Development of tissue culture system for *in-vitro* regeneration, biochemical and molecular characterization of ginger varieties grown in Sikkim

A
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To,

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Sir,

I have the pleasure in forwarding the thesis entitled "**Development of tissue culture system for *in-vitro* regeneration, biochemical and molecular characterization of ginger varieties grown in Sikkim**" submitted by Mr. Sushen Pradhan for the award of degree of Doctor of Philosophy in Biotechnology under the Faculty of Science of Gauhati University.

The thesis embodies the results of a bonafied piece of work carried out under my supervision in the Department of Biotechnology, Gauhati University. The candidate has fulfilled all the requirements under the Ph. D regulation of Gauhati University. This thesis or part thereof has not been submitted for the award of any Degree/Diploma/Associates/Fellowship to this University or any other University/Institutions.

Yours faithfully

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DECLARATION

I, Sushen Pradhan hereby declare that the thesis entitled **"Development of tissue culture system for *in-vitro* regeneration, biochemical and molecular characterization of ginger varieties grown in Sikkim"** submitted for fulfilment of the requirements for the award of the Degree of Doctor of Philosophy in Biotechnology under the Faculty of Technology, Gauhati University.

The research work has been carried out by me, is original and independent work, done during **2009-2013** under the supervision and guidance of **Dr. PJ Handique**, Professor, Department of Biotechnology, Gauhati University.

This thesis or part thereof was not submitted earlier by me for research degree to this University or any other University/Institutions.

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ABBREVATION

RAPD	**Random Amplified Polymorphic DNA**
PCR	**Polymerase Chain Reaction**
%	**Percentage**
NAA	**Napthelic Acetic Acid**
MS	**Murashige and Skoog**
Kin	**Kinetin**
BAP	**6- Benzyl Purine**
IAA	**Indole Acetic Acid**
μ	**Micro**
μg	**Microgram**
DNA	**Deoxyribonucleic acid**
RNA	**Ribonucleic Acid**
RFLPs	**Restriction Fragment Length Polymorphisms ()**
SSR	**Simple Sequence Repeats**
(STMS)	**Sequence Tagged Microsatellite Sites**
(STS)	**Sequence Tagged Sites**
(DAF)	**DNA Amplification Fingerprinting**
(MP-PCR)	**Microsatellite Primed-PCR**
GIS	Geographic **Information System**
GPS	**Global Positioning System**
SH	**Schenk & Hildebrandt**
TAE	**Tris Acetic buffer**
DPPH	**2,2-diphenyl-1-picryl-hydrazyl (DPPH)**
TPC	**Total Phenolic content**
PEY	**Plant Extract Yield**
AA	**Antioxidant Activity**
ANOVA	**Analysis of Variance**
BHT	**Butylated Hydroxylamine**
EDTA	**Ethylene *diamine tetra-acetate***
EtBr	**Ethidium Bromide**
GAE	**Gallic Acid *Equivalent***
H2O2	**Hydrogen Peroxide**
HCL	**Hydrochloric acid**
OD	**Optical density**
OH	***Hydroxyl Radical***
QE	**Quercetin Equivalent**
ROS	**Reactive oxygen Species**
rmp	**Revolution per minute**
RS	**Remote Sensing**
TAE	**Tris Acetate EDTA buffer**

TF	**Total Flavonoids**
(FIC)	**Ferrous ion chelating**
AC	**Antimicrobial Activity**
NaOH	**Sodium Hydroxide**
UV	**Ultraviolet**
RT	**Room Temperature**
TBE	**Tris-Borate-EDTA**
TCA	**Trichloro acetic acid**
LR	**Linear Relationships**
SD	**Standard Deviation**
SQRT	**Square Root Transformed**
bp	**Base pair**
PCA	**Principal Component Analysis**

Chapter 1

Introduction

Ginger is one of the most important cash crop in the state of Sikkim after *Lime (Citrus reticulate)* and *Boda elachi (Amomum subalatum)* and now cultivated in every part of Sikkim for commercial purpose. The hilly state of Sikkim (India) is situated on the Eastern Himalayas, in the shadow of the towering Mount Kanchenjunga, located between 27º 00' 46" n - 28º 07' 48" N latitudes and 88º 00 58" E - 88º 55 25" E longitudes and is sandwiched by Nepal in the West, China in the North, Bhutan in the East and part of West Bengal in the South. The total area of Sikkim is 7096 sq. Km that is 0.22 % of the total area of the country. The stretch from north south is 112 Kilometers and that from east to west is 64 Km. The altitude varies from 300 meters above sea level to 8598 meters above sea level, the areas available for cultivation are not more than 12 % of the total area. Teesta and Rangit are the two major rivers of the state. The major occupation of the people is agriculture and large cardamom, ginger and citrus are the major cash crops of Sikkim (Subba, 1984).

1.1 Distribution and nomenclature of ginger

Ginger has numerous medicinal properties and it is known from ancient times and cultivation of ginger has unrecorded history. In ancient times ginger was more valued for its medicinal properties than spices and played an important role in primary health care in ancient India and China. In European medicine ginger was also among the most highly valued of all mild carminatives and it was an important constituent of many pharmaceutical products.

Ginger, botanically known as *Zingiber officinale* Rosc. family Zingiberaceae and in the natural order Scitamineae (Zingiberales of Cronquist, 1981). The Latin term *Zingiber* was originated from the ancient Tamil root, *ingiver* (Ravindran *et al.,* 2005). The term ingiver spread to different place through Arab traders. The present day names for ginger in most of the western countries were derived from this. Examples are *ingefaer* (Danish), *Gember* (Dutch), ginger (English), *Zingibro* (Esperanto), *harilik ingver* (Estonian), *inkivaari* (Finnish), *gingerbre* (French), and *ingver* (German).

Most of the authors thought that the term *Zingiber* was derived from the Sanskrit term *singavera* (Watt, 1872; Rosengarten, 1969; Purseglove *et al.,* 1982), meaning antler like or horn-shaped, indicating the shape of the rhizome. Mahindru (1982) was of the opinion that the original word for ginger was in all probability a pre-Dravidian one and that it is found with minor variations in about 20 languages extending from China and the islands of the Pacific Ocean to England horn shaped, indicating the shape of the rhizome. In certain languages there are separate terms for fresh ginger and dried ginger, which indicate the importance of both commodities as well as the fact that they are put to uses that are often distinct and different manner.

1.2 Brief botanical account

The family *Zingiberaceae* consists of 51 genera and 1500 species worldwide (Newman, 2001). About 150 species of the genus *Zingiber* are distributed in tropical and subtropical Asia. Roxburg (1810) described and reported a total of 11 Indian species and placed them in two sections based on the character of the spike. Section 1 spikes radical and Section 2 spikes terminal. Baker (1882) carried out an exhaustive survey of the Zingiberaceae of Indian Peninsula for *The Flora of British India* (J.D. Hooker) and his work was fully supported by other workers including Schumann

(1904). Earlier, Zingiberaceae was divided into the sub-families Costoideae and Zingiberoideae, which were later given independent family status as Costaceae and Zingiberaceae.

Ginger rhizomes are pale yellow within or with a red external layer. Leafy stems to about 50 cm tall, 5 mm diameter, glabrous except for short hairs near base of each leaf blade, commonly about 17 by 1.8 cm; rather dark green, narrowed evenly to slender tip; ligule broad, thin, glabrous, to 5 mm tall, slightly bilobed. Scape slender, upto 12 cm tall, the upper sheaths with or without short leafy tips; inflorescence approximately 4.5 cm long and 15 mm diameter; bracts approximately 2.5 by 1.8 cm; green with pale. Bracteoles as long as bract; calyx with ovary 12 mm long; corolla tube 2.5 cm long, lobes yellowish, dorsal lobe 18 by 8 mm (flattened), curving over the anther and narrowed to the tip, laterals narrower. Lip (mid lobe) nearly circular, approximately 12 mm long, and wide, dull purple with cream blotches and base, side lobes about 6 by 4 mm; free almost to the base, colored at mid lobe; anther cream, 9 mm long, appendage dark purple, curved, 7 mm long (Holttum, 1950).

1.3 Morphology

Among all the species, Ginger (*Zingiber officinale* Roscoe) is one of the most economically important cash crops of the world. *Zingiber officinale* is diploid 2n=22 chromosome numbers (Raghavan and Venkatasubban, 1943).

It is a perennial herbaceous plant, grown as an annual crop for its spicy underground rhizomes or stems. Ginger plant is erect, aerial shoots (pseudostems) with leaves, has numerous fibrous and few fleshy roots that emerge from the branched rhizomes. The fibrous roots are thin, contains many root hairs and their main function is

to absorption of nutrient and water. Fleshy roots are developed from the base of matured ginger which is thicker, white in color, with few root hairs and absence of lateral roots. Such roots help ginger for support and absorption (Ravindran.*et al.,* 2005) (Figure 1).

The pseudostems or aerial shoots reach a height of 50–120 cm. The aerial shoots have many narrow leaves borne on very short petioles and with sheaths that are long and narrow, and the overlapping sheaths produce the aerial shoot. Leaves are dark green, narrow, simple, lanceolate and smooth, alternate and approx 25 cm long with a prominent midrib. It flowers only in favorable climatic condition. Flowers are usually yellow, red, with dark purplish or white spots, bisexual, epigynous. Fruits are oblong capsules; slender glabrous and fairly large, flowers are usually sterile and rarely seed set. Gingers are asexually propagated from the portion of the rhizome. It prefers to grow on red soils having pH from 5.5 to 6.5 an altitude ranging up to 1500 m (Pruthy, 1993)**.**

1.4 History of ginger in Sikkim

In Sikkim ginger is an essential crop of for 14 Sikkimese tribes namely, *Lepcha, Bhutia, Sherpa, Khombu Rai, Limboo, Yakha, Sunwar, Mangar, Gurung, Tamang, Majhi, Bhaun, Chettri, Kami and Newar.* Ginger is considered as one of the most important items by these tribes for their religious activities besides culinary and medicinal uses (Subba, 2009; Pradhan *et al.,* 2012). Seven species of *Zingiber* were recorded in Sikkim Himalaya by Kumar and Raju (1983). Five cultivars of *Zingiber officinale* Roscoe, namely, *Bhaisay, Gorubthangey, Jorethangey, Nangrey, and Majhauley* were reported from Sikkim and Darjeeling hills (Subba, 2012). Generally, cultivars of ginger were named after the name of the region or area where they grow (Govindaranjan, 1982), whereas, in Sikkim, cultivars of ginger were named according

Figure 2 Mixed cropping between ginger and maize in Sikkim.

Figure 1 Showing primary roots and secondary roots of *Z.officinale*.

to the size of ginger rhizome, viz., *Majhauley* (medium in size), *Bhaisay (*big in size), *Chari Nangrey* (nail of bird) except *Jorethangey* and *Gorubthangey* representing the name of places (Pradhan *et al.,* 2012).

In Sikkim ginger is intercropped with mandarin orange (*Citrus reticulata* Blanco) and mixed with maize (Figure 2). Many farmers believe that raising maize and ginger together gives more yield than sole crops (Patiram, 1995). Partial shade provided by the mandarin orange provides a congenial atmosphere for ginger. After harvesting of maize, ginger plants become exposed to more sunlight and that favors development of bolder rhizomes. Mixed cropping is more efficient and productive than sole cropping because of higher combined yields (of calories and proteins) and better energy use efficiency (Willey, 1979).

1.5 Agro-climatic conditions required for ginger cultivation

Ginger requires a warm and humid climate adapted for growth in tropical and sub-tropical areas. It can be cultivated in a region that practice light frost as long as rhizomes are not exposed to freezing temperature. The crop is cultivated right from the sea level to an altitude of 1500 meters. The most favorable elevation for its thriving cultivation is 300-900 meters. It shows most suitable production in full sun, but it is also adapted to thrive well under partial shade when growing in intercropping system. Vegetative growth is promoted with long day lengths and rhizome enlargement is promoted under shorter day lengths. Day length response varies among different ginger varieties (Adaniya *et al.,* 1989). The most suitable temperatures for ginger cultivation is 25-28ºC with moist conditions. Good water drainage and aeration system with ideal soils pH is 5.5 to 6.5 is optimum for ginger growth. Steady rainfall is preferable during

growing season of ginger with drier period at the end of the growing seasons prior to and during harvest. Excessive rainfall and water logging may result in soil borne disease (Dhoro, 1997).

1.6 Methods of propagation

World over 25 varieties of ginger are grown. Most of the varieties have not been properly characterized. Varieties differ in the size of the rhizome, flavor, aroma, pungency, color, and fiber content. The ginger plant rarely carry true seeds, due to this ginger are vegetatively propagated from the portions of rhizome. About 2,000 kg of rhizome “seed piece” are required to plant a hectare of ginger, using seed piece of 115-230g in size. Each piece should possess minimum of 3-4 eyes or nodal section. However larger seed piece may result in greater yield when ginger is planted late in the season.

The crop is cultivated in weed free bed with proper soil texture. The following aspects are considered as important (Ravindran *et al.,* 2005).

a) Proper soil fertility and moisture levels during the growth of the crops will be key determinants of crop quality and yields.

b) Organic mulches and rotation with green manures, or other cover crops can also improves and complement the fertility of the soil.

c) Proper irrigation system, such as drip irrigation, may be necessary to maintain proper soil moisture during the production cycle.

d) Prior to planting, soils are typically ploughed to a depth of 45- 60 cm.

e) Lime is incorporated to adjust the pH and furrows are prepared in rows to a depth of 30-45cm.

f) The ginger crop, as it is grows from the bottom of the furrows, is hilled 3-5 times during the growing seasons, resulting in raised hill/beds, allowing for the proper development of the rhizomes.

1.7 Diseases of ginger

Ginger is affected by many numbers of pest and disease (Dhoroo and Edison, 1989). The common diseases are rhizome rot, bacteria wilt, yellow, leaf spot and storage rots that highly affect in economic loss (Nadda *et al.,* 1996). Srivastava *et al.,* (1998) reported thirty nine potent pathogen microorganism causing soft rot, yellow, bacteria wilt and dry rot in the field or in storage. The diseases of ginger are divided into two categories parasitic and non-parasitic. The main parasitic diseases are bacteria wilt (*Rolstonia solanacearum*); bacteria soft rot (*Erwina* sp., *Fusarium yellow)* and rhizome rot *(Fusarium oxysporium* sp. *and Pythium* sp.) and nematodes (*Meloidogyne incognita*), which exist in entire plant or in the part on the plant, which makes the ginger plant to survive difficult. Non-parasitic diseases are caused by different unfavorable environmental condition like drought, temperature, and soil nutrient deficiencies. Information on these dieses of ginger is available in several published literature (Butler, 1907; Simmonds, 1955; Trujillo, 1963; Haware and Joshi, 1973; Pegg *et al.,* 1974; Joshi and Sharma, 1980; So, 1980; Sinha and Mukhopadhya, 1988; Rajan and Agnihotri, 1989; Manicom, 1998; Nepali *et al.,* 2000).

1.8 Uses of ginger

Ginger has been used as one of the raw drug by different ancient medicine systems viz., Ayurveda, Chinese, Japanese, Tibetan and others. In Ayurveda, ginger is called as "*Mahaoushadha"* the great cure. This shows an extensive usage of ginger in

Ayurveda in ancient times. For the Chinese, it is invaluable as a cure for a variety of illnesses and maladies. The properties and uses of ginger in Ayurvedic medicine are available from authentic ancient treatises like *Charaka Samhitha* and *Susrutha Samhitha,* which are the basics for this system and of both treatises/text given the importance of dry and fresh ginger. Due to its warming property, ginger is capable of caring dryness and thus is anti-diarrheal, anti-arthritic and anti-filarial effects of dry ginger. It is also good in asthma, bronchitis, piles, eructation, and ascitis. Ginger has been consumed socially and habitually by people for so long (since ± 2000 BCE), aside from the astringent taste and boost it provides its medicinal properties are often over-looked. Often used as a means to treat digestive, relieve heartburn, or even alleviate symptoms of the common cold, ginger has many versatile uses. However, traditional healers have long believed that ginger is a means of prolonging life (Chopra, 2000). It helps cure digestive complaints, infections and pain relief. Ginger is a natural immune booster and is considered to be a “hot spice "meaning that, when ingested, it quickly has a warming effect on the human body. Ginger has also been found to relieve arthritis-related joint pain.

1.9 Problems of ginger cultivation

Identification and characterization of ginger germplasm (varieties) are very important for the conservation and utilization of plant genetic resource. Certain amount of work is available on identification of different varieties of ginger in Orissa and Coimbatore, India (Rout, 2007; Harisaranraj, 2009). Selection and breeding for the production of desirable traits in this crop have resulted in severe erosion of its genetic base over the time. Lack of proper conservation programmes have caused major reduction in its gene pool, as most of the breeding and conservation programs are still

Table 1: Nutritive value of ginger infusion

Parameters	Value
Moisture	80.900 gm.
Protein	2.300 gm.
Fat	0.900 gm.
Minerals	1.200 gm.
Fiber	2.400 gm.
Carbohydrates	12.300 gm.
Energy	67.000 K cal
Calcium	20.000 mg
Phosphorus	60.000 mg
Iron	3.500 mg
Vitamins	
Carotene	40.000 µg
Thiamine	0.060 mg
Riboflavin	0.030 mg
Niacin	0.600 mg
Vitamin C	6.000 mg
Minerals & Trace Elements	
Magnesium	405.000 mg
Copper	0.740 mg
Manganese	5.560 mg
Zinc	1.930 mg
Chromium	0.057 mg

Percentages are relative to US recommendations. Source: USDA Nutrient Database

based on conventional morphological and agronomical descriptors, which are dependent on environmental and developmental factors thus reflecting the base of the gene pool with no true genetic relatedness (Green, 1971; Wilkremaratne, 1981). The breeding of experimental planting material of a perennial, heterozygous crop like ginger is difficult. However, like any other crop, the main objective of ginger breeding is to improve the quantity and quality of the end product. The methods of introduction, selection, and hybridization have been used with success for ginger improvement. The different varieties have been developed to suit the requirements of the various agro climatic regions. However, proper selection criteria have not yet been established. This apart, the prediction of the performance of mature ginger based on their evaluation in the early years has not been perfected. Application of biotechnological techniques, as has been

done in other crops, it is a greater challenge to ginger breeders and ginger biotechnologists.

Since the *in-vitro* micro-rhizome production in ginger was successfully produced (Sharma, 1995) callus induction and organogenesis have been reported in several papers from the year 1995 to 2010. In ginger research the previous era held major emphasis on standardizing parameters of the *in-vitro* protocol, such as using suitable explants, overcoming microbial contamination, and optimizing media composition combined with growth regulation for better proliferation (Sharma, 1997). Following this era the efforts turned towards hardening micro-shoots to achieve a higher survival percentage. Accordingly, several non-conventional approaches, such as a CO_2 enriched hardening chamber, biological hardening, were developed till 2007 Jamil (2007). Presently, attention is increasingly focused on evaluating field performance of the transformed *in-vitro* grown whole plantlets. However, there is no stable technique available for *in-vitro* multiplication of ginger.

In order to stop further reduction in its gene pool and to breed for new ginger types with more productiveness; less prone to natural calamities, diseases, as well as higher contain of ginger oil, a thorough knowledge of the existing genetic diversity, *in-vitro* culture studies and improvement of the existing varieties through various molecular biology, tissue culture and biotechnological techniques is a pre-requisite in ginger research.

1.10 Objectives of the present study

Sikkim is known for its ginger production as well as for different varieties, but organized study is not available particularly in the area of characterization of ginger

germplasm and tissue culture studies. Considering the importance of this important cash crop, the present programme of study have been designed with the following objectives-

Objectives

1) To enumerate ginger varieties of Sikkim state using morphological descriptors and ecological information.
2) To study antibacterial and antioxidant properties of various extracts of ginger rhizome.
3) To standardize tissue culture protocol for *in-vitro* regeneration of selected germplasm.
4) To molecular characterization of ginger germplasm and study of genetic fidelity of *in-vitro* grown plants using RAPD markers.

Chapter 2

Review of Literature

Underground stem (rhizome) of *Zingiber officinale* (Ginger) has been used as a medicine in Asian, Indian, and Arabic herbal traditions since ancient times (Altman and Marcussen, 2001). It has been used extensively for more than 2500 years in China for headache, nausea, and colds (Grant and Lutz, 2000) and in Mediterranean (Sharma and Clark, 1988) 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).

During the thirteenth and fourteenth centuries, next to pepper, ginger was the commonest and most precious of spices, costing nearly seven scrolling per pound, or about the price of a sheep (Watt, 1872; Mahindru, 1982). The literature also indicates that ginger preserved in syrup (called Green Ginger) was also imported to the Western World during the middle ages and was regarded as a delicacy of the choicest kind. In Africa, ginger is regarded as auspicious, which is absolutely necessary to the Savaras tribe for their religious and marriage functions (Mahindru, 1982).

During the Middle Ages and until the end of the nineteenth century English tavern keepers used to have ground ginger in constant supply for thirsty customer to sprinkle on top of their beer or ale and then stir into the drink with a red-hot poker (Rosengarten, 1969). The Western herbalists and naturalists knew the great qualities of ginger as confirmed by the well-known British herbalist John Gerad. He writes in his herbal

(1577) that "ginger is right good with meat in sauces," and says that this spice is "of an eating and digesting quality (Parry, 1969).

2.1 History of ginger in India

In olden India, ginger was not important as a spice, but it was named as *mahabheshaj*, *mahaoushadhi*, literally meaning the great cure, the great remedy. For the ancient Indian, ginger was the God given universal remedy for a number of ailments. That may be the reason why ginger found a prime place in the ancient Ayurvedic texts of Charaka *(Charaka samhita)* and Susruth *(Sushrutha samhita).* In *Ashtangahridyam* of Vagbhatt (a very important ancient Ayurvedic text), ginger is recommended along with other herbs for the cure of elephantiasis, gout, extenuating the juices, and purifying the skin from all spots arising from scorbutic acidities. Ginger is also recommended when exotic faculties were impaired due to indigestion.

Rabbi Benjamin Tudella, who travelled between 1159 and 1173 A.D and gave an account of ginger cultivated on the west coast of India. Tudella gave a vivid description of the place and trade in spices as well as cultivation of spices in and around the port of Quilon in the State of Kerala (Mahindru, 1982). Marco polo (A.D. 1298), in his famous travelogue, writes "good ginger also grows here in known by the name of Quilon ginger. Another traveller, Friar Odoric (A.D. 1322), writes. Quilon is at the extremity of pepper forests towards the south. Ginger is grown here, better than anywhere else in the world and huge quantities.

Linschotten (1596) presented a very interesting description of the spices. He observed that ginger grew in many parts of India, but the best and the most exported grew on the coast of Malabar.

Ridley (1912) gave a detailed description about ginger and turmeric practices prevalent in nineteenth-century India. Buchanan (1807) made many references on the cultivation of various spices, including ginger, during the journey to south India. Later on ginger cultivation spread from Kerala to various others parts of India, mainly to Bengal and North-eastern states.

2.2 Taxonomical review of ginger

Roscoe (1807) described *Z. officinale* from a plant in the Botanic Garden at Liverpool as "*Bracteis ovato-lanceolatis, laciniis corolla revolutis, nectario trilobato*" and referred to *Amomum zingiber* Willd. Willdenow (1797) extended Linnaeus description *"Amomum scapo nude, spica ovata*" with "*squamis ovatis, foliis lanceolatisbad apicemmargine ciliates*."

Linnaeus (1753) *Amomum zingiber* is the basionym for the species. The genus *Amomum* of Linnaeus is a nomenclatural synonym of the conserved generic name, *Zingiber* Boehm (Burt and Smith, 1968). The specific epithet *zingiber* could not be used in the genus *Zingiber*. Thus, *Z. officinale* was adopted as the correct name for ginger. The specimen available in most herbaria are without flowers, and it is assumed that Linnaeus based his description on the account and figure given by Rheede in *Hortus Malabaricus.* The figure given by Rheede (Vol., 11, plate 12, 1692) is the designated lecto type of the species *Z. officinale* Rosc. (Jansen, 1981). The species epithet *officinale* was derived from Latin, meaning "work shop," which in early Latin was used to mean pharmacy, thereby implying that it had a medicinal use.

2.3 Tissue culture work

In-vitro plant tissue culture has become more powerful tool after the discovery of "Growth Regulator" auxin (Arteca, 1996) and Cytokinins (Haberlandt, 1913) followed by formulation of artificial nutrient medium (Murashige and Skoog, 1962) which was the breakthrough of plant tissue culture because most of the horticulture crops can be micropropagated in this medium.

The potential of tissue culture in various aspects of plant improvement has already been recognized and attracted the attention of scientists. Rapid multiplication of propagation materials through tissue culture, particularly in the initial selection, assumes importance in plant improvement programmes. The potential of tissue culture in various aspects of plant improvement has already been recognized and attracted the attention of scientists. Rapid multiplication of propagation materials through tissue culture, particularly in the initial selection, assumes importance in plant improvement programmes. It also holds promise in the development of pure lines through haploid technology, production of triploids through endosperm culture, culture of embryos of incompatible crosses and isolation of somaclonal variants (Singh, 1978).

Tissue culture as a tool for vegetative plant propagation is relevant for crop plants that resist conventional asexual propagation (Hackett, 1966). The various explants such as axillary bud, shoot tips, meristem tips, root tips are commonly used. *In-vitro* ginger multiplication, dormant buds on excised rhizomes can be forced to form shoots which can be rooted. This method is rather slow, particularly for plant breeders, as on an average only 20 plants can be produced per year from single, one year old plant (Leffring, 1971).

Literatures on ginger tissue culture are limited. Hosoki and Sagawa (1977) first reported on the induction of maximum of 6 shoots per explant under *in-vitro* culture of ginger. Later Illahi and Jabeen (1987) conducted an experiment in ginger using different explant materials *viz.* immature buds, rhizome cutting with shoot bud primordia and juvenile shoots and observed/ obtained efficient plant rejuvenation. Haung (1995) observed that ginger plants were regenerated from the shoot tip explants of 0.2 to 0.9 mm in length were best for *in-vitro* propagation.

Most of the earlier work showed that the shoot tips or new emerging buds are the right explants for *in-vitro* propagation of ginger (Sharma and Singh, 1997; Pandey *et al.,* 1997; Rout. *et al.,* 2001; Kambaska and Santilata, 2009). Lange *et al.,* (1987) reported elimination of nematodes infection from the rhizomes of ginger using *in-vitro* techniques. Inden *et al.,* (1988) reported that each shoot bud explant produced more than four shoots within nine weeks on MS medium containing BA 5 mg/l and NAA 0.5 mg/l. Balchandran *et al.,* (1990) reported successful *in-vitro* propagation *Curcuma longa* using rhizome buds as explants on MS medium with different combinations of BA and kinetin.

Malmug *et al.,* (1991) reported that shoot proliferation of the regenerated shoots was induced with the addition of NAA 1 mg + BA 5 mg/l. Dogra *et al.,* (1994) achieved *in-vitro* propagation of *Zingiber officinale* using rhizome buds. The buds produced multiple shoots when cultured on MS medium with BA 2.5 mg/l and NAA 0.5 mg/l.

Palai *et al.,* (1997) observed that when *Zingiber officinale* cultivars cultured on medium supplemented with increased concentration of BA from 6 to 8 mg/l, there was decreased multiplication of shoots.

Pandey *et al.,* (1997) reported multiple shoot production on MS medium with BA 5 mg/l + NAA 0.5 mg/l using pseudo-stems of ginger as explants. Sharma and Singh (1997) conducted *in-vitro* culture in rhizomes of ginger cv Himachal local and found 7.7 multiple shoots per bud on MS medium fortified with kinetin 2 mg/l and sucrose 20 g/l after four week of culture and 6.8 cm shoot length and 7.0 cm root length was found in MS medium kinetin 2 mg/l, NAA 2 mg/l and 20 g sucrose per litre. Simultaneously, obtained well developed rhizomes from micropropagated plants which was not affected by *Fusarium oxysporum* during storage for six months and developed a method in checking storage rot caused by *F.oxysporum.*

Pandey *et al.,* (1997) obtained disease free ginger plantlets was developed through plant tissue culture protocol with very less cost effective in MS medium with BA 5 mg/l combination with NAA 0.5 mg/l concentration, the highest number of shoots 5.33 shoots per pseudostems after 5 weeks of culture. In same medium highest shoots length and 4.33 numbers of roots were recorded simultaneously.

In 2001 from Orissa (India) an efficient protocol was standardized on (*Zingiber officinale* cv. V_3 S_{18}) ginger in production of (92.2 % of plant show) shoot multiplication in MS medium combination with BAP 26.6 µ*M*, IAA 8.57 µ*M* and adenine sulphate 111.1µ*M* with 3% sucrose, and *in-vitro* rhizome formation was found in MS medium supplemented with BA 4.44µ*M*, IAA 5.71 µ*M* and sucrose 3-8% after eight week of culture. They observed that successful production of multiple shoots and *in-vitro* rhizome formation depended on the nutrient medium and the culture environment Rout *et al.,* (2001).

Khatun *et al.,* (2003) studied a rapid shoots multiplication through new shoot tip of ginger rhizome on MS medium with 3 % sucrose and 0.5 % agar fortified with BAP

2.5 mg/l and kinetin 0.5 mg/l. Rooting was also observed on same culture medium after 45 days of culture.

Rha *et al.,* (2007) standardized a systematic protocol for complete plant regeneration using shoot apical meristems culture for (*Zingiber officinale*) ginger. Wanju of Korea. Callus was observed on explant in culture medium MS fortified with a combination of NAA 1 mg/l and kinetin1.0-2.0 mg/l and IAA 0.1 mg/l and BAP 1.0-2.0 mg/l. Most of the shoot differentiation from callus occurred on MS fortified with IAA 0.1 mg/l and BAP 1.0 mg/l. Regenerated plants were exposed to CO_2 concentration and observed a 400-4000 ppm increase in atmospheric CO_2 concentration leads to increase in adventitious bud and shoot primodium and reduced at the concentration of 8000 ppm.

Zeng *et al.,* (2008) observed in increase in *in-vitro* micro rhizome production of (*Zingiber officinale* Roscoe) ginger on combination of 80 g/l sucrose, 2X MS microelements and 1 X micro element, with a photoperiod of 24 L: 0D (Light/Dark).

Kambaska and Santilata (2009) observed an efficient protocol of *Zingiber officinale* Roscoe, Suprava and Suruchi of Orissa (India) using fresh rhizome bud/sprout culture/micropropagated on semi solid Murashige and Skog's medium with different growth regulator with various concentration and combination of BAP and NAA for shoot and root induction. Multiple shoots of 7.5 numbers found at MS medium supplemented with in BAP 2 mg/l and NAA 0.5 mg/l combination with average shoot length of 6.2 cm and best rooting medium was half MS with NAA 2 mg/l concentration was observed.

Hassan *et al.,* (2009) reported on micropropagation of (*Zingiber officinale* Roscoe) ginger cv. Suruchi and Bari (Ada) using three different explant i.e. leaf, shoot

tip and root, using different combination and concentration of growth regulator in MS medium. Studied on callus induction through five quantative traits i.e. days required for callus induction, size of callus induction, colour of callus and percentage of callus induction. Cultivar variety Suruchi observed 62.64% callus induction, 63.98% shoot induction and 68.76% root induction. Leaf explant found the best explant over the root and shoot tip explant, with the callus, shoot and root induction were 62.64%, 63.98 and 68.76% respectively in MS medium fortified with Dicamba 0.5 mg/l for callusing, MS with kin 1.0 mg/l + BAP 1.0 mg/l for shooting and MS + IBA. 1 mg/l Regenerated *in-vitro* plants were successfully established in pot and to field.

