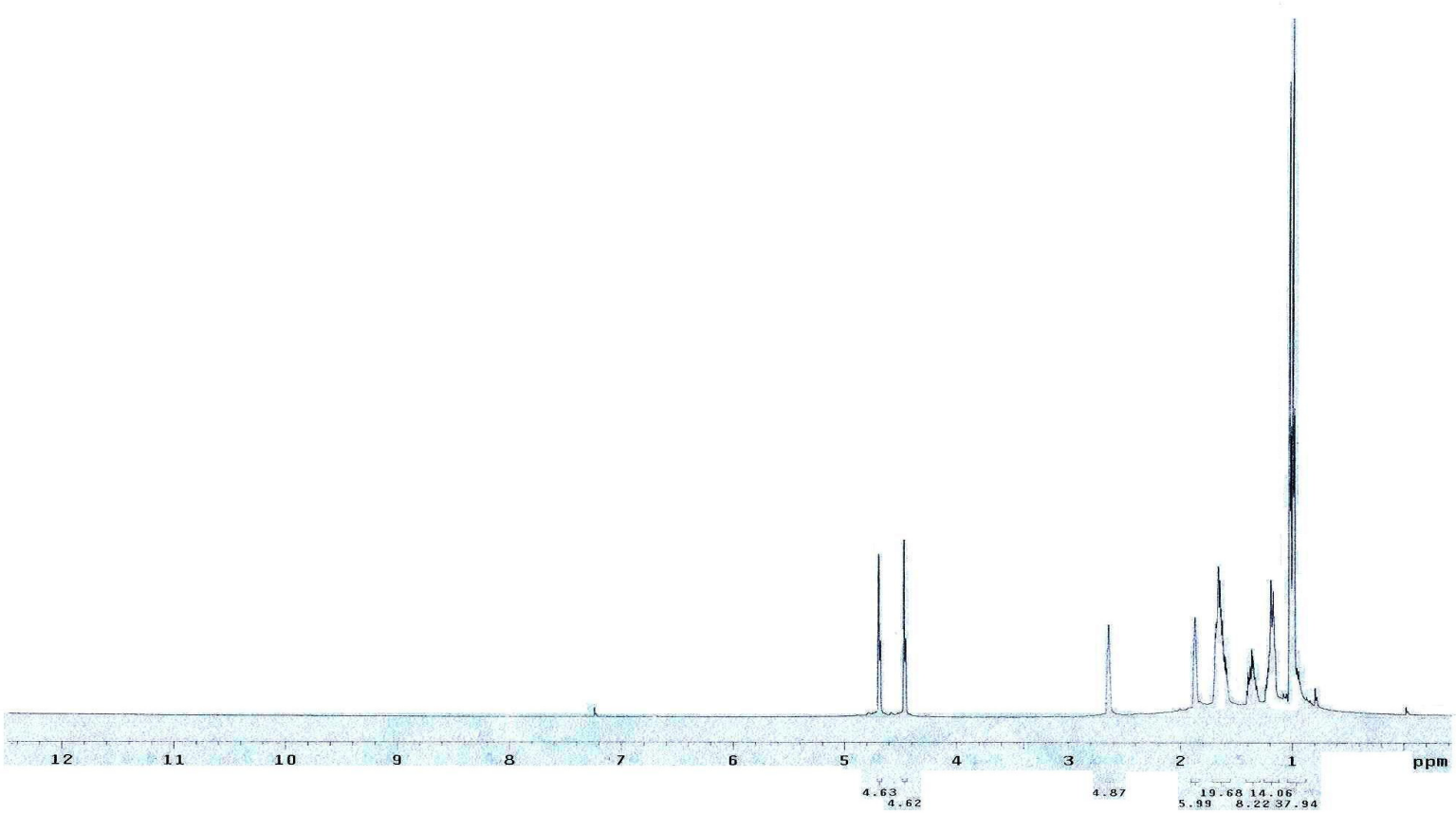
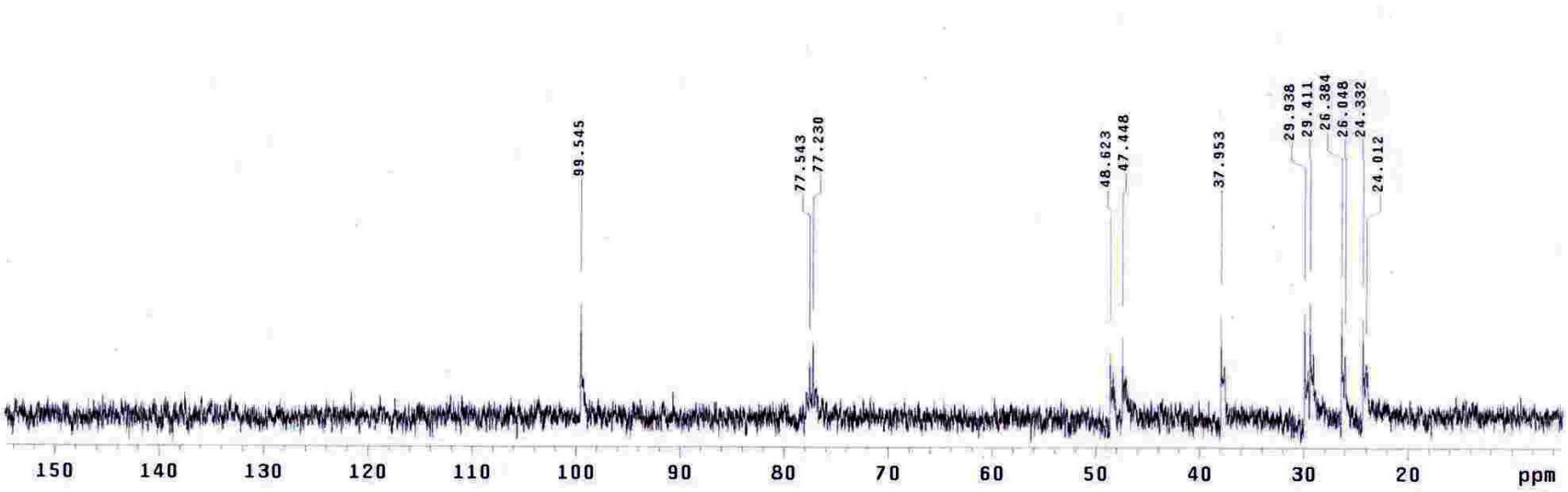
Annexure

Annexure 1. [1]HNMR spectrum of isolated fraction 1



Annexure 2. ^{13}CNMR spectrum of isolated fraction 1



Annexure 3. FTIR spectrum of isolated fraction 1

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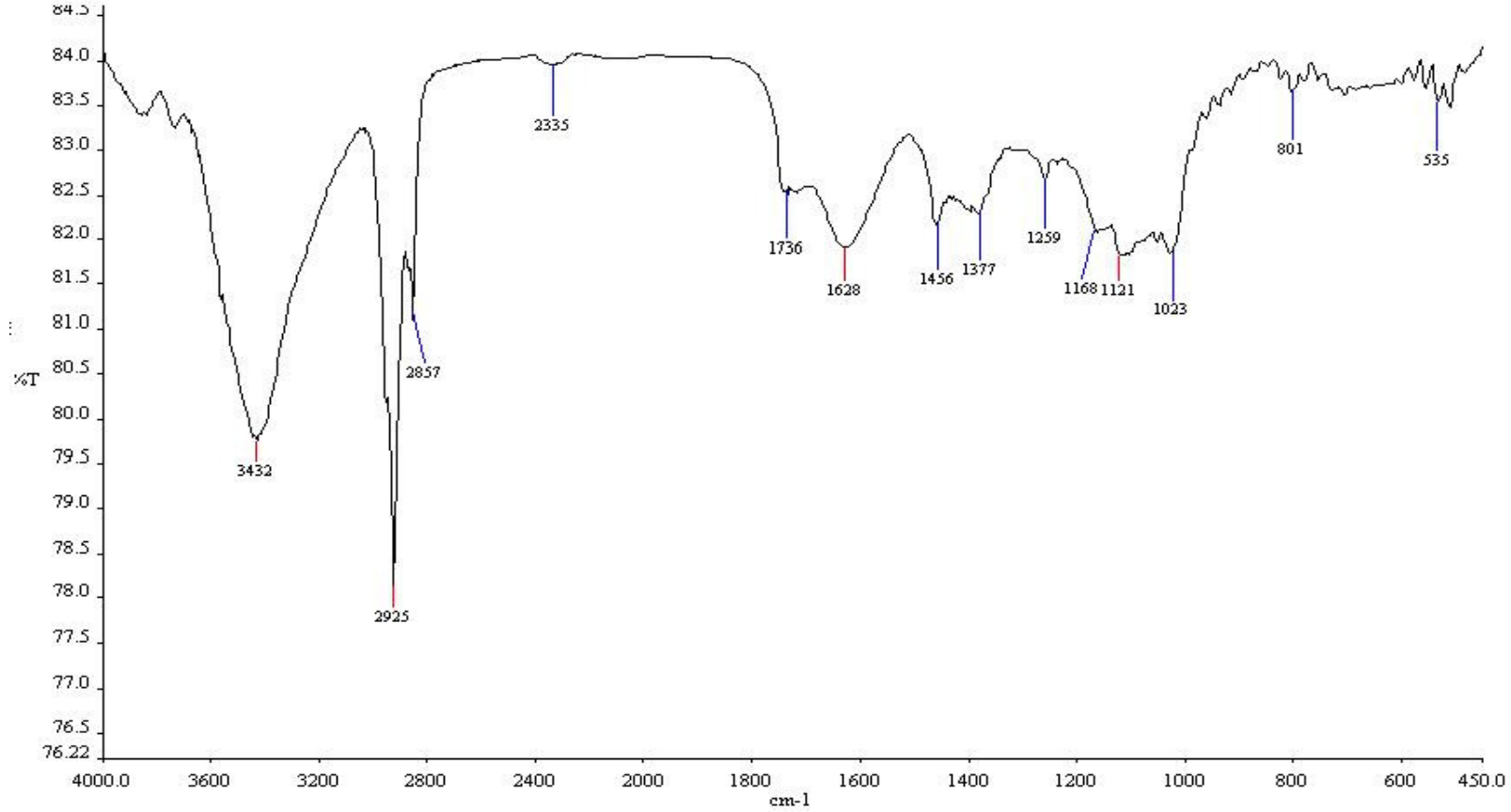
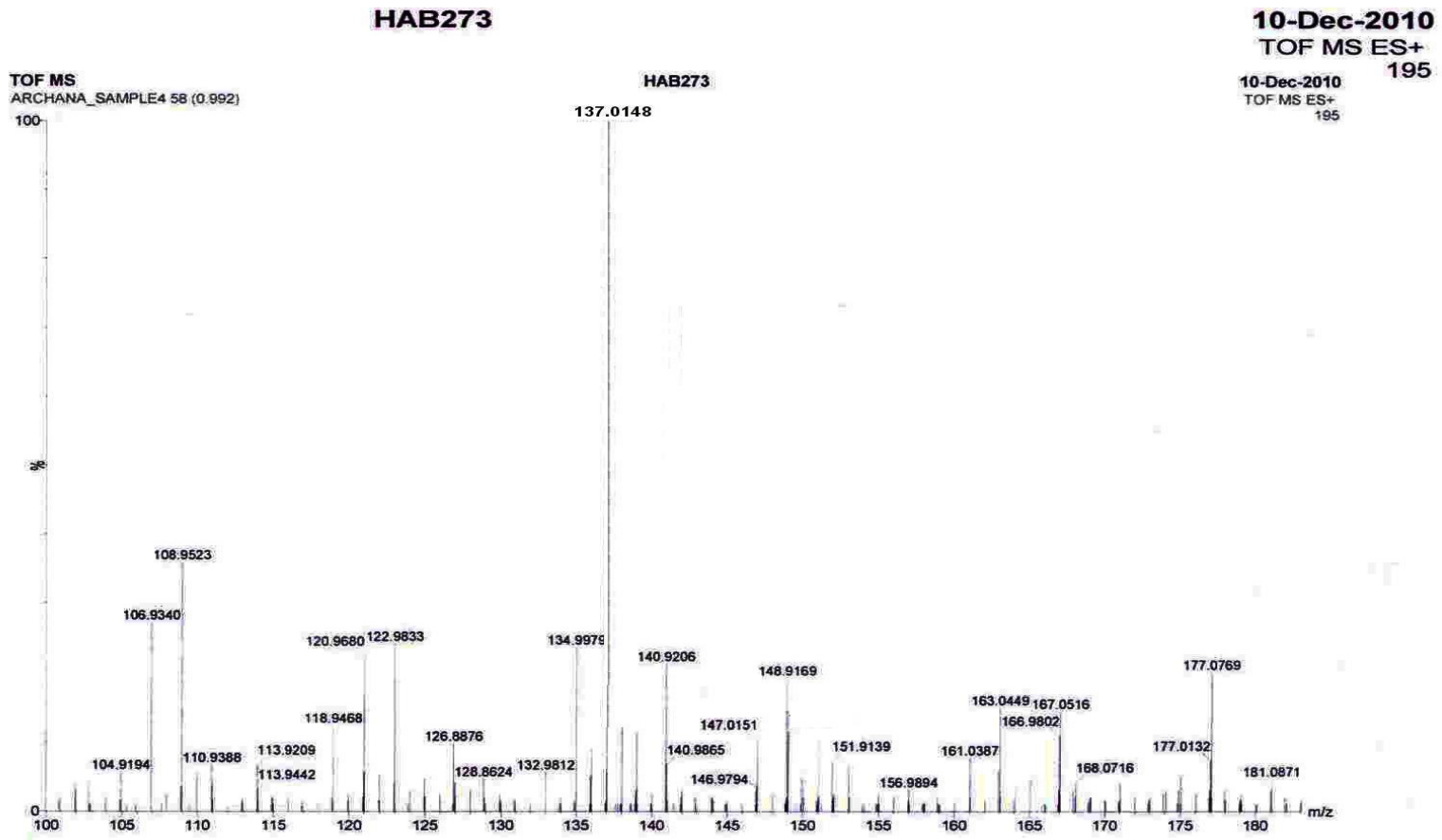
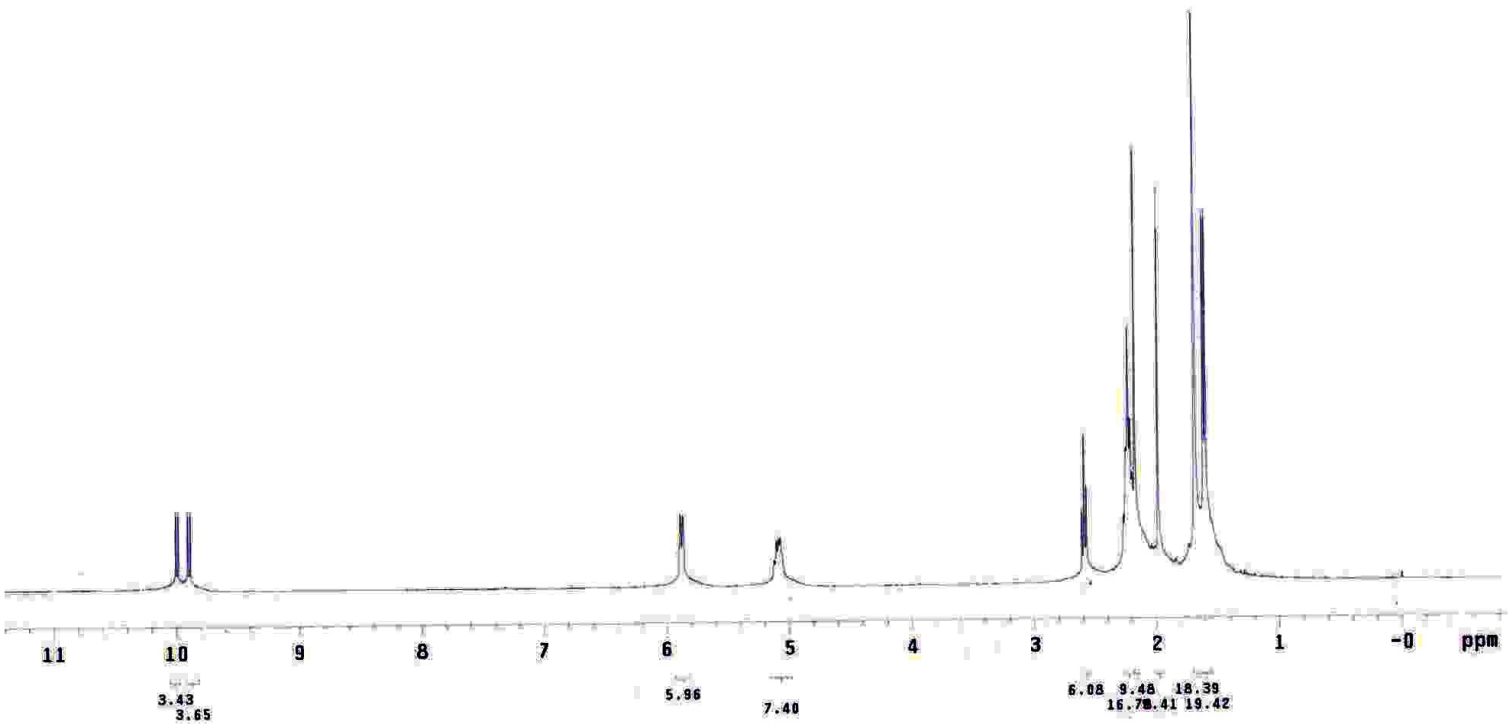