An efficient *in-vitro* protocol was developed on *Zingiber officinale* Roscoe., *var-* Varda through direct regeneration of vegetative buds on LSBM medium supplemented fortified with BAP (17.76µ*M*) with 96 % initiation response (Kavyashree, 2009). Rapid shoot multiplication was observed at the average rate of 4 fold per culture. This efficient protocol for multiple shoots and roots on the same medium after 2-3 subcultures eliminated the steps of *in-vitro* rooting. The statically analysis pertaining to multiple shoots and roots gave highest mean number of 19.1 and 12.3 respectively. The regenerated plants were successfully established in the field with 86% survival frequency.

Bhaskaran *et al*., (2009) have reported a protocol for indirect and direct somatic embryogenesis from aerial stem explants of ginger (*Zingiber officinale* Roscoe.) using aerial stem explant of two ginger varieties were cultured on different concentration of 2, 4-D to induced callus. Two types of callus were found, type I callus was observed with hard, nodular and yellowish in colour and type II observed soft, sticky with pale white colour. The somatic embryo was found in the medium MS

supplemented with BAP 2 mg/l. Direct somatic embryo was observed in the medium MS supplemented with thidiazuron alone or combination with IAA. Histological studied found that the somatic embryo in ginger has a distinct single layered epidermis, scutellum, coleoptile, shoot apex and root apex.

Vilamour *et al.,* (2010) studied *in-vitro* the effects of media strength and source of nitrogen on shoot and root growth of ginger, native variety. Ginger plant was observed significantly proliferation in nitrogen source in the form of KNO_3 in full and half strength media.

2.4 Genetic fidelity of micropropagated clones

Tissue culture techniques plays very important role in rapid multiplication of desired clones, simultaneously provide great contribution in conservation of rare and endangered species. These techniques are highly space efficient, minimize disease and pest problems and allow for the manipulation and control of all external variables, which may cause inimitable loss of important mother plants when collections are maintained outdoors.

True-to-type clonal fidelity is one of the most important pre-requisites in micropropagation of crop species (Sharma *et al.,* 2009). Axillary branching or somatic embryogenesis way give rise to genetically uniform and true-to-type plants, as the structured meristems have generally been considered to be immune to genetic changes that might arise during cell division or differentiation under *in-vitro* conditions (Vasil, 1985; Shenoy and Vasil, 1992).

Somaclonal variation mostly occurs from plantlets derived from *in-vitro* culture is manifested in the form of DNA methylations, chromosome rearrangements, and point

mutations (N Swkocroft, 1981; Phillips *et al.,* 1994) such variations are heritable too (Breiman, 1987) and is therefore not desirable in clonally propagation. Several studies were conducted to screen somaclonal variations produced in tissue cultured plants such as in turmeric, *Lillium* species, neem, tea and soya bean in case of oil palm, where aberrant flowering patterns were observed among the regenerated plants (Matthes, 2001). Reports of somaclonal variation in tissue culture derived plant material have been described for many species including, horseradish, pecan and alfalfa (Hofmann, 2004; Singh, 2002).

It is very important to detect genetic fidelity at early stage of micropropagated plants because variations may be detected only at late developmental stages, or even in the offspring. Variation can also occur when plants are placed under different culture conditions, which may induce stress like responses. These include media with high sugar concentrations and temperature reduction. Thus it would be very important to monitor these variations quite early in the life of plant to prevent from adverse effect which may prove to be economically disastrous. Researchers tried to assess tissue culture induced variations can be determined at the morphological, cytological, biochemical, and molecular levels with several techniques, but most of the techniques have their own limitations. Cytological analysis cannot study in specific rearrangements of genes in chromosome level (Isabel *et al.,* 1993). Using polymerase chain reaction (PCR), DNA based markers are the best markers which are not influenced by environmental factors and generate reliable, reproducible results. Though Restriction Fragment Length Polymorphism (RFLP) can be used for screening genetic stability of tissue cultured plants, the method involve use of expensive enzymes, radioactive labelling and extensive care, therefore appears unsuitable. Among various methods used

for such determination of genetic fidelity in that Random Amplified Polymorphic DNA (RAPD) is the simplest, cheapest, quick, requires only small amounts of DNA detects rare single base mutations and deletions at the level of primer target and within the amplified fragment and appears to be a useful tool for the analysis of genetic fidelity of *in-vitro* propagated plants. RAPD analysis used successfully for genetic analysis of *in vitro*-raised plant materials (Isabel *et al.,* 1993; Rani *et al.,* 1995; Taylor *et al.,* 1995; Munthali *et al.,* 1996; Rani and Raina, 1998; Al-Zahim *et al.,*1999).

There are reports available on genetic fidelity of micropropagated plants of other species. Sharma *et al*., (2009) assessed genetic fidelity of *in-vitro* raised 45 plants of gerbera (*Gerbera amesonii Bolus*) cultured from three different explants i.e. capitulum, leaf and shoot tip. For investigation 32 ISSR markers was assessed for genetic stability from that 15 markers observed clear, distinct and scorable bands with an average of 5.47 bands per marker. The elite clones form capitulum and shoot tip explants did not observed genetic variation whereas, one of the leaf derived micropropagated clones revealed some degree of variation.

Modgil *et al.,* (2005) reported that RAPD marker detected the genetic similarities and dissimilarities in micropropagated clones of 10 micropropagated clones revive through axillary buds of clonal apple (*Malus pumila* Mill.). RAPD markers results revealed that 99 were monomorphic and 30 were showed polymorphic with 23.2% polymorphism out of 129 scorable fragments.

Sahoo *et al.,* (2010) investigated genetic fidelity and essential aromatic oil content of rapid regenerated clones of Patchouli, *Pogostemon cablin* (Blanco) using RAPD marker and Gas Chromatogram. Both markers showed same banding pattern

using. Results ensured that the efficiency of the protocol standardized for the production of this industrially important aromatic plant.

Mathur *et al.,* (2008) experimented biological hardening and genetic fidelity testing of micro-cloned progeny of *chlorophytum borivilianum.* The genetic fidelity testing of micro-cloned, bio hardened progeny based on a RAPD analysis using 40 random decamers DNA primers indicated a strong uniformity in relation to the parent genotype.

Martins *et al.,* 2004 studied genetic stability of micropropagated almond plantlets using RAPD and ISSR markers. Total 22 plantlets was analyzed using 64 RAPD and ISSR primers, 326 distinct and reproducible bands pattern was recorded and all bands found monomorphic exhibiting homogenous patterns for the plant tested.

Joshi *et al.,* (2007) validated the genetic fidelity of *Swertia chirayita* micropropagated clones. Sixteen ISSR markers produced 102 amplicons and homogenous amplification profiles were observed in all micropropagated clones, concluded the safest mode for multiplication of true to type plants. Similarly, Latoo *et al.,* (2006) standardized an organogenetic *in-vitro* protocol for *Chlorophytum arundinaceum* using shoot tip explants. Genetic fidelity RAPD markers, result showed no genomic variation in regenerated plants through shoot bud differentiation and ensures the effectiveness of the protocol developed for the production and conservation of medicinal herb. Gagliardi *et al., (*2007) concurred with above reports that RAPD markers showed the genetic stability of micropropagated clones of *Archisretusa*. Total 90 bands were recorded from RAPD and 372 from AFLP marker. All amplified homogenous bands showed by both the techniques were monomorphic and results signified that recovered shoots are genetically stable.

Furthermore, Rival *et al.,* (1997) recorded monomorphic 8900 (bp) bands using RAPD marker of somatic embryogenesis derived regenerants of oil palm (*Elaeis guineensis* Jacq), followed by Lakshmikumaran *et al.,* (2001) utilized RAPD marker for identification of genetic stability of micropropagated clones of *Lilum* sp. (Asiatic hybrids) derived through adventitious mode of propagation showed no genetic varations in all regenerants clones of *Lilum* sp.

Goto *et al.,* (1998) determined genetic stability of more than 10 years micropropagated shoots of Japanese black pine (*Pinus thunbergi* Parl.). Total 36 shoots consisting three morphotypes (short, medium and long needles) were randomly chosen from 4000 micropropagated clones. Out of 126 primers, 30 primers gave 134 clear scorable bands. Total 4824 bands were obtained from this experiment, no aberration was observed in RAPD banding pattern between the experienced shoots.

Rout *et al.,* (1997) evaluated the genetic stability of micropropagated plants of *Zingiber officinale* (V3S18) using RAPD marker. Fifteen arbitrary decamers were used to assess the genetic fidelity. All RAPD banding profile were monomorphic and similar to those of field grown plants, no aberration were observed within the micropropagated clones.

Nayak *et al.,* (2007) assessed the genetic stability of micropropagated replica of *Cucurma longa* L. by Cytophotmetry and RAPD of 26 months old culture plants. Both assessed methods showed uniformity among micropropagated clones of *C.longa*. This result induces the production of disease free planting material of turmeric for commercial utilization.

Tanwar *et al.,* (2008) used RAPD markers for determination of somaclonal variation in micro-propagated plants of Sugarcane varieties (Co94012) and VSI434. The RAPD banding patterns of both varieties were monomorphic and similar banding pattern was detected in all bands. Studies indicated that somaclonal derived clones showed high genetic fidelity with no genetic variation among the plantlets produced *in vitro*. The results revealed that RAPD analysis can be efficiently to assess the genetic purity of sugarcane clones derived from tissue culture.

2.5 Antioxidant activity of *Zingiber officinale* and other medicinal plants

A large amount of work is available particularly on evaluation of antimicrobial and antioxidant properties of various plants. Antioxidants have been reported to prevent from oxidative damage during generation of free radicals in an integral part of normal metabolism Shahidi *et al.,* (1992). The potential reactive oxygen species (R O S) is generated *in- vivo*, through various physiological and biochemical processes such as mitochondrial respiration, activation of phagocytes, enzymatic oxidation, UV and ionizing radiations. Reactive oxygen species attack various bio molecules like protein, lipids, DNA etc., and inflict damage breaking down various cellular processes (Farber, 1994). Although, natural antioxidant defences are generally adequate to neutralize the radicals, their concentration and rate of generation decreases with age, as well as under inflammation. Under these conditions, exogenous addition of antioxidant becomes necessary to prevent the oxidative stress that has been implicated for various pathological condition and degenerative diseases. As a result antioxidants have assumed great interest in medical science (Buyukokuroglu *et al.,* 2001).

Ginger has a high content of antioxidants activity and has been grouped as one of the spices with good antioxidant rating (Chipault *et al.,* 1952). Sethi and

Agarwal (1957) observed that dried ginger has low antioxidant properties and increase in concentration of crude gingerol increases the antioxidant activity but the gingerol constituent at 165ºC for 30 min indicated the withholding of the antioxidant activity only to 10 percent.

Fugio *et al.,* (1969) investigated the antioxidant activity of the chemical constituents of many spices and observed that the shogaol and zingiberene found in ginger revealed strong antioxidant activities. The free radical scavenger activity of ginger is fully dependent on the side chain structures and substitution patterns on the benzene ring.

Nakatani and Kikuzaki (2002) observed zingerone, 6-gingerol, and 6-shogaol found moderate antioxidant activity and antioxidant activity will decreases with the increasing chain length. Tsushida *et al.,* (1994) observed that antioxidant activity is mostly exerted by gingerol and hexahydro curcumen. Due to this it is a free radical scavenger (Lee and Ahn, 1985).

Ahmed *et al.,* (2001) investigation diet containing ginger showed a more protective effect against the malathion-induced oxidative damage exhibiting the antioxidant activity and incorporation of salt and ginger extract to precooked lean beef retarded rancidity during storage, increased the tenderness, and extended shelf life (Kim and Lee, 1995).

Stoyanova *et al.,* (2006) studied the antioxidant effect and total phenols of *Zingiber officinale* from Vietnam. The carbon dioxide extract of ginger was analyzed with DPPH (2, 2- diphenyl-1-picryl hydrazyl), lipid oxidation and pro-oxidant activity with regards to hydroxyl radicals at body temperature (37˚C). The total phenol obtained

from the alcohol extract was 870.1 mg/g from dry extract, DPPH scavenging activity found/reached 90.1% exceeds that of butylated hydroxytoluene (BHT), IC_{50} concentration was found for inhibition of DPPH was 0.64 μ g/ml. The antioxidant activity in a linoleic acid/water was highest at 37˚C–73.2%, and 71.6% was found when the formation of conjugated dienes was inhibited. The ginger extract inhibited the hydroxyl radicals 79.6% at 37º C and 74.8% at 80º C190 and 2.78 μg/ml.

Jaafar *et al.,* (2010) reported the antioxidant activities, total phenol and flavonoids content in two varieties of Malaysian young ginger (*Zingiber officinale*) Halia-Bentong and Halia-Bara. The antioxidant activities of methanol extract from leaves, stem, and rhizomes were analyzed to compare validates the medicinal potential of the young ginger. The antioxidant activity and total phenol content of leaves reported higher than stem and rhizome. Analysis of FRAP activity found higher in rhizome than leaves and stem. All analysis report concludes that Halia Bara variety contain higher antioxidant activities than Halia Bentong.

Bhattacharya *et al.,* (2009) studied the antioxidant activities of different solvent fractions of ginger (*Zingiber officinale* Rosc). Out of 34 solvent fractions, 10 fractions found free radical scavenging activity ranging from 5.88 % to 80 %. Diethyl ether and ethyl acetate (1:1) showed maximum inhibition percent (80 %) of antiradical activity and liver protective, solvent chloroform fraction observed maximum hydroxyl radical activity, the maximum nitric oxide (NO) generation activity found at benzene fraction (27.27 %) and concluded that ginger flavonoids have some contributory roles in scavenging free radical activity.

Mishra *et al.,* (2011) studied correlation of phytochemical characteristics and antioxidative properties have been studied in hot and cold extracts of *Canna edulis*

rhizome. Total phenol and flavonoid in hot extracts (42.71 mg GAE /g and 21.92 mg QE /g) and in cold extracts (33.7 mg GAE /g and 15.12 mg QE /g). IC_{50} value of DPPH and H_2O_2 and electron donation ability (EDA) observed higher in hot extracts than cold extracts. Analysis observed that hot extracts of *C. edulis* exerts more effective antioxidant property as compared to cold extract.

Lim *et al.,* (2008) screened 26 ginger species belonging to nine genera. Analysis was performed with total phenolics content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC). Out of 26 species, leaves of *Etlingera* species found the highest TPC and AEAC. Ferrous ion chelating (FIC) abilities of leaves and rhizomes of eight species were compared and six of the eight species showed higher values in leaves than rhizome. Analyzed value of FIC showed *Alpinia galanga* leaves which were more than 20 times higher than that of rhizomes. From five species of Etlingera, leaves of *E. elatior* showed strongest tyrosinase inhibition activity than *E. flugens* and *E. maingayi* and this three also found high antioxidant activity and antibacterial properties.

Goyal *et al.,* (2010) studied the antioxidant activity with spectrophotometrically analyze the ability of the plant extracts to scavenging activity of DPPH, TPC and flavonoid content from methanolic leaf extract of *Bambusa vulgaris*. Analyzed results showed the presence of carbohydrates, reducing sugars, flavonoids, steroids, sapionins, alkaloids, tannins, anthraquinones and glycosides. The antioxidant activity of the investigated extract found a scavenging ability of hydroxyl peroxide radicals (421.74 ± 25.61 mg/ml) and DPPH radical activity (95%). The TPC and flavonoid content was measured (GAE22.69 ± 0.084 mg/g of dry extract) and (Quercetin 159.80 ± 0.047 mg/g of dry extract) indicated that these compounds contribute to the antioxidative activity.

Fagbenro and Jauncey (1994) observed that during study of the chemical and nutritional quality of fermented fish silage containing potato extracts, formalin, and ginger extracts, it was observed that ginger extract showed to be effective as an antioxidant in fermented *tilapia* silage (*Oreochromis niloticus).* Nakatani and Kikuzaki (2002) observed zingerone, 6-gingerol and 6-shogaol found moderate antioxidant activity and antioxidant activity will decreases with the increasing chain length.

Nishimura (1995) investigated the volatile compounds for the aroma of fresh rhizomes of ginger and the compounds with high dilution factor were linalool, geraniol, geranial, neral, isobor-neol, borneol, 18-cineol, 2-pinenol, geranyl acetate, 2-octenal, 2-decenal, and 2-dodecenal. The pungent principle of ginger, 6-gingerol, has been reported to be a potential antioxidant among 10 phenolics compounds separated by TLC.

2.6 Antimicrobial activity

Medicinal plants have been used since antiquity to treat common diseases because they contain bioactive constituent for remedial value to treat various diseases. This plant-based, traditional medicine system continues to play an essential role in health care, with about 80% of the world's inhabitants relying mainly on traditional medicines for their primary health care (Owolabi *et al.,* 2007). According to World Health Organization, medicinal plants would be the best source to obtain a variety of drugs. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (Nascimento *et al.,* 2000). Many researchers reported that some medicinal plants contains many components such as peptides, unsaturated long chain aldehydes, alkaloidal constituents, some essential oils, phenols and water, ethanol, chloroform, methanol and butanol soluble compounds. These plants have

potential in therapeutic application against human pathogens, including bacteria, fungi or virus (Elastal *et al.,* 2005). Some application were used by others researchers are the use of bearberry (*Arctostaphylos uv aursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tea tree (*Melaleuca alternifolia*) are described as broad spectrum antimicrobial agents (Rio and Recio, 2005).

Nguanpuag *et al.,* (2011) investigated antimicrobial activity both *in-vitro* and *in-vivo* using ginger oil extracted by hydro distillation and solvent extraction methods. The bioactive compounds obtained from hydro distillation were camphene, 1, 8-cineol and a-pinene and from solvent extraction were ß-phellandrene and 1, 8-cineol. Both extraction oils inhibited *Bacillus subtilis*, *Bacillus nutto*, *Pseudomonas aerugenosa*, *Rhodoturola* sp., *Samonella newport* DMST 15675, *Samonella enteritidis* DMST 15676 and *Fusarium* sp. No inhibition was found on *Escherichia coli*, *Campylobactor coli* NTCT 11353 and *Campylobacteor jejuni* ATCC 33291. *In vivo* growth of microorganism was suppressed significantly in shredded green papaya with 5 and 10 5 and 10 µ L. Major volatile ginger oils detected were a-pinene, camphene, ß-phellandrene and 1, 8-cineolin shredded package papaya and observed that ginger oils can be used for reducing population of microorganism in shredded papaya and other fresh produce processed products.

Adeshina *et al.,* (2011) reported antibacterial activity of fresh red and white *Allium cepa* (Onion) and *Zingiber officinale* (Ginger) juice against multidrug resistant bacteria viz *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli and*

Salmonella typhi isolated from salad was using agar well diffusion and agar dilution methods.

Ayse *et al.,* (2008) reported an antimicrobial and cytotoxic activities of chloroform and ethanol extracts of *Zingiberi officinale* against *Klebsiella pneumoniae, Salmonella thyphimurium, Bacillus cereus, Enterococcus fecalis and Staphylococcus aureus* and cytotoxic effects on human cervical cancer (HeLa) and mouse fibroblast (L929) cell lines. Schnitzler *et al.,* (2001) investigated acyclovir resistant clinical isolates of herpes simplex virus type 1 (HSV-1) were analyzed *in-vitro* for their susceptibilities to essential oils of ginger, thyme, hyssop, and sandalwood. Similarly, Patel *et al.,* (2011) observed that aqueous ginger extracts which was used to check antimicrobial activity found to be efficient.

Anjan *et al.,* (2012) experimented recorded that *in-vitro* antimicrobial potential of 10 % ginger extract against *Streptococcus mutans (S.mutans), Candida albicans (C. albicans)* and *Enterococcus faecalis (E.faecalis)* are mostly causation of oral infections microorganism, during study results revealed that 10% ethanolic ginger extract possess high antimicrobial potential.

Indu and Nirmala (2010) reported that Zingiberene was the main chemicals constituents for an antimicrobial activity of fresh and dry ginger against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Candida albicans*, *Trichoderma* spp, *Aspergilus niger, Pencillium* spp. and *Saccharomyces cescerevisiae* microorganism. Furthermore, Tagoe *et al.,* (2011) compared the antifungal properties of Onion, Ginger and Garlic against *Aspergilus flavans*, *Aspergilus niger* and *Cladosporium herbarum* using pour plate technique in PDA medium and results found that ginger showed highest antifungal activity on all test fungi with a mean diameter 1.40 cm followed by garlic

(1.70 cm) and onion (1.80 cm). Likewise, Ekwenye and Elegalam, (2005) recorded aqueous extracts found high antimicrobial activity against *E.coli* and *S. typhi* microorganism.

Nader *et al.,* (2009) reported antimicrobial activity of ginger extracts of cold-water, hot- water and ethanolic and essential oil against pathogenic bacteria *Escherichia coli, Salmonella* sp, *Klebsiella* sp, *Serratia marcescens, Vibrio cholerae, Staphylococcus aureus, Streptococcus* sp. was examined disc diffusion method, results showed that ginger extracts were more effective on gram positive bacteria than gram negative bacteria and ethanolic extract observed highest antibacterial activity (11 to 28mm) than other extracts. Phytochemical analysis of ethanolic extracts revealed the presence of glycosides, terpenoids, flavonids and phenolic compounds.

Kumar *et al.,* (2011) studied antibacterial potential of natural food preservatives against *Staphylococcus aureus* isolated from different food samples. *Six different* spices were used for study such as *Cucurma longa* (Turmeric), *Zingiber officinale* (ginger), *Piper nigrum* (black pepper), *Trigonella foenum graecum* (methi), *Syzygium aromaticum* (clove) and *Ferula assafoetida* (hinge). All sample showed significant antibacterial activity against *S.aureus* isolated from food samples. It was observed that, ethanolic extracts of all the spices showed highest inhibited effect against *S.aureus* followed by methanolic and aqueous extracts. Pedgee *et al.,* (2012) recorded chloroform extracts of both sample of turmeric and ginger rhizome found potent antimicrobial activity against *Pseudomonas* and *E. coli than* distil water extracts.

However, Premlata *et al.,* (2011) recorded chloroform and water extracts of *Withania somnifera* (RUBL-20668) and *Cenchrus setigerus* (CAZRI-76) showed high antimicrobial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Enterobactor*

aerogens and *Aspergillus flavus*. Water extract of stems of *C. setigerus* (IZ-21.83 ± 0.24 mm, ai- 0.780) and chloroform extract of calyx of *W. somnifera* (iZ-16.17 ± 0.24 mm, ai 1.078) showed highest activity against *B. subtilis.* Auta *et al.,* (2011) reported that ethanol extract of *Zingiber officinale* at the concentration of 20 mg/ml showed high antimicrobial activity against *E.coli* and *Pseudomonas aeruginosa* pathogens for gastrointestinal tract. Similar results was concurred by Chand (2013) found antibacterial effect of garlic and ginger against bacteria *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Bacillus cereus.*

2.7 Molecular studies

Morphological markers such as leaf area, dry matter production, length and weight of secondary rhizomes, tiller number and leaf number differed significantly (Yadav, 1999). Biochemical markers such as total gingerol, shogaol, polyphenol content etc. are used to identify the superior ginger plant (Ravindran *et al.,* (1994); Manmohan das *et al.,* (2000). However, ginger breeders are often unable to use these markers effectively because they are greatly influenced by environmental factors and show a continuous variation with a high degree of plasticity. A lucid knowledge of genetic variability is essential for formulating a meaningful breeding strategy. Nybe and Nair (1982) reported that morphological characters are not reliable to classify the types, although some of the types can be distinguished to a certain extent from rhizome characters. Hence, to overcome these problems, research has shifted to using more sensitive DNA marker technology. Molecular markers very efficiently enhance morphological, cytological, and biochemical characters in germplasm characterization, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation

of genetic relationship, phylogenetic and evolutionary studies etc. Williams *et al.,* (1990).

2.7.1 Morphological marker

Generally ginger has been classified into different species or varieties by morphological characters. Most of the vegetatively propagated crop species the amount of genetic variations will be limited unless samples are drawn from distinctly different agro-ecological situations (Ravindran *et al.,* 1994). Minute variability exists among the genotypes that are grown in the same area; however, good variability has been reported among cultivars that came from widely divergent areas (Ravindran *et al.,* 1994). Most of studies were carried out in morphological characters, rhizome yield and quality parameter.

Ravindran *et al.,* 1994 reported characterization of 100 accessions of ginger germplasm based on morphological, yield, and quality parameters. They observed moderate variability for many yield and quality traits, reported tiller number per plant had the highest variability, followed by rhizome yield/plant and in biochemical trait, the shogaol content traced the highest variability, followed by crude fiber and oleoresin.

Yadav (1999) reported a high genotypic coefficient of variation for length and weight of secondary rhizomes, weight of primary rhizomes, number of secondary and primary rhizomes, and rhizome yield/plant. Furthermore, Sasikumar *et al.,* (1992) studied 100 accessions of ginger germplasm for variability, correlation, and path analysis. They found that rhizome yield was positively correlated with plant height, tiller number, leaf number, leaf length and leaf width.

Nybe and Nair (1982) studied the morphological characters of ginger accessions and reported that phenotypic studies are not reliable to classify the types, although some of the types can be distinguished to a certain extent from rhizome characters. All the morphological characters were found to vary among types except for breadth of leaf, leaf area index, and number of primary fingers. Quality parameters such as dry recovery and oleoresin and fiber contents are known to vary with the soil type, cultural conditions, and climate (Ravindran *et al.,* 1994).

2.7.2 Biochemical marker

Biochemical markers were widely used for characterization of different plant germplasm (Das *et al.,* 2002). The bioactive constituent of gingers plays a very important role in characterization of different ginger germplasm. Oleoresin of ginger is the total extract of ginger containing all the bioactive principles as well as the pungent constituents. The oleoresin contains two important compounds gingerol and shogaol that contribute to the ginger pungency (Ravindran *et al.,* 2005). The quality and characterization of ginger was classified by amount of gingerol and shogaol present in the extract of ginger.

Zachariah *et al.,* (1993) also classified 86 ginger accessions into high, medium, and low quality types of oleoresin based on the relative contents of the quality components. There are many ginger cultivars with high oleoresin, a few them, such as Rio de Janeiro, Ernad Chernad, Wynad, Kunnamangalam, and Meppayyur, also had high gingerol content. The inter character association showed a positive correlation with oleoresin, gingerol, and shogoal.

Shamina *et al.,* (1997) investigated the variability in total free amino acids, proteins, total phenols, and isozymes, using 25 cultivars. Moderate variations were recorded for total free amino acids, proteins, and total phenols. Isozyme variability for polyphenol oxidase, peroxidase, and SOD was reported to below, indicating only a low level of polymorphism. Jiang *et al.,* (2005) experimented metabolic profiling analysis from different origin. They observed that no qualitative difference among major volatile bioactive compounds whereas low varations was observed in non-volatile composition, particularly regarding the content of 6, 8 and 10 gingerols. Singh *et al.,* (1999) grouped 18 cultivars into three clusters under Nagaland conditions based on D^2 analysis. The major forces influencing divergences of cultivars were rhizome yield per plant and oleoresin and fiber contents.

2.7.3 Molecular Marker

Williams *et al.,* (1990) described a novel type of genetic marker based on DNA amplification, which does not require prior information of target DNA sequences. These markers called RAPD (Random Amplified Polymorphic DNA) markers are generated by the amplification of random DNA segments with single primers of arbitrary chosen primers. Use of random amplified polymorphic DNA (RAPD) markers, detected by PCR amplification of small inverted repeats scattered throughout the genome, adds a new technology of DNA fingerprinting to the molecular analysis of relatedness between genotypes. The PCR based RAPD technique (Williams *et al.,* 1990) is an attractive complement to conventional DNA finger printing. RAPD analysis is conceptually simple. Nano gram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotide of random sequence. The amplification protocol differs from the standard PCR conditions (Erlich, 1989) in that only a single random

oligonucleotide primer is employed and no prior knowledge of the genome subjected to analysis is required. When the primer is short (e.g. 10 mer), there is a high probability that the genome contains several priming sites close to one another that are in an inverted orientation. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. The profile of amplification products depends on the template-primer combination and is reproducible for any given combination. The amplification products are resolved on agarose gels and polymorphisms serve as dominant genetic markers, which are inherited in a Mendelian fashion (Williams *et al.,* 1990; Carlson *et al.,* 1991; Welsh, Peterson and McClelland, 1991). Amplification of non-nuclear RAPD markers is negligible because of the relatively small non-nuclear genome sizes. Since discovered, random amplified polymorphic DNA (RAPD) assay (Williams *et al,* 1990) is being used for a number of areas in plant taxonomy. At the present it is the most preferred DNA markers due to greater speed, easy-to-perform and non-requirement of radioactive materials etc. In ginger and other species of *Zingiber*, a considerable amount of work has been carried out which are summarized below.

Rout *et al.,* (2007) analyzed genetic fingerprinting among eight varieties of *Zingiber officinale* using RAPD markers, the investigation showed the distant variation within the varieties, similar result was obtained by (Harisaranraj *et al.,* 2009) within the eight varieties of ginger of Orissa.

Pattanayak *et al.,* (2010) assayed forty nine ginger clones cultivated in North East India using RAPD markers. The high polymorphism detected in a cultivated species in this study exhibit aptness the resolving power of the RAPD markers selected for genetic diversity investigation. Similarly, Wahyuni *et al.,* (2003) investigated

genetic diversity of morphological distinct (big and small) Indonesian gingers using AFLP markers and analysis of genetic variation reported that there was no clear genetic variation between small and big form gingers. Results also showed there was higher genetic diversity in small size ginger than big size variant.

Nayak *et al.,* (2005) characterized 16 elite cultivars of gingers using cytological and RAPD markers, and assayed result reported that significant genetic variations were detected in gingers variant.

Jaing *et al.,* (2006) studied genetic diversity using phylogenetic analysis and metabolic profiling among and within ginger species and result found that gingers variant from different geographical origins were indistinguishable.

In a study of Jatoi *et al.,* (2008) genetic diversity in gingers and relationships among the *Zingiber* species using rice ISSR markers as RAPD markers. They observed significant allelic diversity in ginger from Myanmar. Result showed higher genetic variability were observed in gingers collected from farmers' fields in comparison with gene bank accession and market collection. Similar study was carried out by Watanabe *et al.,* (2006) using rice SSR marker as RAPD marker for genetic diversity analysis in *Zingiberaceae.* They reported that high variation was found among ginger, turmeric and galangal species.

Kizhakkayil and Sasikumar (2010) investigated Indian gingers diversity using RAPD and ISSR markers. They found gingers diversity are geographical bias and significant similarity among the clones. Subramanian *et al.,* (2007) successfully identified using RAPD markers for characterization of diseases resistant to *Fusarium oxysporium* f.sp *Zingiberi* and susceptible varieties of gingers.

2.7.4 Cytological marker

Studying the structural properties and spatial organization of chromosomes is important for the understanding and evaluation of the regulation of gene expression, DNA replication and repair, and recombination. Different facets of chromosomal research are gaining significance for the analysis of genomes in plant taxonomy (Bennett, 1987; Mowforth and Grome, 1989; Ceccarelli *et al.,* 1992; Das *et al.,* 1995; Roser *et al.,* 1997).

Cytological markers of the genus *Zingiber* were elaborately studied in the early 1920s with many interesting features. The somatic chromosome number of *Zingiber officinale* 2n=22 was first studied by Sugiura 1928 followed by Sharma and Bhattacharyya, (1959); Ramachandran, (1969); Rai *et al.,* (1997); Kihara *et al.,* (1931) and 2n=66 Bisson *et al.,* (1968) were reported. The nuclear DNA content varies considerably not only among species, but also among and within populations of species Bennett and Leitch (1995). Das *et al.,* (1998) examined karyotype and estimated 4C DNA in ginger. They found significant differences of 4C DNA between the cultivars.