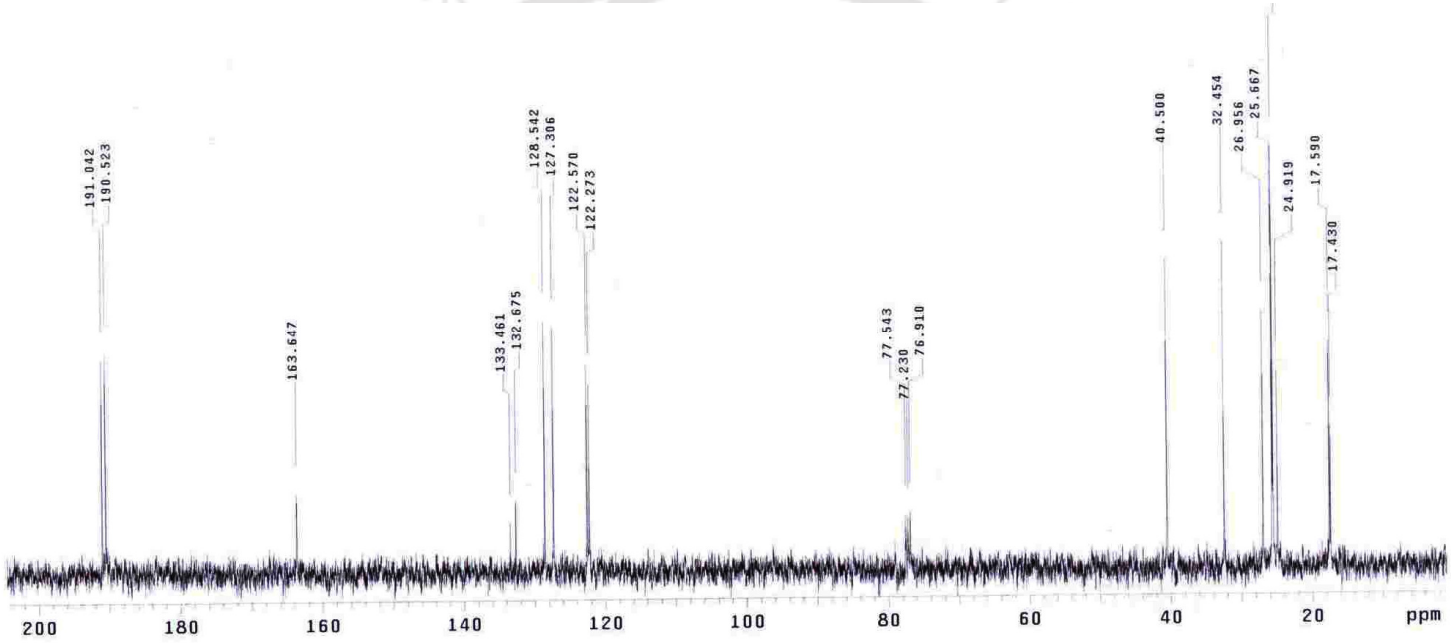
Annexure 4. HRMS spectrum of isolated fraction 1



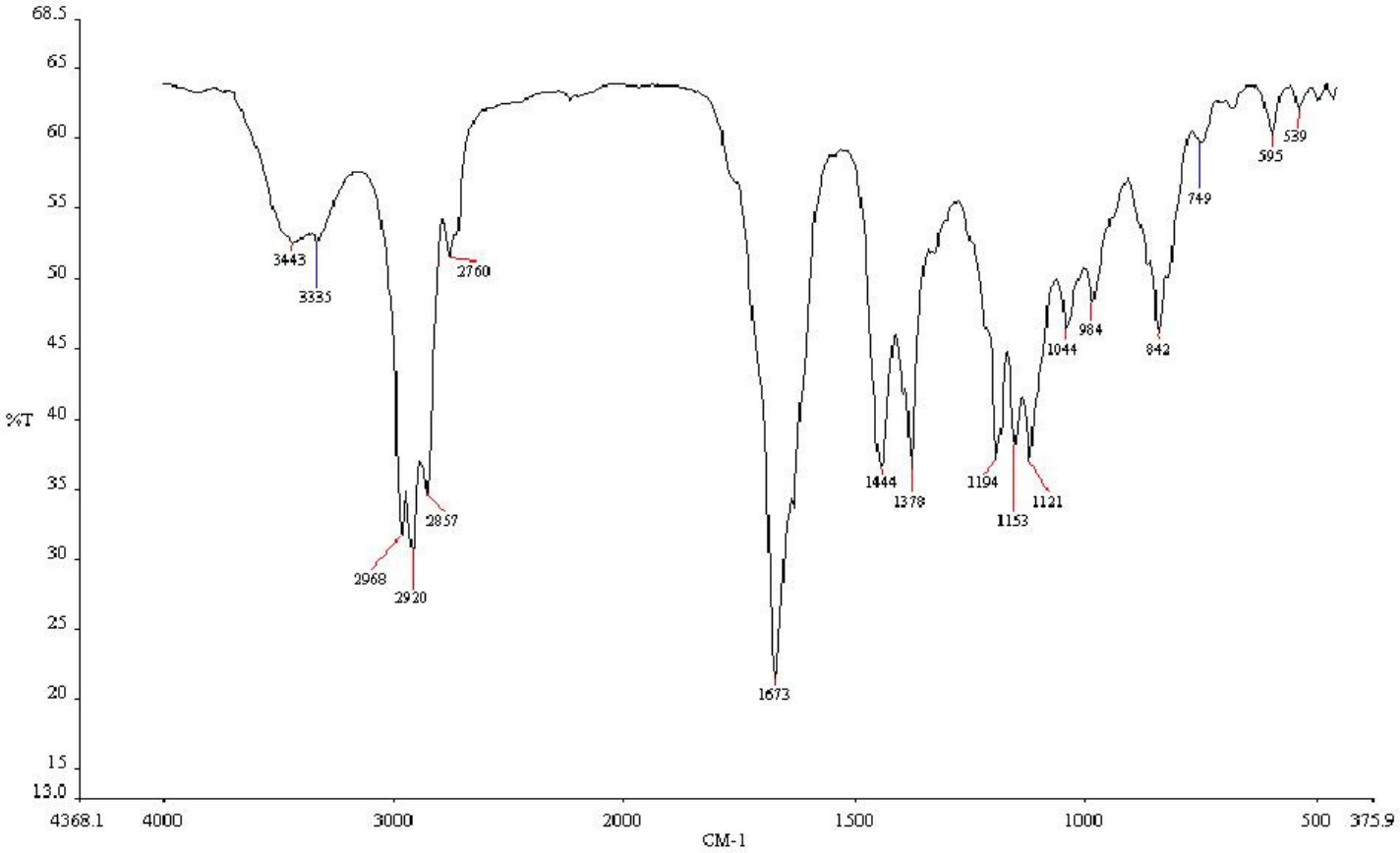
Annexure 5. [1]HNMR spectrum of isolated fraction 2



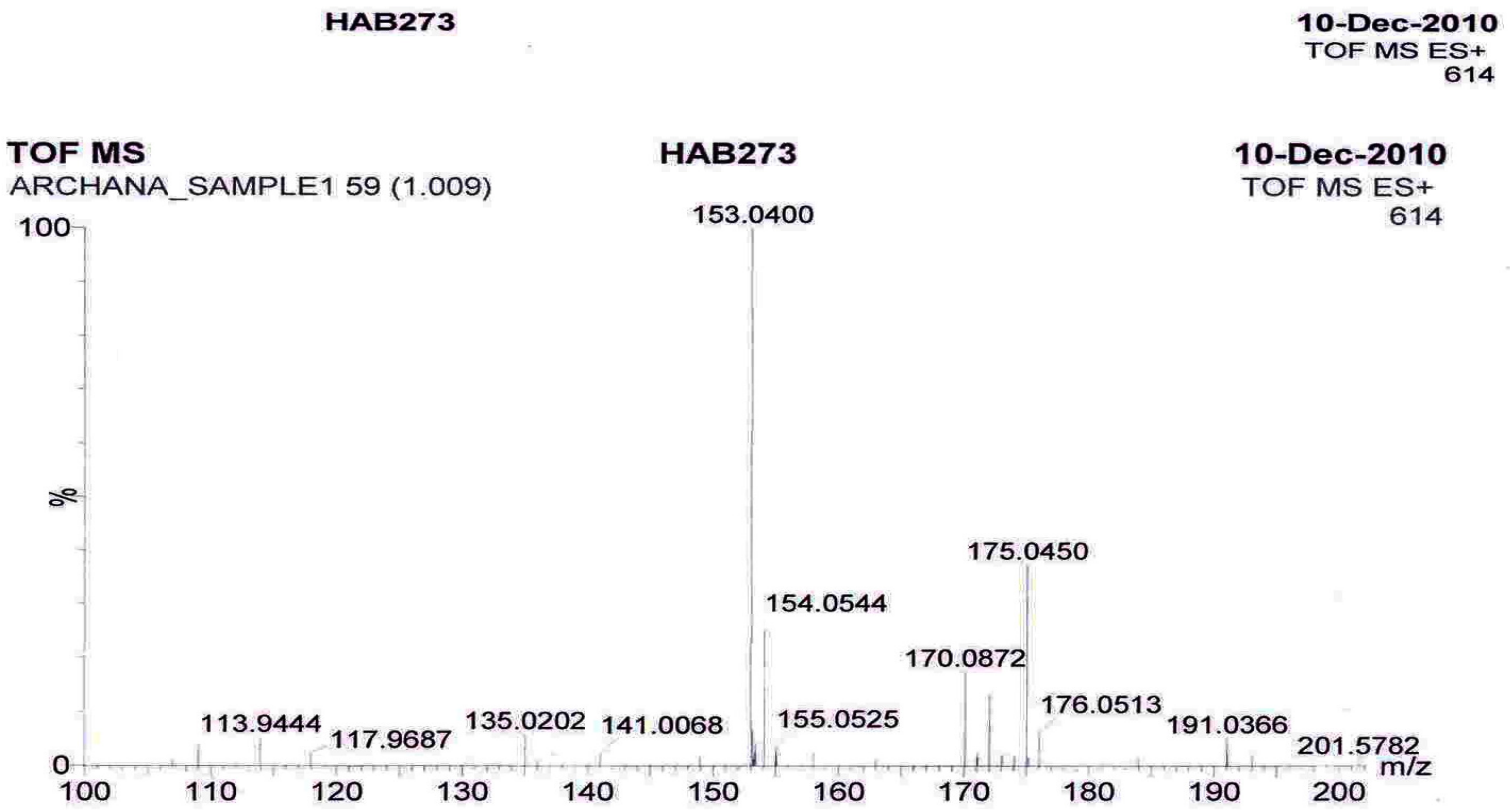
Annexure 6. ^{13}CNMR spectrum of isolated fraction 2



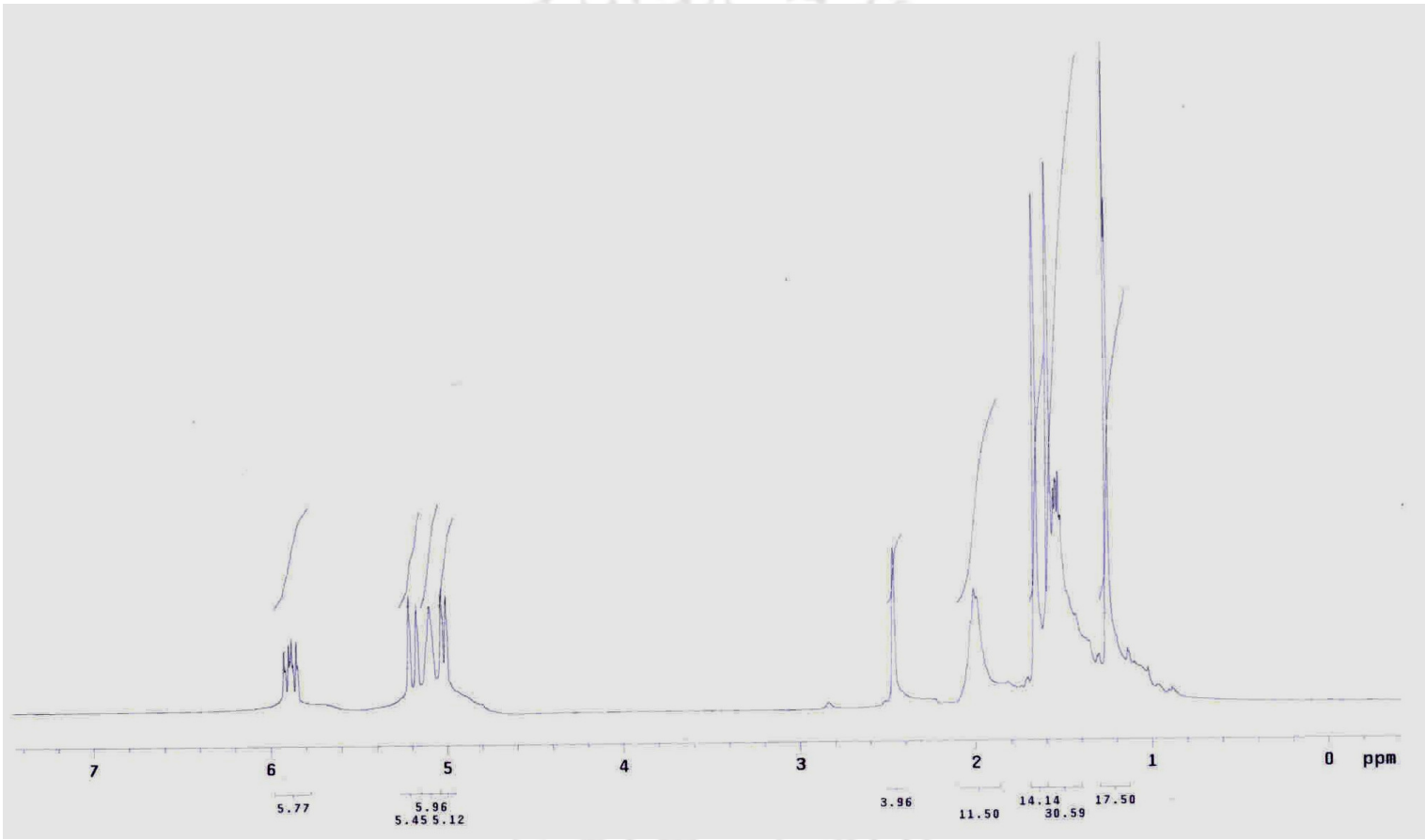
Annexure 7. FTIR spectrum of isolated fraction 2