2.7.5 Morphological study

Knowing morphological characters of horticultural crops is very necessary for formulating a fruitful breeding strategy. The most easily obtained assessment of genetic variation is that of measuring morphological or phenotypic variation. The sharing of phenotypic characters is interpreted as an indication of relatedness. Morphological characters are however, often influenced by environmental conditions Jasienski, (1997); Kercher and Sytsma (2000), which in turn may influence the estimation of genetic

variation and relatedness. Morphological variation studies were carried out by many researchers on ginger and other plants, few are of them are as follows.

Mohanty and Sarma (1979) reported on morphological studies of ginger that expected genetic advance and heritability estimates were high for the number of secondary rhizome and total root weight.

Mohanty *et al.,* (1981) observed significantly varietal differences for all the characters except for the number of tillers per plant and number of leaves per plant. Nybe and Nair (1982) suggested that morphological characters are not reliable to classify the types, although some of the types can be distinguished to a certain extent from rhizome characters. All the morphological characters were found to vary among types except for breadth of leaf, leaf area index, and number of primary fingers.

Rattan *et al.,* (1988) reported on ginger that plant height was positively and significantly correlated with number of leaves, leaf length, rhizome length, rhizome breadth, and yield per plot.

Pandey and Dobhal, (1993) observed a wide range of variability for most of the characters studied by them. Rhizome yield per plant was positively associated with plant height, number of fingers per plant, weight of fingers, and primary rhizome.

Ravindran *et al.,* (1994) characterized 100 accessions of ginger germplasm based on morphological, yield, and quality parameters. Moderate variability was observed for many yield and quality traits. Tiller number per plant had the highest variability, followed by rhizome yield/plant.

At IISR, Sasikumar *et al.,* (1992) studied 100 accessions of ginger germplasm for variability, correlation, and path analysis. They found that rhizome yield was

positively correlated with plant height, tiller and leaf number, and leaf length and width. Manmohandas *et al.,* (2000) found that all the cultivars differed significantly in tiller number and leaf number.

Ravindran *et al.,* (2005) found little variability among the ginger genotypes that are grown in the same area and high variability was estimated among cultivars that came from widely divergent areas. Terratas *et al.,* (2007) observed that stem trait was identification characteristics of 21 pithaya genotypes. Yadav, (1999) found high genotypic variation for length and weight of secondary rhizomes, weight of primary rhizomes, number of secondary and primary rhizomes, and rhizome yield/plant of gingers. Singh, (2001) observed during morphological studies of ginger, plant height, number of tiller per plant and leaf length had a maximum direct effect on rhizome yield.

2.8 Remote sensing and GIS in agriculture

Food Security is a big bang question for all developed or developing countries. Agricultural sustainability has the uppermost priority in all countries. Remote Sensing and GIS technology are gaining importance as useful tools in sustainable agricultural management and development. The sustainable agriculture is to maintain the natural land resources with crop requirement towards achievement of sustained productivity over a long period Lal and Pierce, (1991). The key for providing food security to all people of the world without affecting the agro ecological balance lies in the adaptation of new research tools, particularly from aerospace Remote Sensing, and combining them with conventional as well as frontier technologies like Geographic Information Systems (GIS).

In 1971 first time remote sensing technology was used for large area crop survey in USA under Corn Blight Watch Experiment (CBWE). In 1973 Crop Identification Technology Assessment for Remote sensing (CITARS) was started for to quantify the Crop Identification Performance (CIP). From 1974 to 1977 (NASA) major wheat growing areas of the world was forecast under Large Area Crop Inventory Experiment (LACIE). The Monitoring Agriculture through Remote Sensing (MARS) project was developed for crop growth and monitoring system (CGMS), which helps into crop simulation models, agro meteorological models, and real time data for crop predicting and assessment. Lepoutre (1991) used remote sensing satellite imagery for monitoring crop production, for estimating losses due to drought in France in 1998 to 1991and to monitor potential crop yields throughout Europe.

In context of Indian scenario pioneer application of remote sensing and GIS technology was carried out by Dakshinamurti *et al.,* (1971) experimented on coconut root wilt disease using colour infrared aerial photography. Later mega project of agriculture was experimented about agricultural resources under Agriculture Resources Inventory and Survey Experiment (ARISE) in Anantpur (1974-75) and Patiala (1975-77). Subsequently identification and classification of paddy and sugarcane crops in Madhya Pradesh (1975-77) were experimented.

Systematic crop productions are of vital importance to country such as India, where the agricultural production is highly susceptible to the vagaries of monsoon. Acreage estimation using RS technology was first demonstrated by Mc Donald (1984) and Renondo *et al.,* (1985) in various parts of world. In India Dadhwal and Parihar (1985) estimated wheat acreage estimation of Karnal district using Land sat MSS digital data and supervised classification. Rai *et al.,* (2004) experimented on land use statistics

through integrated modelling using GIS. Mishra *et al.,* (2005), developed an integrated approach for estimation of crop acreage using remote sensing data, GIS and field survey of hilly region.

Sukhatme and Panse (1951) developed the estimation procedure of estimating average yield crop production based on crop cutting experiments. Singh and Goyal (1993) have used spectral vegetation indices like Normalized Difference Vegetation Index to obtain improved crop yield estimators. Rai *et al.,* (2007) developed spatial models for small area levels crop yield estimation. Singh and Ibrahim (1996) examined the use of multidate satellite spectral data for crop yield modelling using Markov chain model. Saha (1999) used satellite data and GIS for developing several yield models for forecasting.

Utpala *et al.,* (2006) analyzed the environmentally most site suitable for ginger cultivation in India using Eco-crop model of DIVA-GIS. Three parameters were studied i.e. growing periods, temperature and rainfall. Study found that most suitable areas were Orissa, West Bengal, Kerala, Western Ghat region of Karnataka and Maharashtra for ginger cultivation in India. In north-eastern states Assam, Mizoram, Tripura, Western Ghat of Meghalaya showed most site suitability for ginger cultivation.

Utpala *et al.,* (2007) studied the site suitability location of turmeric cultivation in India, researcher found that site suitability is an important factor to determine the productivity of the crop, determine suitable areas which are useful to have the greatest success for growing a particular crop in a region. Land suitable analysis was carried out in such a way that local needs and condition. Experiment found that most suitable were Andra Pradhesh for turmeric cultivation leaving small patch in central and western part

of Andra Pradesh, Assam due to high suitable environmentally for turmeric cultivation, Bihar, Kerala is most suitable for turmeric cultivation.

Kris Sunato, (2011) analyzed the site suitability for cultivation of ginger (*Zingiber officinale* Roscoe) in Indonesia, using geographical data, analysis tools and overlay technique on Geographic Information System (GIS). Analyzed data were produced and displayed in maps, graphs, images and tables. The study recorded about 11.36% of total area comprising about 33874 Ha found high suitable for ginger cultivation.

Utpala *et al*., (2008) reports on identification of suitable areas and effect of climate change on ginger cultivation in India. Reports suggest that increase in area is not always directly proportional to the increase in production. Records of data for thirty years on area and production were compared with Eco-crop suitability model, which showed that suitability has direct impact on production. The most suitable areas are Orissa, West Bengal, Mizoram and Kerala. North western states like Gujarat, Rajasthan, Uttar Pradesh and Madhya Pradesh are marginally suitable or unsuitable. The north-eastern states of Assam, Mizoram, Tripura, and Meghalaya showed most site suitability for ginger cultivation.

Analysis of future prediction of Eco-crop model showed that the temperature increases by 1.5 to 2°C will reduce drastically from high suitability to marginally suitable, showed the effect of climate change.

Chapter 3

Materials and Methods

3.1 Sample collection areas

The ginger samples were collected from various location of Sikkim state. The hilly state of Sikkim (India) is situated on the Eastern Himalayas, located between 27º 00' 46" n - 28º 07' 48" N latitudes and 88º 00 58" E - 88º 55 25" E longitudes. The total area of Sikkim is 7096 sq. Km. The stretch from north south is 112 Kilometers and that from east to west is 64 Km. The altitude varies from 300 meters above sea level to 8598 meters above sea level, the areas available for cultivation are not more than 12 % of the total area J.R. Subba (1984).

3.2 Plant material

Five cultivars of *Zingiber officinale* i.e. *Majhauley*, *Bhaisay*, *Gorubthangey*, *Jorethangey* and *Charinangrey* were selected for this experimental study. All samples were collected from the farmer's field from different location of Sikkim and maintained in the department experimental polyhouse and experimental field (Figure 3).

Rhizome samples of *Majhauley*, *Bhaisay, Gorubthangey*, *Jorethangey* and *Charinangrey* were collected from different ginger fields representing four districts of Sikkim. *Majhauley* and *Bhaisay* were collected from East, West and South Sikkim districts. *Gorubthangey* and *Jorethangey* were collected from west and South Sikkim and *Charinangrey (Sanuaduwa)* was collected from Sudur, Ravang and Hee-gyathang of North Sikkim only. The morphological characters of *Sanuaduwa* were found

different from other variety of Sikkim. Location of the samples collected was recorded with GPS (Garmin) and the map for sampling sites was prepared by using GIS (Geographic information system) Software Arc 10.1 (Figure 3.24). All the samples were brought to the laboratory of Sikkim State Council of Science and Technology, Gangtok to raise in nursery.

3.3 Ecological study

Ecological studies of ginger were carried out in four district of Sikkim. Total 237 samples were collected from different places (Figure 4) of Sikkim. The following parameter was taken for studies altitude, latitude, longitude, aspect, soil moisture, soil pH, soil colour, soil texture, org. carbon, nitrogen kg/ha, phosphorous kg/ha, potassium kg, seed size (cm), approx weight (gm.), depth of seed grown (cm), average distance between two seeds (cm), number of buds/ tillers, quantity of seed used in one acre land (Kg), land preparation from how many days before sowing ginger seed (days), mulching materials, ginger disease, fertilizer, treatment of seed during sowing, cultivation practice and medicinal use of ginger.

3.3.1Application of remote sensing in ecological studies of *Zingiber officinale*

Different map were prepared viz. distribution of five different cultivar varieties of ginger in Sikkim, location of bacterial and fungal diseases recorded in ginger field, soil texture map found in ginger cultivation, elevation wise distribution of ginger cultivation, districts wise distribution of five cultivars of gingers using GIS Remote Sensing Technology and ARC 10. The methodology for generation of different map was presented in (Figure 5).

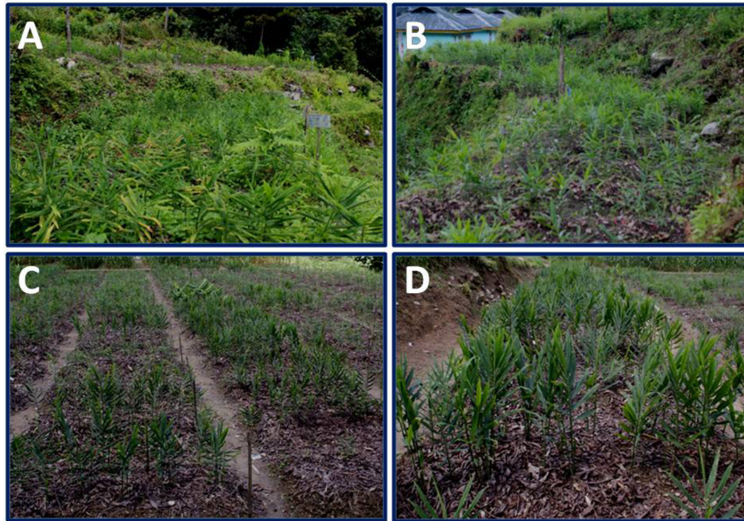


Figure 3 Maintenance of five ginger cultivars germplasm in experimental field of Sikkim State Council of Science and Technology.

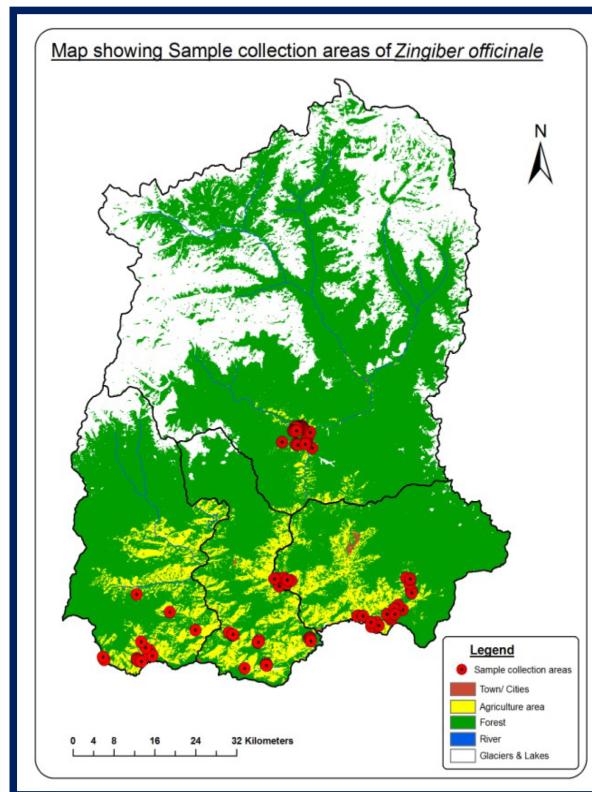


Figure 4 Representing sample collection areas of five different cultivars of Zingiber *officinale* from four districts of Sikkim Himalaya.

3.3.2 Morphological studies of five different cultivar variety of ginger

Morphological studies of five cultivar variety of ginger (*Zingiber officinale* Rosc) was studied using guidelines and methods developed by "Indian Institute of Spices Research, Calicut was finalized by the Task Force (7/2007) constituted by PPV & FR Authority". The following morphological characters are taken/chosen for studies are plant: growth habit, plant: height (cm), plant: number of shoots, plant: height of shoot (cm), shoot: diameter (cm), shoot: intensity of green color, shoot: number of leaves on main shoot, leaf: length (cm), leaf: width (cm), leaf: intensity of green color, leaf: petiole length (cm), spike: length (cm), colour of the bract tip of fully developed spike, rhizome: thickness (cm), rhizome: shape, crop duration (days). Details of all characters are listed in Table 4.4.

3.3.3 Soil analysis

Edaphic factors play an important role in ginger cultivation and have been measured as the foremost determinants of plant growth and rhizome yield. In ginger quality parameters such as dry recovery and oleoresin and fiber contents are known to vary with the soil type, cultural conditions, and climate (Ravindran *et al.,* 1994). To characterize the ecological preferences and the role of edaphic factors of *Zingiber officinale*, physical and chemical properties of the soil were determined.

3.3.3.1 Soil sampling

The soil samples were collected from different layers at a depth of 8–10 inches below the surface (after removing the topmost soil) were stored in a Jam bottle/Ziploc

plastics tightly, labelled and brought down to State Soil laboratory, under Department of Agriculture and Horticulture, Government of Sikkim for chemical analysis using their

Methodology

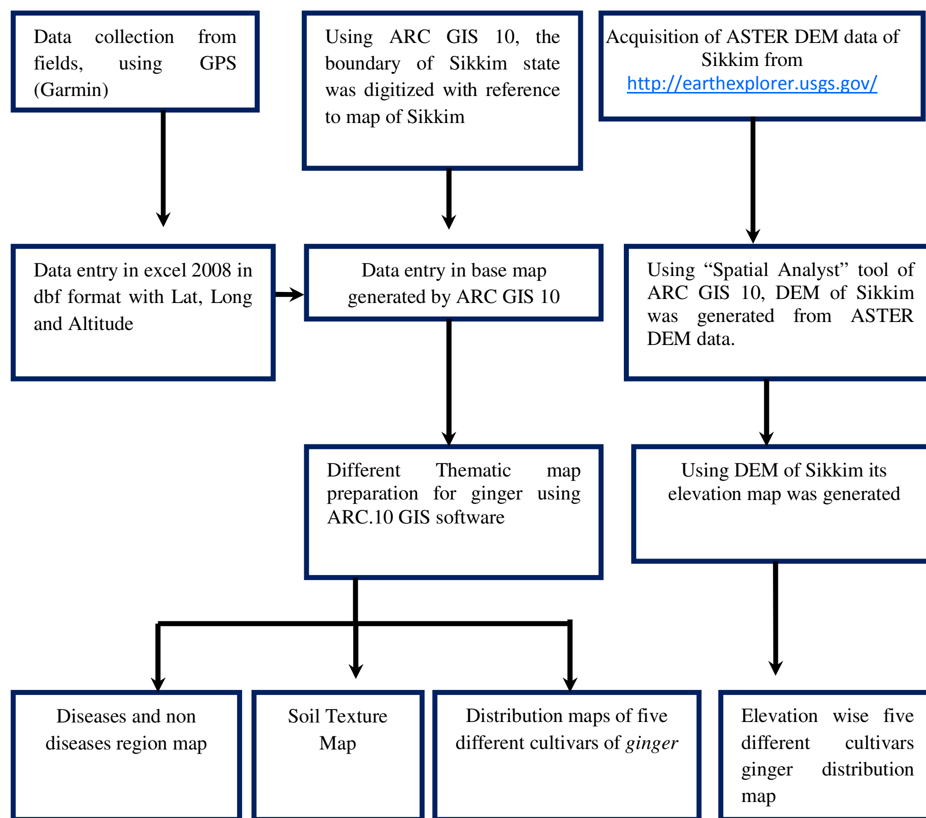


Figure 5 Flow chart showing methodology adapted during map

standard protocol. Different parameters of the soil like pH, Organic carbon, Potassium, Phosphorous, Soil texture and Nitrogen, were tested and depicted in (Appendix III).

3.3.4 Statistical analysis of ecological data

Mean variation of different morphological characters of five different cultivars of ginger found in Sikkim Himalaya was carried out with respect to different soil texture wise, districts wise, among five cultivars wise using Excel 2007 and SPSS 16.0 software. Simultaneously, regression analysis was carried out between different morphological characters to observe relationship between them with Excel 2007 and SPSS 16.0 Furthermore, principal component analysis of five different cultivars of ginger found in Sikkim Himalaya was analyzed with thirteen (13) different morphological characters using (TIBCO Spotfire S+8.1 for Windows) software, TIBCO Software Inc, USA).

3.4 Micropropagation of *Zingiber officinale*

3.4.1 Culture medium

For the selection of best medium and sucrose concentration, five different media/ basal salts MS (Murashige and Skoog 1962), ½ MS (Murashige & Skoog, 1962), White's (White, 1963), B5 (Gamborg's, 1976) and SH (Schenk & Hildebrandt, 1972). Plant growth hormones used were 6- Benzyl purine (BAP), Kinetin, Indole acetic acid (IAA), α- napthyl acetic acid (NAA) in various combinations as per treatment. Basal media devoid of growth regulators was used as control far all the tissue culture experiments carried out.

3.4.2 Standardization of medium

In all the five basal media, BAP (0.5-5 mg/l), Kinetin (0.5-5 mg/l) and NAA (0.5) were various concentration of growth regulators were added for standardization of the best medium. Four different sucrose concentrations (1 %, 2 %, 3 % and 4 %) were fortified with various concentrations of growth regulator to each treatment. These different concentrations of culture media were carried out for the standardization of best medium for multiplication and production of disease free planting materials of *Zingiber officinale*. On the basis of number of shoots and roots developed after 30-45 days of inoculation, the best medium was selected and used for further studies.

3.4.3 Media preparation

Media constituents for five media used for experimental studies are provided in the Appendix I.

3.4.3.1 Media preparation (MS)

The Three/five stock solutions were formulated in required proportion along with growth regulators and sucrose concentration. The volume was made up by adding double distilled water. The pH of the medium was adjusted between 5.6-5.8 by using either 0.1 N HCl or NaOH with the help of a digital pH meter (Lab India model No.PH102260-634). The volume was finally adjusted with 0.72 % agar (Himedia) and 2 mg/l activated charcoal. Agar in the medium was completely melted by gentle heating upto 90˚C and 15-20 ml of medium was poured into 25 x 150 mm pre sterilized glass culture tubes and bottle. The media was autoclaved at

121˚C at 15 lbs/square inch pressure for 20 min and then allowed to cool to room temperature and stored in culture rooms until further used.

3.4.4 Preparation of stocks (MS)

Murashige and Skoog (MS) medium was commonly used for all the experiments. The stock solutions (10x) were prepared as given below with double distilled water, poured into well stoppered bottle and were stored in refrigerator at 4˚C for Stock A and stock B and Stock C are stored in -20ºC. (Appendix Table I)

Stock A: Macro nutrients – 1000 ml (10x)

Stock B: Micro nutrients – 1000 ml (10x)

Stock C: Vitamin – 100 ml (50x)

Stock D; Vitamin

Stock E: Vitamin

3.4.5 Culture condition

Laminar air flow was thoroughly cleaned with cotton dipped in 70 % alcohol and all the surgical instruments were sterilized/autoclave prior to use. All the required materials like media, spirit lamp, lighter, glass ware etc. were transferred on to the clean laminar air flow. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet where all manipulations were conducted. All the aseptic manipulations such as surface disinfection of explants, preparation and inoculation of explants and subsequent sub-culturing were carried out in the laminar air flow cabinet.

3.4.6 Incubation

All the cultured tubes were incubated under a 16 hr. /8 hr. light and dark photoperiod at 25 ± 2°C light intensity in the culture room.

3.4.7 Preparation of explants

Explants for tissue culture of the cultivars variety of *Zingiber officinale* was collected from the nursery bed raised in the Sikkim State Council of Science and Technology, Gangtok. Young shoot tips of ginger 2-3cm long were excised and collected in a petridish. The explants were soaked in water adding two drops of tween-20 for 15 min. To remove soil particles the explants were washed several times with running tap water. The explants were rinsed with single distilled water for 7-8 times under the laminar airflow. Outer scales of the explants were excised by sharp blade and surface sterilized with 0.1 % mercuric chloride for 10-15 min. The explants were washed with double distilled water for 7-8 times and its outer scales were removed, followed by sterilization with 6 % sodium hypochlorite for 5-8 min. This step was repeated for once and thoroughly washed with double distilled water. The explants were sterilized again with 70 % alcohol for 2 to 3 min and it was washed with double distilled water for 2-3 times. The sterilized explants were kept for drying in the sterilized blotting paper and collected in sterilized in a petridish for inoculation to different culture media.

3.4.8 Culture initiation

3.4.8.1 Shoot tip culture

Shoot tip sterilized explants of *Zingiber officinale* of five cultivar variety were inoculated in MS medium with various combinations and concentration of BAP, Kinetin, and NAA as shown in (Table 4.11).

3.4.8.2 Observations

Response of explants with medium started from 10 days and number of shoots developed initiated was recorded at 21 days to 49 days of culture. After six weeks of inoculation, both shoot and root was found in the same medium with multiple shoots. Each treatment was represented by 10 cultures and all the experiments were carried out for three replicates.

3.4.8.3 Subculture

The multiple shoots were sub-cultured in the same medium for 18 months and after 40 days of sub-cultured plantlets were taken for acclimatization for hardening process. Data were scored based on visual observation and length of shoot and root was measured with graded measuring scale.

3.4.8.4 Root formation

Root initiation in days and root numbers (15) was recorded.

3.4.8.5 Shoot formation

Days to bud initiation and shoot initiation, mean number of shoots, mean number of roots, mean. Numbers of shoots, mean numbers of leaf, days for shoot induction, mean shoots length (cm), was recorded after 60 days of culture.

3.4.8.6 Subculture

Micro shoots formed in the test tubes/ bottles were taken out 7-8 weeks after inoculation. The shoots were separated by dissecting them in the sterile environment

of laminar air flow cabinet with sterile dissecting needle and forceps. They were placed in the test tubes containing fresh media.

3.4.8.7 Leaf explants culture

No response was observed when culturing leaf explants.

3.4.8.8 Hardening and transfer of rooted plants

The *in-vitro* rooted ginger propagules were removed from the culture bottle and washed thoroughly with running tap water to remove media attached with the propagules. These propagules were transferred to petridish and kept in bavistin (methyl-2-benzinmidazole car bate, $C_9H_9N_3O_2$) for 5 min. The freshly developed plantlets were then taken for acclimatization to polybags by placing transparent polythene over polybags for one week for maintaining humidity. Different media/substrates were used for acclimatization of plantlets. A sun dried mixture of (**a**) soil and farm yard manure (FYM) with 1:1 ratio, (**b**) perlite, soil and farm yard manure with ratio of 1:1:1 and (**c**) soil, farm yard manure, sand and perlite with 1:1:1:1 ratio was used. All polybags containing ginger micro-propagules were kept in shade house for primary hardening for one week, followed by removing polythene bag for secondary hardening for another one week and then transferred to field.

3.4.5 Experimental setup and statistical analysis

Statistical rules and modules must support any experimental findings so that it cannot be treated as invalid. The statistical analysis of the data of biological experimentation has tremendous ecological importance and it accepted internationally in the field of quantitative bio-ecology. Following are the applied statistical methods for data analysis.

1. Analysis of Variance (ANOVA)
2. T- test

3.4.5 DNA fingerprinting study

RAPD was done for characterization of various ginger germplasm and also for the test of genetic fidelity of micropropagated ginger. The following protocol (Syamkumar *et al*., 2003) was followed throughout the work with slight modifications.

3.5.1 Ginger DNA extraction

- Young tender leaves were taken weighing approx. 5 gms in a mortar and pestle and ground into a fine powder with the help of liquid nitrogen.
- The pulverized material was taken in a 30 ml Oakridge tube (Tarsons, Cat#41040) containing 15 ml of prewarmed (65ºC) CTAB extraction buffer (Refer Appendix II for composition).
- The tube was then vortexed for 5 seconds and incubated in a water bath (Genei, Cat#107931) for 1 hr. at 65ºC with occasional mixing by gentle swirling.
- Following the 1 hr. incubation an equal volume of chloroform (E Merck Ind. Ltd., Cat#822265)/Isoamyl alcohol (E Merck Ind. Ltd., Cat#8.18969.1000) (24:1) was added and the mixture was mixed gently by inverting the tube upside down.
- The extract was centrifuged (Innova Eppendroff) for 10 min at about 10,000 rpm (room temperature) and the supernatant was carefully transferred to a fresh tube.
- The chloroform/Isoamyl alcohol step was repeated twice and 0.6 volume of ice cold Isopropanol (E Merck Ind. Ltd., Cat#17813) was added to the final supernatant.

- Upon gentle swirling the DNA-CTAB complex precipitated as a whitish network was formed
- Centrifuged at 12000 rpm for 10 min and pellet was taken.
- It was then washed thrice in a washing solution containing 70% alcohol and 10 mM ammonium acetate (SIGMA, Cat#A-7330).
- It was then dried and dissolved in 1ml of 1X TE buffer (pH 7.4) Refer Appendix II for composition.
- The dissolved DNA was extracted with an equal volume of equilibrated phenol (pH 8.0) (SRL, Cat#1624262) mixed properly and centrifuged at 10000 rpm for 15 min.
- The upper aqueous phase was taken in a fresh tube and to it an equal volume of chloroform-Isoamyl alcohol (24:1) was added and then centrifuged at 10000 rpm for 15 min at room temperature.
- The upper aqueous phase was taken in a fresh tube and to it 0.1 volume of 7.5 M ammonium acetate and 2 volume of absolute alcohol (E Merck Germany, Cat#K29824783) was added and precipitated at 4ºC for 30 min in a cooling centrifuge (Innova Eppendroff) at 12000 rpm.
- The pellet obtained was washed in 70% alcohol, dried with vacuum pump (Tarsons make, Model No.Rocker300) and dissolved in 500 µl of 1X TE buffer (pH 7.4).

3.5.2 Purification of DNA

Core contaminants in DNA sample preparation are RNA, proteins and polysaccharides. Inclusion of CTAB in DNA extraction buffer helps in elimination of polysaccharides from DNA preparations to a large extent. The RNA was removed by treating the sample with RNase. Extraction with phenol: chloroform following RNase treatment was also employed for eliminating RNA and most of proteins. Following protocol was used to purify DNA.

- RNase A (50 µg/ml) (SIGMA, Cat#R-4875) was added to the genomic DNA of ginger dissolved in 500 µl of 1X TE buffer (pH 7.4) and it was incubated at 37ºC for 1 hr. in a Dry water bath (Genei make, Cat#107173).
- An equal volume of chloroform/Isoamyl alcohol (24:1) was added and mixed properly.
- Centrifuged at 10000 rpm for 15 min at room temperature.
- The aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat#500010).
- To the aqueous phase 2 volumes of absolute alcohol and 0.1 volumes of 3M Sodium acetate (pH 5.2) (SIGMA, Cat#S-9513) was then added for DNA precipitation. It was centrifuged at 12000 rpm for 30 min.
- The DNA pellet obtained was dried and finally dissolved in 500 µl of 1XTE (pH 7.4) buffer.

3.5.3 Quantitation of DNA

A reliable measurement of DNA concentration is important for many applications in molecular biology including complete digestion of DNA by restriction enzymes and amplification of target DNA by polymerase chain reaction. DNA quantitation is carried out by using agarose gel electrophoresis.

3.5.3.1 Agarose gel electrophoresis analysis

- Agarose gel (0.8%, gelling temperature 36°C) (SIGMA, Cat#A9539) was casted in 1XTBE (Tris-Borate-EDTA) buffer (Refer Appendix II for composition) on gel platform (100X70mm) (Tarsons, Cat#7024).
- Sample DNA (5 µl) was loaded.
- An unknown amount of λ DNA/*Hind III* (Genei, Cat#106000) was loaded as control in the adjacent well.
- The gel was run at 50 V for 1hr in a Mini Submarine Gel Electrophoresis Unit (Tarsons, Cat#7030) and made to run at a constant volt of 50 V applied with Electrophoresis Power Supply Unit (Tarsons, Cat#7090).
- After cooling it to a temperature of 40°C, the gel was stained with 0.5µg/ml Ethidium bromide (2, 7-diamino-10-ethyl-9-phenylphenanthridinium bromide, $C_{12}H_{20}BrN_3$, M_r 394.33, (SIGMA Cat#E8751) for 10 min, Washed with distilled water and the gel was visualized under UV light on a UV Transilluminator (Genei, Cat#SF850).

- The DNA quality was judged by presence of a single compact band at the corresponding position to λ DNA/*Hind III* (Genei, Cat#106000) indicating high molecular weight of the DNA.
- The quantity of the DNA was estimated by comparing the sample DNA with the control by eye judgement.

This pure DNA was used for various fingerprinting studies like:

3.5.4. RAPD of ginger (*Zingiber officinale*)

A total of 63 random 10 decamers primers (CLONI TECH, Genuine Chem. Corp., New Delhi, India) given in a tabular form (Table 2) were screened for 5 cultivars variety.

3.5.4.1. RAPD-PCR amplification

In a sterile 0.2ml PCR tube (Tarsons, Cat#500050) the 25µl PCR master mix was prepared with the following components:

- Template DNA -5 µl (50 ng)
- Primers -2 µl (0.25 µM) each of forward and reverse primer
- Taq DNA polymerase -2 unit (Fermentas).
- PCR Mix (dNTPs, Buffer, $MgCl_2$ -) -12.5 µl (Fermentas make)
- Nuclease free water- To a final volume of 25µl
- One negative control tube was prepared containing PCR mix without DNA.

The PCR reactions were performed on a Thermo cycler (Perkin-Elmer). The amplification cycle consisted of the following specifications:

- Cycle 1: Denaturation at 94ºC for 4min, Primer annealing at 35ºC for 1min., Primer extension at 72ºC for 2min.
- Cycle 2-29: Denaturation at 94ºC for 1min., Primer annealing at 35ºC for 1min., Primer extension at 72ºC for 2min.
- Cycle 30: Denaturation at 94ºC for 1min., Primer annealing at 35ºC for 1min., Primer extension at 72ºC for 5 min.
- The PCR products were separated on 1.5% (W/V) agarose gel run in 1XTBE buffer.
- PCR product (10µl) was mixed with 5µl of Gel loading dye (6X), (Fermentas, Cat#R0611).
- The samples were loaded and electrophoresis was carried out at 50V for 1hr.
- The gel was stained with Ethidium bromide solution (0.5µg/ml).
- The gels were visualized with a UV transilluminator (Genei, Cat#107161) and photographed with Gel Documentation System (Vilber lourmat France, Model No. DP-001.FDC). A DNA ladder (lambda DNA *HindIII* digest) (Genei, Cat#106000) was used as a molecular size marker. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc and POPGENE freeware).