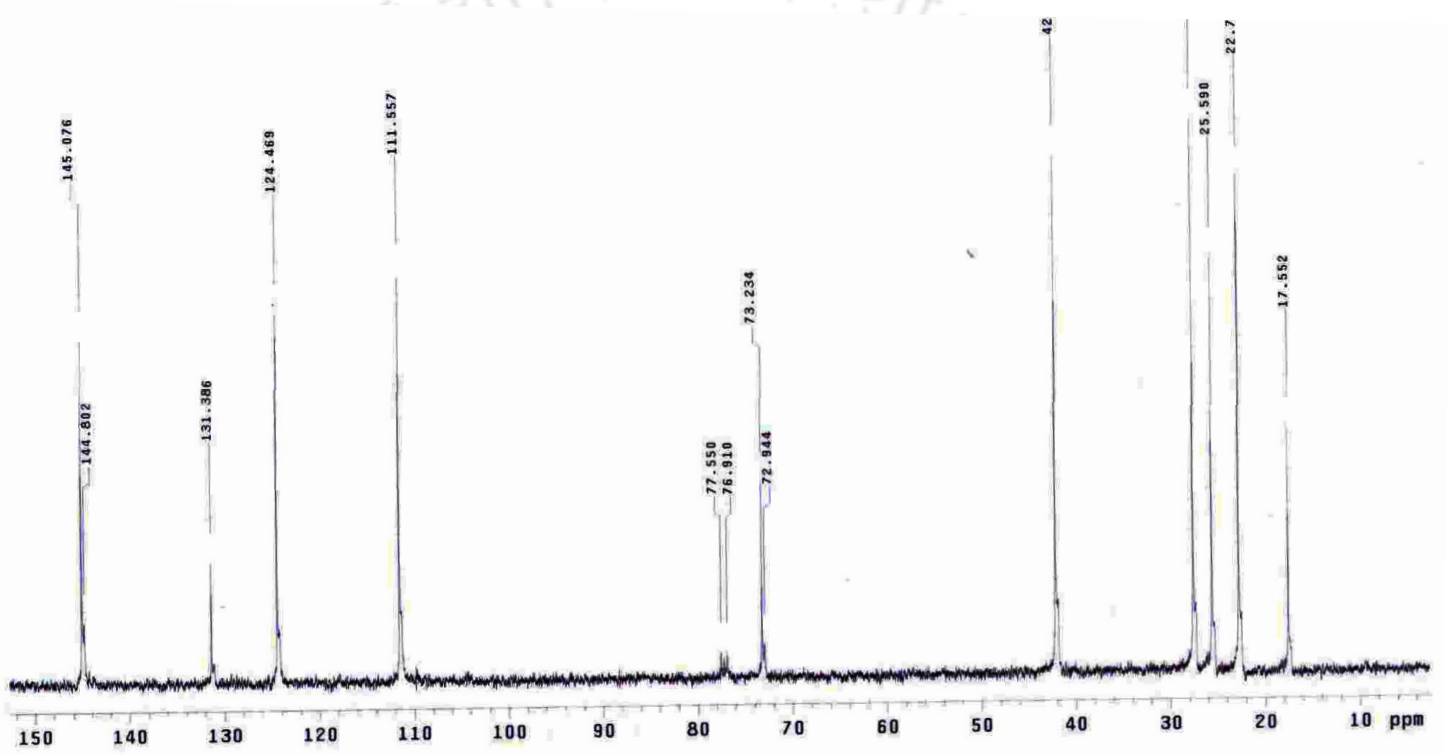
Annexure 8. HRMS spectrum of isolated fraction 2



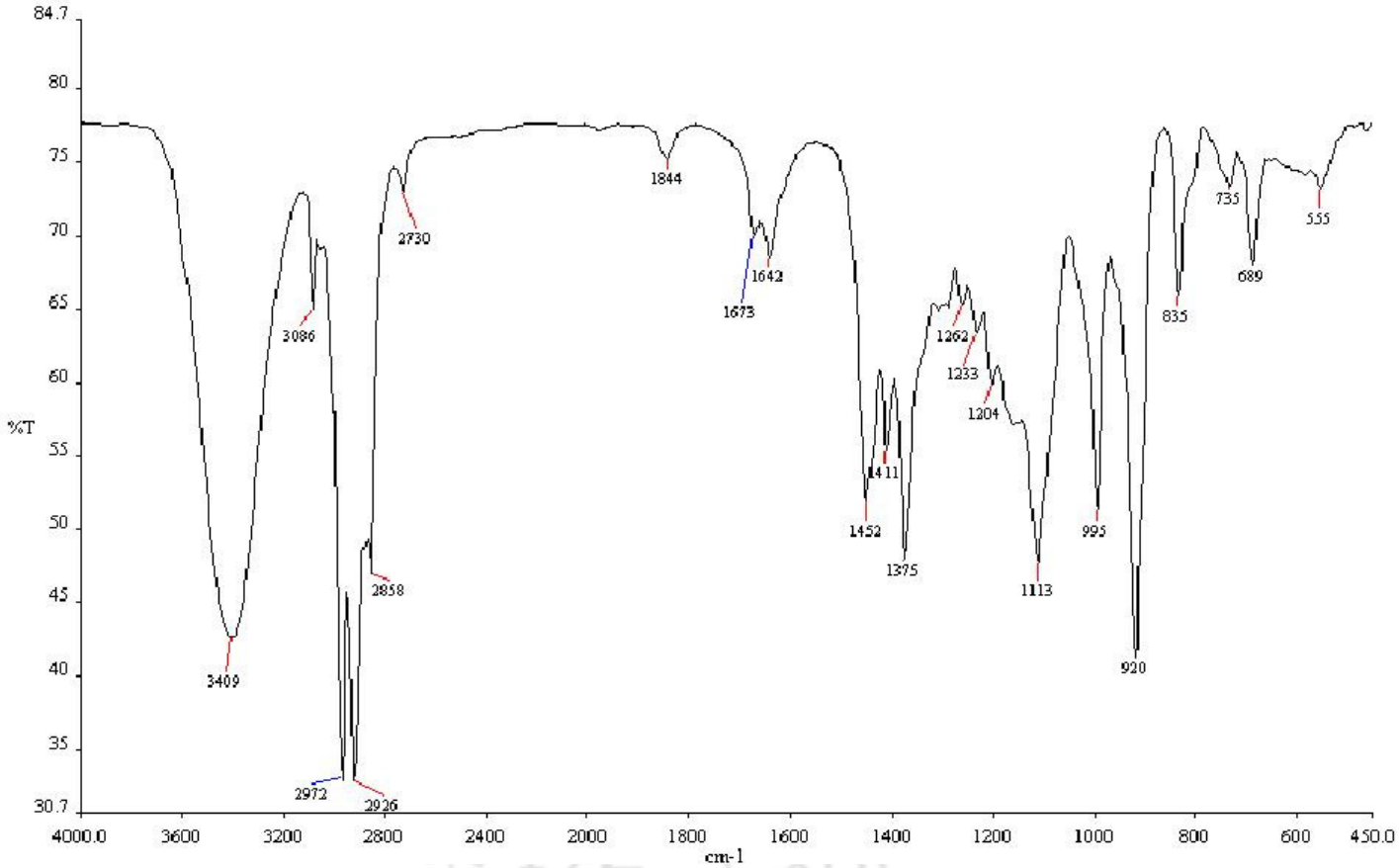
Annexure 9. [1]HNMR spectrum of isolated fraction 3



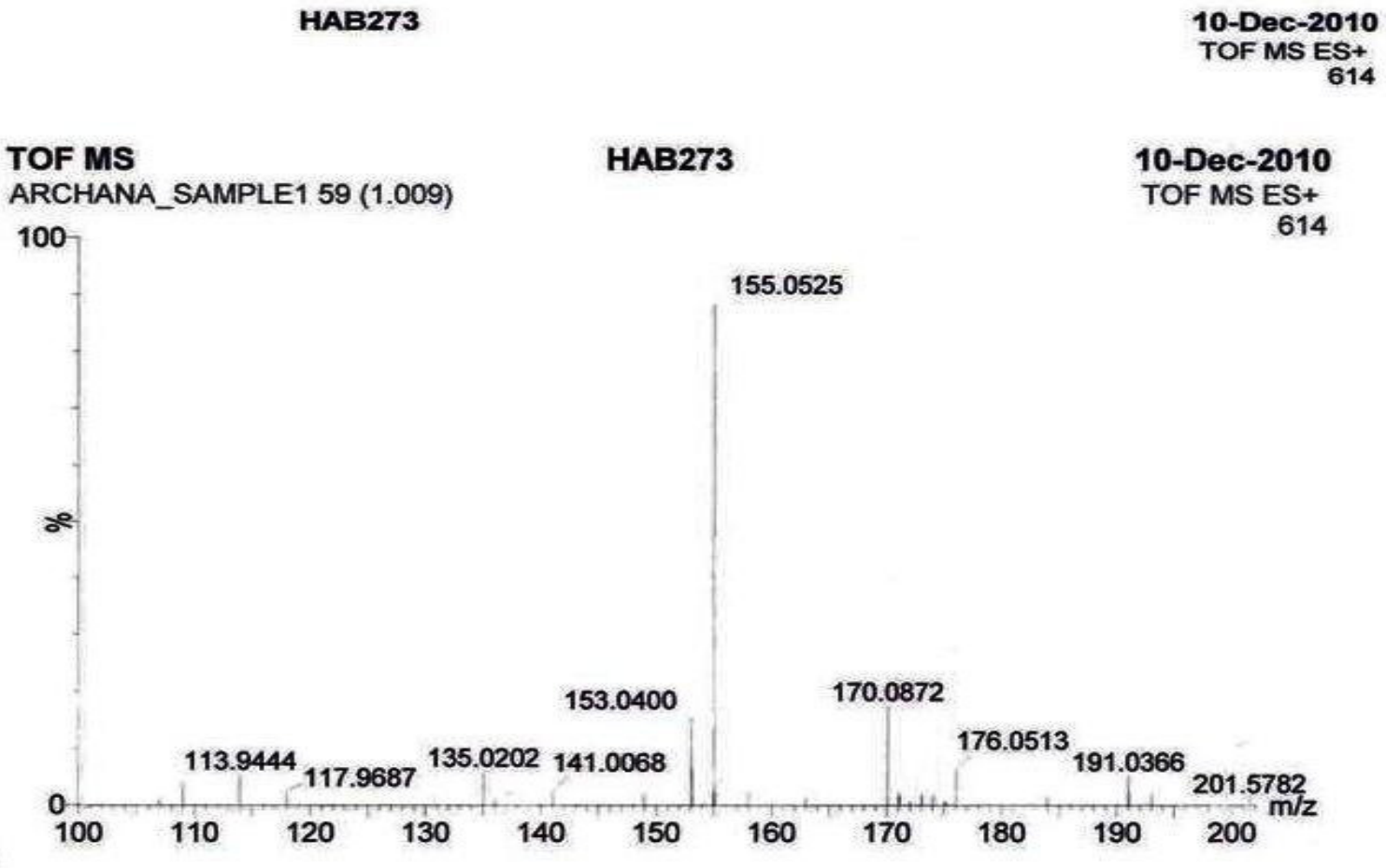
Annexure 10. ^{13}CNMR spectrum of isolated fraction 3



Annexure 11. FTIR spectrum of isolated fraction 3



Annexure 12. HRMS spectrum of isolated fraction 3



List of Publications

Publications

Publications from Ph.D. research work

(A) Publications in Journals

1. **Das A,** Kesari V, Rangan L (2011) Genetic relationship of *Curcuma* species from Assam India using PCR based markers- RAPD, ISSR, and AFLP. *Molecular Biotechnology*. DOI: 10.1007/SI2033-01109379-5. (Publisher: Springer).
2. **Das A,** Kesari V, Rangan L (2010) Plant regeneration in *Curcuma* species of Northeast India and assessment of genetic stability of regenerated plants by biomolecular techniques. *Biologia Plantarum* 54:423-429.

(B) Under Review

1. **Das A,** Rangan L (2011) Rapid plant regeneration and cytogenetic analysis of micropropagated plants of two wild gingers, *Zingiber moran* and *Zingiber zerumbet* - medicinally important plants from Northeast India. Journal of Herbs Spices and Medicinal Plants.
2. **Das A,** Rangan L (2011) Cytological studies in two significant members of Zingiberaceae from Northeast India. Chromosome Science.
3. **Das A,** Parida A, Vinod S M, Rangan L (2011) Evaluating genetic relationships in endemic *Zingiber moran* ecotypes from Northeast India, by means of RAPD, ISSR and AFLP profiling. Plant Systematics and Evolution.
4. **Das A,** Rangan L (2011) Antimicrobial activity and micro-Raman spectroscopy of selected Zingiberaceae species from Northeast India. Journal of Crop Science and Biotechnology.

(C) Conference presentations

1. **Das A,** Kasoju N, Bora U, Rangan L (2010) Biochemical, antimicrobial and pharmacological screening of flavanoids from *Z. moran* of Northeast India. Oral

presentation in *National Conference on Emerging Trends in Biopharmaceuticals: Relevance to Human Health & 4th Annual Convention of Association of Biotechnology and Pharmacy* **Thapar University, Patiala, India,** November 11–14, 2010.

2. Nath A, **Das A,** Rangan L, Khare A (2010) Antibacterial activity of copper oxide nanoparticles synthesized via laser ablation in liquids. Poster presentation in *Xth International Conference on Fiber Optics and Photonics.* **Indian Institute of Technology Guwahati, Assam, India,** December 12-15, 2010.
3. **Das A,** Kesari V, Rangan L (2009) Antimicrobial activity of rhzome extracts of some important gingers of Northeast India. Poster presentation in *TWAS Regional Young Scientist Conference*, **Petaling Jaya, Selangore, Malaysia**, November 2-5, 2009.
4. **Das A,** Rangan L (2009) Effect of plant growth regulators and culture conditions on shoot multiplication in three medicinally important Zingiber species of Northeast India. In: *Proceedings of International Conference on Emerging Trends in Biotechnology*, **Banaras Hindu University, Varanasi, India.** December 4-6, 2009.
5. **Das A,** Kesari V, Vinod MS, Parida A, Rangan L (2008) Genetic diversity analysis of *Curcuma* species of Northeast India using RAPD and SSR markers. Poster presentation in *International conference on Plant Biotechnology and Molecular Biology*. **Kakatiya University Warangal, India,** August 15-17, 2008.
6. **Das,A,** Agarwal S, Kesari V, Sarma GC, Rangan L (2008) Use of DNA Barcodes to identify *Curcuma* sp of Northeast India. Oral presentation in *International Conference on Emerging Technologies and Applications in Engineering, Technology and Sciences (ICETAETS)*, **Saurashtra University, Rajkot, India,** January 13-14, 2008.

(D) Book Chapter

1. Rangan L, **Das A**, Agarwal S, Kesari V, Sarma GC (2009). DNAB- A molecular systematic approach for species identification and bioresource protection. *In*

Diversity of Plant- A Molecular Approach (J. S. Britto ed.) The Rapinat Herbarium, Tiruchirapalli. pp. 19-39.

(E) Others

1. Nath A, **Das A**, Khare A, Rangan L (2011) Screening of antimicrobial activity of Copper nanoparticles against pathogenic bacteria. *Science of Advanced Materials* 34:234-237.
2. **Das A**, Tushar, V Kesari, L Rangan (2010) Aromatic Joha Rice of Assam- A Review. *Agriculture Reviews* 31:1-10.
3. Kesari V, **Das A**, Rangan L (2010) Effect of genotype and auxin treatments on rooting response in stem cuttings of CPTs of *Pongamia pinnata*, a potential biodiesel legume crop. *Current Science* 98(9):1227-1230.
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Plant regeneration in *Curcuma* species and assessment of genetic stability of regenerated plants

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Abstract

An efficient plant regeneration protocol was developed from rhizomes of two *Curcuma* species *C. longa* and *C. amada*. Response was highly dependent on the season, with above 69 % of culture developing adventitious shoots during spring. Greatest regeneration and multiplication was observed in modified Murashige and Skoog (MS) medium supplemented with 13.31 μM benzyladenine and 2.68 μM α-naphthalene acetic acid (NAA) in *C. longa* or 2.46 μM indolebutyric acid in *C. amada*. Effect of sugars and agar at different concentrations were also studied and 2 % maltose and 0.7 % agar were found optimum for shoot multiplication and regeneration. Most plantlets developed roots simultaneously but others formed roots when subcultured in ½ MS medium supplemented with 2.68 μM NAA. Plants were successfully hardened in greenhouse with 80 % survival. The genetic purity of micropropagated plantlets was analyzed using RAPD and protein profiles.

Additional key words: axillary bud, micropropagation, RAPD, SDS-PAGE, *Zingiberaceae*.

Introduction

Curcuma amada Roxb. and *Curcuma longa* L. are the two commercially important plants of the family *Zingiberaceae*. They are propagated vegetatively by underground rhizomes at a slow rate. *In vitro* clonal multiplication of *Curcuma* species through rhizome buds has been reported (Sit and Tiwari 1997, Salvi *et al.* 2002, Prakash *et al.* 2004) but the difficulty has been to establish good regenerating material in a suitable medium. Even attempts at using axillary bud meristem have faced problem at the multiplication stage. Another inherent problem with *Curcuma* rhizome is to establish a good axenic culture. A rapid multiplication method is therefore necessary to provide disease-free planting material in enough quantity especially for endemic species *C. longa* that are available in limited quantities.

The establishment of genetic stability of *in vitro* regenerated plants will be an essential requisite for large scale multiplication. Despite the advantages of the *in vitro* propagation, genetic instability has been observed in micropropagated species hence it is necessary to establish a system that produces genetically stable and identical plants. Several strategies can be used to assess the genetic stability of *in vitro* derived plants such as karyological analysis and isozyme markers, but they have their own limitations (Isabel *et al.* 1993). Bimolecular analytical techniques like polypeptide and DNA polymorphisms profiling facilitates direct and reliable measurements to detect culture-induced variation at the DNA and protein level (Cloutier and Landry 1994). Of several molecular markers used for assessment, random amplified polymorphic marker DNA (RAPD) is the simplest, cheapest and appears to be useful for the analysis of genetic fidelity of *in vitro* propagated plants and have been well established in many plants (Williams *et al*. 1990, Salvi *et al.* 2001, Panda *et al.* 2007, Hussain *et al.* 2008, Tyagi *et al.* 2010). Polypeptide profiling (SDS-PAGE) is a useful biochemical marker system, which has been applied, *e.g.*, to seeds analysis for diagnostics and estimation of outcrossing rates (Ferreira *et al.* 2000).

In the present communication we report an efficient regeneration from mature rhizomes for two *Curcuma* species from Northeast India with the aim to multiply, conserve and establish a disease free material for further

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Abbreviations: BA - benzyladenine; bp - base pairs; IBA - indolebutyric acid; KIN - kinetin; MS - Murashige and Skoog; NAA - α-naphthalene acetic acid; RAPD - random amplified polymorphic DNA; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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studies. We investigated the effect of modified Murashige and Skoog (1962; MS) media with different hormone combination for multiplying *Curcuma* species. In addition, we performed RAPD and SDS-PAGE analysis of the micropropagated plants for genetic purity assessment.

Materials and methods

Mature rhizomes of *Curcuma amada* Roxb. and *Curcuma longa* L. collected from different districts of Assam (Kamrup, Barpeta, Jorhat and Sivasagar) having axillary buds were used as starting material. Outer scales were removed and buds were cut into small pieces (1 - 2 cm long) and washed under running tap water to remove dust and adhering soil particles. The explants were treated with 0.1 - 0.2 % *Bavistin* solution along with (2 % m/v) *Tween 20* for 5 min and rinsed with water. Explants were pretreated with 70 % ethanol for 2 min before surface sterilizing with 0.1 % (m/v) mercuric chloride for 10 min followed by five rinses with sterile double distilled water.

Both ends of the explants were trimmed and incubated in culture tubes (*Borosil*, Mumbai, India) containing the standard MS medium and modified MS medium (MSR) containing additional supplements like yeast extract (300 mg dm^{-3}) and casein hydrolysate (100 mg dm^{-3}) with 0.8 % agar (*Himedia*, London, UK) and 3 % sucrose. The pH of the media was adjusted to 5.8 and tubes were autoclaved at 121 °C for 15 min. Cultures were incubated at temperature of 25 ± 2 °C, 16-h photoperiod and irradiance of 40 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes. Different concentrations of benzyladenine (BA) and kinetin (KIN) were tried singly and in combination with NAA and IBA for shoot multiplication and maintenance (MM medium).

To study the effect of gelling concentration on shoot multiplication, shoot tips were cultured on MM medium solidified with 0.6, 0.7 and 0.8 % agar. The effect of sugars at varying concentrations on *in vitro* shoot multiplication was also carried out by supplementing MM medium with 1, 2 or 3 % sucrose. Subculturing was carried out after 3 and 8 weeks of culture, the number of shoots per explant, shoots length, number of roots per shoot and root length were recorded.

Rooted plantlets were removed from culture tubes and washed thoroughly with tap water to remove the adhering medium and planted in poly-bags containing sand and clay at the ratio of 1:4, and kept in the mist chamber. Plants were hardened under a 16-h photoperiod, irradiance of 55 µmol m^{-2} s^{-1}, 28 ± 2 ºC and relative humidity of 80 % in the greenhouse. Intermittent mist was supplied for 30 s at 15-min intervals. The percentage survival was determined after two months.

For RAPD analysis, 10 regenerated plants of *C. amada* and *C. domestica* collected from 60-d-old plantlets selected randomly and mother plant were analyzed. Total genomic DNA was extracted from fresh tender leaves using SDS protocol with slight modifications (McCouch 1992). Leaf tissue (1 g) was ground in liquid nitrogen and suspended in 10 cm^3 of extraction buffer (100 mM Tris, 0.5 M NaCl, 50 mM EDTA) containing 1 % β-marcaptoethanol. The suspension was incubated at 65 °C for 30 min, extracted with 5 M potassium acetate and centrifuged at 2 795 *g* at 4 °C for 30 min. The aqueous phase was precipitated with isopropanol and again centrifuged at 2 795 *g* at 4 °C for 20 min. The pellet was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and treated with RNAse. DNA was purified by ethanol precipitation. The quality and quantity of the extracted DNA was confirmed to be consistent both spectrophotometrically and by running the extracted DNA on 1.0 % agarose gels containing 0.5 µg cm^{-3} of EtBr.

PCR amplification of the genomic DNA was carried out using 10 arbitrary decamer oligonucleotide primers (*Operon Technologies*, Almeda, USA). Each reaction mixture of 0.02 cm^3 contained 50 µg cm^{-3} of template DNA, 1× assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1 % gelatin), 0.2 mM each dNTPs (*Banglore Genei*, Bangalore, India), 5 pmol of each primer and 0.05 U of *Taq* polymerase (*Banglore Genei*). The reaction was performed in 0.2 cm^3 microfuge tubes (*Dialabs*, Austria). PCR amplification was carried out in a mini thermal cycler (*Applied Biosystems*, CA, USA) programmed for 40 cycles. The first amplification cycle consisted of initial denaturation step of 5 min at 94 °C. This was followed by 40 cycles of 45 s at 94 °C, annealing for 1 min at 32 °C, and extension at 72 ºC for 2 min. An additional cycle of 10 min at 72 ºC was used for primer extension. The amplification products were electrophoresed in 1.3 % agarose gels in 1× TAE (50× stock contained 2 M Tris, 0.5 M EDTA and glacial acetic acid). The gels were visualized and photographed under UV radiation by a gel documentation system (*BioRad*, Hercules, USA). The size of amplification products was estimated using λ DNA marker (*Banglore Genei*). PCR amplification was repeated twice and only primers producing reproducible bands were considered for analysis.

Protein extraction was carried out for the same regenerated plants from which DNA was extracted. Young leaves were homogenized in 10 mM Tris-EDTA buffer (pH 8.0) containing 10 % SDS, 5 mM β-mercapto-ethanol and 0.1 mg cm^{-3} phenylmethanesulphonyl fluoride (PMSF). After centrifugation at 10 000 *g* for 10 min (4 ºC), the supernatant was boiled for 10 min. Protein content was measured by Bradford method (1976) using bovine serum albumine as a standard. Proteins were separated using discontinuous SDS-PAGE (10 % running gel, pH 8.8 and 5 % stacking gel, pH 6.8) at 4 ºC according to Laemmli (1970). After electrophoresis, gels

were stained overnight with 0.25 % Coomassie Brilliant Blue R250, destained, fixed and photographed. Molecular mass of polypeptides was determined according to the mobility of the standard proteins.

Three replicates (10 explants per replicate) were inoculated per treatment to test the effects of medium on multiplication rate. All experiments were carried out independently and repeated thrice. The data were analyzed using one-way *ANOVA* (*SPSS 16.0* version, 2008) and significant differences between treatment means were assessed using Duncan's multiple range test (DMRT) at a 5 % probability level ($P < 0.05$). Amplified DNA and polypeptide fragments were scored as present or absent both in the regenerated and in mother plants. Electrophoretic bands of low visual intensity that could not be readily distinguished as present or absent were considered as ambiguous and were not scored.

Results

Establishment of the *in vitro* cultures in *Curcuma* species posed considerable problems with contamination in primary cultures, which reappeared even after repeated subculturing. The problem was overcome by treating the explants with 0.2 % (m/v) *Bavistin* for 2 h and then with 70 % ethanol for 2 min.

No organogenic response was observed after 4 weeks, in the excised meristem cultured *in vitro* on MS medium supplemented with vitamins and carbon source but devoid of growth regulators. The addition of BA and KIN (4.43 µM each) to the basal medium induced axillary buds sprouting (Fig. 1*A*). Multiple shoots were induced from *in vitro* raised explants on MS and MSR media with varying concentrations and combinations of cytokinins and auxins. The greatest response in *C. amada* were recorded after 2 weeks in MS (93.33 %) and MSR medium (100 %), both supplemented with 13.31 µM BA + 2.46 µM IBA (Table 1).

Rhizome buds of *C. amada* when cultured on MSR medium containing 13.31 µM BA + 2.46 µM IBA started to proliferate producing new shoots within 10 d whereas in MS medium with the same hormone combination, buds started to proliferate only after 3 weeks of culturing. The highest mean number of buds per explant (4.3 in MS and 8.9 in MSR medium) was recorded at the same hormone combination (Fig. 1*B*). This medium also showed the highest length of the longest shoot (Table 1). The buds of *C. longa* showed a regeneration frequency of 80 and 100 % in MS and MSR medium, respectively (Table 1). Response was 100 % in MSR medium with 13.31 µM BA + 2.46 µM IBA, but with a lesser average number of shoot per explants. The highest mean number of shoots per culture (8.2) was in MSR medium supplemented with 13.31 µM BA + 2.68 µM NAA (Fig. 1*C*), showing greater response than in MS medium having the same hormone concentration (4.2). The highest length of the longest shoot in MSR medium was also recorded in the same medium (Table 1). BA was found to be a more effective than KIN for multiple shoot regeneration when used alone. Subsequent subculturing on the optimal multiplication medium repeatedly for two or three cultures did not increase the multiplication rate.