3.5.4.2 RAPD data analysis

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

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

The POPGENE freeware (Yeh *et al*., 1997) was used to partition genetic diversity among the 40 sample.

The same software was used to calculate genetic distances and similarities between populations and to draw a dendrogram based on Nei's (1972) genetic distances using the UPGMA to show the genetic relations between the different cultivars.

Graphical representation of the results was done on the basis of these studies.

Shannon's index was also calculated, the degree of polymorphism among the ginger varieties collected from various regions of Sikkim was calculated for each primer from the binary data matrix using Shannon's index of phenotypic diversity from the following equations as mentioned by (Pattanayak *et al*., 2002).

$H_0 = - \Sigma P_i \text{ In } P_i$ ------------------------ (1)

Where P_i is the frequency of phenotype I and H_o is genetic diversity within the ginger varieties detected by a particular primer.

H_{pop}= 1/2 Σ [$H_{0(hill)}$ + $H_{0(plane)}$] -------(2)

Where H_{pop} is average diversity in different varieties of ginger revealed by a particular primer.

H_{sp} = - Σ P_i In Pi ---------------------- (3)

Where H_{sp} is diversity in all the five cultivars varieties considered for a particular primer.

$H_{0,}$ H_{pop} and $_{Hsp}$ were calculated for all the primers and the average estimate over 21 primers was calculated. Proportion of diversity present within populations, H_{pop} / H_{sp}, was compared with that between populations, (H_{sp}-H_{pop}) / H_{sp}. The result was given in a tabular as well as graphical form.

3.6 Antioxidant study

3.6.1 Chemicals and reagents

Following chemicals were used for the study of presence of antioxidant in different cultivar variety of *Zingiber officinale* Roscoe.

- Of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), Quercetin, Sodium Nitrite ($NaNO_2$), Trichloroacetic acid (TCA), Ascorbic acid, Ferric chloride ($FeCl_3$), Gallic acid were procured from HI Media Laboratories Pvt. Ltd, Mumbai, India.
- Potassium di-hydrogen phosphate (KH_2PO_4), di-Potassium hydrogen phosphate (K_2HPO_4), Sodium hydroxide (NaOH), Gallic acid, Potassium ferricyanide ($K_2Fe (CN)_6$), Sodium carbonate (Na_2CO_3), Hydrogen peroxide (H_2O_2) and Methanol were obtained from Merck, Mumbai, India.

- Folin-Ciocalteau reagent from Sisco Research Laboratory, Mumbai, India. Aluminium Chloride ($AlCl_3$) was obtained from SD fine Chemicals Ltd., Mumbai, India.
- All chemicals and solvents were of analytical grade.

3.6.2 Plant materials and extraction

3.6.3 Determination of plant extract yield (PEY)

Based on dry weight, the yields of extracts were calculated using following equation:

Yield (g/100 g of dry plant material) = $(W1 \times 100) / W2$

Where, W1 = the weight of the extract after the solvent evaporation and,

W2 = the weight of the dry plant material.

3.6.4 Determination of total phenolic content (TPC)

The method of Singleton and Rossi (1965) using Folin-Ciocalteau reagent (FCR) was opted to determine the total soluble phenols in the extracts. Using Thermo UV1-Vis spectrophotometer, the absorbance of the blue colour that developed was read at 760 nm. Gallic acid was used as a standard. The concentration of total phenolic compounds in Gallic acid was determined in µg of Gallic acid equivalent using an equation obtained from the standard Gallic acid graph.

3.6.5 Determination of total flavonoid content (TFC)

In this case Quercetin was used as standard in mg QE. With some modifications, method opted by Goyal *et al.*, (2010) was opted for the total flavonoid contents of the

extracts with Aluminium Chloride ($AlCl_3$). Here the absorbance was recorded at 510 nm and all the experiments were carried out in triplicates.

3.6.6 Antioxidant properties of extracts *in vitro*

An antioxidant property of ginger was evaluated using the following techniques.

3.6.6.1 Free radical scavenging activity (DPPH Method)

Based on the scavenging effect of the stable DPPH free radical, anti-oxidant activity of all the standards and extracts were determined following the methods of Hasan *et al*., (2006). Spectrometric absorbance of each solution was determined at 517 nm after taking blank reading. The remaining DPPH scavenging activity in percentage was calculated using the formula:

DPPH scavenging activity (%) $= \frac{Ao - A_1}{Ao}$ X 100

Where A_0 = the absorbance of the control and, A_1 = the absorbance in the presence of the sample. To scavenge 50% free radical, IC50 value concentration of the sample required.

3.6.6.2 Reducing power assay

The method of Oyaizu (1986) was followed to determine the reducing power of the extracts. The absorbance was measured at 700 nm and compared with standards. Indication of increased reducing power could be assessed by increase in absorption value of the reaction mixtures.

3.6.6.3 Scavenging of hydrogen peroxide

The scavenging ability of the extracts in this experiment was determined following the methodology of Ruch *et al.,* (1989). The scavenging activity percentage was calculated using the formula:

$$\text{Hydrogen peroxide scavenging activity} = \frac{\text{Abs (control)} - \text{Abs (standard)}}{\text{Abs (control)}} \times 100$$

Where, Abs (control) is the absorbance of the H_2O_2 (2 mM) as control and Abs (standard) is the absorbance of the extract/standard.

3.6.6.4 Statistical analysis

Experiments as mentioned above were conducted in triplicates. Experimental results were expressed as means ± standard deviation (SD) of three parallel measurements. The differences between the extracts were analyzed using one-way analysis of variance (ANOVA) with a significant difference was adjudged at a level of $P < 0.05$.

3.7 Antimicrobial activity of *Zingiber officinale* Roscoe.

Antimicrobial activities of extracts of ginger rhizome were evaluated against authentic test organism.

3.7.1 Microbial cultures

All the microbial cultures, used for antimicrobial screening were produced from MTCC, Chandigarh, India. A total of six bacterial species were tested including *Staphylococcus aureus (MTCC 96), Bacillus subtilis (MTCC 441), Escherichia coli (MTCC 739), Klebsiella pneumonia (MTCC 432), Enterobacter aerogenes (MTCC III)* and *Pseudomonas aeruginosa (MTCC 424).*

3.7.2 Maintenance of microorganism and pure culture

The pure culture was maintained by transferring inoculums into slant or petriplate time to time and incubated at 25 ± 2ºC. The bacterial culture was maintained on nutrient agar slants which were stored at -4°C.

3.7.3 Antibacterial assays

The antibacterial activity was based on agar well diffusion method using bacterial cell suspension whose concentration was equilibrated to a 0.5 McFarland standard. A 100 µl of each bacterial suspension was spread on a Mueller Hinton agar plate. Well (6 mm diameter) were impregnated with 50 µl of each extract dissolved in DMSO at a concentration of 100 mg/ml. The wells were allowed to dry and then placed in the incubated at 37˚C for 24 h. Wells with the solvent used for dissolution were used as negative control and 1mg/ml amoxicillin were used as positive controls. After incubation time, zone of inhibition was measured.

3.7.4 Statistical analysis

All the assays were carried out using triplicate. Experimental results were expressed as means ± standard deviation (SD) of three parallel measurements. The differences between the extracts were analyzed using one-way analysis of variance (ANOVA) with a significant difference was adjudged at a level of $P < 0.05$. All the statistical analyses were performed using the SPSS version 16 software and the correlation coefficient (R^2) was determined for correlations.

Chapter 4

Results

4.1 Ecological study and enumeration of ginger in Sikkim

4.1.1 Ginger cultivation, ecology and genetic resources in Sikkim

Ginger is a major cash crop in Sikkim and is cultivated by all the communities of Sikkim (Subba, 2000). Majority of the farmers in Sikkim use a standard farming procedure introduced by the Department of Horticulture and Cash Crops Management, Government of Sikkim. However, three ethnic communities, namely, Lepcha, Rai and Limboo have been using age old traditional methods for ginger cultivation.

4.1.2 Ginger cultivation in Sikkim

In north Sikkim, majority of the ginger farmers are belong to Lepcha community. They preserve ginger seeds in a traditional ways for next year cultivation. Size of the ginger seeds ranged from 10 to 25 cm in length and 75 to 900 gm. in weight with approximately 3 to 5 number of shoot buds (Figure 6). It is to be noted that the ginger in north Sikkim is almost free from fungal and bacterial diseases. In Sikkim ginger cultivation is found in different landscapes (Figure 7). In south Sikkim, ginger is mainly cultivated for commercial purpose. The method of cultivation is a standard farming procedure introduced by the competent authorities. In east and west Sikkim, ginger has been cultivated both for commercial and religious purposes.

4.1.3 Spatial distribution of five different ginger cultivars of Sikkim

In the present investigation, ecological data were analyzed using (ARC.10 software), and different thematic maps was generated based on elevation, districts, location of disease and non-disease areas, soil texture and distribution of five different cultivars of ginger grown in Sikkim.

4.1.4. Elevation wise distribution of five different cultivars of *Zingiber officinale* of Sikkim Himalaya using GIS overlay technique

Geographic Information System (GIS) is fixed in logical practices, occupied by data and motorized by mathematical analysis, the use of GIS is needed to collect data, store, manage, analyse and produce useful information. Schuurman (2004) suggested that currently, the main use of GIS is for spatial analysis, predictive modelling, cartography and visualisation. GIS maps showed the exact location and survey coordinates of an object in space to provide answer to queries using a computer system (Ibid, 2004). In the present study, distribution of ginger with an elevation wise (Figure 8 and 9) was recorded with an altitude range form 1019-5784 ft., and having total area (323.85 acre) with different range from (0.05-8.00 acre) land (plot) size. This result revealed that significant ginger cultivation areas was observed at an altitude range from (2290-3220 ft.), this include 66 plots with total area 18.00 acres, with range from 0.1-3.5 acre, as shown in (Table 4.1). Furthermore, second major ginger growing altitudinal range was recorded from 3230-4070 ft., with total (57.00) plot, which include 80.80 acres of ginger cultivation land (plot). With reference to lowest ginger cultivation area was recorded at altitude range from 1410-2280 ft., which comprises 19 plots includes 2.5 acres of land.

Figure 6 Ginger seed of Majhauley cultivar. Figure on left showing; seed has two to four new shoots and the figure on right side showing 5 to 10 cm seed distance between two ginger seeds. This above information clearly suggest that ginger seed should have two to four new buds and the distance should be maintained upto range from 5 cm to 10 cm between two seeds.

Figure 7 Different landscapes used for ginger cultivation in Sikkim Himalaya. A) Showing ginger field of Gayzing, West Sikkim; B) Ginger field of Dzongu, North Sikkim; C) Ginger field Manaybhangyang, South Sikkim; D) Rolep, East Sikkim.

From the current investigation, results from (Table 4.1) showed that *Majhauley* cultivars were cultivated by most gingers farmers of Sikkim. The utmost twenty one (21) numbers of plots recorded at an altitude range above 4080 ft., followed by twenty (20) Numbers of plots at an altitude range from (3230-4070 ft.) and next nineteen (19) numbers of plots at an altitude range from 2290-3220 ft. Moreover, about distribution of cultivar *Gorubthangey* was second foremost grown by gingers farmers of Sikkim, par fifteen (15) numbers of plots distribution of ginger was recorded at an altitude range from 2290-3220 and 3230-4070 ft. respectively (Table 4.1 and Figure 10). Lowest number of *Gorubthangey* cultivar plot was distributed at an altitude 1410-2280 ft. range. Regarding distribution of two ginger cultivars *Bhaisay* and *Jorethangey*, maximum sixteen (16) and fourteen (14) numbers of plots recorded at an altitude range from 2290-3220ft followed by 12 and 8 numbers of plots was recorded at an altitude 3230-4070 ft. and 4080 ft above range respectively. Concerning about lowest ten (10) numbers of plots recorded in (Figure 10) revealed that cultivar *Charinangrey* was grown by minority gingers farmers of Sikkim Himalaya at an altitude range.

4.1.5 District wise distribution of five different cultivars of *Zingiber officinale* in Sikkim using GIS overlay technique

In this current study, using GIS overlay technique five different cultivars of *Zingiber officinale* distributed in four districts as showed in (Figure 11). Maximum sixty four (64) number of plots recorded from north districts, followed by west districts includes sixty (60) numbers of plots, subsequently by south districts includes fifty eight (58) numbers of plots, at last east districts enclosed fifty six (56) numbers of plots as recorded in (Figure 12).

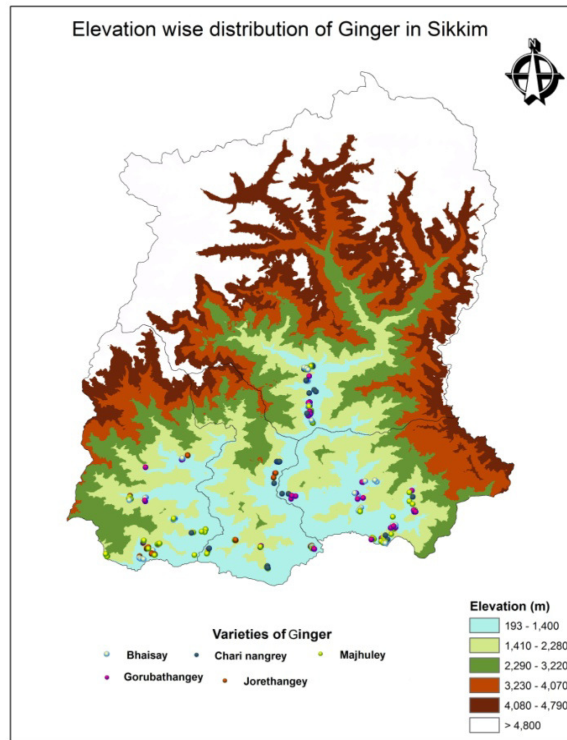


Figure 8 Elevation wise distribution of *Zingiber officinale* in Sikkim.

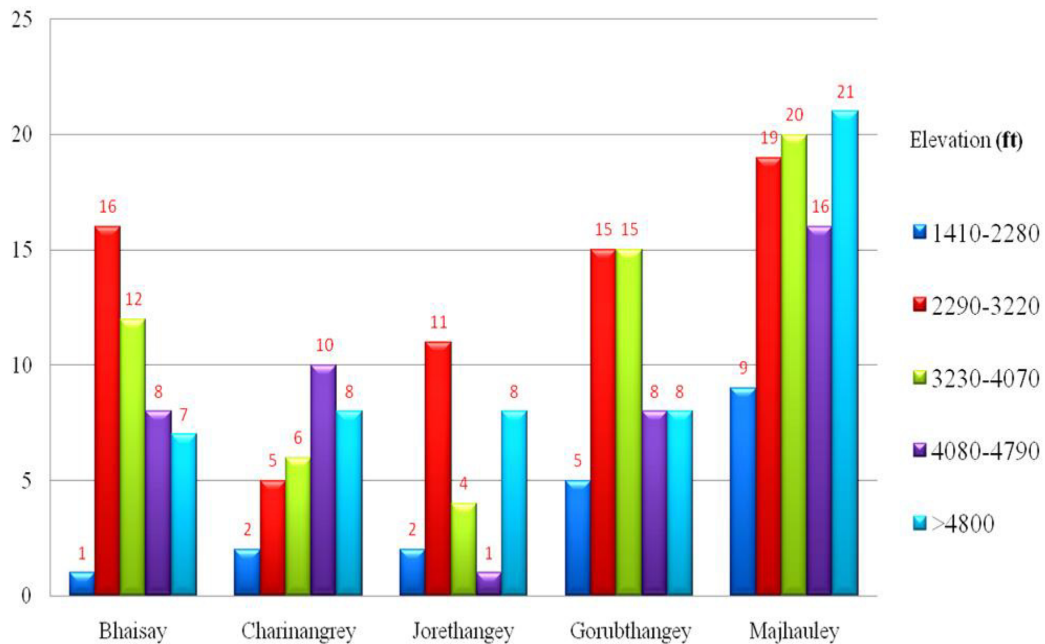


Figure 9 Elevation wise distributions of five different cultivars of *Zingiber officinale* of Sikkim Himalaya

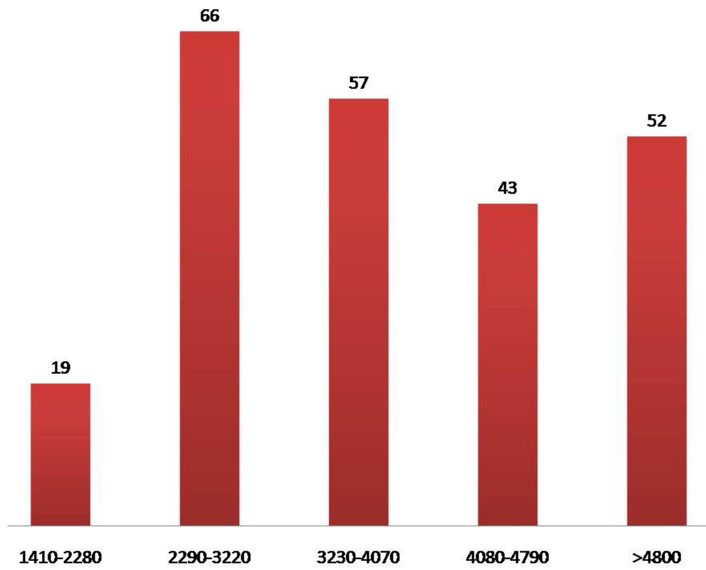


Figure 10 Histogram showing elevation wise distribution of ginger in Sikkim

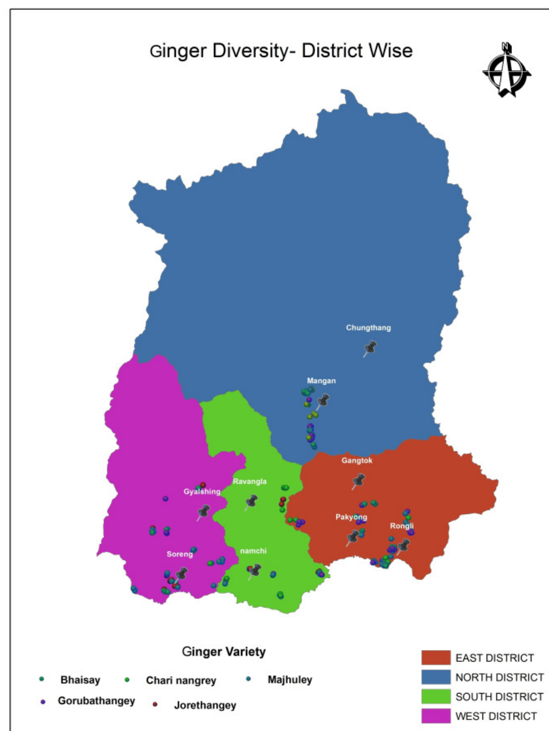


Figure 11 District wise distributions of five different cultivars of *Zingiber officinale* of Sikkim Himalaya using Geographic Information System (GIS) overlay technique**.**

Table 4.2. Showing distribution of five different cultivars of *Zingiber officinale* of Sikkim Himalaya. Table shows that ginger *Majhauley* cultivars were used my most of ginger farmers of Sikkim at all the altitudinal range.

Altitudinal Range (ft.)	Number of plots	Area of plots	*Bhaisay*	*Charinangrey*	*Jorethangey*	*Gorubthangey*	*Majhauley*
1410-2280	19	2.5	01	02	02	05	09
2290-3220	66	18	16	05	11	15	19
3230-4070	57	80.8	12	06	04	15	20
4080-4790	43	100.1	08	10	01	08	16
>4800	52	61.25	07	08	08	08	21
Total		**323.85**	**44**	**31**	**26**	**51**	**85**

Table 4.2 Showing district wise distribution of five different cultivars of *Zingiber officinale* of Sikkim Himalaya. The table exhibits that all districts farmers favour *Majhauley* cultivar for cultivation followed by *Gorubthangey* cultivar**.**

Districts	Numbers of plot	*Bhaisay*	*Charinangrey*	*Jorethangey*	*Gorubthangey*	*Majhauley*
East	56	11	6	Nil	19	20
North	64	15	15	Nil	12	22
South	58	04	11	11	12	20
West	60	13	01	15	08	23

Table 4.3 Evaluation of diseases and non-disease areas of five different cultivars, *Zingiber officinale* of Sikkim Himalaya. Table showing that maximum diseases recorded in *Jorethangey* and *Bhaisay* cultivar at all the altitudinal range.

	plot		**Range (ft.)**			***y***		
Fungal and Bacterial Diseases	112	113	1719-5784	25	3	16	26	40
No diseases	126	211.8	1019-5733	44	33	26	51	85
			Percentage (%)	36.23	8.33	38.09	33.766	32

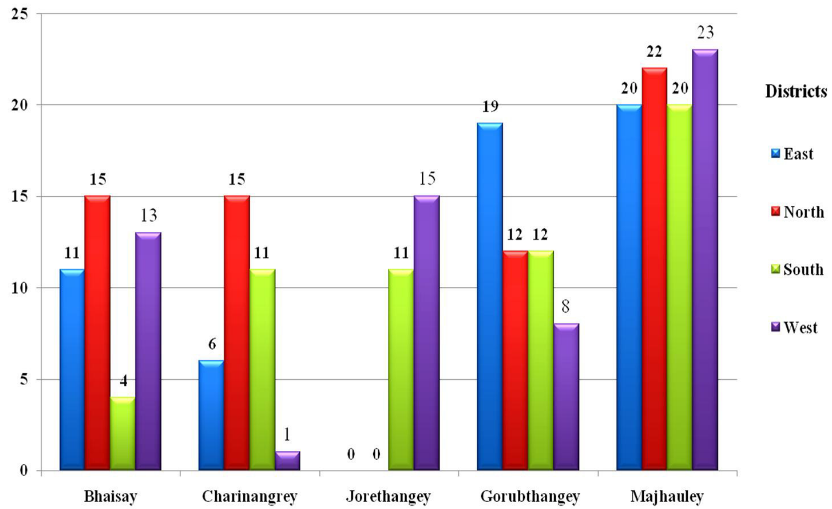


Figure 12 District wise distributions of five different cultivars of *Zingiber officinale* of Sikkim Himalaya

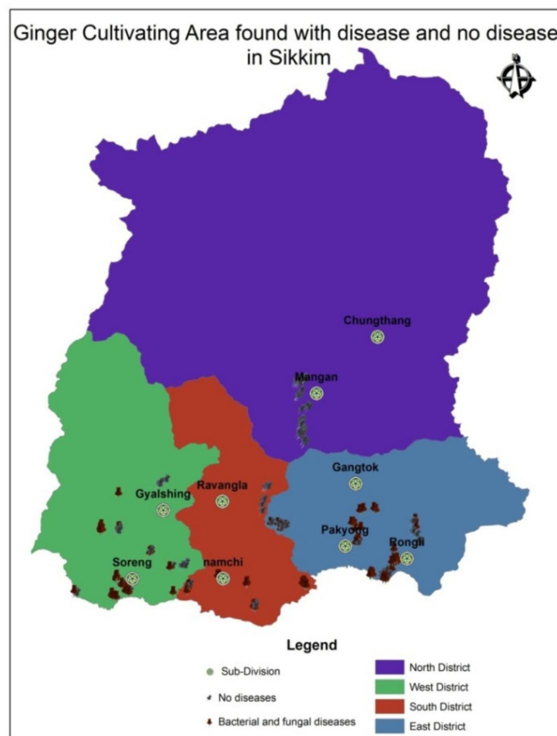


Figure 13 Showing of diseases and non-disease areas of five different cultivars, *Zingiber officinale* of Sikkim Himalaya using Geographic Information System (GIS) using overlay technique

Among five cultivars majority of plots were recorded from *Majhauley* cultivars as shown in Table 4.2. From figure showed that *Majhauley* cultivar evenly distributed in all four districts with maximum number of plots. According to district wise distribution next cultivar *Gorubthangey* found with modest type of distribution in all districts subsequently followed by *Bhaisay* and *Charinangrey*. At last *Jorethangey* recorded least numbers of plots recorded from district wise distribution study and zilch numbers of plots were recorded from north and east districts.

4.1.6 Evaluation of diseases and non-disease areas of five different cultivars, *Zingiber officinale* in Sikkim using GIS overlay technique

Bacterial and fungal diseases have been reported to be common in Sikkim ginger cultivation (Rai, 2009). In present investigation, using GIS overlay technique, disease and non-disease regions were identified as showed in Figure 13. Current study revealed 126 diseases less plots out of a total of 237 plots. The rest 112 plots were found with various bacterial and fungal diseases (Table 4.3).

4.1.7 Morphological characterization of five ginger cultivars

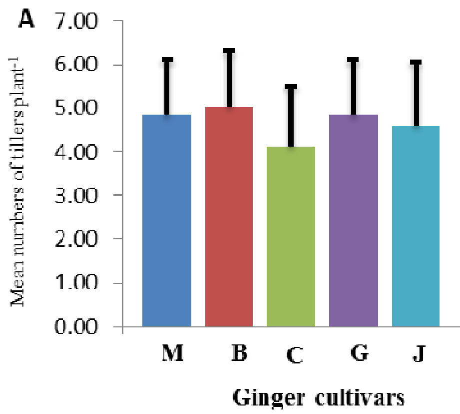
4.1.7.1 Quantitative characterization

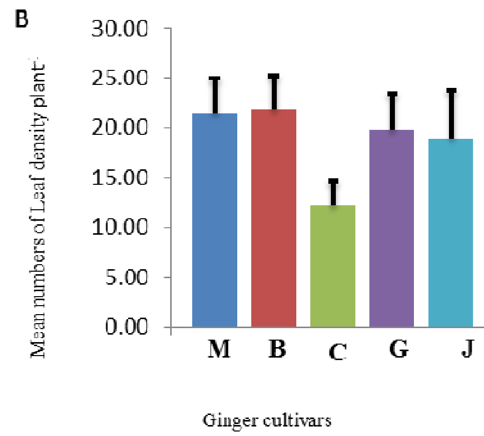
Study on morphological characters showed that cultivars, viz., *Majhauley*, *Bhaisay* and *Charinangrey* have erect growth habit, whereas *Gorubthangey* and *Jorethangey* the semi-erect growth habit. Maximum mean plant height recorded for *Bhaisay* cultivar was 78.13 ± 12.67 cm (Table 4.4), followed by *Majhauley* cultivar (69.90 ± 8.26 cm), *Gorubthangey* (69.6 ± 17.31 cm), *Jorethangey* (66.87 ± 9.29 cm) and *Charinangrey* (64.87 ± 12.67 cm) (Figure 14 A). One-way ANOVA showed statistically significant difference of means for plant height among five cultivars (F_{value}

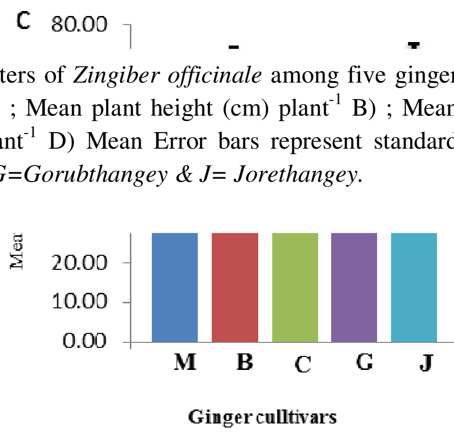
= 6.54, $P < 0.000052$) (Table 4.5), among four districts $F_{value} = 2.55$, $P < 0.045$) and among different soil types ($F_{value} = 2.588$, $P < 0.038$) (Tables 4.6 and 4.7). Maximum number of shoots was recorded for *Majhauley* cultivar with a mean value of 17.65 ± 4.56 (Figure 15 A), followed by *Bhaisay* cultivar (16.78 ± 3.27), *Gorubthangey* (15.70 ± 4.56), *Jorethangey* (9.65 ± 3.60) and *Charinangrey* (8.59 ± 3.69) (Table 4.5). One-way ANOVA showed statistically significant difference in means of number of shoots per plant among five ginger cultivars ($F_{value} = 7.48$, $P < 8.84E\text{-}05$) (Table 4.5), among four districts ($F_{value} = 4.40$, $P < 0.0049$) and among different soil types ($F_{value} = 3.057$, $P < 0.017$) (Tables 4.6 and 4.7).

Mean shoot diameter per plant was recorded highest in *Bhaisay* cultivar (4.25 ± 0.38), followed by *Jorethangey* (3.71 ± 0.53), *Majhauley* (3.33 ± 0.62), *Gorubthangey* (3.33 ± 0.72) and *Charinangrey* (3.16 ± 0.32) (Figure 14 D).

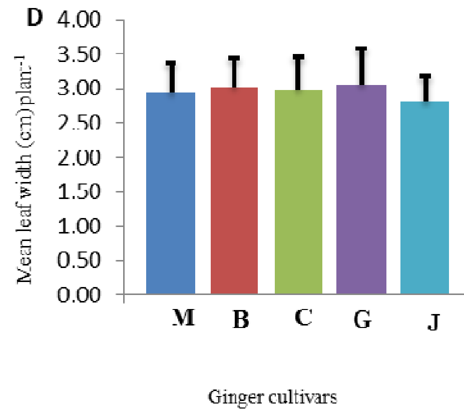
One-way ANOVA revealed mean shoot diameter per plant was statistically highly significant among five ginger cultivars ($F_{value} = 25.73$, $P < 1.07E\text{-}17$) (Table 4.5), among four districts ($F_{value} = 6.69$, $P < 0.0002340$) and among different soil types ($F_{value} = 3.097$, $P < 0.016$) (Tables 4.6 & 4.7). Maximum number of leaf-density per plant was recorded in *Bhaisay* cultivar with mean value of 21.80 ± 3.41, followed by *Majhauley* (21.45 ± 3.59), *Jorethangey* (19.53 ± 3.59), *Gorubthangey* (18.90 ± 4.92) and among five ginger cultivars ($F_{value} = 33.54$, $P < 3.60E\text{-}22$) (Table 4.5). However, the difference in means of leaf-density per plant was statistically insignificant among four districts ($F_{value} = 0.608$, $P < 0.610197$) and among different soil types ($F_{value} = 1.155$, $P < 0.331$) (Tables 4.6 and 4.7). Leaf length was recorded maximum in *Charinangrey* cultivar with a mean value of 24.04 ± 2.16 cm, followed by *Bhaisay* (23.56 ± 4.09), *Jorethangey* (22.93







rent morphological characters of *Zingiber officinale* among five ginger mbers of tillers plant^{-1} (A) ; Mean plant height (cm) plant^{-1} B) ; Mean n shoot diameter (cm) plant^{-1} D) Mean Error bars represent standard *aisay, C=Charinangrey, G=Gorubthangey & J= Jorethangey.*



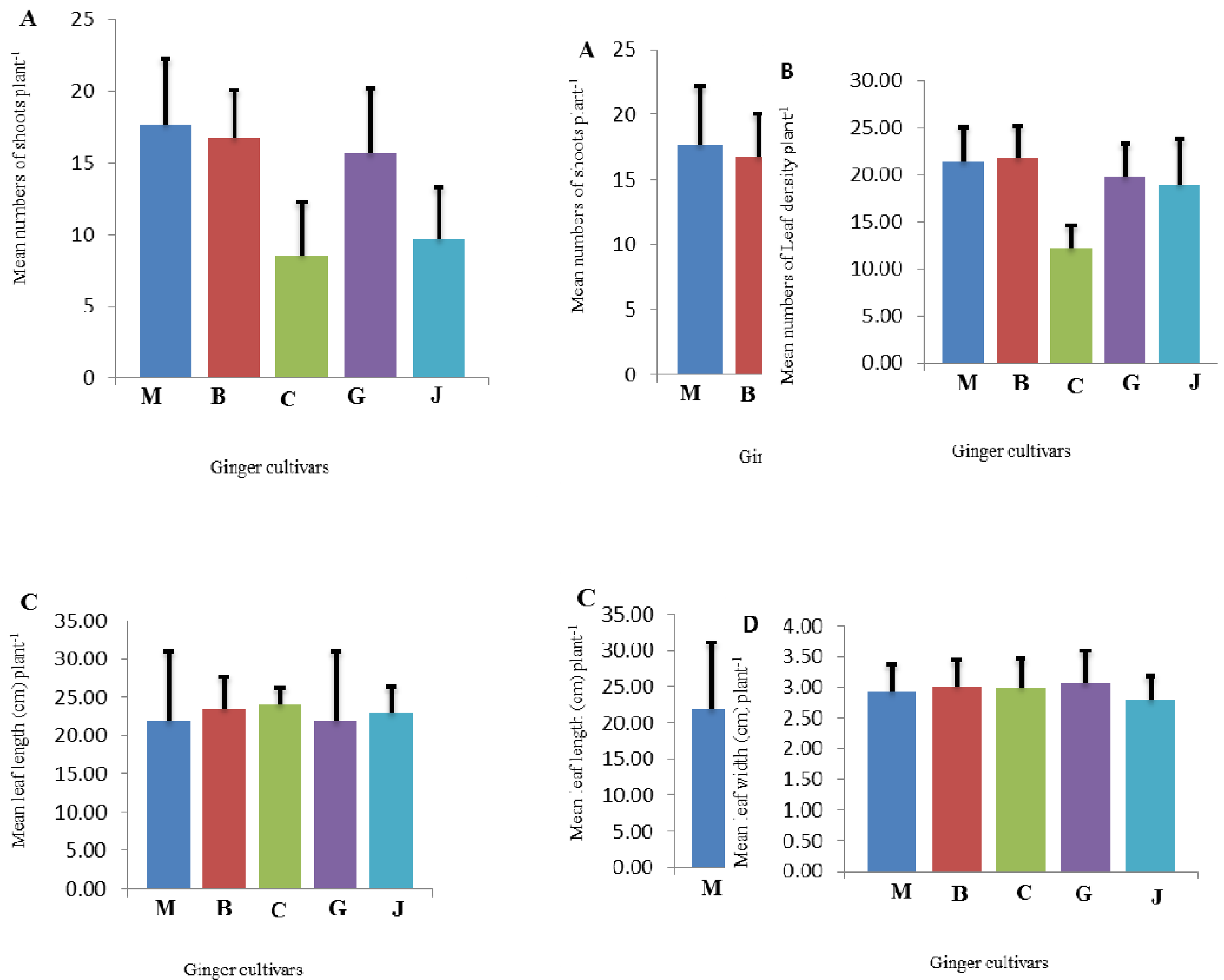


Figure 15 Comparison of different morphological characters of *Zingiber officinale* among five cultivars. Number of shoots per plant (A); Mean number of leaf density per plant (B); Mean numbers of Leaf density plant^{-}1; (C) Mean leaf length (cm) plant^{-1} (D) Mean leaf width (cm) plant^{-1} .Error bars represent standard deviation. *M=Majhauley, B=Bhaisay, C=Charinangrey, G=Gorubthangey and J= Jorethangey.*

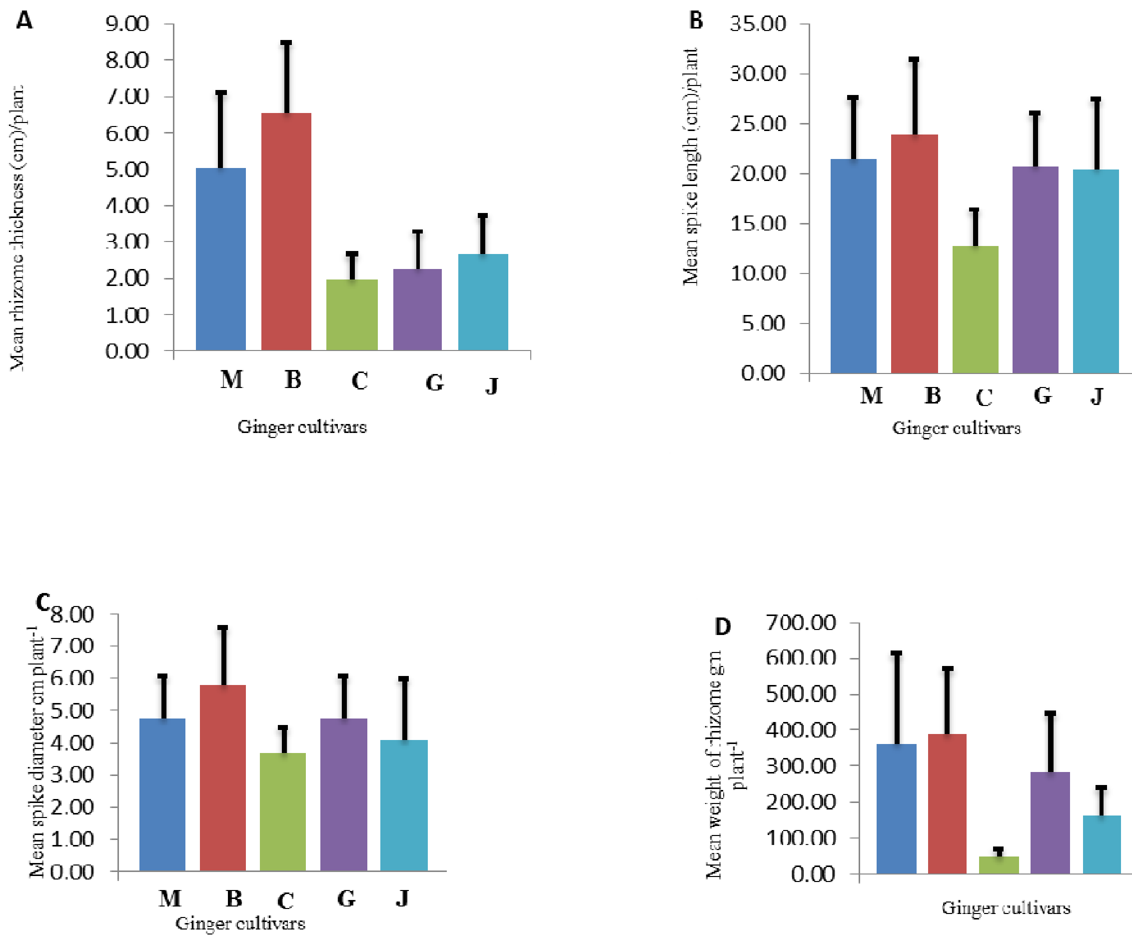


Figure 16 Comparison of different morphological characters of *Zingiber officinale* among cultivar variety wise. Mean rhizome thickness (cm)/plant A); Mean spike length (cm)/plant B); Mean spike diameter cm plant^{-1} D); Mean weight of rhizome gm. plant^{-1} .Error bars represent standard deviation. *M=Majhauley, B=Bhaisay, C=Charinangrey, G=Gorubthangey & J= Jorethangey.*

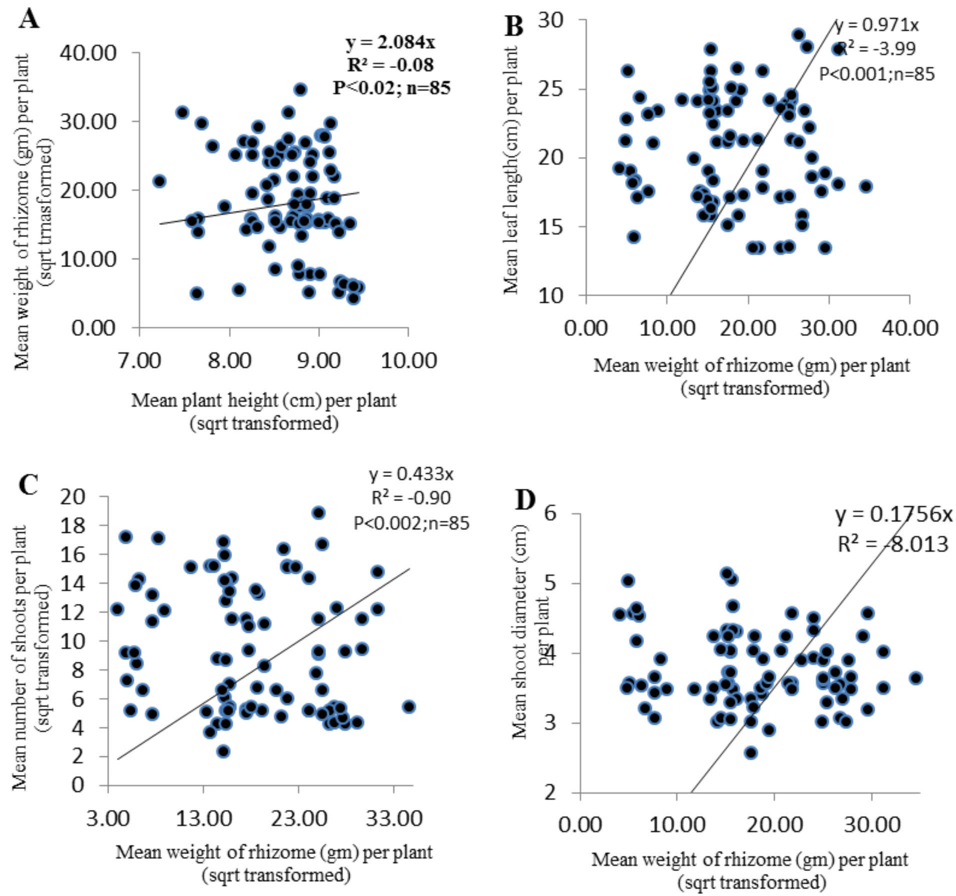
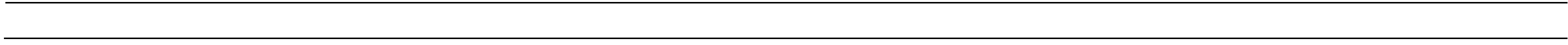


Figure 17 Linear relationships between Mean weight of rhizome (gm.) per plant (sqrt transformed) and Mean plant height (cm) per plant (sqrt transformed), Mean canopy (cm) per plant (sqrt transformed) and Mean weight of rhizome (gm.) per plant (sqrt transformed), Mean number of shoots per plant and Mean weight of rhizome (gm.) per plant, Mean shoot diameter (cm) and Mean weight of rhizome (gm.) per plant (sqrt transformed) and Mean rhizome thickness per plant and mean spike length per plant

Sl No.	Characteristics	Value	States	Example varieties	Stage of observation	Type of Assessment
1	**Plant: Growth habit**		Erect	*Majhauley*	At the end of the growing phase	VG
	Erect		Erect	*Bhaisay*		
	Semi erect		Semi Erect	*Gorubthangey*		
	Spreading		Semi Erect	*Jorethangey*		
			Erect	*Charinangrey*		
2	**Plant: Height (cm)**					
	Short (< 100)	69.906	Short	*Majhauley*	At the end of the growing phase	MS
	Medium (100 – 120)	78.529	Short	*Bhaisay*		
	Tall (>120)	69.608	Short	*Gorubthangey*		
		70.142	Short	*Jorethangey*		
		64.875	Short	*Charinangrey*		
3	**Plant: Number of shoots**					
	Few (<10)	17.654	Many	*Majhauley*	At the end of the growing phase	MS
	Medium (10-15)	16.785	Many	*Bhaisay*		
	Many (>15)	15.700	Medium	*Gorubthangey*		
		9.657	Few	*Jorethangey*		
		8.597	Few	*Charinangrey*		
4	**Shoot Diameter (cm)**	3.33	Medium	*Majhauley*	At the end of the growing phase	MS
	Narrow (<3)	4.25	Medium	*Bhaisay*		
	Medium (3-5)	3.33	Medium	*Gorubthangey*		
	Broad (>5)	3.71	Medium	*Jorethangey*		
		3.16	Medium	*Charinangrey*		

S No.	Characteristics	Value	States	Example varieties	Stage of observation	Type of Assessment
5	**Shoot: Intensity of green color**				At the end of the growing phase	
	Light green		Green	*Majhauley*		**VG**
	Green		Green	*Bhaisay*		
	Dark Green		Green	*Gorubthangey*		
			Green	*Jorethangey*		
			Light Green	*Charinangrey*		
6	**Shoot : Number of leaves on main shoot**				At the end of the growing phase	**VG**
	Few (<25)	21.45	Few	*Majhauley*		
	Medium (25-35)	21.80	Few	*Bhaisay*		
	Many (>35)	18.90	Few	*Gorubthangey*		
		19.53	Few	*Jorethangey*		
		12.16	Few	*Charinangrey*		
7	**Colour of the bract tip of fully developed spike**		Red & white	*Majhauley*	At the end of the growing phase	
	Crimson		Crimson	*Bhaisay*		**VG**
	Yellow white tip		Crimson	*Gorubthangey*		
			Crimson	*Jorethangey*		
			Pale yellow	*Charinangrey*		
8	**Leaf: Length (cm)**					
	Short (<25)	21.89	Medium	*Majhauley*	At the end of the growing phase	
	Medium (25-30)	23.56	Medium	*Bhaisay*		**MS**
	Long (>30)	21.89	Short	*Gorubthangey*		
		22.93	Short	*Jorethangey*		
		24.04	Short	*Charinangrey*		



S No.	Characteristics	Value	States	Cultivars	Stage of observation	Type of Assessment
9	**Leaf: Width (cm)**	2.93	few		At the end of the growing phase	
	Few (<10)	3.02	few	*Majhauley*		**MS**
	Medium (10-15)	3.07	few	*Bhaisay*		
	Many (>15)	2.81	few	*Gorubthangey*		
		2.99	few	*Jorethangey*		
				Charinangrey		
10	**Leaf: Intensity of green colour**		Green	*Majhauley*	At the end of the growing phase	**VG**
	Light green		Green	*Bhaisay*		
	Green		Dark Green	*Gorubthangey*		
	Dark green		Light Green	*Jorethangey*		
			Light Green	*Charinangrey*		
11	**Leaf: Petiole length (cm)**				At the end of the growing phase	
	Short (<0.5)	00.60	Medium	*Majhauley*		**MS**
	Medium (0.5-0.7)	00.60	Medium	*Bhaisay*		
	Long(>0.7)	00.50	Short	*Gorubthangey*		
		00.40	Short	*Jorethangey*		

00.50	Medium	*Charinangrey*

	Characteristics	Value	States	Cultivars	Stage of observation	Type of Assessment
12	**Spike: Length**	21.26	Short	*Majhauley*	At the end of the growing phase	**MS**
	Short (<25)	23.86	Short	*Bhaisay*		
	Medium (25-35)	20.50	Short	*Gorubthangey*		
	Long (>35)	20.92	Short	*Jorethangey*		
		12.78	Short	*Charinangrey*		
13	**Rhizome: Thickness (cm)**	5.06	Bold	*Majhauley*	At the end of the growing phase	**MS**
	Thin (<2)	6.53	Bold	*Bhaisay*		
	Medium (2-3)	2.28	Medium	*Gorubthangey*		
	Bold(>3)	2.67	Medium	*Jorethangey*		
		1.98	Thin	*Charinangrey*		
14	**Rhizome: Shape**		Straight	*Majhauley*	At the end of the growing phase	
	Straight **Zigzagged)**		Straight Straight	*Bhaisay* *Gorubthangey*		
	Curved		Zigzagged Curved	*Jorethangey* *Charinangrey*		
15	**Crop duration (days)**				At the end of the growing phase	**MS**

Short (<200)			
Medium (200-210)	200-210	Medium	*Majhauley*
Long (>210)	200-220	Medium	*Bhaisay*
	190-230	Medium	*Gorubthangey*
	200-220	Medium	*Jorethangey*

MG: Measurement by a single observation of a group of plants or parts of ants **MS:** Measurement of a number of individual plants or parts of plants **VG:** Visual assessment by a single observation of a group of plants or parts of ants **VS:** Visual assessment by observations of individual plants or parts of plants. **NR:** Not Recorded

Table 4.5 One-way ANOVA for different morphological characters among five cultivars of *Zingiber officinale* of Sikkim Himalaya. Except the leaf width, all other morphological characters are statistically significant which suggest that characters are important for differentiation of cultivars in ginger.

Morphological character	Source of variation	DF	Sum of squares	Mean of Squares	*F* value	*P* value
Plant height	Between Groups	4	5945.24	1486.31	6.54	0.000052
	Within Groups	237	53841.74	227.18		
No of shoots	Between Groups	4	299.54	99.84716	7.48	8.84E-05
	Within Groups	237	2775.95	13.34595		
Shoot diameter	Between Groups	4	27.57158	6.892896	25.73	1.07E-17
	Within Groups	237	62.94541	0.267853		
Canopy	Between Groups	4	7419.35	1854.84	6.22	0.0001
	Within Groups	237	70672.75	298.20		
Leaf density	Between Groups	4	2285.16	571.29	33.54	3.60E-22
	Within Groups	237	4036.85	17.03		
Leaf length	Between Groups	4	305.66	101.89	2.68	0.0478
	Within Groups	237	8017.02	38.00		
Leaf width	Between Groups	4	18.20	4.55	1.41	0.23
	Within Groups	237	766.54	3.23		
Spike length	Between Groups	4	2479.39	619.85	14.51	1.28E-10
	Within Groups	237	10118.83	42.70		
No. of tillers	Between Groups	4	18.1398	4.5350	2.63	0.0350
	Within Groups	237	408.3507	1.7230		
Rhizome thickness	Between Groups	4	28.626	7.157	2.92	0.022
	Within Groups	237	579.119	2.444		
Weight of Rhizome	Between Groups	4	259016.4	64754.11	1.06	0.0373
	Within Groups	237	1437445	60651.7		

Table 4.6 One-way ANOVA for district-wise different morphological characters among five cultivars of *Zingiber officinale* of Sikkim Himalaya. Majority of the morphological characters differed among cultivars districts wise suggesting the role of ecological factors.

character	variation	DF	squares	Squares	value	*P* value
Plant height	Between Groups	3	1366.901	455.6336	2.55	0.045
	Within Groups	237	42224.84	178.1639		
Canopy	Between Groups	3	2114.557	704.8522	2.37	0.071
	Within Groups	237	70461.15	297.3044		
Leaf density	Between Groups	3	44.2508	14.75027	0.60	0.610197
	Within Groups	237	5746.58	24.24717		
Leaf length	Between Groups	3	181.0231	60.34102	1.68	0.170373
	Within Groups	237	8478.751	35.77532		
Leaf width	Between Groups	3	2.150124	0.716708	3.25	0.022366
	Within Groups	237	52.16546	0.220107		
Spike length	Between Groups	3	455.7782	151.9261	3.02	0.030455
	Within Groups	237	11916.75	50.28165		
No. of tillers	Between Groups	3	13.22	4.417541	2.62	0.05
	Within Groups	237	398.90	1.683424		
Rhizome thickness	Between Groups	3	77.42154	25.80718	2.85	0.037994
	Within Groups	237	2143.942	9.046168		
Weight of Rhizome	Between Groups	3	15783.98	526234.66	7.98	0.000043
	Within Groups	237	156422.16	65925.831		
No of shoots	Between Groups	3	168.5572	56.1857	4.40	0.0049
	Within Groups	237	2972.2950	12.7566		
Shoot diameter	Between Groups	3	7.865169	2.621723	6.69	0.000234 0
	Within Groups	237	92.77488	0.391455		
Spike diameter	Between Groups	3	25.90819	8.636062	3.30	0.021029
	Within Groups	237	619.6768	2.61467		

Table 4.7 One-way ANOVA for different morphological characters among five cultivars of *Zingiber officinale* based on soil types of Sikkim Himalaya. Maximum morphological traits were not differed among ginger cultivars soil texture wise, which indicate that soil texture do not have any role for differentiation of ginger cultivars.

Plant height	Between Groups	4	2502.229	625.557	2.588	0.03
	Within Groups	237	57284.74	241.708		
Canopy	Between Groups	3	242.756	80.9187	0.255	0.85
	Within Groups	214	67754.53	316.609		
Leaf density	Between Groups	4	120.945	30.2364	1.155	0.33
	Within Groups	237	6201.071	26.1648		
Leaf length	Between Groups	4	190.297	47.5743	1.288	0.27
	Within Groups	237	8752.166	36.9289		
Leaf width	Between Groups	4	4.15029	1.03757	0.175	0.95
	Within Groups	237	1403.578	5.92227		
Spike length	Between Groups	4	17.0423	4.26057	0.080	0.98
	Within Groups	237	12581.91	53.0882		
No. of tillers	Between Groups	4	1.75313	0.43828	0.244	0.91
	Within Groups	237	424.7374	1.79214		
Rhizome thickness	Between Groups	4	182.791	45.6977	5.686	0.00021
	Within Groups	237	1904.689	8.03666		
Wt. of Rhizome	Between Groups	4	757777.3	189444.3	2.836	0.025
	Within Groups	237	1582997	66793.1		
No. of shoots	Between Groups	4	185.092	46.2732	3.075	0.01
	Within Groups	237	3565.51	15.0443		
Shoot dia.	Between Groups	4	5.090529	1.27263	3.097	0.01
	Within Groups	237	97.35835	0.41079		
Spike diameter	Between Groups	4	18.522	4.63057	1.749	0.13
	Within Groups	237	627.437	2.647413		

±3.48), *Gorubthangey* (21.89 ± 2.21) and *Majhauley* (21.89 ± 2.34) (Table 4.4 and Figure 15 C). One-way ANOVA showed the difference in means of leaf length per plant was statistically significant among five ginger cultivars (F_{value} = 2.68, $P < 0.0478$) (Table 4.5), whereas the difference in means of leaf length per plant was statistically insignificant among four districts (F_{value} = 1.68, $P < 0.1705373$) and soil types (F_{value} = 1.28, $P < 0.275$) (Tables 4.6 and 4.7). Highest leaf width was recorded in *Gorubthangey* cultivar with a mean value of (3.07 ± 0.52), followed by *Bhaisay* (3.02 ± 0.43), *Charinangrey* (2.99 ± 0.46), *Majhauley* (2.9 ± 0.44) and *Jorethangey* (2.81 ± 0.38) (Table 4.4 and Figure 15 C). The difference in means of leaf width per plant was found statistically insignificant among five ginger cultivars (F_{value} = 1.41, $P < 0.23$) and soil types (F_{value}= 0.175, $P < 0.950$), whereas it was found statistically significant among four districts (F_{value} =3.25, $P < 0.022$) (Tables 4.6 & 4.7). The petiole length was recorded high in *Bhaisay* (0.6 ± 0.03), followed by *Majhauley* (0.6 ± 0.04), *Charinangrey* (0.5 ± 0.12), *Gorubthangey* (0.5 ± 0.02) and *Jorethangey* (0.4 ± 0.04) (Table 4.4). (F_{value} = 1.25, $P < 0.002$).The longest spike length per plant was recorded for *Bhaisay* cultivar with a mean value of 23.86 ± 7.66 cm, followed by *Majhauley* (21.42 ± 6.14), *Jorethangey* (20.92 ± 5.28), *Gorubthangey* (20.50 ± 6.95) and *Charinangrey* (12.78 ± 3.64) (Table 4.4 and 4.19 B). The difference of means for spike length per plant was found statistically significant among five ginger cultivars (F_{value} = 14.51, $P < 1.28027E-10$) and among four districts (F_{value} = 3.02, $P < 0.030455$) (Tables 4.6 and 4.7).

The rhizome thickness per plant was recorded high for *Bhaisay* cultivar with a mean value of 6.53 ± 1.96, followed by *Majhauley* (5.06 ± 2.03), *Gorubthangey* (2.28 ±

1.00), *Jorethangey* (2.67 ± 1.06) and *Charinangrey* (1.98 ± 0.69) (Table 4.4 and Figure 16 A). One-way ANOVA showed that the difference in means of rhizome thickness per plant was statistically significant among five ginger cultivars (F_{value} = 2.92, P <0.0224) (Table 4.5), among four districts (F_{value} = 2.85, P < 0.037994) and different soil types (F_{value} = 2.836, P < 0.0251) (Tables 4.6 and 4.7). Weight of ginger rhizome per plant was recorded maximum in *Bhaisay* cultivar with a mean value of 390.00 ± 18.81, followed by *Majhauley* (362.53 ± 252.36), *Gorubthangey* (284.16 ±1 64.04), *Jorethangey* (161.29 ± 78.43) and *Charinangrey* (48.77 ± 19.86) (Figure 16 D). The difference in means of ginger rhizome weight per plant was statistically significant among five different cultivar (F_{value} =1.06, P < 0.0373) (Table 4.5), among four districts (F_{value} = 7.98, P < 0.000043) and soil types (F_{value} = 2.836, P < 0.0251) (Tables 4.6 and 4.7) and various thickness of five cultivars of ginger rhizome is depicted in (Figure 25).

In order to reveal the functional relationships between quantitative morphological characters recorded for five ginger cultivars, a simple linear regression analysis was used. Of the several characters analysed on quantitative characters (Table 4.4), the significant positive and negative relationships were recorded between the (i) mean plant height per plant and mean weight of rhizome (R^2=0.08; P < 0.0001), (ii) mean canopy per plant mean weight of rhizome per plant (R^2=-9.65; P < 0.0001), (iii) mean number of shoots per plant and weight of rhizome per plant (R^2=-0.90; P < 0.0001, (iv) mean number of shoots per plant and mean number of leaf density per plant (R^2=0.997; P < 0.0001), (v) mean shoot diameter per plant and weight of rhizome per plant (R^2=-8.01; P < 0.0001). (vi) mean shoot diameter and mean number of shoots per plant (R^2=0.999; P < 0001), (vii) mean canopy per plant and mean number of shoots per plant (R^2= 0.143; P < 0.001) and (viii) mean weight of rhizome per plant and mean

number of leaf density per plant (R^2= 0.248; P < 0.05) mean leaf density per plant and mean weight of rhizome per plant (R^2= 6.53; P < 0.001) (ix) mean leaf length per plant and weight of rhizome per plant (R^2= 3.99; P < 0.001), (x) mean rhizome thickness per plant and mean spike length per plant. (xi) Mean number of shoots per plant and mean weight of rhizome weight (R^2= -1.09; P < 0.001) (Figure 17 and 18).

4.1.7.2 Qualitative characterization

Variations in qualitative morphological characters were recorded in different cultivars of ginger. The shoots were dark green in *Majhauley* and *Bhaisay* cultivars, green in *Gorubthangey* and light green in *Jorethangey* and in *Charinangrey* (Table 4.4). Leaf intensity colour was green in *Majhauley* and *Bhaisay* cultivars, dark green in *Gorubthangey* and light green in *Jorethangey* and *Charinangrey* (Table 4.4). Matured ginger spike bract tip was recorded with red and white in *Majhauley* cultivar (Figure 19 and 20), pale yellow in *Charinangrey* and Crimson in *Bhaisay*, *Gorubthangey* and *Jorethangey* cultivars. Shape of ginger rhizome was recorded as straight in *Majhauley*, *Bhaisay* and *Gorubthangey* cultivars and curved and zigzag in *Charinangrey* and *Jorethangey* cultivars (Figure 21). The different morphological characters measured among different cultivars with the mean values are shown in (Figure 22, 23 and 24).

4.1.7.3 Crop duration of ginger cultivars in Sikkim

Variation was also recorded in crop duration pattern. Medium crop duration pattern of 200-210 days was recorded for the cultivars *Majhauley*, *Bhaisay* and *Gorubthangey*. The cultivars *Jorethangey* and *Charinangrey* were recorded for long duration of more than 210 days (Table 4.4).

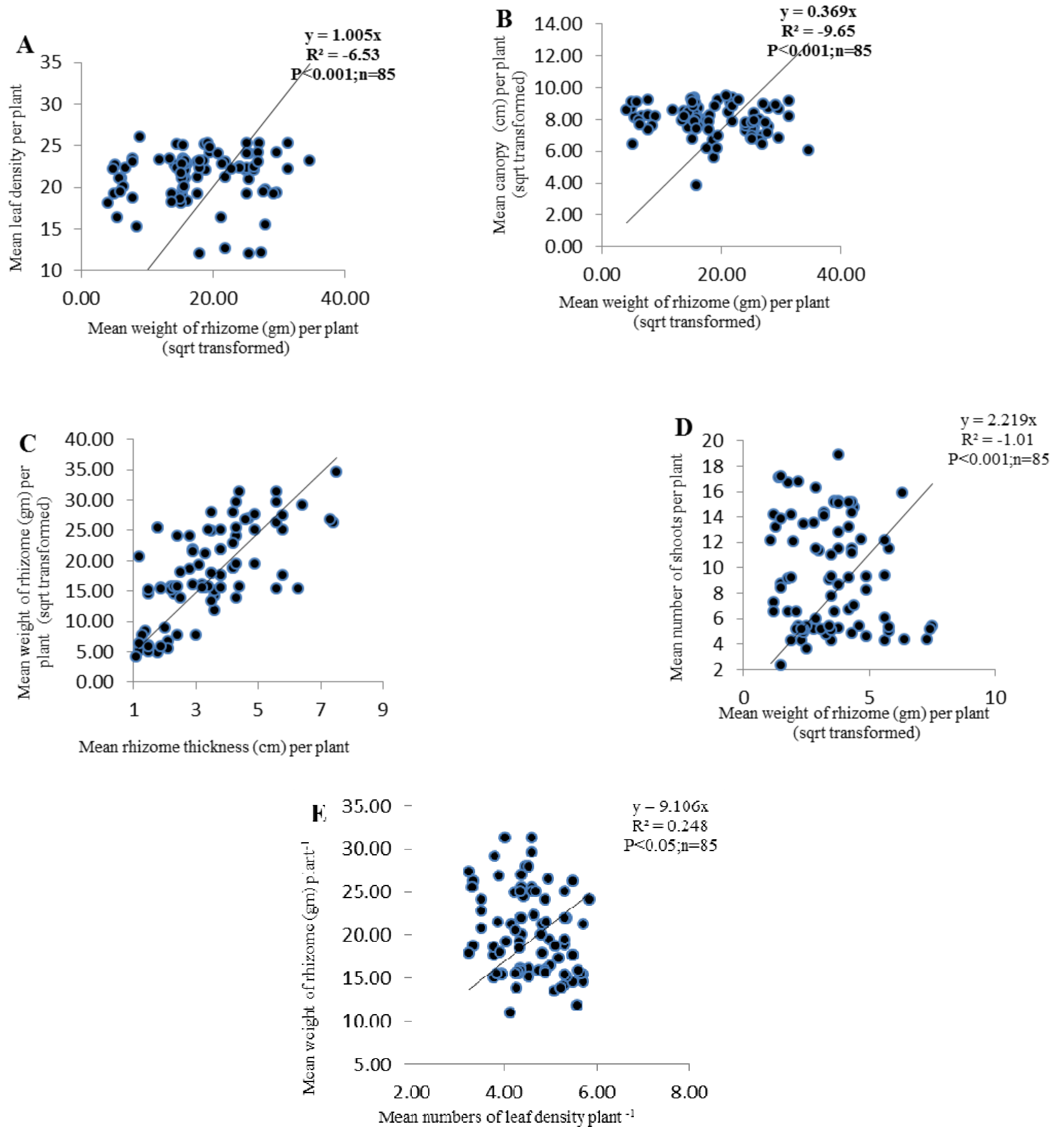


Figure 18 Linear relationships between Mean leaf density pe
rhizome (gm.) per plant (sqrt transformed), Mean leaf lengt
weight of rhizome (gm.) per plant (sqrt transformed), Mean w
plant (sqrt transformed) and Mean rhizome thickness (cm) p
shoots per plant and Mean weight of rhizome (gm.) per plant (
weight of rhizome (gm.) plant^{-1} and Mean numbers of leaf densit

Figure 19 Ginger flower and fully developed spike of *Bhaisay* cultivar at North Regu, East Sikkim. Showing crimson colour bract tip with red and white flowers.