When different concentrations of agar were tested, 0.7 % was found to be better than the others for shoot

Table 1*A*. Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma amada* (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [µM]	MS medium response [%]	shoot number [explant^{-1}]	shoot length [cm]	MSR medium response [%]	shoot number [explant^{-1}]	shoot length [cm]
BA	4.43	0	-	-	30.0 ± 10.0	1.80 ± 0.11b	1.47 ± 0.05a
	13.31	43.3 ± 6.67	1.33 ± 0.13a	2.70 ± 0.85c	56.6 ± 6.67	2.40 ± 0.15cd	2.68 ± 0.07c
	22.19	16.6 ± 3.33	1.00 ± 0.00a	2.10 ± 0.17b	63.3 ± 3.33	1.50 ± 0.11ab	2.01 ± 0.06b
KIN	2.32	0	-	-	53.3 ± 3.33	1.50 ± 0.12ab	1.66 ± 0.08e
	4.64	46.6 ± 6.67	1.50 ± 0.13abc	2.60 ± 0.08c	70.0 ± 0.00	2.00 ± 0.16abc	2.90 ± 0.06d
	9.29	56.6 ± 3.33	1.16 ± 0.34cd	1.60 ± 0.08a	56.6 ± 6.67	1.30 ± 0.11a	2.60 ± 0.12b
BA + NAA	13.31 + 2.68	73.3 ± 6.67	3.40 ± 0.19f	4.00 ± 0.10e	93.3 ± 3.33	7.60 ± 0.25g	4.96 ± 0.07f
	13.31 + 5.37	60.0 ± 0	2.40 ± 0.14de	3.10 ± 0.09d	56.6 ± 6.67	2.10 ± 0.12bc	3.23 ± 0.08e
KIN + NAA	4.64 + 2.68	53.3 ± 3.33	2.30 ± 0.13de	2.50 ± 0.09c	90.0 ± 5.78	3.20 ± 0.15e	3.18 ± 0.07e
	4.64 + 5.37	50.0 ± 0	1.90 ± 0.18bcd	1.70 ± 0.08a	66.6 ± 3.33	2.40 ± 0.13cd	2.03 ± 0.11b
BA + IBA	13.31 + 2.46	93.3 ± 6.67	4.30 ± 0.29g	4.90 ± 0.09 f	100.0 ± 0.00	8.96 ± 0.43h	5.56 ± 0.10g
	13.31 + 4.92	53.3 ± 3.33	2.30 ± 0.12de	2.64 ± 0.81c	83.3 ± 8.82	3.90 ± 0.16f	2.50 ± 0.05c
KIN + IBA	4.64 + 2.46	90.0 ± 10.0	2.70 ± 0.18ef	2.60 ± 0.07c	93.3 ± 6.67	2.70 ± 0.13cde	2.13 ± 0.07b
	4.64 + 4.92	63.3 ± 3.33	2.40 ± 0.14de	2.03 ± 0.07b	66.6 ± 3.33	2.90 ± 0.22de	2.11 ± 0.08b

Table 1*B*. Effect of medium and growth regulators on percentage response, number of shoots and length of the longest shoot in *Curcuma longa* (after 6 weeks of culture). Means ± SE, n = 10. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [μM]	MS medium response [%]	shoot number [explant^{-1}]	shoot length [cm]	MSR medium response [%]	shoot number [explant^{-1}]	shoot length [cm]
BA	4.43	0	-	-	56.67 ± 3.33	1.10 ± 0.09a	1.57 ± 0.04b
	13.31	46.67 ± 8.82	1.20 ± 0.20a	1.37 ± 0.12abc	90.00 ± 5.77	2.71 ± 0.11e	2.02 ± 0.11cd
	22.19	40.00 ± 5.77	1.57 ± 0.03abc	1.62 ± 0.02bcd	66.67 ± 3.33	2.00 ± 0.15cd	1.57 ± 0.18c
KIN	2.32	13.33 ± 3.33	1.00 ± 0.00a	1.08 ± 0.06a	56.67 ± 6.67	1.29 ± 0.06a,b	1.49 ± 0.20a
	4.64	46.67± 3.33	1.85 ± 0.08bc	1.89 ± 0.05de	86.67 ± 8.82	2.63 ± 0.26e	2.22 ± 0.06de
	9.29	50.00 ± 5.77	1.08 ± 0.08a	1.27 ± 0.07ab	60.00 ± 5.77	1.78 ± 0.04bc	1.82 ± 0.24bc
BA + NAA	13.31 + 2.68	80.00 ± 10.0	4.27 ± 0.30f	4.25 ± 0.07h	100.00 ± 0.00	8.17 ± 0.17h	4.54 ± 0.13i
	13.31 + 5.37	60.00 ± 5.77	1.71 ± 0.27bc	1.68 ± 0.11c,d	60.00 ± 5.77	2.57 ± 0.14e	2.91 ± 0.08g
KIN + NAA	4.64 + 2.68	70.00 ± 5.77	2.25 ± 0.39cd	2.24 ± 0.02ef	66.67 ± 3.33	3.17 ± 0.12f	3.10 ± 0.05g
	4.64 + 5.37	56.67 ± 3.33	1.37 ± 0.19ab	1.37 ± 0.01abc	63.33 ± 3.33	1.74 ± 0.30bc	2.53 ± 0.01f
BA + IBA	13.31 + 2.46	73.33 ± 3.33	3.61 ± 0.22e	3.58 ± 0.12g	100.00 ± 0.00	7.03 ± 0.15g	3.96 ± 0.10h
	13.31 + 4.92	53.33 ± 3.33	1.87 ± 0.13bc	1.93 ± 0.04de	56.67 ± 3.33	2.42 ± 0.21de	2.42 ± 0.04ef
KIN + IBA	4.64 + 2.46	56.67 ± 3.33	2.49 ± 0.16d	2.50 ± 0.07f	80.00 ± 0.00	3.33 ± 0.11f	3.72 ± 0.06h
	4.64 + 4.92	53.33 ± 3.33	1.81 ± 0.12bc	1.82 ± 0.04d	60.00 ± 5.77	1.58 ± 0.21bc	1.76 ± 0.13bc

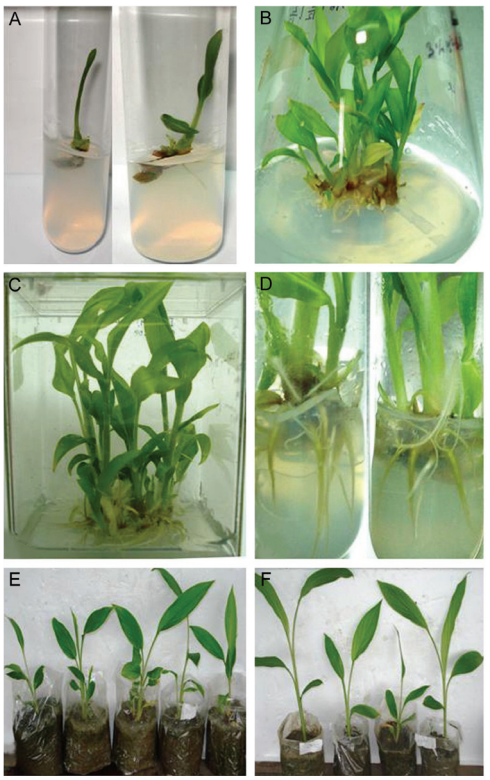

Fig. 1. Plant regeneration in *Curcuma* species: *A* - shoot induction from axillary bud in MSR medium supplemented with BA and kinetin after 2-week culture period in *C. amada* and *C. longa*. *B* - multiple shoots formed from axillary bud of *C. amada* in MSR medium (13.31 μM BA + 2.46 μM IBA) after 8 weeks, *C* - simultaneous rooting and shooting in *C. longa* in MSR medium (13.31 μM BA + 2.68 μM NAA) after 8 weeks, *D* - rooting of shoots in ½ MS + NAA (2.68 μM) after 4 weeks, *E, F* - 2-month-old hardened plants of *C. amada* (*E*) and *C. longa* (*F*).

multiplication, while 0.6 % agar produced the highest shoot length. Shoots produced on medium with 0.8 % agar were long, slender, elongated with some shoots showing signs of drying and browning (data not shown). Carbon source in culture medium had also a significant effect on the growth of *Curcuma* species. MSR medium containing maltose was giving best result as far as shoot induction and multiplication was concerned in both the *Curcum*a species studied when compared to MS medium containing sucrose. Further, a significant increase in shoot length, and number and length of roots were observed when the concentration of sugar was 2 % suggesting that low concentration of sugar could be best for *in vitro* multiplication of *Curcuma* species.

Rooting was observed in all combination of BA with IBA and NAA in both *C. amada* and *C. longa*. This indicated the inherent root inducing tendency of explants of rhizome origin rather than the influence of hormones applied exogenously. Such response has been reported earlier in *C. haritha* (Bejoy *et al.* 2006). 83.3 % of well developed shoots (5 - 6 cm) when excised and transferred to ½ MS medium supplemented with 2.68 μM NAA produced well developed roots (Table 2, Fig 1*D*).

Plantlets developed *in vitro* could be hardened in the greenhouse for two months suggesting the suitability of the *in vitro* protocol (Fig. 1*E*,*F*). Hardened plants of *C. amada* and *C. longa* recorded about 80 and 100 % survival and were reared to maturity.

To confirm the genetic stability of regenerated plants maintained in culture for a period of 2 months, RAPD analysis was carried out. Out of the 10 different RAPD primers tested, 9 and 7 primers produced clear and scorable bands in *C. amada* and *C. longa*, respectively. Nine selected RAPD primers utilized in this study for *C. amada* gave rise to a total of 46 scorable bands, ranging from 300 - 1 800 bp in size. The number of bands

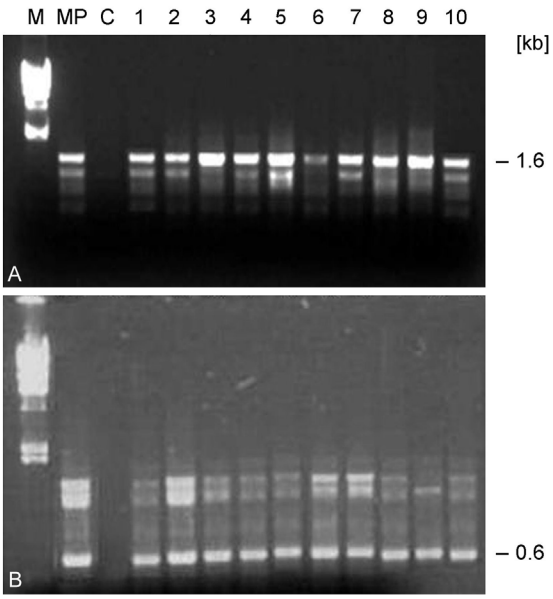

Fig. 2. RAPD profiles of micropropagated plants of *Curcuma* species using the decamer primers: *A* - *C. amada* (OPO-04), *B* - *C. longa* (OPA-03). M - DNA marker, *lane* MP - DNA from the mother plant, *lane* C - water control, *lanes* 1 - 10, DNA from micropropagated plants.

for each primer varied from 1 - 8, with an average of 5.1 bands per primer. Similarly, the 7 selected RAPD primers utilized in this study for *C. longa* gave rise to a total of 36 scorable bands, ranging from 500 - 2 000 bp in size and the number of bands for each primer varied from 1 - 5, with an average of 5 bands per primer. Each primer generated a unique set of amplification products that were monomorphic across all the micropropagated plants. Number of monomorphic bands was highest, six in case of primer OPA-03 for *C. amada* and was lowest in case of the primer OPA-10 in *C. longa*. Overall, no changes in the amplified fragments were detected among all micropropagated plantlets with reference to donor plant, which confirmed the genetic stability of these plantlets derived *in vitro*. Fig. 2 shows representative examples of amplified banding patterns produced by RAPD primers.

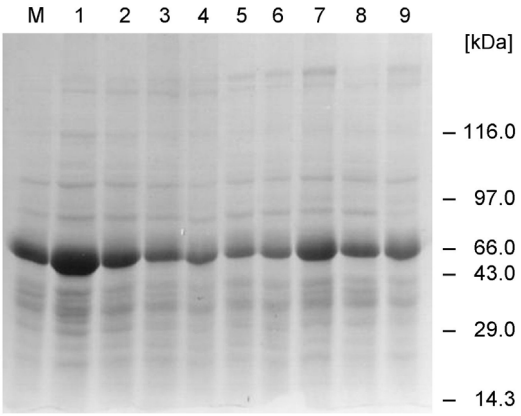

Fig. 3. Polypeptide profiles from leaves of micropropagated plants of *C. amada* using SDS-PAGE analysis. *Lane* M - proteins from the mother plant, *lanes* 1 to 9 - proteins from micropropagated plants, molecular mass marker (14 - 116 kDa) *on the right*.

In SDS-PAGE analysis, 12 unique bands were observed and the protein patterns of both micropropagated plants and the mother plant exhibited relatively high degree of identity (Fig. 3). The difference among polypeptides is in the relative intensity of the stained bands rather than in their numbers. It was observed that the low molecular mass proteins produced high intensity bands while the high molecular mass proteins were present in lower concentrations.

Table 2. Effect of auxin treatments on *in vitro* rooting in shoots of *C. amada* and *C. longa* cultured in half-strength MS medium (after 6 weeks of culture). Means ± SE, $n = 10$. Means followed by the same letters in each column are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Growth regulators	Concentration [μM]	*C. amada* response [%]	root number [shoot^{-1}]	root length [cm]	*C. longa* response [%]	root number [shoot^{-1}]	root length [cm]
NAA	2.68	76.6 ± 3.33	5.2 ± 0.18e	4.4 ± 0.12d	83.3 ± 3.33	7.1 ± 0.24d	5.2 ± 0.12e
	5.37	56.6 ± 6.67	1.6 ± 0.12ab	1.7 ± 0.11a	66.6 ± 6.67	3.0 ± 0.13b	3.3 ± 0.09d
IAA	2.85	60.0 ± 5.78	3.8 ± 0.15d	3.1 ± 0.09c	70.0 ± 5.78	3.8 ± 0.20c	2.5 ± 0.10b
	5.70	50.0 ± 5.78	1.2 ± 0.12a	2.1 ± 0.06b	46.6 ± 3.33	2.0 ± 0.15a	1.8 ± 0.12a
IBA	2.46	73.3 ± 3.33	3.0 ± 0.15c	3.0 ± 0.10c	56.6 ± 6.67	3.5 ± 0.15bc	3.0 ± 0.14c
	4.92	56.6 ± 8.29	1.8 ± 0.13b	2.2 ± 0.08b	43.3 ± 3.33	2.1 ± 0.14a	1.5 ± 0.10a

Discussion

In rhizomatous plants, contamination is a major problem during initiation and further successful establishment of aseptic cultures (Balachandran *et al.* 1990, Borthakur *et al.* 1999). It was observed that for *Curcuma* species, percentage response and number of explants showing contamination was highly dependent on the season during

which the material was collected. Spring (March - May), when plants are actively growing, was found to be the most favorable season for initiation of culture; 69 % of cultures developed adventitious shoots and rate of contamination was also less. *In vitro* seasonal effect on bud growth has been reported in rhizomatous species such as *Curcuma zedoaria* (Stanly and Keng 2007) and *Curguligo orchioides* (Wala and Jasrai 2003).