Figure 20 Ginger flower bud of Majhauley cultivar. Showing red and white bract tip colour of matured spike.

Figure 21 Representing shape of ginger rhizome showed straight rhizome in Majhauley, Bhaisay and Gorubthangey cultivars and curved and zigzag in Charinangrey & Jorethangey cultivars

Figure 22 Plant heights of five different ginger cultivars of Sikkim Himalaya. A) Majhauley cultivar 69.90 cm B) Charinangrey (64.87 cm), C) Gorubthangey (69.60 cm) D) Bhaisay (74.52 cm) and E) Jorethangey (70.142 cm).

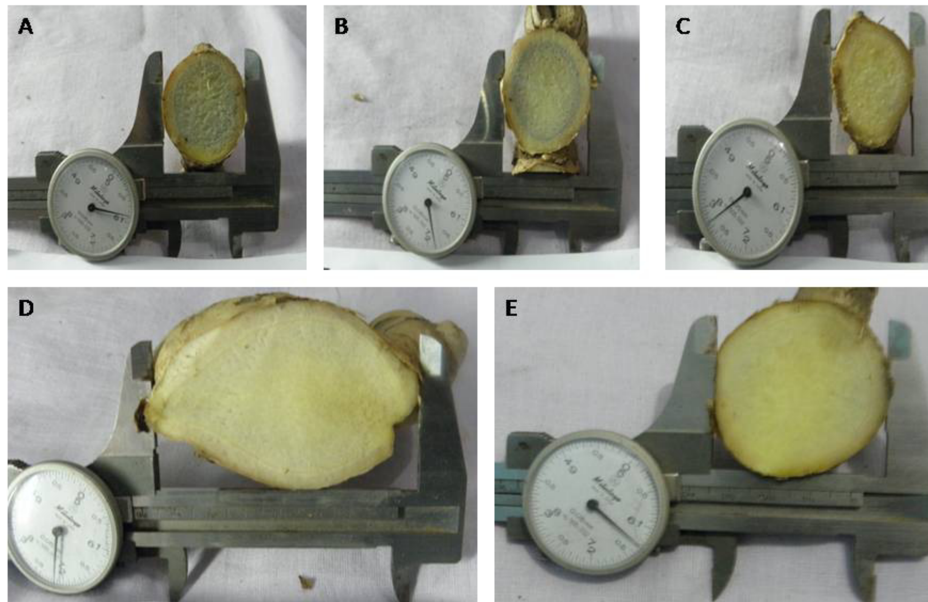


Figure 25 Thickness of ginger rhizome of five different cultivars of Sikkim. Thin rhizome of Charinangrey (1.98 cm) A); Medium rhizome of Gorubthangey (2.67 cm) B); Medium rhizome of Jorethangey (2.68 cm) C); Bold rhizome of Bhaisay (6.53 cm) D); and Bold rhizome of Majhauley (5.05 cm) E). The mean different size of rhizome thickness suggesting that rhizome thickness is one of the important traits for differentiation among ginger cultivar.

Figure 23 Leaf lengths of five cultivars of *Zingiber officinale* of Sikkim Himalaya. Majhauley 22.5 cm A); Bhaisay 23.2 cm B) ; Charinangrey 24.3 cm C) Gorubthangey cm 22.1 cm.

Figure 24 Leaf widths of five cultivars of *Zingiber officinale* of Sikkim Himalaya. Majhauley 2.6 cm A); Bhaisay 3.5 cm B); Charinangrey 2.8 cm C); Jorethangey 2.7 cm D) Gorubthangey 2.5 cm D).

4.1.7.4 Principle Component Analysis

In order to show the relatedness in morphological characters of 5 different ginger cultivars, data on quantitative and qualitative morphological characters was used. The result of the 237 X 13 correlation matrix data set showed ten Eigenvectors (Principal Components, PCs) (Figure 27). The components loading of thirteen variables along the three axes are given in (Table 4.8 and Figure 26). The first three PCs (Comp 1, Comp 2, and Comp 3) accounted for the total variance of 51.78 % differing five cultivars. The first axis (PC1) explained 27.64 % of the variance. Only one qualitative character (Number of shoots), and quantitative characters Leaf Density, Spike Length and Shoot Diameter were found considerably significant taxonomic values along the component one axis. The second axis (PC2) explained 13.79% of the variance in which only one qualitative character (Rhizome Shape) and two quantitative characters were (Spike Length and Leaf Density) was found to have taxonomically significant values. In the third axis (PC3), which accounted for 10.3 % of the variance, only two quantitative characters were Rhizome thickness and Leaf Length revealed taxonomically significant differentiating character to discriminate between the five cultivars of *Zingiber officinale*. The scatter plot of first PC1 and PC2 based on thirteen morphological characters are illustrated in (Figure 26 and 28). Based on 13 morphological variables of 237 samples of five cultivars of *Zingiber officinale*. Three separate groups were formed, large group include cultivars *Majhauley* and *Bhaisay* (BS+MJ), second large group comprising cultivars *Gorubthangey* and *Jorethangey* (GN+JT) and smallest separate single group representing cultivar *Charinangrey* (CN). The PCA showed the

Charinangrey form a distinct group and other four cultivars shares overlapping variations in morphological characters (Figure 28).

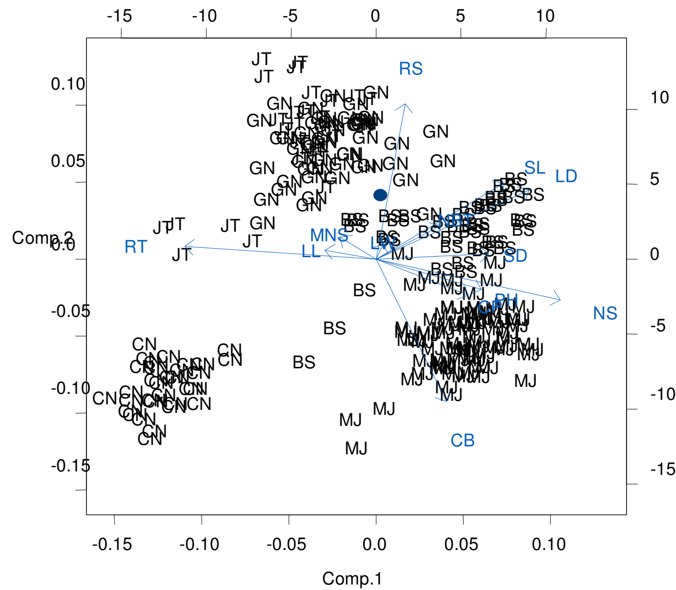


Figure 26 Showing principal component analysis (PCA) of first two components based on 13 variables of 237 samples of five cultivars of *Zingiber officinale* includes Majhauley, Bhaisay, Charinangrey, Gorubthangey, and Jorethangey. Three separate groups were formed, in one group contains cultivars Majhauley and Bhaisay (BS+MJ), second group comprise cultivars Gorubthangey and Jorethangey (GN +JT) and single group representing cultivar Charinangrey (CN).

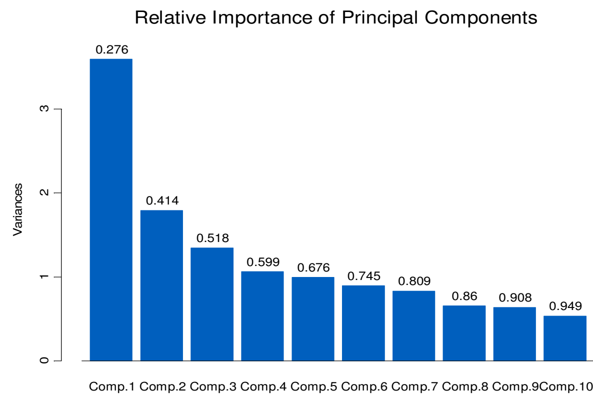


Figure 27 Histogram showing relative importance of principal components among 10 components includes five different cultivars of *Zingiber officinale*. Numerical data represents Eigen values ordered from largest to the smallest.

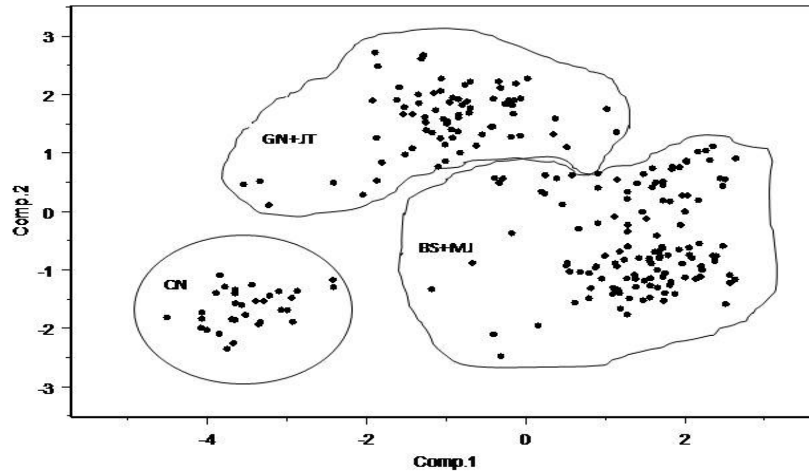


Figure 28 Scatter plot of first two components from principal component analysis (PCA) based on 13 variables of 237 samples of five cultivars of *Zingiber officinale*, includes Majhauley, Bhaisay, Charinangrey, Gorubthangey, and Jorethangey. Three separate groups were formed, large group include cultivars Majhauley and Bhaisay (BS+MJ), second large group comprising cultivars Gorubthangey and Jorethangey (GN +JT) and smallest separate single group representing cultivar Charinangrey (CN). The PCA showed the Charinangrey form a distinct group and other four cultivars shares overlapping varations in morphological characters.

Table 4.8 Loadings of the first three principal components (PCs) for thirteen characters (quantitative and qualitative) from the analysis of 237 individuals of *Zingiber officinale* collected from Sikkim Himalaya, India.

	Comp.1	Comp.2	Comp.3
No of Buds	0.1647	0.1354	-0.0963
Plant Height	0.2662	-0.1236	0.0960
Canopy	0.2332	-0.1459	-0.0768
Mean number of shoots	-0.0905	0.0886	-0.2650
Shoot Diameter	0.2846	0.0214	0.1984
Leaf Density	0.3866	0.2794	0.0545
Leaf Length	-0.1291	0.0362	0.6288
Rhizome Thickness	0.0181	0.0614	0.6615
Spike Length	0.3235	0.3084	-0.0216
Leaf Width	-0.4831	0.0505	0.0050
Colour of Bract	0.1780	-0.5773	-0.0938
Rhizome Shape	0.0733	0.6290	-0.1417
No. of Shoots	0.4657	-0.1646	0.0265
Proportions of Variations	27.64	13.79	10.35
Cumulative Proportions	27.64	41.43	51.78

4.2 Tissue culture

4.2.1 Micropropagation of five cultivars of *Zingiber officinale*

One of the major objectives of the present study was to produce disease-free planting materials of ginger cultivars of Sikkim Himalayan region and to standardize an efficient protocol for multiplication of ginger micropropagules (Figure no. 29 and 30). Different stages of *in-vitro* micropropagation ginger are depicted in (Figure 31, A-F).

4.2.2 Source of explants for micropropagation of ginger

Not all the tissues or organs of a plant are equally capable of exhibiting morphogenesis (Hartmann *et al.,* 1997). In the present investigation to identify the true explants for micropropagation of ginger, various plants parts were tried as explants. Among various explants, shoot tips of underground rhizome gave the good response for initial growth and produced highest number of multiple shoots.

4.2.3 Standardization of the sterilization procedure for different explants

The explants were cleaned under running tap water and then dipped in double distilled water with tween-20 (100:2) for 15 min. The explants were surface sterilized with 0.1 % mercuric chloride for 10-15 min, followed by 6 % sodium hypochlorite for 5-8 min. Explants were then rinsed in 70 % alcohol for two to three min and finally washed in double distilled water.

4.2.4 Identification of suitable basal medium and sucrose concentration

For the selection of best medium and sucrose concentration, sterilized ginger explants were cultured in five different media namely, full and half strength MS medium (Murashige and Skoog, 1962), White's medium (White, 1963), B5 medium

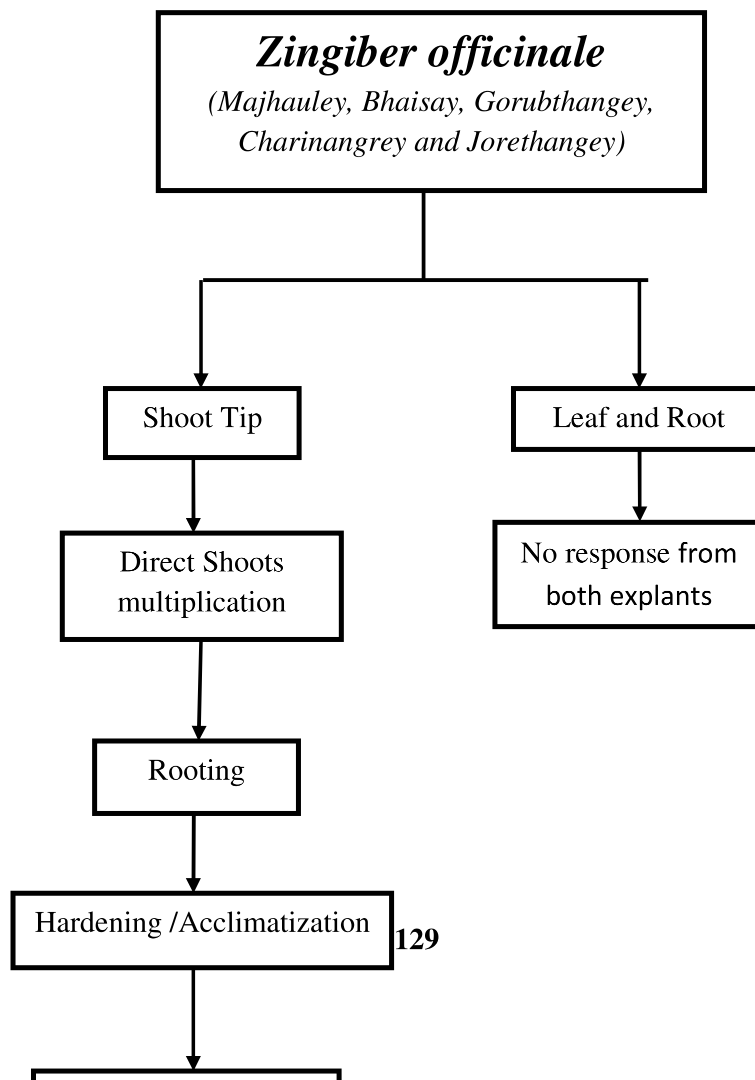


Figure 29 Representing of *in vitro* micropropagation protocol of *Zingiber officinale*

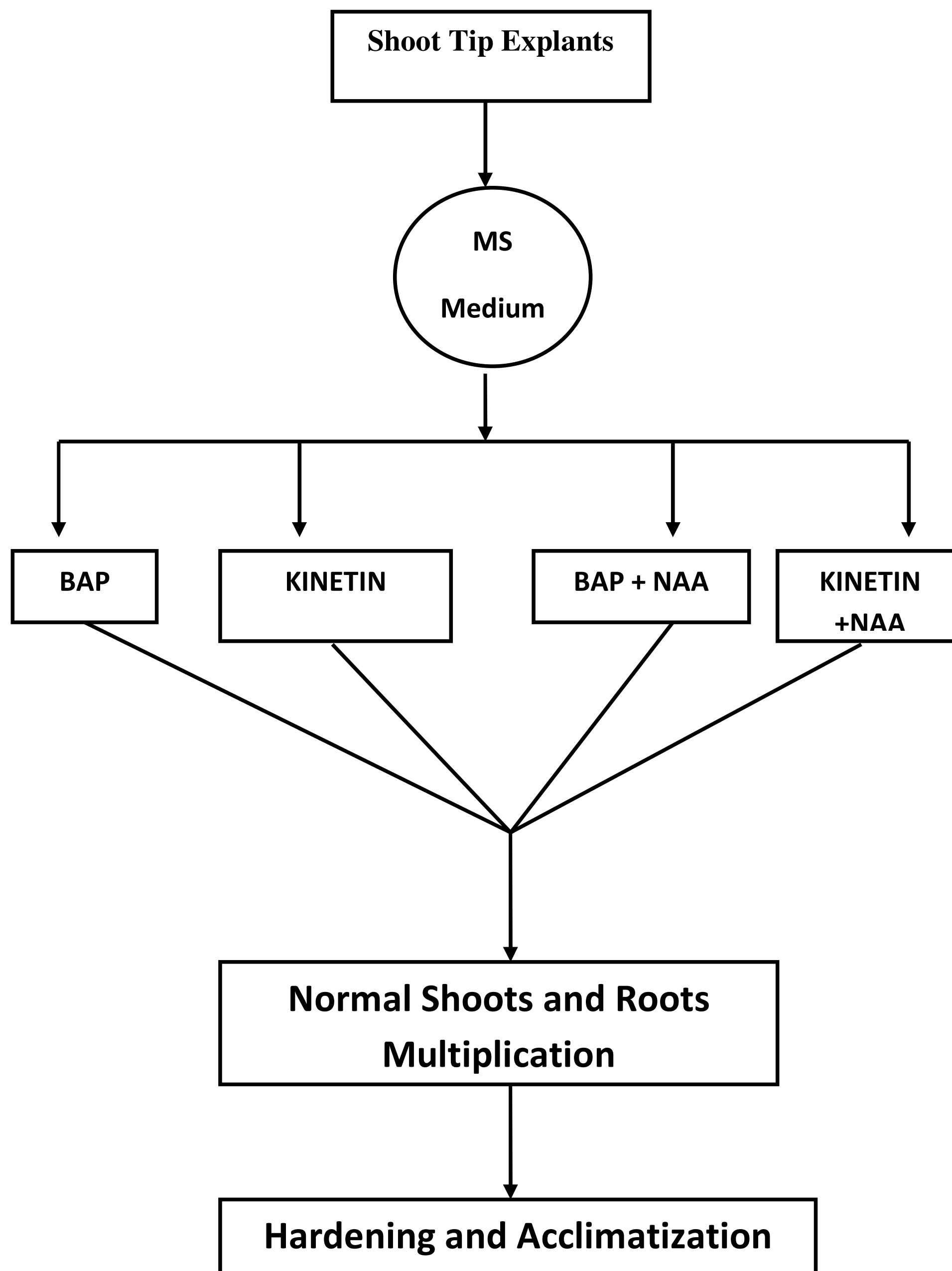


Figure 30 Flow chart showing protocol of *in vitro* micropropagation *Zingiber officinale* of Sikkim Himalaya.

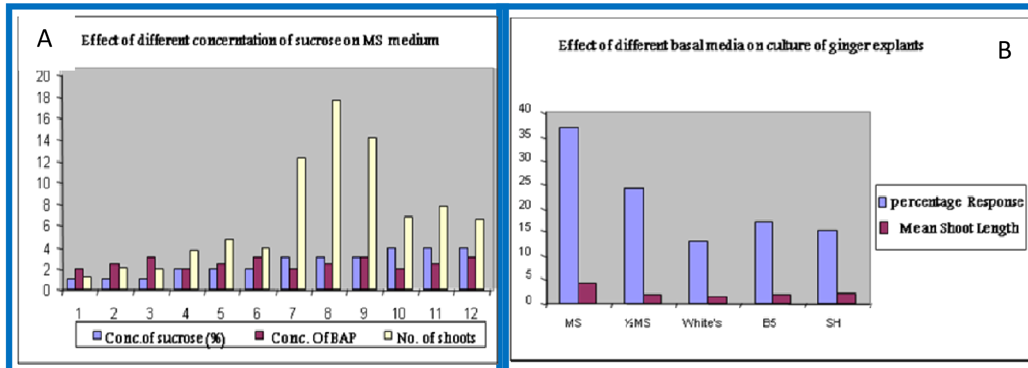


Figure 30.1 A-B. (A) Effect of different Basal media on *Zingiber officinale.* MS medium showed maximum shoots with higher percentage responses; (B) Effect of different sucrose concentration on MS medium 3% sucrose showed maximum number of shoots.

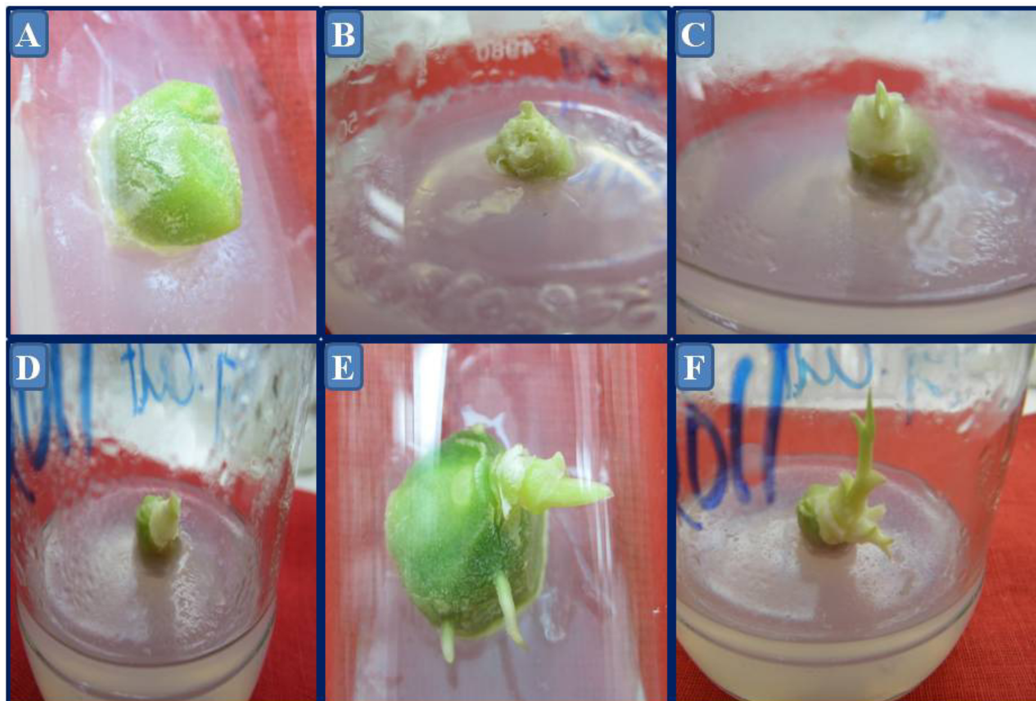


Figure 31 (A-F) Showing different stages of invitro micropropagation of *Zingiber officinale.* Figure (A) Enlargement of explant. (B) Juvenile stage (C-D) Development of shoots at MS+2.5 mg BAP/l +0.5 NAA (E) Development of roots and shoots simultaneously MS+2.5 mg BAP/l +0.5 NAA (F) Initiation of multiple shooting**.**

(Gamborg, 1976) and SH medium (Schenk and Hildebrandt, 1972). Among the different concentrations of culture media, MS medium with 3% sucrose concentration showed good response in multiplication of ginger explants (Figure no. 30.1 A and B), compared with other four media formulations.

4.2.5 Effects of different growth regulators on regeneration of explants

Total 37 (named as GM-1 to GM-37) numbers of different media compositions were formulated for the study of shoot tip culture (Table 4.11). Out of these, GM-23 (MS + BAP 2.5 mg/l + NAA 0.5 mg/l) showed best response in shoot induction within 8-10 days of culture.

4.2.6 Effects of BAP on shoot initiation and multiplication

4.2.6.1 Mean shoots number

Highest average number of shoots (9.25) per explants was recorded in MS medium containing BAP (2.5 mg/l) after 50-60 days of culture (Table 4.9 and Figure 32, C). With the increase or decrease concentration of BAP on the medium the number of shoot decreases. When BAP concentration was increased at 3.0 mg/l and decreased at 2.0 mg/l, numbers of shoots was decreased to 5.87 and 6.15 respectively. While BAP concentration was reduced to 1.5 mg/l concentration and 1 mg/l concentration, shoots number was decreased to 4.67 mg/l and 01.56 respectively (Table 4.9 & Figure 33).

4.2.6.2 Mean number of leaves per plant

Maximum average number of leaves (7.33) was recorded in MS medium fortified with BAP (1.5 mg/l) followed by 6.45 numbers of leaves in MS medium with BAP (1.0 mg/l.). Increase of BAP concentration resulted in the reduction of leaf numbers (Table 4.9).

4.2.6.3 Mean shoots Length

Highest average shoots length (5.79 cm) was recorded in MS medium containing 1.5 mg/l BAP. The minimum mean shoot length (2.52 cm) was recorded in MS containing BAP 2.5 mg/l. (Table 4.9)

4.2.6.4 Mean number of roots

Maximum average number of roots (4.21) was recorded in MS medium fortified with BAP 3.0 mg/l. Least mean root number was traced (0.90) in MS medium in combination with BAP 1 mg/l (Table 4.9).

4.2.6.5 Mean root length

In MS medium with BAP 3.0 mg/l gave highest root length (2.98 cm) and at BAP 2.0 mg/l and 2.5 mg/l concentration, mean root length was recorded 2.51 cm and 2.87 cm (Table 4.9).

4.2.7 Effect of kinetin on shoot initiation and multiplication

4.2.7.1 Mean number of shoots

In the present investigation, maximum mean number of shoots (6.03) was recorded in MS medium supplemented with 2 mg/l kinetin after 50-60 days of culture (Table 4.10)**.** No increase in number of shoots was recorded with further increase or decrease in kinetin concentration.

4.2.7.2 Mean number of leaves

Maximum average number of leaves (7.87) was recorded in MS medium fortified with kn 1.5 mg/. Lowest mean number of leaves was recorded in MS medium fortified with BAP 4.5 mg/l (Table 4.10).

Figure 33 Micropropagation of *Zingiber officinale* on MS medium

- **A.** Kinetin 2.5 mg /l, 20-25 days after culture
- **B.** Kinetin 3 mg /l, 20-25 days after culture
- **C.** Kinetin 3.5 mg /l, 20-25 days after culture
- **D.** BAP 1.0 mg/l, 20-25 days after culture
- **E.** BAP 3.0 mg/l, 20-25 days after culture
- **F.** BAP 3.5 mg/l, 20-25 days after culture

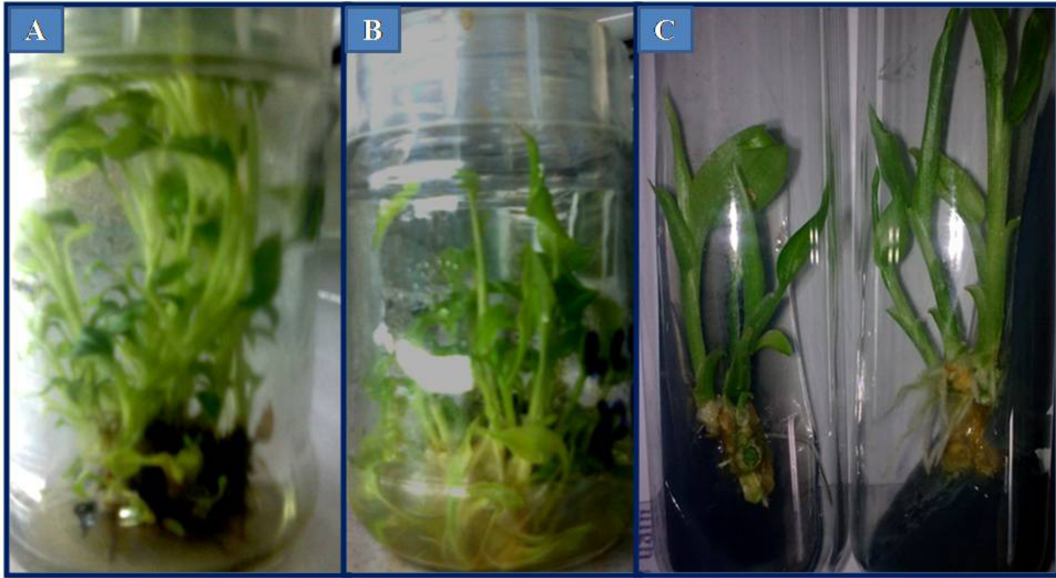

A
B
C

Table 4.9 Effect of different concentration of BAP on MS medium for shoot and root induction from shoot tip of *Zingiber officinale* Rosc of cultivar varieties Charinangrey, Bhaisay, Gorubthangey, Jorethangey and Majhauley of Sikkim Himalayas

Name of media	BAP	Mean No. of Roots	Mean No. of Shoots	Mean No. of leaf	Mean shoots Length (cm)	Mean Root Length (cm)
GM 1	0	0	0	0	0	0
GM 2	1.0	00.90 ±0.01	01.56 ±0.05	06.45 ±0.01	05.76±0.01	01.01 ±0.02
GM 3	1.5	02.48 ±0.02	04.67 ±0.03	07.33 ±0.01	05.79±0.02	01.03 ±0.03
GM 4	2.0	02.51 ±0.03	06.15 ±0.02	04.72 ±0.02	04.52±0.03	02.51 ±0.01
GM 5	2.5	02.23 ±0.01	09.25 ±0.04	05.87 ±0.05	02.52±0.03	02.87 ±0.03
GM 6	3.0	04.21 ±0.05	05.87 ±0.03	04.66 ±0.03	03.76±0.01	02.98 ±0.03
GM 7	3.5	03.67 ±0.04	02.18 ±0.04	05.55 ±0.04	02.97±0.07	01.98 ±0.02
GM 8	4.0	00.00 ±0.0	02.67 ±0.03	06.32 ±0.02	03.94±0.02	00.00 ±0.04
GM 9	4.5	00.00 ±0.0	01.21 ±0.04	02.02 ±0.02	05.54±0.08	00.00 ±0.08
GM 10	5.0	00.00	00.00	00.00	00.00	00.00

Table 4.10 Effect of different concentration of Kinetin on MS medium for shoot and root induction from shoot tip of *Zingiber officinale* Rosc of cultivar varieties Charinangrey, Bhaisay, Gorubthangey, Jorethangey and Majhauley of Sikkim Himalayas.

Name of media	Kinetin	Mean No. of Roots	Mean No. of Shoots	Mean No. of leaf	Mean shoots Length (cm)	Mean Root Length (cm)
GM 11	1.0	01.08 ±0.09	01.08 ±0.01	6.5.37 ±0.05	05.98 ±0.02	03.26 ±0.04
GM 12	1.5	02.05 ±0.03	01.05 ±0.06	07.87 ±0.02	07.70 ±0.02.	03.96 ±0.07
GM 13	2.0	02.03 ±0.02	06.03 ±0.03	04.34 ±0.07	04.63 ±0.05	05.78 ±0.01
GM 14	2.5	04.30 ±0.01	04.80 ±0.02	02.63 ±0.03	05.43 ±0.03	06.56 ±0.02
GM 15	3.0	03.10 ±0.03	04.10 ±0.07	04.08 ±0.02	03.43 ±0.01	06.75 ±0.01
GM 16	3.5	01.05 ±0.04	01.05 ±0.06	05.03 ±0.03	04.63 ±0.05	03.50 ±0.03
GM 17	4.0	01.03 ±0.03	01.03 ±0.02	02.63 ±0.06	03.76 ±0.01	04.98 ±0.01
GM 18	4.5	00.00 ±	00.00	02.03 ±0.03	02.03±0.05	00.00
GM 19	5.0	00.00 ±	00.00	00.00	02.01 ±0.03	00.00

4.2.7.3 Mean shoots length

Highest shoots length (7.70 cm) was recorded in MS medium containing kn 1.5 mg/l followed by (5.98 cm) in MS medium containing kn1 mg/l. The minimum mean shoot length (2.03) was recorded in MS plus kn 4.5 mg/l. Further increase in concentration of Kn, no increase in mean shoot length. At kn 2.5 mg/l concentration, mean shoot length (5.43 cm) was recorded. When concentration of kn to 4.0 mg/l, mean shoot length reduced to 3.76 cm was recorded (Table 4.10).

4.2.7.4 Mean number of roots

Maximum number of mean roots (4.30) was recorded in MS medium fortified with kn 2.5 mg/l, next highest mean root length (3.10) in MS plus kn 3 mg/l. Least mean root number was traced (1.80) in MS medium in combination with kn 1 mg/l (Table 4.10).

4.2.7.5 Mean root length

In MS medium with kinetin 3.mg/l gave highest root length (6.75 cm) and Kinetin at 2.5 mg/l and 2.0 mg/l concentration, mean root length was recorded 6.56 cm and 5.78 cm. At kinetin (3.5 and 4.0 mg/l) concentration, mean root length 3.50 cm and 4.98 cm respectively (Table 4.10).

4.2.8 Effect of growth regulator BAP and NAA on *in-vitro* micropropagation of *Zingiber officinale*

4.2.8.1 Mean number of shoots

The maximum mean number of shoots (19.98) per explant was obtained in MS medium fortified with BAP 2.5mg/l plus NAA 0.5 mg/l, further increase in concentration of BAP 3 mg/l and NAA 0.5 mg/l recorded (17.88) mean number of shoots. However increase or decrease in the concentration of BAP and NAA, recorded

Table 4.11 Effect of different concentration of BAP, Kinetin and NAA on MS medium on shoot induction from shoot tip of *Zingiber officinale* Rosc of cultivar varieties Charinangrey, Bhaisay, Gorubthangey, Jorethangey and Majhauley of Sikkim Himalayas.

Name of media	BAP	Kinetin	NAA	Days for shoot induction
GM 1	0	0.0	0.0	0
GM 2	1.0	0.0	0.0	15-20
GM 3	1.5	0.0	0.0	15-20
GM 4	2.0	0.0	0.0	10-15
GM 5	2.5	0.0	0.0	10-15
GM 6	3.0	0.0	0.0	15-20
GM 7	3.5	0.0	0.0	15-20
GM 8	4.0	0.0	0.0	20-25
GM 9	4.5	0.0	0.0	25-30
GM 10	5.0	0.0	0.0	25-30
GM 11	0.0	1.0	0.0	15-20
GM 12	0.0	1.5	0.0	15-20
GM 13	0.0	2.0	0.0	15-20
GM 14	0.0	2.5	0.0	15-20
GM 15	0.0	3.0	0.0	15-20
GM 16	0.0	3.5	0.0	30-35
GM 17	0.0	4.0	0.0	45-50
GM 18	0.0	4.5	0.0	40-45
GM 19	0.0	5.0	0.0	40-45
GM 20	1.0	0.0	0.0	10-15
GM 21	1.5	0.0	0.5	10-13
GM 22	2.0	0.0	0.5	08-12
GM 23	2.5	0.0	0.5	08-10
GM 24	3.0	0.0	0.5	10-12
GM 25	3.5	0.0	0.5	15-18
GM 26	4.0	0.0	0.5	15-20
GM 27	4.5	0.0	0.5	30-35
GM 28	5.0	0.0	0.5	30-35
GM 29	0.0	1.0	0.5	20-25
GM 30	0.0	1.5	0.5	10-15
GM 31	0.0	2.0	0.5	10-15
GM 32	0.0	2.5	0.5	10-12
GM 33	0.0	3.0	0.5	10-12
GM34	0.0	3.5	0.5	20-25
GM35	0.0	4.0	0.5	25-30
GM 36	0.0	4.5	0.5	30-35
GM 37	0.0	5.0	0.5	30-35

decrease in mean number of shoots. At BAP 2 mg/l with NAA 0.5 mg/l concentration, recorded 12.66 mean numbers of shoots (Table 4.12 & Figure 32, A-B).

4.2.8.2 Mean shoots length

The present results revealed that mean shoots length (5.83 cm) was highest in MS medium containing combination of BAP (1.0 mg/l) and NAA (0.5 mg/l). With further increase in BAP concentration the mean of shoot length decreases. When BAP concentrations at (2.0 mg/l, 2.5 mg/l, and 3 mg/l) recorded mean shoots length was (4.64 cm, 3.09 cm and 3.97 cm) respectively (Table 4.12).

4.2.8.3 Mean number of leaves

Maximum mean number of leaves was recorded in MS medium supplemented with BAP 2.0 mg/l and NAA 0.5 mg/l. At MS medium with combination of NAA 0.5 mg/l and increased concentrations of BAP (2.5 mg/l, 3.0 mg/l 3.5 mg/l) recorded a reduction in mean number of leaves (6.96, 6.55 and 6.05) respectively (Table 4.12).

4.2.8.4 Mean number of roots

Maximum number of mean roots (6.78) was recorded in MS medium fortified with BAP (2.5 mg/l) and NAA (0.5 mg/l), next highest mean root length (5.56) in MS plus BAP 3 mg/l and NAA 0.5 mg/l. Least mean root number was traced (1.05) in MS medium in combination with BAP 1 mg/l and NAA 0.5 mg/l (Table 4.12 and Figure 34).

4.2.8.5 Mean root length

In MS medium with BAP 2.0 mg/l plus NAA 0.5 mg/l gave highest root length (07.01 cm) and at BAP 2.5 mg/l and 3.0 mg/l concentration, mean root length was recorded 5.53 cm and 6.86 cm. At BAP (3.5 and 4.0 mg/l) and NAA (0.5 mg/l) concentration, mean root length 4.07 cm and 5.68 cm respectively (Table 4.12).

4.2.9 Effect of growth regulator kinetin and NAA on *in-vitro* micropropagation of *Zingiber officinale*

4.2.9.1 Mean number of shoots

The maximum mean number of shoots (14.15) per explant was obtained in MS medium fortified with kinetin 2.0 mg/l plus 0.5 NAA, further increase in concentration of kinetin 3.0 mg/l and NAA 0.5 mg/l recorded (13.22) mean number of shoots. However, increase or decrease in the concentration of BAP and NAA, recorded decrease in mean number of shoots. At BAP 3.5 mg/l with NAA 0.5 mg/l concentration, recorded 6.18 mean numbers of shoots (Table 4.13).

4.2.9.2 Mean shoots length

The present work highest mean shoots length (5.98 cm) was recorded in MS medium in combined effect of kinetin 1.0 mg/l and NAA 0.5 mg/l. With further increase in kinetin concentration the mean of shoot length decreases. When kinetin concentrations at (2.0 mg/l, 2.5 mg/l, 3 mg/l) recorded mean shoots length was (4.63 cm, 4.43 cm and 4.70 cm) respectively (Table 4.13).

4.2.9.3 Mean number of leaves

The current finding revealed that maximum mean number of leaves was recorded in MS medium supplemented with kinetin 3.5 mg/l and NAA 0.5 mg/l. At MS medium in combination of NAA 0.5 mg/l and increased concentrations of BAP (2.5 mg/l, 3.0

mg/l 3.5 mg/l) recorded reduced mean number of leaves (6.96, 6.55 and 06.05) respectively (Table 4.13).

4.9.4 Mean number of roots

Maximum number of mean roots (5.03) was recorded in MS medium fortified with kinetin 2.0 mg/l and NAA 0.5 mg/l, next highest mean root length (4.80) in MS with kinetin 2.5 mg/l and NAA 0.5 mg/l. Least mean root number was traced (1.08) in MS medium in combination with kinetin 1 mg/l and NAA 0.5 mg/l (Table 4.13).

4.2.9.5 Mean root length

In MS medium with Kinetin 3 mg/l plus NAA 0.5 mg/l gave the highest root length (6.75 cm) and at further increased or decreased in concentration of kinetin with NAA 0.5 mg/l, recorded no further increased in mean root length. At MS medium in combination with kinetin 2.0 mg/l and 2.5 mg/l concentration, mean root length was recorded 5.78 cm and 6.56 cm respectively (Table 4.13).

4.2.9.6 Rooting of *in-vitro* raised shoots

The present investigation revealed that the regenerated micropropagules produced a good number of roots in the same multiple shoots hormonal concentration. The maximum mean number of roots (6.78) was recorded per explant in MS medium with BAP (2.5 mg/l) and NAA (0.5 mg/l), and lowest (04.21) in MS medium fortified with BAP mg/l (Table 4.13 and Figure 34).

4.2.10 Hardening and acclimatization

Healthy *in-vitro* grown plantlets having 5-6 cm long shoot with a good number of roots were taken for the acclimatization or hardening of plantlets. The rooted plantlets were transferred to small earthen pots containing combination of soil, farmyard manure,

Figure 35 Ac

A. *Zingi* ing viz. soil, farmyard
manu

B. Accli

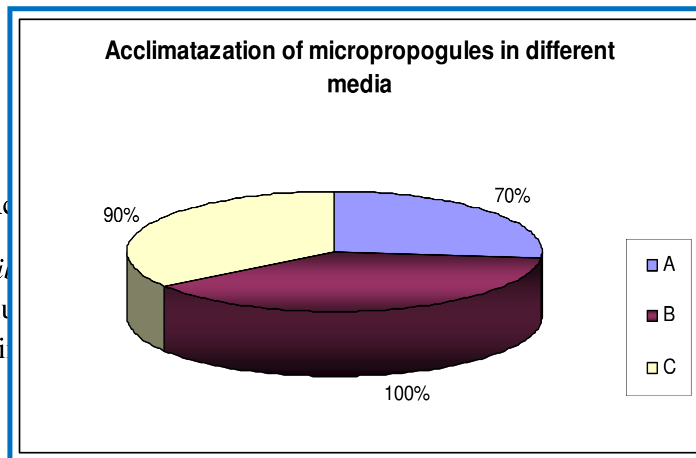


Figure 36 Acclimatization of micropropagules of *Zingiber officinale* 100% were survived in media B, followed by A and C.

Table 4.12 Effect of different concentration of BAP and 0.5mg/l NAA on MS medium for shoot and root induction from shoot tip of *Zingiber officinale* Rosc of cultivar varieties Charinangrey, Bhaisay, Gorubthangey, Jorethangey and Majhauley of Sikkim Himalayas.

Name of media	BAP	NAA	Mean No. of Roots	Mean No. of Shoots	Mean No. of leaf	Mean Root Length (cm)	Mean shoots Length (cm)
GM 20	1.0	0.5	01.05 ±0.03	02.88 ±0.03	05.35 ±0.01	02.86 ±0.01	05.83 ±0.02
GM 21	1.5	0.5	03.84 ±0.01	05.87 ±0.05	06.85 ±0.02	03.03 ±0.03	04.21 ±0.02
GM 22	2.0	0.5	03.58 ±0.03	12.66 ±0.02	07.25 ±0.01	07.01 ±0.03	05.64 ±0.01
GM 23	2.5	0.5	06.78 ±0.01	19.98 ±0.01	06.96 ±0.03	05.53 ±0.05	04.09 ±0.04
GM 24	3.0	0.5	05.56 ±0.02	17.88 ±0.01	06.55 ±0.01	06.86 ±0.02	04.97 ±0.07
GM25	3.5	0.5	02.68 ±0.02	03.43 ±0.01c	06.05 ±0.01	04.07 ±0.02	03.76 ±0.01
GM26	4.0	0.5	01.08 ±0.01	02.31 ±0.02c	05.78 ±0.04	05.68 ±0.02	03.43 ±0.02
GM 27	4.5	0.5	00.00	02.03 ±0.07	05.63 ±0.03	00.00	03.50 ±0.03
GM 28	5.0	0.5	00.00	02.02 ±0.01	06.61 ±0.02	00.00	03.35 ±0.03

Table 4.13 Effect of different concentration of Kinetin and 0.5mg/l NAA on MS medium for shoot and root induction from shoot tip of *Zingiber officinale* Rosc of cultivar varieties Charinangrey, Bhaisay, Gorubthangey, Jorethangey and Majhauley of Sikkim Himalayas.

Name of media	Kinetin	NAA	Mean No. of Roots	Mean No. of Shoots	Mean No. of leaf	Mean Root Length (cm)	Mean shoots Length (cm)
GM 29	1.0	0.5	01.08 ±0.01	03.56 ±0.02	05.37 ±0.02	03.26 ±0.04	05.98 ±0.02
GM 30	1.5	0.5	01.05 ±0.06	02.67 ±0.03	05.70 ±0.08	03.96 ±0.07	03.86 ±0.01
GM 31	2.0	0.5	05.03 ±0.03	14.15 ±002	06.34 ±0.06	05.78 ±0.01	04.63 ±0.05
GM 32	2.5	0.5	04.80 ±0.02	13.25 ±0.01	05.63 ±0.03	06.56 ±0.02	05.43 ±0.03
GM 33	3.0	0.5	04.10 ±0.07	11.87 ±0.02	04.08 ±0.01	06.75 ±0.01	07.70 ±0.02
GM34	3.5	0.5	01.05 ±0.06	06.18 ±0.01	08.03 ±0.02	03.50 ±0.03	02.87 ±0.01
GM35	4.0	0.5	01.03 ±0.02	02.67 ±0.03	06.63 ±0.03	04.98 ±0.01	03.76 ±0.01
GM 36	4.5	0.5	00.00	02.07±0.01	05.61 ±0.04	00.00	02.03±0.05
GM 37	5.0	0.5	00.00	02.04±0.02	04.97 ±0.05	00.00	02.01 ±0.03

sand and perlite at the ratio of 1:1:1:1 (Figure 35). They survival percentage was recorded as 100%.

4.2.11 T Test for number of shoots

The data were analyzed statistically using the t-test to determine the variations between and within treatment with respect to number of shoots and roots.

The results of statistical analyses on the data revealed that there exists significant difference between and within treatments in explants treated with single growth regulator (BAP and kinetin), (*P* (*T*<=t) two tail = 0.01356997). Whereas, no significant difference (*P* (*T*<=t) two tail =0.321765267) between and within treatments, of the when the explants treated with different combination of growth regulators, However, the number of shoots were recorded highest in second condition.

4.2.11.1 ANOVA for numbers of shoots

The data on number of shoots among the cultivars were analyzed using one-way analysis of variance (ANOVA) to determine the variations between and within treatment with respect to number of shoots and roots. The statistical analysis of the experimental data revealed that there exists no significant difference between and within treatments ($P < 0.0778$). But MS medium with different concentration of growth regulator gave good number of shoots.

4. 3 Screening of ginger for antimicrobial and antioxidant properties

4.3.1 Different solvent extraction of five cultivars of *Zingiber officinale* of Sikkim Himalaya

The rhizome of five different cultivar of *Zingiber officinale* were extracted using five different solvent, starting with the low polar solvent like petroleum ether (PE), ethanol (E) and chloroform (C) for extraction of lower polar compounds, towards higher polarity of solvents like methanol (M) and acetone (A) for extraction of higher polar compounds. The weight of different solvent extraction of ginger rhizome extract is depicted in (Table 4.14).

The weight of extracts (ginger oil) varied across samples (cultivar) and solvents (Table 4.14). The minimum ginger rhizome extract was recorded in ethanol extracts of *Charinangrey* i.e. 69.9 mg (0.70%) and maximum was found in methanol extracts of *Gorubthangey* i.e. 1548.4 mg which was 15.48 %.

In petroleum ether extraction (PEE), maximum yield of plants extracts (ginger oil) was recorded in *Majhauley* (292.4 mg), *Bhaisay* (289.7 mg) and *Jorethangey* (267.5 mg) respectively as shown in (Table 4.14).

In chloroform extraction, *Charinangrey* (559.9 mg) was found to have maximum yield followed by *Gorubthangey*, *Jorethangey*, *Bhaisay* and *Majhauley* (Table 4.14).

With increase in polarity of the solvent for extraction of high polarity of compounds, methanol extraction (ME) of *Gorubthangey* found maximum yield, contain higher polarity compounds followed by *Jorethangey*, *Majhauley*, *Bhaisay* and *Charinangrey* (Table 4.14). The yield of the acetone extraction was as *Gorubthangey*> *Charinangrey*> *Majhauley*>*Bhaisay*> *Jorethangey*.

4.3.2 Antimicrobial activity of five different cultivars of *Zingiber officinale* of Sikkim Himalaya

The statistical analysis using one way ANOVA revealed that the five different gingers cultivars had highly significant effect ($P < 7.69778E\text{-}15$) on the level of inhibition recorded (Table 4.15). Antimicrobial activity of the extracts was determined by disc diffusion assays.

Twenty five extracts tested showed antibacterial activity towards four microorganisms (*Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441), *Klebsiella pneumoniae* (MTCC 432), and *Pseudomonas aeruginosa* (MTCC 424). Only one microorganism (*Escherichia coli* (MTCC 739) did not exhibit any inhibitory activity for all the extracts under study.

The zone of inhibition was recorded to be 12-26 mm in case of MTCC 96 and 9-24 mm in MTCC 441 for tested gram positive bacterial strains. Similarly the zone of inhibition was recorded to be 9-14 mm and 11-18 mm in case of MTCC 424 and MTCC 432 for gram negative bacteria respectively.

The result of antimicrobial activities of the 25 extracts on the test organism is shown in (Table 4.17). The result of this investigation indicates that the extracts of *Zingiber officinale* have antimicrobial activities.

4.3.2.1 Activity against gram-positive bacteria

Each microorganism was examined *in-vitro* with 25 ginger samples consisting of five varieties of ginger each extracted with five different solvent. The microorganism *Staphylococcus aureus* (MTCC 96) was most susceptible to chloroform extract of *Majhauley*, which showed widest zone of inhibition of 26 mm, followed by sample SG 8 (24 mm zone of inhibition) and 23 mm by sample SG 23.

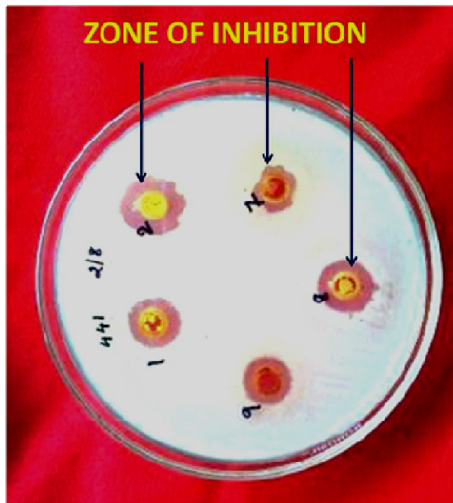


Figure 38 Showing Zone of Inhibition of Z.*officinale* sample 1, 2, 7, 8 and 9 against Gram Negative Bacteria (GNB)

Figure 40 Showing Zone of Inhibition of sample of *Z.officinale* 6, 7, 8 9 and.10 against Gram Positive Bacteria.

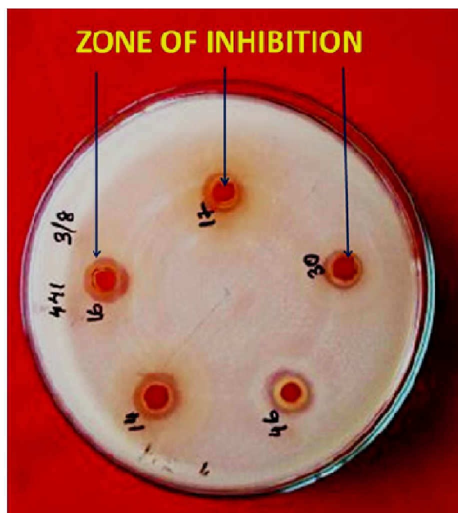


Figure 37 Showing zone of inhibition of Z.*officinale* against (*Bacillus subtilis*) Gram Positive Bacteria (GPB).

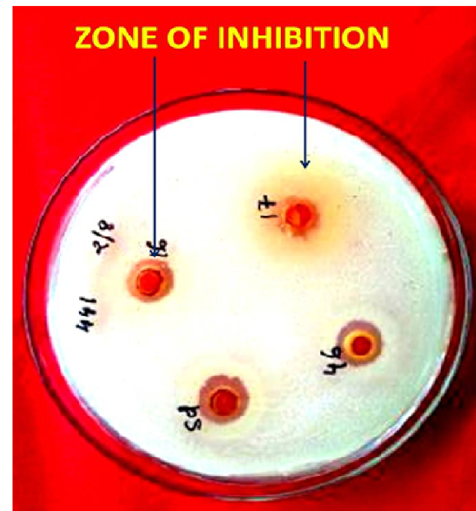


Figure 39 Showing zone of inhibition of Z.*officinale* against *Pseudomonas aerugi*nosa (GNB).

Table 4.14 Showing weight & percentage of different solvents extracts of five indigenous *Zingiber officinale* of Sikkim Himalaya to determine Antimicrobial and Antioxidant activity

Sl. No.	Solvent	Sample	Dried extract (mg)	Percentage %
SG 1	Petroleum ether	*Majhauley*	126.2	1.26
SG 2	Ethanol	*Majhauley*	93.4	0.93
SG 3	Chloroform	*Majhauley*	104.3	1.04
SG 4	Methanol	*Majhauley*	908.6	9.09
SG 5	Acetone	*Majhauley*	838.4	8.38
SG 6	Petroleum ether	*Bhaisay*	289.7	2.90
SG 7	Ethanol	*Bhaisay*	236.9	2.37
SG 8	Chloroform	*Bhaisay*	357	3.57
SG 9	Methanol	*Bhaisay*	891.3	8.91
SG 10	Acetone	*Bhaisay*	629	6.29
SG11	Petroleum ether	*Gorubthangey*	292.4	
SG 12	Ethanol	*Gorubthangey*	311	
SG 13	Chloroform	*Gorubthangey*	392.3	
SG 14	Methanol	*Gorubthangey*	1548.4	15.48
SG 15	Acetone	*Gorubthangey*	1059	10.59
SG 16	Petroleum ether	*Jorethangey*	267.5	2.68
SG 17	Ethanol	*Jorethangey*	270.4	2.70
SG 18	Chloroform	*Jorethangey*	384.6	3.85
SG 19	Methanol	*Jorethangey*	929.1	9.29
SG 20	Acetone	*Jorethangey*	212.8	2.13
SG 21	Petroleum ether	*Charinangrey*	159	1.59
SG 22	Ethanol	*Charinangrey*	69.9	0.70
SG 23	Chloroform	*Charinangrey*	559.9	5.60
SG 24	Methanol	*Charinangrey*	610	6.10
SG 25	Acetone	*Charinangrey*	956.4	9.56

Figure 40 Showing Zone of Inhibition of sample of *Z.officinale* 6, 7, 8 9 and.10 against Gram Positive Bacteria.

The most susceptible/sensitivity to growth inhibition of *Staphylococcus aureus* was recorded in chloroform extract of *Zingiber officinale* irrespective of the cultivar variety. Furthermore, in ethanol extract the highly susceptible growth of microorganism (*Staphylococcus aureus*) was observed in *Bhaisay* followed by *Charinangrey*, *Gorubthangey*, *Majhauley* and *Jorethangey* respectively (Table 4.17).

The microorganism *Bacillus subtilis* (MTCC 441) was most susceptible to ginger extract (Figure 37). The highest zone of inhibition were obtained with chloroform extract of *Majhauley* (24 mm), followed by *Bhaisay* (21 mm) and *Gorubthangey* (12 mm), *Charinangrey* (11 mm) respectively.

The least sensitivity to *Bacillus subtilis* was recorded with *Jorethangey* (9 mm). The other solvents extracts also showed modest sensitivity to microorganism with *Bacillus subtilis* except sample SG 17 and 18 showed least sensitivity as shown in (Figure 37). Twenty five ginger extracts showed statistically significant difference ($P < 0.01790$) with effect on the level of inhibition observed on gram positive bacteria. Gram positive bacteria (*Staphylococcus aureus* and *B. subtilis*) were the most susceptible to growth inhibition by the plant extracts (Figure 41 and 40).

Furthermore an inversely significant correlation between total phenol content and antimicrobial activity were recorded against microorganism *Staphylococcus aureus* (R^2=*-0.53,* $P < 0.001$) and *B. Subtilis* (R^2=*-0.58,* $P < 0.001$) and presence of total flavonoids content (Table 4.15) in different cultivars of ginger showed an inversely significant correlation between microorganism *Staphylococcus aureus* (R^2=*-0.75,* $P < 0.001$) *B. Subtilis* (R^2=*-0.71,* $P < 0.001$) (Figure 44).

Table 4.15 Summary statistics of one way ANOVA of antibacterial activity of *Zingiber officinale* against four microorganisms.

Microorganisms	Source of variation	DF	Sum of Sq.	Mean of Squares	*F* value	*P* value
All	Between Groups	3	807.23	269.0766	33.29	7.69778E-15
	Within Groups	96	775.76	8.080833		
Gram positive	Between Groups	1	81.92	81.9	6.011	0.01790
	Within Groups	48	654.08	13.6		
	Within Groups	48	121.68	2.535		

Table 4.16 One way ANOVA among five different solvent used in antimicrobial activity. The significant difference in five difference solvent suggests that the solvent used for antimicrobial study were highly significant for the study.

Solvent	Source of variation	DF	Sum of squares	Mean of Squares	F value	P value
Petroleum Ether	Between Groups	3	583.56	194.52	23.29	6.21E-11
	Within Groups	80	668.19	8.35		
Chloroform	Between Groups	3	623.27	207.76	24.22	3E-11
	Within Groups	80	686.29	8.58		
Ethanol	Between Groups	3	630.42	210.14	24.25	2.92E-11
	Within Groups	80	693.14	8.66		
Methanol	Between Groups	3	670.52	223.51	24.72	2.041E-11
	Within Groups	80	723.43	9.04		
Acetone	Between Groups	3	647.48	215.83	31.58	1.44E-13
	Within Groups	80	546.76	6.83		

4.3.2.2 Activity against gram-negative bacteria

Least susceptibility towards Gram negative bacteria was observed (Figure 42), may be due to bacterial cell wall outer membrane appears to act as a barrier to many substances including antibiotics (Tortora *et al*., 2001). The results of this investigation against *Klebsiella pneumonia* (Gram-negative bacteria) revealed that the chloroform extract of all ginger were found susceptible the most sensitivity inhibition was measured with *Majhauley* (14) mm, followed by *Bhaisay* and *Gorubthangey* has par activity against this microorganism (12 mm), *Charinangrey* (11mm) respectively. The least sensitivity to *Klebsiella pneumonia* was recorded with *Jorethangey* (9 mm). The other solvents extracts also showed modest sensitivity to microorganism with *Klebsiella pneumonia* except sample SG 17 and SG 18 that showed least sensitivity (Figure 38).

Another gram negative bacteria *Pseudomonas aeruginosa* which was used to be resistant to many conventional drugs (Ayse *et al.,* 2008). The present study observed that most susceptible with chloroform extract of *Majhauley* (18mm zone diameter) (Figure 39), acetone extract of *Charinangrey* (17 mm zone diameter), and chloroform extract of *Bhaisay* (16 mm zone diameter) followed by chloroform extract of *Charinangrey* and par susceptible with chloroform extracts of *Gorubthangey* and *Jorethangey*.

The statistically differences among twenty five ginger extracts showed statistically significant difference ($P < 1.06101E\text{-}09$) with effect on the level of inhibition observed on gram positive bacteria microorganism (Table 4.15). The linear correlation showed highly negative significant difference in the means between total phenol content and antimicrobial activity against *Pseudomonas aeruginosa* (R^2=*-1.02,*

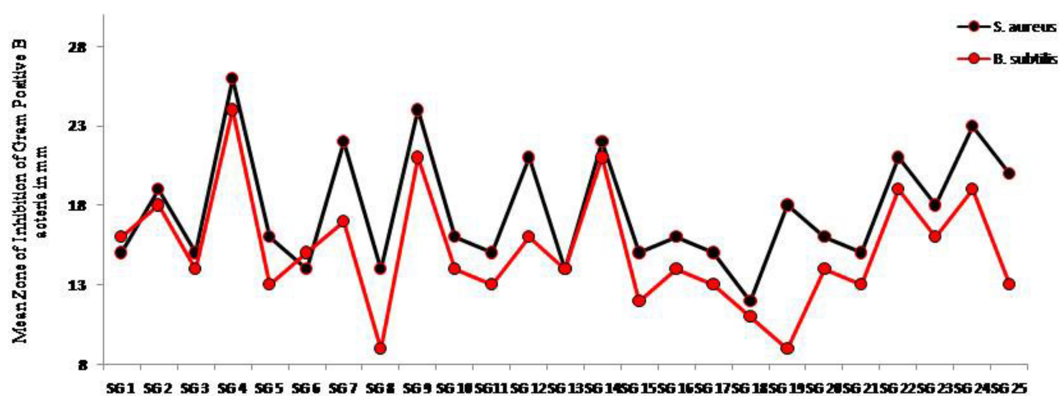


Figure 41 Mean inhibition zone of gram positive bacteria at 1mg/ml concentration of different solvent extracts of five cv-*Zingiber officinale* of Sikkim Himalaya.

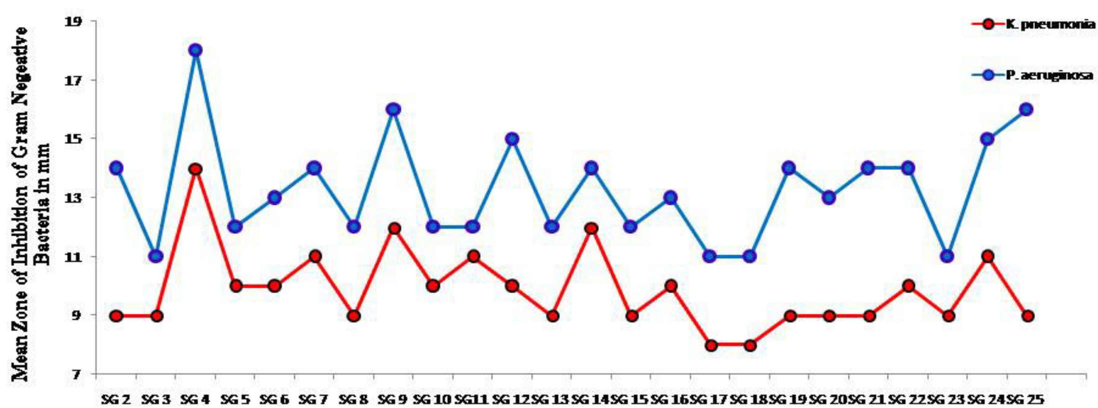


Figure 42 Mean inhibition zone of gram negative bacteria at 1mg/ml concentration of different solvent extracts of five cv-Zingiber officinale of Sikkim Himalaya.

Table 4.17 Antibacterial activity of rhizome extracts of *Zingiber officinale* of Sikkim Himalaya, Numbers indicate the mean diameters of inhibition of triplicate experiments ± standard deviation, -indicate no growth inhibition. NM indicate zone of inhibition not measured.