In the present investigation, multiplication was found to occur by development of axillary buds which is ideal for maintaining genetic stability. However, the rate of bud multiplication was significantly different according to the various concentrations of growth regulators and combination of BA with lower concentration of auxin (NAA or IBA) was found to be ideal for shoot multiplication. However, persistence of explants in culture media containing higher concentration of cytokinins suppressed shoot elongation in contrast to other researchers who used rather high concentrations of plant growth regulators for the multiple shoot formation for some of the *Zingiberaceae* species (Chan and Thong 2004, Bharalee *et al.* 2005). Loc *et al.* (2005) reported that MS medium supplemented with 20 % (v/v) coconut water, 3 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA induced the formation of 6 shoots per explants in *C. zedoaria*. Bharalee *et al.* (2005) found that MS medium supplemented with 4 mg dm^{-3} BA and 1.5 mg dm^{-3} NAA was the best medium for shoot multiplication of *C. caesia* (3.5 shoots per explants) and MS with 1 mg dm^{-3} BA + 0.5 mg dm^{-3} NAA for *C. zedoaria* (4.5 shoots per explants). Balachandaran *et al.* (1990) reported that *C. domestica* could produce 3.4 shoots per explants, *C. caesia* produced 2.8 shoots per explants using MS medium supplemented with 3 mg dm^{-3} BAP. Nayak (2000) reported MS medium supplemented with 5 mg dm^{-3} BAP to be effective for shoot multiplication in *C. aromatica* producing an average of 3.3 shoots per explants whereas Mohanty *et al.* (2008) found 3 mg dm^{-3} BAP and 0.5 mg dm^{-3} NAA to be optimum medium for multiple shoot induction *via* indirect regeneration in the same species. Our results indicated that 13.39 µM BA and 2.68 µM NAA in the MSR medium was sufficient for the induction of multiple shoots from the buds and shoots of *C. amada* and *C. longa.*

Sucrose is widely used as a standard carbon source for plant tissue culture, and different concentrations and different osmotic environments have been used. However, a significant increase in shoot length and number was observed when maltose was added to the medium suggesting that non reducing sugars such as maltose could be best carbon source for *in vitro* multiplication of *Curcuma* species. Sugars (sucrose or maltose) at concentration 2 % were found to be most suitable for shoot multiplication. At concentration above 3 % *Curcuma* plantlets were etiolated and died. Similar observations were recorded in ginger species (Barthakur and Bordoloi 1992). However higher concentration of sugar source has been found to be ideal for *in vitro* microrhizome production in *Zingiber officinale* (Zheng *et al.* 2008).

RAPD based genetic assessment of genetic stability of *in vitro* grown micropropagated plants has been reported in many other plant species (Rout and Das 2002, Hussain *et al.* 2008, Tyagi *et al*. 2010). The source of the explants and mode of regeneration (somatic embryogenesis/ organogenesis/axillary bud multiplication) are known to play a major role in determining the presence or absence of variation (Salvi *et al.* 2001). We have used rhizomatous buds as explants for micropropagation of *C. longa* and *C. amada* because it lowers the risk of genetic instability. The results concur with earlier reports (Suri *et al.* 1999) that the micropropagation through explants containing organized meristem is generally more resistant to genetic changes that might occur indirect regeneration (Salvi *et al.* 2002).

The results obtained from our study suggest that *in vitro* regeneration and multiplication of *C. longa* and *C. amada* from rhizome with axillary buds could be used for rapid clonal propagation of these two valuable medicinal plants with the least possibility of genetic variations. These can also be used as a source of disease-free planting material for the farmers.

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RESEARCH

Genetic Relationship of *Curcuma* Species from Northeast India Using PCR-Based Markers

Archana Das · Vigya Kesari · Vinod M. Satyanarayana · Ajay Parida · Latha Rangan

Abstract Molecular genetic fingerprints of nine *Curcuma* species from Northeast India were developed using PCR-based markers. The aim involves elucidating there intra- and inter-specific genetic diversity important for utilization, management, and conservation. Twelve random amplified polymorphic DNA (RAPD), 19 Inter simple sequence repeats (ISSRs), and four amplified fragment length polymorphism (AFLP) primers produced 266 polymorphic fragments. ISSR confirmed maximum polymorphism of 98.55% whereas RAPD and AFLP showed 93.22 and 97.27%, respectively. Marker index and polymorphic information content varied in the range of 8.64–48.1, 19.75–48.14, and 25–28 and 0.17–0.48, 0.19–0.48, and 0.25–0.29 for RAPD, ISSR, and AFLP markers, respectively. The average value of number of observed alleles, number of effective alleles, mean Nei's gene diversity, and Shannon's information index were 1.93–1.98, 1.37–1.62, 0.23–0.36, and 0.38–0.50, respectively, for three DNA markers used. Dendrograms based on three molecular data using unweighted pair group method with arithmetic mean (UPGMA) was congruent and classified the *Curcuma* species into two major clusters. Cophenetic correlation coefficient between dendrogram and original similarity matrix were significant for RAPD ($r = 0.96$), ISSR ($r = 0.94$), and AFLP ($r = 0.97$). Clustering was further supported by principle coordinate analysis. High genetic polymorphism documented is significant for conservation and further improvement of *Curcuma* species.

Keywords AFLP · *Curcuma* · Genetic diversity · ISSR · Northeast · RAPD

Abbreviations

AFLP	Amplified fragment length polymorphism
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ISSR	Inter simple sequence repeat
PCA	Principle coordinate analysis
PIC	Polymorphic information content
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
TE	Tris–EDTA buffer
UPGMA	Unweighted pair group method with arithmetic mean

Introduction

Curcuma belonging to the family Zingiberaceae has immense medicinal value and finds extensive use in the indigenous system of medicine [1–4]. Northeast (NE) India houses the vast majority of *Curcuma* species, many of which are endemic to the region [4]. However, the species are gradually facing the fear of being endangered in NE region as a result of ruthless uprooting of the rhizomes by the tribal people from their wild habitats for its extreme medicinal value and use in spices. Some of the species descriptions are

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without Latin diagnosis or type specimen, therefore the legitimate status of many species is suspicious and remains unclear (Per. Comm., Dr. Sharma, GU). Sustainable management has so far not succeeded further aggravating the extinction. For any future analysis using *Curcuma*, it may be useful to have information regarding its genetic diversity and relationship between cultivated and wild species. The knowledge of genetic variability is a pre requisite to study the evolutionary history of a species, as well as for other studies like intraspecific variations, genetic resources conservation, etc [5]. Thus, molecular markers based characterization of *Curcuma* species may contribute to analyze taxonomic relationships and intraspecific diversity of the genus. Further, the study will be useful for planning strategies for their conservation and optimal utilization.

The usefulness of molecular markers in genetic diversity studies has been convincingly established [6–12]. Random amplified polymorphic DNA (RAPD) analysis is the simplest and least laborious method and has been used to estimate the genetic distances and diversity in a wide range of plant species especially at the sub-species and cultivar level [13–18]. New technological developments have expanded the range of DNA polymorphisms assays for genetic mapping, marker assisted breeding, genome finger printing, and investigating genetic relatedness [19]. Amplified fragment length polymorphism (AFLP) is considered more powerful than RAPD in discriminating genetic diversity [20]. On the other hand, inter simple sequence repeats (ISSRs) markers are abundant throughout the genome and show a higher level of polymorphism than any other genetic markers [21]. The sequences flanking specific microsatellite loci in a genome are considered to be conserved within species, across species in a genus and perhaps even across the related genera [22]. Recently, microsatellite markers have been used in genetic diversity studies in certain members of Zingiberaceae [23]. Studies are available on the genetic diversity analysis using RAPD, SSR, and ISSR markers on the members of genus *Curcuma* [23–25], but there has been no effort to analyze the endemic *Curcuma* species of NE India. The main goal of the present study was to access the degree of genetic diversity and to analyze the genetic proximity among the nine species of *Curcuma* from NE region including endemic ones using three different sets of molecular markers, viz., RAPD, ISSR, and AFLP, respectively.

Materials and Methods

Plant Material

The materials for the present study consists of nine species of *Curcuma*, both wild and cultivated collected from various regions of NE India (Fig. 1; Table 1). The species were collected and maintained in the departmental green house of IIT Guwahati and Botanical Garden, Gauhati University. Fresh, healthy green leaves of these samples were used for DNA extraction and subsequent finger-printing analysis.

DNA Extraction

Total genomic DNA was extracted from fresh tender leaves using modified SDS method as described by Kesari et al. [26]. The quality and quantity of the extracted DNA was confirmed to be consistent both spectro-photometrically and by running the extracted DNA on 1.0% agarose gels stained with ethidium bromide.

RAPD Analysis

PCR amplification of the genomic DNA was carried out using 20 arbitrary decamer oligonucleotide primers (Operon Tech, USA) (Table 2). The reaction mixture of 20 µl contained 50 ng/µl of template DNA, 1× assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl, and 0.1% gelatin), 0.2 mM each dNTPs (B'Genei, India), 5 pmol of each primer, and 0.5 U of *Taq* polymerase (B'LGenei, India). The reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems 9700). Thermal cycling conditions were as follows: pre-denaturing step of 5 min at 94°C, followed by 35 cycles each of 45 s at 94°C, annealing for 1 min at 32°C, extension for 1 min at 72°C, and followed by one final extension cycle of 5 min at 72°C. The amplification products were electrophoresed in 1.5% agarose gels in 0.5× TBE (10× stock contained 0.8 M Tris, 0.8 M boric acid, 0.5 M EDTA). The gels were photographed under a UV transilluminator.

ISSR Analysis

PCR amplification was carried out for 20 ISSR primers (Table 2). The PCR composition was same as that used for RAPD analysis with a final volume of the reaction mixture being 20 µl. The steps of temperature cycling were as follows: 94°C for 4 min, followed by 35 cycles of 45 s at 94°C, 1 min with varied temperatures as per the melting temperature of the ISSR primers used, 1 min 30 s at 72°C, and 10 min final extension step at 72°C. The amplified products were visualized in a 1.5% agarose gel containing ethidium bromide and photographed for further analysis.

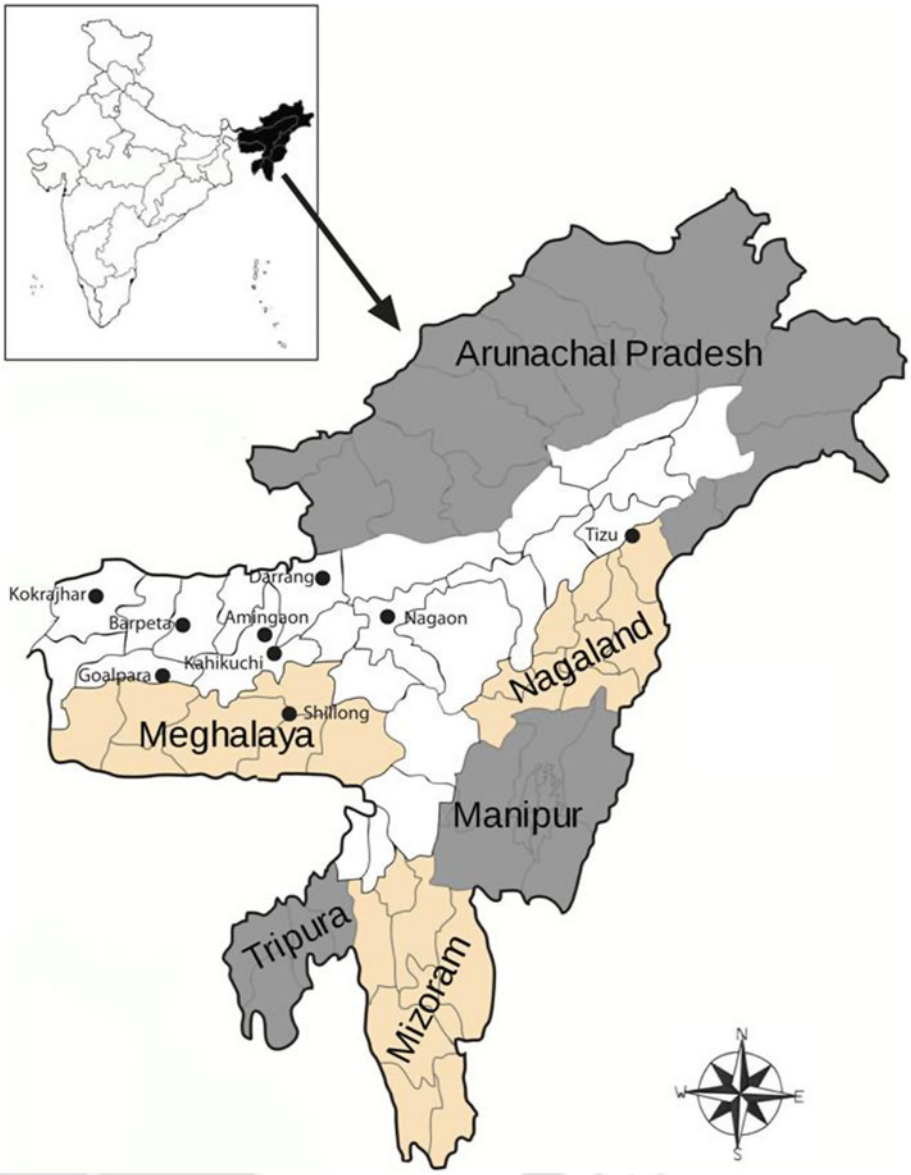

Fig. 1 Map locations of collection site of different *Curcuma* species used in the current study

AFLP Analysis

AFLP analysis was performed as described by Vos and his coworkers [27] with minor modifications. DNA (500 ng) was double digested with *Eco*R1 and *Mse*I, and then ligated with adapters using T4 DNA ligase (New England Biolabs). The pre-selective-PCR product was diluted in a ratio of 1:10 with TE buffer and then used as a template for the selective amplification. The pre-selective amplification was performed at 72°C for 2 min followed by 20 cycles of denaturation (at 94°C for 20 s), primer annealing (at 56°C for 30 s) and primer extension (at 72°C for 2 min) and finally maintained at 60°C for 30 min. An aliquot of the pre-selective PCR product was electrophoresed on 1.5% agarose gel and checked for amplification. It was then diluted (1:20) with DNase free water and used as a template for the selective amplification.

The selective amplification was performed using primers from the AFLP selective primer kit. The selective amplification involved the following thermal cycling conditions: denaturation at 94°C for 2 min followed by 11 cycles of 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min. The annealing temperature was reduced by 1°C every cycle till it reached 56°C. This was followed by another 20 cycles of amplification at 94°C for 20 s, 56°C for 30 s, and 72°C for 2 min, with a final extension at 60°C for 30 min. The PCR product of selective amplification (1 µl) was mixed with 0.5 µl of the GeneScan 500 ROX internal size standard (Applied Biosystems P/N 402985) and 8.5 µl of Hi-Di Formamide (Applied Biosystems P/N 4311320). The

Table 1 Plant materials used in the study

Species	Place of collection	Latitude and altitude	Rhizome character	Habitat
C. amada; SK 130	Amingaon	26°11′N, 56 m	Light yellow, creeping, soft, strong smell like raw mango.	Hilly slopes and moist grasslands
C. angustifolia; SK 261	Shillong	25°34′N, 1540 m	White, tuber is hard with characteristic odor.	Hilly moist slopes
C. caesia; SK270	Goalpara	26°10′N, 63.3 m	Whitish, soft tuber with mild smell, bluish inside	Moist, shady lands
C. zedoaria; SK160	Darrang	20°9′N, 104 m	Cone shaped tuber towards the end, Yellowish, with strong smell	Shady, humid places
C. aromatica; SK 335	Nagaon	26°11′N, 55 m	Hard, whitish in color with strong smell	Both shady and hot-humid places
C. longa; SK145	Barpeta	26°10′N, 56 m	Yellowish, smaller tuber, with characteristic smell	Shady, moist places
C. domestica; GS 205	Kahikuchi	26°9′N, 55.5 m	Reddish in color, larger tubers with strong smell	Shady, hot and humid places
C. domestica; GS 466	Nagaland (Tizu)	27°4′N, 900 m	Dark yellow in color, tuber has strong odor	Shady, moist, hilly places
C. spp. (wild); SK435	Kokrajhar	26°24′N, 65 m	White and hard tuber with mild smell	Hilly moist slopes

Table 2 Sequence information of RAPD and ISSR oligonucleotide primers used for amplification and polymorphism study amongst nine *Curcuma* species from Northeast India

S. No.	RAPD primer	Sequence (5′–3′)	ISSR primer	Sequence (5′–3′)
1	OPC 07	GTCCCGACGA	HB 12	CACCACCACGC
2	OPL 11	ACGATGAGCC	HB 13	GAGGAGGAGGC
3	OPO 08	GCTCCAGTGT	HB 14	CTCCTCCTCGC
4	OPAH 15	CTACAGCGAG	HB 15	GTGGTGGTGGC
5	OPAM 20	ACCAACCAGG	P 3	AGAGAGAGAGAGAGAGTG
6	OPAN 01	ACTCCAGGTC	P 6	CCACCACCACCACCA
7	OPAO 01	AAGACGACGG	P 8	CACCACCACCACCAC
8	OPAP 20	CCCGGATACA	807	AGAGAGAGAGAGAGAGT
9	OPAN 05	GGGTGCAGTT	809	AGAGAGAGAGAGAGAGG
10	OPAP 10	TGGGTGATCC	811	GAGAGAGAGAGAGAGAC
11	OPAA 01	AGACGGCTCC	816	CACACACACACACACAT
12	OPAB 01	CCGTCGGTAG	817	CACACACACACACACAA
13	OPAB 05	CCCGAAGCGA	818	CACACACACACACACAG
14	OPAB 14	AAGTGCGACC	824	TCTCTCTCTCTCTCTCG
15	OPAH 13	TGAGTCCGCA	825	ACACACACACACACACT
16	OPAF 02	CAGCCGAGAA	826	ACACACACACACACACC
17	OPAJ 19	ACAGTGGCCT	844	CTCTCTCTCTCTCTCTAC
18	OPX 20	CCCAGCTAGA	872	GATAGATAGATAGATA
19	OPA 08	GTGACGTAGG	17898A	CACACACACACAAC
20	OPA 12	TCGGCGATAG	17898B	CACACACACACAGT

mixture was then denatured prior to separation by capillary gel electrophoresis on an automated DNA sequencer (ABI 3130, Applied Biosystems). The electropherograms generated by the sequencer were interpreted with Gene scan software. Fragments sized from 50 to 500 base pairs (bp) with a peak height >50 in the electropherogram were retained for subsequent analysis.

Data Analysis

For all the three types of marker systems, duplicate samples from each individual were tested and only clear, unambiguous, and reproducible bands amplified in both cases were considered for the scoring and data. The numbers of polymorphic and monomorphic amplification

products were determined for each primer for nine *Curcuma* species. Scoring was carried out as 1/0 for the presence or absence of each fragment in each sample. To avoid taxonomic ambiguities, the intensity of bands was not taken into considerations, only the presence of band was taken as indicative. To compare the efficiency of primers polymorphic information content (PIC); as a marker discrimination power, was computed using the formula $\mathrm{PIC} = 1 - \sum p_i^2$, where p_i is the frequency of *i*th allele at a given locus [28] and also marker index (MI) was calculated as given by Powell et al. [20]. The basic parameters for genetic diversity were calculated in the POPGENE application [29]. The polymorphism of amplification products (P), the number of observed alleles (n_a), the mean number of effective alleles (n_e), the mean Nei's gene diversity index (h), and the Shannon index (I) were calculated using the POPGENE software.

Level of similarity among species was established as percentage of polymorphic bands and a matrix of genetic similarity compiled using the Dice's coefficient [30]. Applying the UPGMA [31] method using the SHAN subroutine through the NTSYS-pc (Numerical taxonomy system, 2.2 version) (Numerical taxonomy system, Applied Biostatistics, NY) [32] a dendrogram was generated representing the genetic relationship among nine *Curcuma* species. The correlation between the original similarity indices and cophenetic values were calculated, and the Mantel's test [33] was performed using 250 permutations to check the goodness of fit for nine different species of *Curcuma* to a specific cluster in the UPGMA similarity matrix. Further, principal coordinate analysis (PCA) was undertaken for the families with modules STAND, CORR, and EIGEN of NTSYS-pc [32] using the Euclidean distances derived from the standardized values using the NTSYS-pc-2.2 software.

Results

Efficiency of Polymorphism Detection

RAPD Analysis

The genetic diversity among the nine different *Curcuma* species was evaluated by 12 selected primers which yielded species specific DNA profiles and proved to be informative. A total of 55 mappable RAPD markers were generated by 12 primers. The amplicons ranged between 0.3 and 1.8 kb in size. Amplicon number per primer ranged from 2 (OPAM 20) to 8 (OPC 07) with an average of 4.92. Polymorphism also varied in different species of *Curcuma* with a maximum of eight bands for the primer OPC 07 and a minimum of one band in the primer OPAM 20 with a mean of 4.58 (Table 3). RAPD profile of nine different *Curcuma* species analyzed showed the polymorphic index value of 93.22% across all the species examined in the current study. The details of amplification products, polymorphic fragments generated, PIC, and MI values for each primer were showed in Table 3. The RAPD profile generated by OPAN 01 for the nine different *Curcuma* species is shown in the Fig. 2a. PIC values for RAPD primers varied from 0.17 (OPAM 20) to 0.48 (OPAN 05) whereas marker indices ranged between 8.64 (OPAM 20) to 48.14 (OPAN 05).

ISSR Analysis

Twenty ISSR primers were used to characterize the genetic diversity present among the nine species of *Curcuma.* Nineteen of these primers showed a total of 69 reproducible fragments that ranged from 0.2 to 0.85 kb in size. High percentage of polymorphism with all the 19 primers (98.55%) was displayed among the nine species of *Curcuma* with 68 polymorphic bands. It was observed that the number of visible bands ranged from 1 (807, 818) to 6 (17898 B) with an average of 3.63 whereas the average number of polymorphic bands per primer obtained was 3.58 (Table 3). The percentage of ISSR polymorphism for nine different *Curcuma* species studied ranged from 75 to 100%. Figure 2b displays the ISSR fingerprints using the primer HB 12. The PIC values for ISSR primers ranged from 0.19 (818) to 0.48 (825) with an average of 0.38, whereas MI ranged between 19.75 (818) to 48.14 (825).

AFLP Analysis

A total of 147 AFLP bands were recorded with four primer pair combinations and 143 of these bands were polymorphic (Table 4). The size of amplified products ranged from 50 to 500 bp. Total number of bands per primer ranged from 3 (*Mse*l-CAA/*Eco*RI-ACT) to 81 (*Mse*l-CAT/*Eco*RI-ACC) with an average of 36.75 whereas polymorphic bands per primer ranged from 3 (*Mse*l-CAA/*Eco*RI-ACT) to 78 (*Mse*l-CAT/*Eco*RI-ACC) with an average of 35.75 (Table 4). The PIC of each primer was evaluated which was found highest (0.61) for the primer combination *Msel*-CAG/*Eco*RI-AAC and the lowest (0.25) for the primer combination *Mse*l-CAC/*Eco*RI-ACA (Table 4) with an average of 0.35 per primer combination. The highest MI of 59.8 obtained for the primer pair *Mse*l-CAG/*Eco*RI-AAC and the primer combination *Msel*-CAC/*Eco*RI-ACA showed the lowest MI of 25 with an average of 35.18.

Table 3 Degree of polymorphism and polymorphic information content for RAPD and ISSR primers in nine species of *Curcuma*

Markers	Primer code	Total no. of bands	Total no. of polymorphic bands	POL (%)	PIC	MI
RAPD	OPAN 01	6	5	83.33	0.33	27.43
	OPAO 01	5	5	100	0.39	39.50
	OPC 07	8	8	100	0.44	44.44
	OPL 11	3	3	100	0.41	41.15
	OPAM 20	2	1	50	0.17	8.64
	OPAN 05	4	4	100	0.48	48.14
	OPAB 10	5	4	80	0.38	30.61
	OPO 08	6	6	100	0.36	36.21
	OPA 8	3	3	100	0.42	42.79
	OPA 12	7	7	100	0.45	45.14
	OPAH 15	5	4	80	0.34	26.86
	OPAP 20	5	5	100	0.40	40.43
	Total	59	55	93.22	–	–
	Mean	4.92	4.58	–	–	–
	Range	2–8	1–8	50–100	0.17–0.48	8.64–48.1
ISSR	HB 12	4	3	75	0.33	24.99
	HB 13	4	4	100	0.40	40.74
	HB 14	5	5	100	0.45	45.43
	HB 15	2	2	100	0.41	41.97
	P3	5	5	100	0.43	43.45
	P6	3	3	100	0.32	32.92
	P8	4	4	100	0.46	46.91
	807	1	1	100	0.44	44.44
	809	3	3	100	0.34	34.56
	811	4	4	100	0.25	25.92
	816	4	4	100	0.37	37.04
	817	4	4	100	0.37	37.04
	818	1	1	100	0.19	19.75
	824	5	5	100	0.35	35.55
	825	4	4	100	0.48	48.14
	826	4	4	100	0.32	32.09
	844	4	4	100	0.38	38.27
	17898 A	2	2	100	0.46	46.91
	17898 B	6	6	100	0.38	38.68
	Total	69	68	98.55	–	–
	Mean	3.63	3.58	–	–	–
	Range	1–6	1–6	75–100	0.197–0.481	19.75–48.14

POL polymorphism, *PIC* average polymorphic information content for polymorphic bands, *MI* marker index = POL(%) × PIC

Gene Diversity Between Species

In this study, relatively higher level of polymorphism and genetic diversity among the nine *Curcuma* species was revealed by RAPD, ISSR, and AFLP markers. Screening genetic diversity at the interspecific level, the average values of n_a, n_e, and h were ranged from 1.93–1.99, 1.37–1.62, and 0.24–0.36, respectively (Table 5). The mean Shannon's indexes (I) for *Curcuma* species were 0.53, 0.50, and 0.38 based on RAPD, ISSR, and AFLP, respectively (Table 5).

Genetic Diversity Analysis as Revealed by RAPD, ISSR, and AFLP

Based on the RAPD, ISSR, and AFLP analyses, the similarity coefficient among all the species of *Curcuma* were calculated. The genetic similarity value derived from the

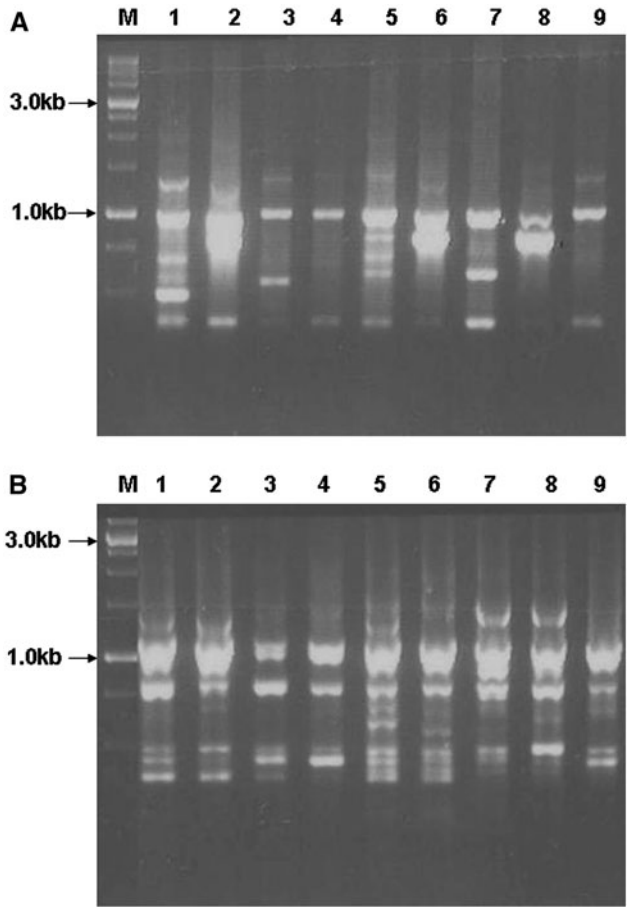

Fig. 2 RAPD and ISSR polymorphic profiles for nine species of *Curcuma* from Northeast India. M, 1 kb DNA ladder; 1, *C. amada*; 2, *C. angustifolia*; 3, *C. caesia*; 4, *C. zedoaria*; 5, *C. aromatica*; 6, *C. longa*, 7, *C. domestica* I; 8, *C. domestica* II; 9, *C.* spp. **a** RAPD (OPAN 01), **b** ISSR (HB 12)

RAPD data ranged from 0.098 between *C. longa* and *C. zedoaria* to 0.806 between *C. domestica* I and *C. domestica* II. The genetic similarity coefficients for all the nine species of *Curcuma* depicted with ISSR markers showed a wide range from 0.278 (between *C. angustifolia* and *C. caesia)* to 0.756 (between *C. aromatica* and *C. longa*). Similarly, for AFLP, the lowest genetic similarity coefficient was for the pair of *C. angustifolia* and *C. longa* (0.04) while the highest value (0.648) was calculated for the pair of *Curcuma* species *C. amada* and *C. zedoaria*, indicating a broad genetic basis.

In the present study, the genetic relationships among nine species of *Curcuma* were analyzed by 12 RAPD, 19 ISSR, and 4 AFLP primer combinations on the basis of Dice genetic distance [30]. Resulting clusters were expressed as UPGMA dendrograms constructed using SHAN neighbor-joining tree separately for each molecular marker used. The coefficients on the *x*-axis represent the similarity indices (DICE) of the different species chosen for the study. Based on UPGMA clustering algorithm from RAPD, the genotypes were grouped into two major clusters at a similarity index value of 0.20 (Fig. 3a). *C. amada* and *C. zedoria* were the two extremes in the dendrogram. Cluster I consisted of only one group having *C. caesia* and *C. zedoria.* Cluster II consists of individuals belonging to domestic species along with *C. angustifolia* and *C. aromatica.* Within cluster II, three subgroups were evident, one containing *C. amada* and *C.* spp., while subgroup 2 included *C. angustifolia* and *C. longa.* In subgroup 3, *C. aromatica* was placed with the domestic varieties *C. domestica* I and II. Similarly, the dendrogram obtained from ISSR profiles showed two distinct groups for nine species of *Curcuma* studied at a similarity index value of 0.34 (Fig. 3b), placing *C. caesia* in first and the rest in the second cluster. Cluster II again formed three distinct subgroups where *C. amada* and *C. zedoaria* are found to form subgroup 1 separately with *C. angustifolia.* The cultivated species are grouped with *C. aromatica* and *C.* spp. in subgroups 2 and 3, respectively. The dendrogram prepared based on AFLP analysis for studied *Curcuma* species where again formed two clusters showing higher level of diversity. The AFLP discriminated most genotypes and grouped individuals together though belonging to two different species (such as *C. amada* and *C. caesia*) (Fig. 3c). *C. angustifolia* alone formed cluster I. In cluster II, cultivated species are grouped together along with other closer species. *C. amada*, *C. zedoaria*, and *C.* spp. were

Table 4 Degree of polymorphism and polymorphic information content for AFLP primers applied to nine species of *Curcuma*

Primer combinations	Total number of bands	POL		PIC	MI
		Number	%		
*Mse*I-CAA/*Eco*RI-ACT	3	3	100	0.28	28
*Mse*I-CAC/*Eco*RI-ACA	12	12	100	0.25	25
*Mse*I-CAG/*Eco*RI-AAC	51	50	98.0	0.61	59.8
*Mse*I-CAT/*Eco*RI-ACC	81	78	96.3	0.29	27.9
Total	147	143	97.27	1.43	140.7
Average	36.75	35.75	–	0.36	35.18

POL polymorphism, *PIC* average polymorphic information content for polymorphic bands, *MI* marker index = POL(%) × PIC

Table 5 Genetic diversity parameters in nine species of *Curcuma*

Parameter	Value		
	RAPD	ISSR	AFLP
The number of observed alleles, n_a	1.93 ± 0.24	1.99 ± 0.12	1.97 ± 0.16
The mean number of effective alleles, n_e	1.62 ± 0.31	1.57 ± 0.30	1.37 ± 0.30
The mean Nei's gene diversity index, h	0.36 ± 0.14	0.34 ± 0.13	0.24 ± 0.15
Shannon index, I	0.53 ± 0.19	0.51 ± 0.17	0.38 ± 0.19

Each value = mean ± SD

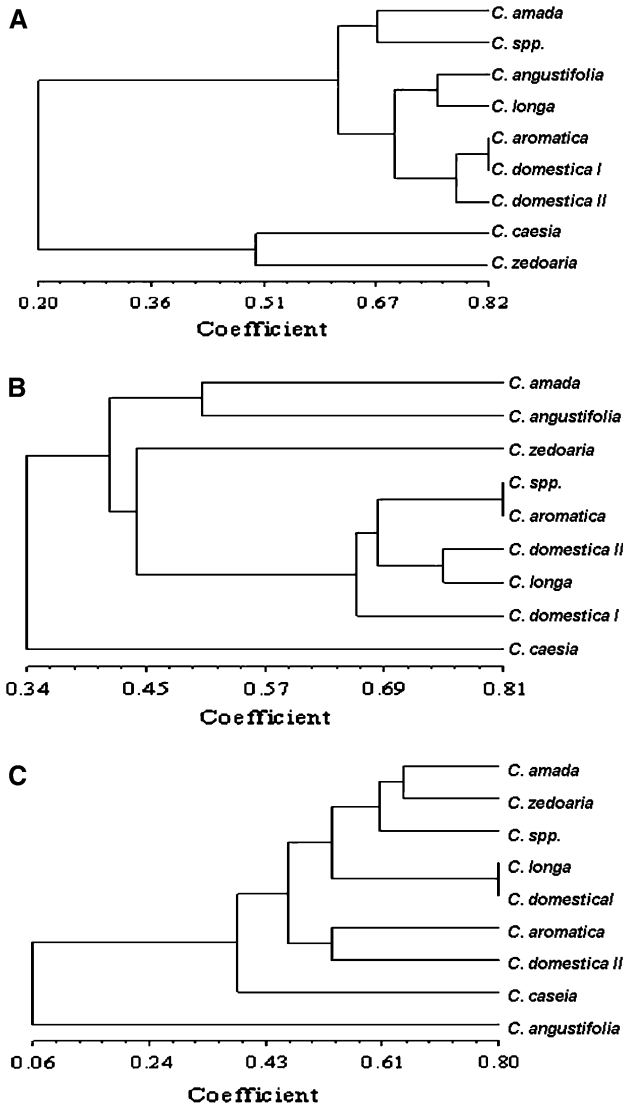

Fig. 3 Dendrograms representing the genetic variability among nine species of *Curcuma* from Northeast India as revealed by UPGMA cluster analysis. The genetic distances were from Dice similarity coefficient. **a** RAPD, **b** ISSR, **c** AFLP

placed in subgroup 1, where as *C. longa* and *C. domestica* I was placed in subgroup 2. In subgroup 3, *C. domestica* II, *C. aromatica*, and *C. caesia* grouped separately.

The separation approach as revealed by the Mantel test comparing the results of RAPD, ISSR, and AFLP, indicated a significant correlation within the nine different *Curcuma* species. The cophenetic correlation coefficient between dendrogram and the original similarity matrix were also significant for RAPD ($r = 0.96$), ISSR ($r = 0.94$), and AFLP ($r = 0.97$) supporting a good degree of confidence in the association obtained for the nine species of *Curcuma*. PCA derived on the basis of RAPD data illustrated that the first three principal coordinate components accounted for 24.99, 21.45, and 13.78% variation, respectively, among the *Curcuma* species. While for ISSR and AFLP marker-based PCA maps showed the three coordinates describing 25.77, 17.36, and 11.91% (for ISSR) and 32.17, 14.25, and 11.64% (for AFLP) of the total variance, respectively. Thus, the first three most informative coordinates accounted for 60.22, 55.04, and 58.06% of the genetic similarity variance based on RAPD, ISSR, and AFLP markers. PCA showed the multidimensional relationships that describe portions of the genetic variance in a data set for *Curcuma* species (Fig. 4a–c).

Discussion

Curcuma along with other species of Zingiberaceae display diversity in habitat, ethno botanical use, and morphology [25]. Very little is known about phylogenetic relationship among taxa and genetic diversity. Detailed knowledge about genetic relationships among wild and cultivated species of *Curcuma* will enhance the utilization value of wild species for any future study. A few studies based on morphological, anatomical, and biochemical characterization of *Curcuma* species and cultivars have been attempted earlier [34–38]. Relying much on the morphological characters alone in species delimitation has its own limitations since they are not always completely representative of the genetic structure [39]. Conventional taxonomic techniques in conjunction with molecular biology tools may go a long way in providing accurate and powerful ways of analyzing genetic relationship in the genus *Curcuma*. However, not much has been done on molecular characterization in *Curcuma* [40] Molecular markers

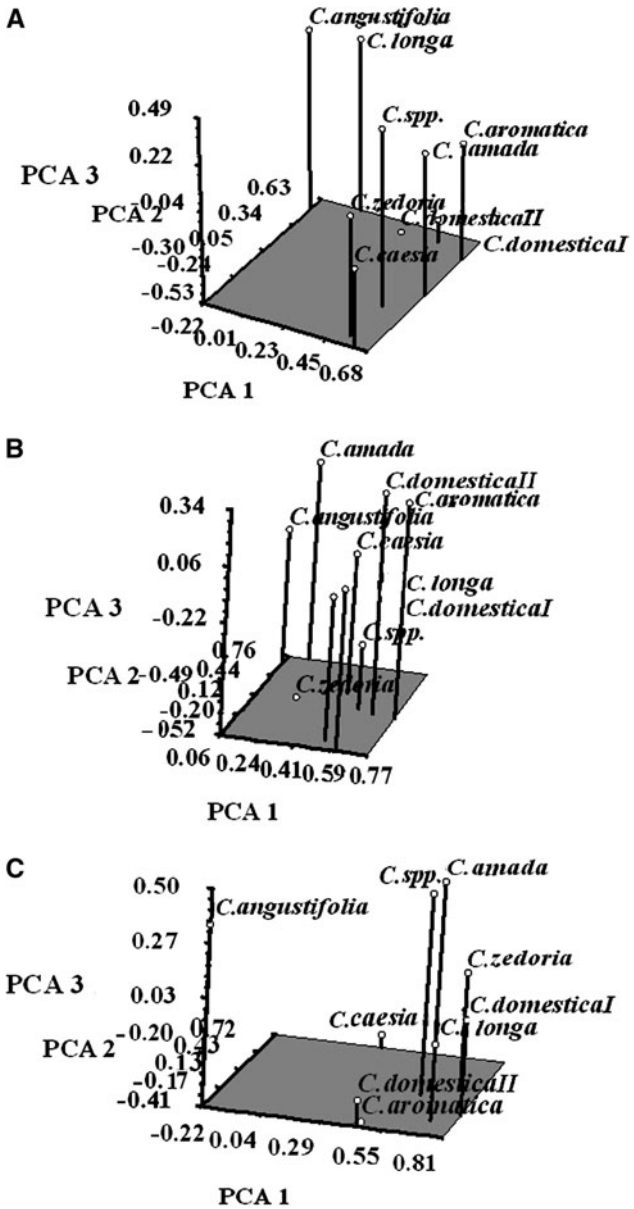

Fig. 4 Principle co-ordinate map for the first, second and third principle coordinate estimated for RAPD, ISSR, and AFLP markers for nine species of *Curcuma* from Northeast India

assume great significance, as these methods detect polymorphisms by assaying subsets of the total amount of DNA sequence variation in a genome. Earlier diversity studies have been reported in *Curcuma* species of all over India by using only RAPD and ISSR markers [25, 41].

The present work is the first attempt to assess the genetic relationship of nine economically important indigenous species of *Curcuma* (cultivated and wild) from NE India using three different sets of molecular markers (RAPD, ISSR, and AFLP). The percentage of polymorphism by ISSR markers was found highest (98.55) as compared to RAPD (93.22%) and AFLP (97.27%) in the studied *Curcuma* species. This indicates that the efficiency of ISSR markers in terms of amplification of a large number of fragments is high, compared to RAPD. This coincides with the observations found in other monocots, viz., rice bean and barley by Muthusamy and Fernandez et al., respectively [42, 43]. AFLP technology is another powerful tool for detection and evolution in germplasm collections and in the screening of biodiversity as well as for fingerprinting studies [44–46]. It is also clear from the present investigation that AFLP technique was able to differentiate intraspecific relations of the *Curcuma* species by a higher number of unique markers compared to RAPD and ISSR techniques. Similar findings were reported among four *Zingiber* species from Western Ghats, South India using AFLP markers [47]. However, ISSR marker demonstrated a different polymorphic capability compared with RAPD and AFLP and was found to be most informative in characterizing closely related *Curcuma* species from NE India. Yang and his coworkers [48] also stated that ISSR assay can provide more informative data than other techniques.

The three marker systems, RAPD, ISSR, and AFLP analyzed different segments of genome and hence revealed different genetic information [45, 49]. Results showed that RAPD, ISSR, and AFLP are very powerful methods of characterization of genetic relationship among species of *Curcuma*. The dendrograms based on three markers data were basically same with minor changes showing interspecific differences were more significant compared to intra-varietal ones. In RAPD cluster analysis, *C. ceasia* and *C. zedoaria* formed cluster I. All other species were grouped into the cluster II along with cultivated species. Cluster II formed 3 subgroups where in *C.* spp. is placed with *C. amada* in subgroup 1 showing that the wild species does not make an independent cluster. The adjoined group of cultivated varieties with wild ones suggests that they have been evolved in course of time. *C. domestica* I, II, and *C. aromatica* have physiological similarity of strong aroma and were also sub-grouped together in the RAPD dendrogram having least genetic distance. Coinciding with the results of RAPD, the clusters based on ISSR analysis divides the *Curcuma* species at their genetic distances segregating them more precisely. ISSR analysis has placed *C. caesia* separately whereas other species were placed together with the cultivated species in another cluster. *C. amada, C. angustifolia*, and *C. zedoaria* were found to be genetically closer to each other and placed in a single group inferring their vegetative and topological similarity also. However, the genetic distance between *C. aromatica* and the domestic varieties of *Curcuma* was found to be the same in both RAPD and ISSR analysis. Different hierarchical positions of the nine *Curcuma* species in the dendrograms elucidated that genomes of each species are

not exactly the same. Like ISSR, AFLP analysis was also found to provide a high resolution for the detection of genetic diversity and structures between and within species of *Curcuma.* AFLP markers separated the three varieties of a single species (*C. longa*, *C. domestica* I and II) in two subgroups depicting that intraspecific variations also exists (Fig. 3c). This supports the findings of Kavitha et al. [47] where intraspecific genetic diversity is studied of four *Zingiber* species using AFLP markers. *C. angustifolia* was placed in a separate cluster I inferring to its wild nature. *C. domestica* II, *C. aromatica*, and *C. caesia* were found to form a subgroup which shows a more precise discrimination among them. This can be explained that *C. domestica* II and *C. aromatica* are highlands species, possess geographic similarity and have a strong aroma; thus are found to be related. Dendrogram revealed that the species that are the derivatives of genetically similar type clustered more together. Similar findings were reported by Vanijajiva and his group in a genetic relationship study among *Boesenbergia* and related genera [50]. In all three cluster patterns *C. domestica* (I and II), *C. longa*, and *C. aromatica* are cohabiting having much similarity in floral, vegetative and rhizome characters. *C.* spp. also share many common vegetative and rhizome traits with the four species mentioned above. *C. longa* and *C. domestica* (I and II) as used in Indian spices and cultivated largely hence can be inferred as evolutionarily very active species. In all the three markers based dendrograms, *C. aromatica* is found to be genetically close to *C. domestica* II, both of which share a similar ecological niche in the highlands of NE India. It is evident from the data that genetic segregation does exist among the species of *Curcuma* both wild and domestic. These data were comparable with many works on other genera of Zingiberaceae [23, 51].

Based on Mantel tests [33], strong correlations observed and were statistically significant for each of the three marker systems used independently to study the diversity patterns of nine *Curcuma* species. Muthusamy [42] observed the same results while studying *Vigna umbellata* landraces for genetic variations. PCA further helped in depicting the variability among the species in the three dimensional modes. In case of RAPD, the first three coordinates accounted for 60.22% at the maximum. Whereas for ISSR and AFLP, the variability accounted for by three coordinates was 55.04 and 58.06% of the total variance, respectively. It was evident from the 3D plot that the species from hilly areas fall close to each other genetically. This analysis allowed understanding the spatial distribution of studied species and broadly classifies them. Similar result was reported by Islam and his group in intraspecific variation study of *C. zedoaria* [52]. From PCA analysis it is evident that both the methods, phenogram and three-dimensional plots of PCA were effective in studying genetic relationships and the groups found were comparable.

Conclusion

In conclusion, employing molecular marker-based study of genetic variations facilitates in the delineation of *Curcuma* species in dendrograms which are suggestive of an evolutionary pattern among *Curcuma*. The results provided an insight into the phylogenetic relationship between cultivated and wild relatives of *Curcuma*. ISSR markers are more discriminating than RAPD and AFLP to evaluate the genetic diversity/relationship among *Curcuma* species from the rich flora of NE India. Although RAPD markers are very quick and easy to develop but reproducibility is less than ISSR which detect at predetermined sites, such as DNA repetitive regions of the genome which are known as fast evolving sequences. ISSR fingerprinting opens new and interesting possibilities in the characterization of the *Curcuma* plants especially from NE region of India which still awaits proper systematic identification. Exploration and evaluation of diversity would be of great significance for in situ conservation of important *Curcuma* species especially for their scientific and commercial programmes. Furthermore, the scientific data presented here indicates that the application of PCR-based fingerprinting using whole DNA and arbitrary primers would provide a rapid and sensitive method for detection of genetic variations among the different species of the genus and also other genera of Zingiberaceae.

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