Sl. No.	Sample No.	*S. aureus (MTCC 96)*	*B. subtilis (MTCC 441)*	*Klebsiella pneumonia (MTCC 424)*	*Pseudomonas aeruginosa (MTCC 432*	*E. coli (MTCC 39)*
1.	SG 1	15±0.3	16±1.2	10±0.1	13±0.1	-
2.	SG 2	19±1.2	18±0.3	09±0.3	14±1.2	-
3.	SG 3	26±0.1	24±0.3	14±0.3	18±0.3	-
4.	SG 4	15±0.3	14±1.2	09±0.4	11±0.3	-
5.	SG 5	16±0.3	13±0.7	10±0.3	12±1.2	-
6.	SG 6	14±0.3	15±0.2	10±0.3	13±0.1	-
7.	SG 7	22±0.2	17±1.1	11±1.3	14±0.3	-
8.	SG 8	24±0.2	21±1.2	12±1.2	16±0.6	-
9.	SG 9	14±1.2	09±1.1	09±0.1	12±0.2	-
10.	SG 10	16±0.2	14±0.3	10±1.1	12±0.21	-
11.	SG11	15±0.3	13±0.5	11±0.1	12±0.3	-
12.	SG 12	21±1.3	16±0.3	10±0.3	15±0.22	-
13.	SG 13	22±0.2	21±0.2	12±0.2	14±0.3	-
14.	SG 14	14±1.1	14±0.4	09±0.3	12±1.1	-
15.	SG 15	15±0.0	12±0.5	09±0.2	12±1.2	-
16.	SG 16	16±0.1	14±0.3	10±1.2	13±0.5	-
17.	SG 17	15±0.3	13±0.2	08±0.3	11±1.2	-
18.	SG 18	18±1.2	09±1.1	09±1.2	14±1.4	-
19.	SG 19	12±0.4	11±0.3	08±0.4	NM	-
20.	SG 20	16±0.6	14±1.1	09±0.3	13±0.4	-
21.	SG 21	15±0.3	13±0.2	09±0.5	14±1.2	-
22.	SG 22	21±0.3	19±0.6	10±0.5	14±1.2	-
23.	SG 23	23±0.5	19±1.1	11±0.2	15±0.3	-
24.	SG 24	18±0.2	16±0.3	09±1.2	11±1.2	-
25.	SG 25	20±0.2	13±0.2	09±0.6	16±0.2	-

P < *0.001*) and *Klebsiella pneumonia* (R^2=*-2.99,* P < *0.001*), furthermore, presence of total flavonoid content also showed negative significant correlation against *Pseudomonas aeruginosa* (R^2=*-0.43,* P < *0.001*) *Klebsiella pneumonia* (R^2=*-3.48,* P < *0.001*) (Figure 43).

In the present study, the analysis of the data on the antimicrobial activity of different solvent ginger rhizome extracts on *S. aureus, B. subtilis*, *Klebsiella pneumonia and Pseudomonas aerugi*nosa revealed that the solvent used in the extraction showed significant (P < 0.05) on the level of inhibition observed (Table 4.16). The maximum variance was observed in acetone solvent with significant value of *(*P < *1.44E*-13), followed by methanol (P <2.04231E-11), ethanol (P < 2.92E-11), Chloroform (P < *3E*-11) and Petroleum ether ((P <6.21E-11) (Table 4.16).

4.3.3 Antioxidant study

4.3.3.1 Total phenol content

Gallic acid is a type of phenolic acid, extensively found in leaves, oak bark, and other plants (Reynolds and NG Wilson, 1991). Gallic acid was reported as a free radical scavenger and as an inducer of differentiation and apoptosis in leukaemia, lung cancer, and colon adenocarcinoma cell lines, as well as in normal lymphocyte cells. (Inoue *et al.,* 1994). It possesses significant antioxidant activity (Rasool *et al.,* (2010), anti-inflammatory properties (Sang-Hyun Kim *et al.,* 2006), antifungal and anti-diabetes activity. (Sohi *et al.,* 2003).

Phenolic compounds may contribute directly to the antioxidative action. Total phenol content in case of ginger (*Zingiber officinale*) ranged in between 99.00 to

228.557 mg/ml GAE (gallic acid equivalent) per gm. of plant extract irrespective of the variety or the solvent type (Table 4.18). Among the different solvents like acetone, chloroform, ethanol, methanol and petroleum ether, chloroform proved to be the best solvent for extraction of phenolic in ginger in most of the varieties under study except for *Bhaisay* and *Charinangrey* where acetone and petroleum ether extract had higher phenolic content respectively compared to other solvent type. The phenolic content varied considerably in varieties with respect to solvent type.

The presence of higher amount of phenolic in the chloroform extract is in accordance with previous report on *Mimusops elengi* (Roa *et al.,* 2011). The total phenolic content of plant is shown in (Table 4.18). The differences in total phenolic content among five cultivars gingers used were statistically significant ($P < 0.0000661$).

4.3.3.2 Total flavonoids content

Flavonoids, a group of phenolic compounds widely occurring in the plant kingdom, have been reported to possess strong antioxidant activity such as Quercetin had anticancer activities and were able to inhibit cancer cell growth Ferry *et al.,* (1996), Ranelletti *et al.,* (1999). In present study the total flavonoid content is depicted in (Table. 8.5). From the table it is clear that the flavonoid content ranged from 45.12 to 621.52 mg/ml QE (Quercetin equivalent) per gram of the plant extract. In different varieties of ginger irrespective of the solvent used. The highest and the lowest flavonoid content were noted in the chloroform extract of *Charinangrey* (621.52 mg/ml QE) and *Bhaisay* (45.12 mg/ml QE). However for the extraction of flavonoid, petroleum ether was found to be more effective solvent in comparison to others except for *Charinangrey* where chloroform out stretched others. The differences in total flavonoids content among five cultivars gingers used were statistically not significant ($P < 0.348$).

4.3.3.3 DPPH scavenging activity

The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 517 nm and also for the visible deep purple colour. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance (Goyal *et al.,* 2010). Figure 45 depicts the DPPH scavenging activity of the different varieties of *Zingiber officinale* with respect to the solvent type. From the figure it is evident that the DPPH scavenging activity ranged from 84.81% to 94.18%. The standard ascorbic acid showed the scavenging activity of 64.76%. This is lower than ginger scavenging activity. Among the *Majhauley* variety the DPPH scavenging activity was found to be as GAM>GEM>GMM>GCM>GPM, while in case of *Bhaisay* it was GEB>GPB>GAB>GCB>GMB and *Gorubthangey* it followed as GCG>GPG>GMG>GEG>GAG. However in case of both *Jorethangey* and *Charinangrey* the DPPH scavenging activity was found to be approximately 93% irrespective of the solvent used for preparing the extract. The high DPPH scavenging activities of ginger have been previously note by some researchers (Stoilova *et al.,* 2007, El- Ghorab *et al.,* 2010).

4.3.3.4 Ferric reducing power assay

The reducing capabilities of the plant extract compared with ascorbic acid have been depicted in (Figure 46). The reductive capability is determined by the transformation of Fe^{3+} to Fe^{2+} in presence of the extract and the ascorbic acid used as standard (Goyal *et al*, 2011a). GCC fraction had the highest reductive capability (OD 700 nm = 1.177)

Table 4.18 Total phenolic content (TPC) and Total flavonoid content (TFC) equivalent to GAE mg/ml and QE mg/ml of 25 different samples of five cultivars of *Zingiber officinale* of Sikkim Himalaya.

SL No.	Variety	Solvent	Sample ID	Phenol GAE mg/ml	Flavonoid QE mg/ml
1.	*Majhauley*	Petroleum ether	GPM	140.241 ±5	521.12 ±1
2.	*Majhauley*	Ethanol	GEM	99.003 ±2	77.92 ±3
3.	*Majhauley*	Chloroform	GCM	216.529 ±5	254.72 ±5
4.	*Majhauley*	Methanol	GMM	99.691 ±3	179.12 ±1
5.	*Majhauley*	Acetone	GAM	158.454 ±2	276.72 ±5
6.	*Bhaisay*	Petroleum ether	GPB	137.835 ±3	304.72 ±1
7.	*Bhaisay*	Ethanol	GEB	167.388 ±2	287.92 ±3
8.	*Bhaisay*	Chloroform	GCB	141.271 ±1	45.12 ±3
9.	*Bhaisay*	Methanol	GMB	149.519 ±3	239.52 ±4
10.	*Bhaisay*	Acetone	GEB	189.381 ±5	278.72 ±3
11.	*Gorubthangey*	Petroleum ether	GPG	149.863 ±2	527.92 ±2
12.	*Gorubthangey*	Ethanol	GEG	174.605 ±3	228.72 ±3
13.	*Gorubthangey*	Chloroform	GCG	201.065 ±5	517.52 ±4
14.	*Gorubthangey*	Methanol	GMG	166.357 ±3	105.12 ±5
15.	*Gorubthangey*	Acetone	GAG	159.485 ±2	331.52 ±2
16.	*Jorethangey*	Petroleum ether	GPJ	193.162 ±4	374.72 ±2
17.	*Jorethangey*	Ethanol	GEJ	213.436 ±3	181.92 ±5
18.	*Jorethangey*	Chloroform	GCJ	214.654 ±4	174.32 ±3
19.	*Jorethangey*	Methanol	GMJ	209.656 ±2	227.92 ±3
20.	*Jorethangey*	Acetone	GAJ	212.749 ±1	264.32 ±4
21.	*Charinangrey*	Petroleum ether	GPC	228.557 ±4	221.92 ±4
22.	*Charinangrey*	Ethanol	GEC	169.450 ±3	465.52 ±5
23.	*Charinangrey*	Chloroform	GCC	179.759 ±6	621.52 ±2
24.	*Charinangrey*	Methanol	GMC	200.378 ±3	358.32 ±2
25.	*Charinangrey*	Acetone	GAC	204.502 ±6	296.32 ±4

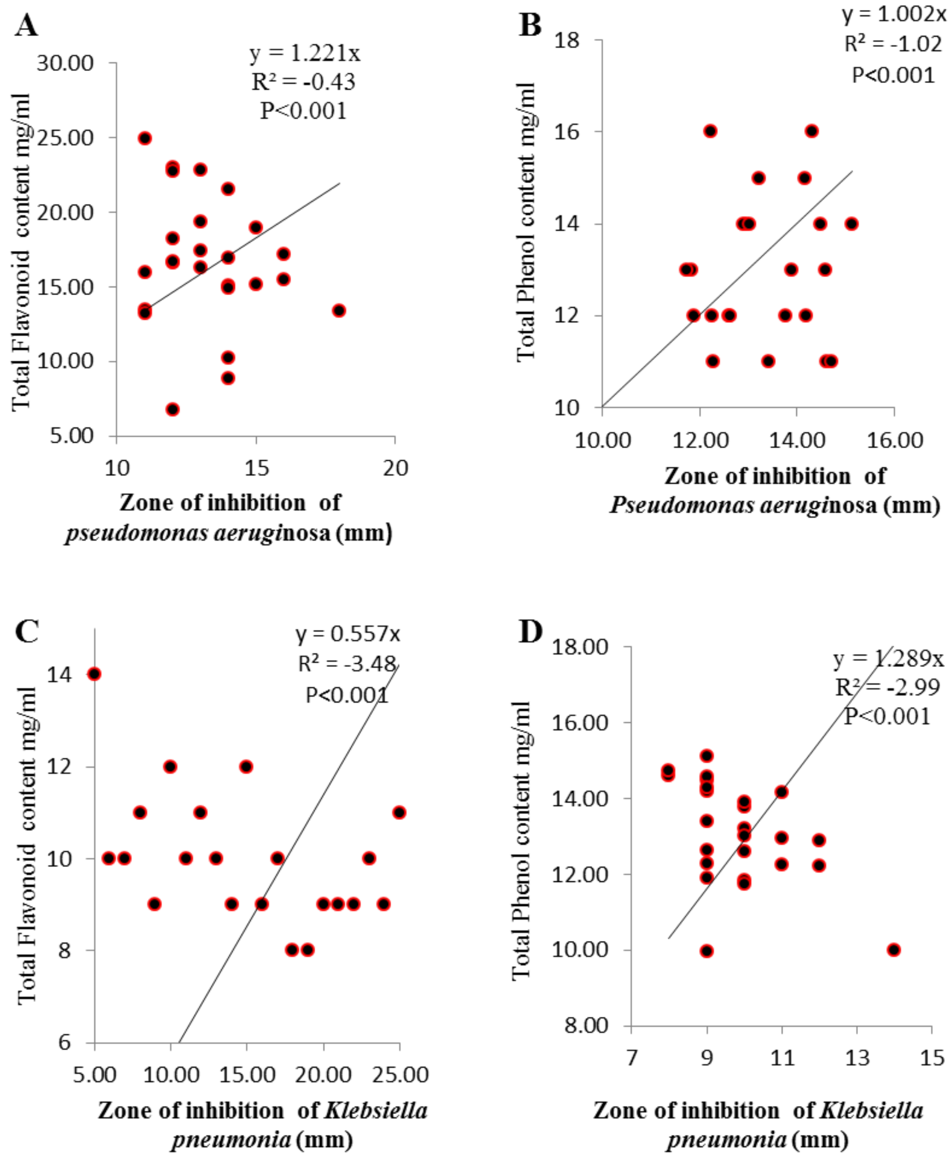


Figure 43 (A-D) Linear regression of A) total flavonoid content (sqrt. transformed) and antimicrobial activity against of *Pseudomonas aeruginosa* (mm), B) total phenol content (sqrt. transformed) and antimicrobial activity against **of** *Pseudomonas aeruginosa* (mm), C) total flavonoid content (sqrt. transformed) and *Klebsiella pneumonia* (mm), and D) total phenol content (sqrt. transformed) *Klebsiella pneumonia* (mm).

Figure 44 (A-D) Linear regression of A) total phenol content (sqrt. transformed) and antimicrobial activity against *Bacillus subtilis* (mm), B) total flavonoid content (sqrt. transformed) and antimicrobial activity against *Bacillus subtilis* (mm), C) total phenol content (sqrt. transformed) and *S.aureus (*mm), and D) total flavonoid content (sqrt. transformed) *S.aureus (*mm).

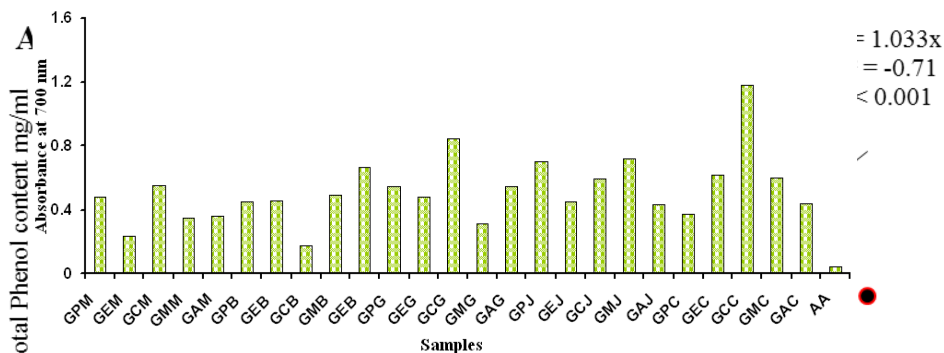
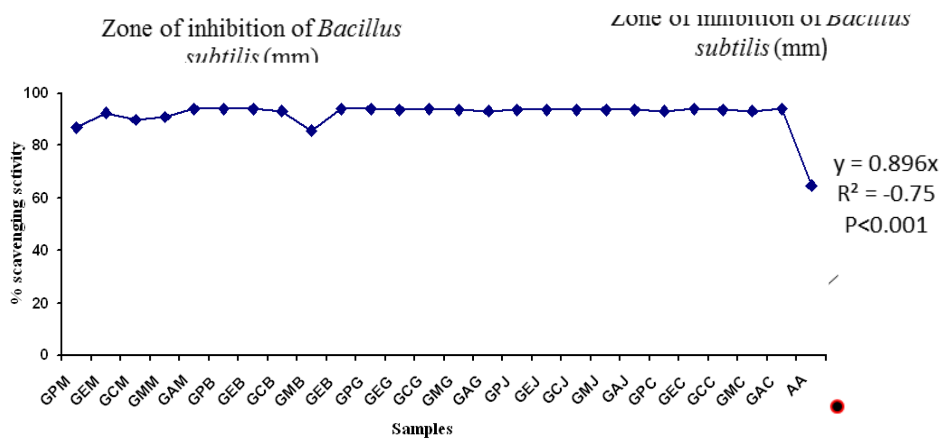


Figure 46 Reducing power activity of different varieties of ginger with respect to solvent type compared to standard ascorbic acid.



Total

Figure 45 DPPH scavenging activity of different varieties of ginger with respect to solvent type compared to standard ascorbic acid

30

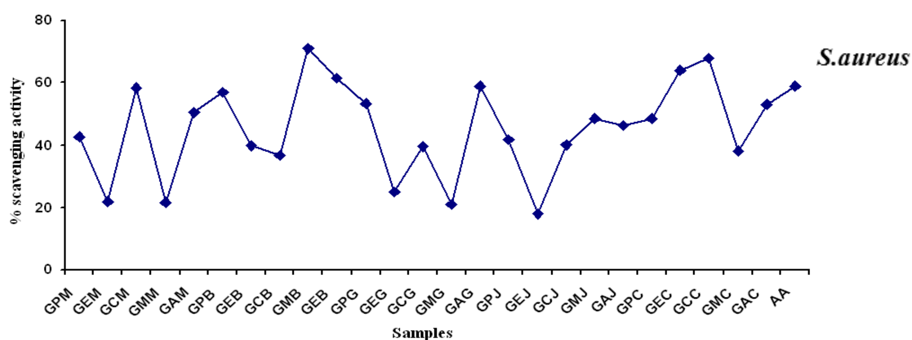


Figure 47 H2O2 scavenging activity of different varieties of ginger with respect to solvent type compared to standard ascorbic acid**.**

while the lowest reducing ability was noted in GCB (OD 700 nm = 0.176), however the standard ascorbic acid (OD 700 = 0.041) showed much lower than GCB.

4.3.3.5 H_2O_2 scavenging activity

Hydrogen peroxide (H_2O_2) is highly diffusible and an important reactive oxygen species (ROS) (Goyal *et al*., 2011b). The ability of the different ginger extract to quench H_2O_2 is shown in (Figure 47). The value for maximum % inhibition was found to be 70.99 % in GMB fraction. It ranged from 21.68% to 58.31%, 36.64% to 70.99%, 21.07% to 58.90%, 17.90 to 48.57% and 38.18 to 67.92 in case of ginger varieties *viz. Majhauley*, *Bhaisay*, *Gorubthangey*, *Jorethangey* and *Charinangrey* respectively. The scavenging of hydrogen peroxide of the standard (ascorbic acid) was found to be 59% at the same concentration.

4. 4 Study of diversity of ginger in Sikkim using RAPD analysis

4.4.1 Genetic variation and its relevance to agronomy

Molecular study using universal markers, such as, RAPD can help in characterization and identification of plant germplasm. To assess the genetic variation in five (5) cultivars of *Zingiber officinale* (ginger), (Table 4.19) a total of 63 RAPD primers were screened, out of which 21 primers showed consistent bands. These 21 primers were used for further amplification. A total of 104 clear and reproducible and scorable RAPD fragments ranging from 150–13000 bp were generated from 21 primers. Of the 104 scorable RAPD bands, 99 were found to be polymorphic. Each primer was tried thrice and the results were reproducible.

The number of polymorphic bands per primer (Clonitech Technologies) ranged from two in CL-80 and Cl-75 to thirteen in CL-28, with an average of 4.95 polymorphic

bands per primer (Table 4.20). Selection of polymorphic alleles was carried out in a careful manner and only the clear, repeatable and polymorphic bands were scored and used in for statistical analyses (Figure 48, A-D). The percentage of polymorphic bands ranged from 50% to 100%. Cultivars, such as, CL-182, CL-192, CL-80, CL-23, CL-1, CL-11, CL-25, CL-40 etc. (Table 4.20) revealed 100% polymorphism, while CL-76 showed 75% polymorphism. Cultivar (CL-105) showed 50% polymorphism, followed by CL-75, CL- 54 with 66% polymorphism (Table 4.20). Cultivar (CL-28) showed the highest number of bands (13) and the CL-188 produced only one band (Table 4.20). For all primers, the average polymorphism was recorded to of 95.20 %; however, a few primers (Figure 49 and 50) also showed 100% polymorphism among the varieties owing to their self-incompatibility, which suggests a highly heterogeneous plants and high genetic variation within *Zingiber officinale*.

Diversity measures were calculated using Nei's (1973) index, which ranged from h = 0.04 to h = 0.09 with a mean value of 0.08 (Table 4.21). The high matrix value indicated that the cultivars considered in the study are distantly related to each other. The percentage of polymorphic loci ranged from 38.46 % in *Bhaisay* to 82.69 % in *Majhauley* cultivars with an average (73.08%) polymorphic locus of per population (Table 4.22). The observed number of alleles (na) per locus varied from 1.3846 ± 0.4889 in *Bhaisay* to 1.8269 ± 0.3801 in *Majhauley* with an average value of 1.6731 ± 0.4714. The effective number of alleles (ne) per locus ranged from 1.2532 ± 0.3757 in *Bhaisay* to 1.4010 ± 0.3857 in *Gorubthangey* with an average of 1.3721± 0.3739 effective number of allele per cultivars. The lowest Nei's gene diversity (h) was recorded in *Bhaisay* (0.1434 ± 0.1992), whereas the highest diversity was observed in

Gorubthangey (0.2329 ± 0.1979) with an average gene diversity value of 0.2322 ± 0.1681 per cultivars. *Bhaisay* cultivars recorded the lowest Shannon's diversity index

Table 4.19 Sources and population of the ginger clones used in the study.

Sl. No	Cultivar variety *Zingiber*	Given code	Altitude	Latitude	Longitude	Location
1.	*Majhauley*	SKG 1	2725 ft.	N 27 20"00.9'	E088 27"01.5 '	Menglee
2.	*Charinangrey*	SKG 2	4106 ft.	N 27 19"45.3'	E088 27"34.8 '	Mangzing
3.	*Majhauley*	SKG 5	3486 ft.	N 27 14"25.6'	E088 26"23.4 '	Tarku Tanak
4.	*Charinangrey*	SKG 6	1991ft	N 27 16"14.4'	E08830"35.6'	Sudhur1
5.	*Charinangrey*	SKG 8	1172 ft.	N27 28" 00.0'	E08836"37.0'	Sudhur 2
6.	*Charinangrey*	SKG 9	1253 ft.	N27 21"21.1'	E08830"0.5'	Mangten
7.	*Gorubthangey*	SKG 11	1470 ft.	N2728"30.4'	E08830"20.7	Hegyathang1
8.	*Majhauley*	SKG 12	1620 ft	N27 28" 6.0'	E088 29" 55.9'	Hegyathang2
9.	*Charinangrey*	SKG 13	4793ft	N27 28"50.5'	E088 29"33.3'	Ravang1
10.	*Charinangrey*	SKG 14	4897ft	N27 28"50.5'	E088 29"33.3'	Ravang2
11.	*Gorubthangey*	SKG 15	2843 ft	N 27 09.273"	E 088 18.137"	Chisopani1
12.	*Gorubthangey*	SKG 16	3133 ft	N 27 09.026"	E 088 18.064"	Chisopani2
13.	*Gorubthangey*	SKG 17	3021 ft	N 27 05.732"	E 088 17. 970"	Babatey
14.	*Majhauley*	SKG 18	2933 ft	N 27 08.742"	E 088 17. 970"	Chisopani3
15.	*Majhauley*	SKG 19	2794 ft	N 27 09 .356"	E 088 18.224"	Sadam
16.	*Majhauley*	SKG 20	1151ft	N 27 11’3.74"	E 088	Sadam
17.	*Majhauley*	SKG 21	4767 ft	N 27 07.223"	E 088 25.312"	Sadam
18.	*Majhauley*	SKG 22	4975 ft	N 27 07.018"	E 088 25.359"	Sadam
19.	*Majhauley*	SKG 23	3495 ft	N 27 9’38.23	E 88 24’28.62	Bhangjayang
20.	*Majhauley*	SKG 24	3544ft	N 27 10 38.67	E 88 20’ 57.91	Kamrang
21.	*Gorubthangey*	SKG 25	3417 ft	N 27 11.939"	E 88 40.925"	Lower
22.	*Bhaisay*	SKG 26	3217 ft	N 27 12.284"	E 88 40.562"	Lower
23.	*Gorubthangey*	SKG 27	3825 ft	N 27 13.265"	E 88 40. 577"	Gangtok
24.	*Gorubthangey*	SKG 28	4274 ft	N 27 11.760"	E 88 40.760"	Middle
25.	*Gorubthangey*	SKG 29	4223 ft	N 27 11.902"	E 88 40.317"	Upper
26.	*Gorubthangey*	SKG 30	4138 ft	N 27.11.117"	E 88 40.382"	Aritar
27.	*Gorubthangey*	SKG 32	3875 ft	N 27 11 924"	E 88 40.385"	Middle
28.	*Gorubthangey*	SKG 33	3487 ft	N 27 11 .939"	E 88 40 924"	Pakyong
29.	*Gorubthangey*	SKG 34	3417 ft	N 2711.939"	E 88 40.925"	Pakyong
30.	*Jorethangey*	SKG 35	3038 ft	N 27 12.248"	E 88 40.908"	Gangtok
31.	*Jorethangey*	SKG 36	5020ft	N 27°10'5.94"	E 88°11'52.42"	Sombarey
32.	*Jorethangey*	SKG 37	5094 ft	N 27°10'7.33"	E 88°11'44.56"	Tashiding
33.	*Jorethangey*	SKG 38	5618 ft	N 7°10'28.89"	E 88°11'46.47"	Tashiding
34.	*Jorethangey*	SKG 39	4850 ft	N 27° 9'57.65"	E 88°11'58.35"	Mangalbarey 1
35.	*Jorethangey*	SKG 40	4580 ft	N 27° 9'45.06"	E 88°11'57.45"	Gayshing
36.	*Jorethangey*	SKG 41	4586 ft	N 7°17'28.61"	E 88° 9'3.09"	Okhrey
37.	*Bhaisay*	SKG 42	4333 ft	N 7°17'21.24"	E 88° 9'12.36"	Mangalbarey 2
38.	*Bhaisay*	SKG 43	5046 ft	N 27°17'0.17"	E 88° 8'48.61"	Okhrey
39.	*Bhaisay*	SKG 44	5046 ft	N 27° 8'12.98"	E 88° 8'43.94"	Tashiding
40.	*Bhaisay*	SKG 45	4732 ft	N 27° 8'13.72"	E 88° 8'53.43"	Okhrey

Table 4.20 Frequency of polymorphic bands generated by RAPD primers

Sl.No	Name of Primer	Sequence 5'-3'	Total No. of bands	No. of poly bands	Size range (Kb)	Polymorphic bands (%)
1.	GCC76	-GAGCACCAGT-	4	3	0.45-1.30	75
2.	GCC 182	-GTTCTCGTGT-	5	5	0.25-1.05	100
3.	GCC 192	-GCAAGTCACT-	3	3	0.25-0.65	100
4.	GCC 80	-GGGCCACGCT-	2	2	0.75-0.80	100
5.	GCC 23	-CCCGCCTTCC-	4	4	0.30-0.75	100
6.	GCC 1	-CCTGGGCTTC-	3	3	0.15-0.50	100
7.	GCC 11	-CCCCCCTTTA-	6	6	0.20-0.85	100
8.	GCC 25	-ACAGGGCTCA-	5	5	0.20-0.60	100
9.	GCC 40	-TTACCTGGGC-	5	5	0.10-0.85	100
10.	GCC 75	-GAGGTCCAGA-	3	2	0.15-0.40	66.66
11.	GCC 105	-CTCGGGTGGG-	2	1	0.15-0.30	50
12.	GCC 112	-GCTTGTGAAC-	7	7	0.15-0.65	100
13.	GCC 54	-GTCCCAGAGC-	6	4	0.30-0.90	66.66
14.	GCC 30	-CCGGCCTTAG-	5	5	0.15-0.50	100
15.	GCC 53	-CTCCCTGAGC-	9	9	0.05-0.85	100
16.	GCC 28	-CCGGCCTTAA-	13	13	0.05-0.85	100
17.	GCC 197	-TCCCCGTTCC-	7	7	0.20-0.85	100
18.	GCC 188	-GCTGGACATC-	1	1	0.125	100
19.	GCC 64	-GAGGGCGGGA-	3	3	0.30-0.60	100
20.	GCC 67	-GAGGGCGAGC-	4	4	0.25-0.55	100
21.	GCC 78	-GAGCACTAGC-	7	7	0.15-0.75	100
22.	**Grand Total**		**104**	**99**		**95.19**
			4.95 average band per primer			

Table 4.21 Nei's genetic distance and genetic identity among different cultivars of *Zingiber officinale* of Sikkim Himalaya

No	SKG1	SKG2	SKG3	SKG4	SKG5
SKG1	****	0.9524	0.9514	0.9190	0.9121
SKG2	0.0488	****	0.9815	0.9142	0.8978
SKG3	0.0498	0.0186	****	0.9042	0.8744
SKG4	0.0845	0.0898	0.1007	****	0.9116
SKG5	0.0920	0.1078	0.1342	0.0925	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal). (SKG1, Charinangrey; SKG2, Gorubthangey; SKG3 Majhauley; SKG4 Jorethangey; SKG4 Bhaisay)

Table 4.22 Five different cultivar populations showing polymorphic loci and the percentage

Sl. No	Cultivars (*Zingiber officinale*)	Polymorphic loci	Percentage of Polymorphic loci
1.	*Charinangrey*	54	51.92 %
2.	*Gorubthangey*	76	73.08 %
3.	*Majhauley*	86	82.69 %
4.	*Jorethangey*	70	67.31 %
5.	*Bhaisay*	40	38.46 %

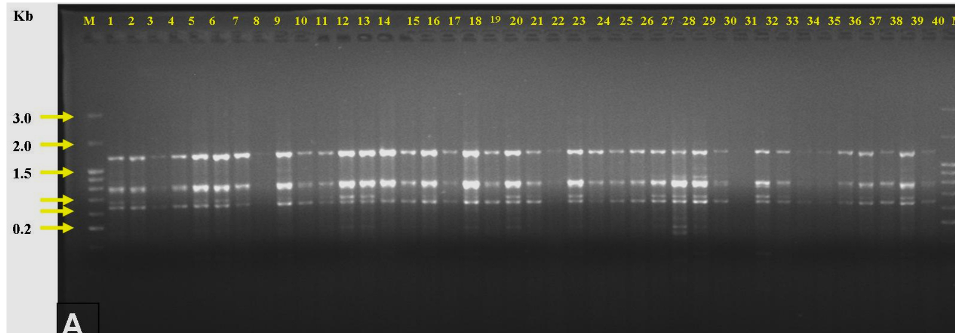


Figure 48 A Representative profiles amplified with CL 11 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1- 40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya.

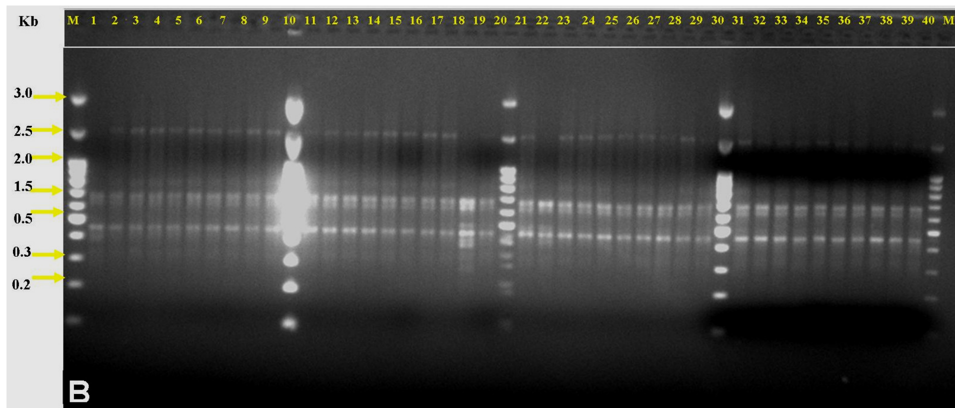


Figure 48 B Representative Profiles amplified with CL 53 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1- 40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya.

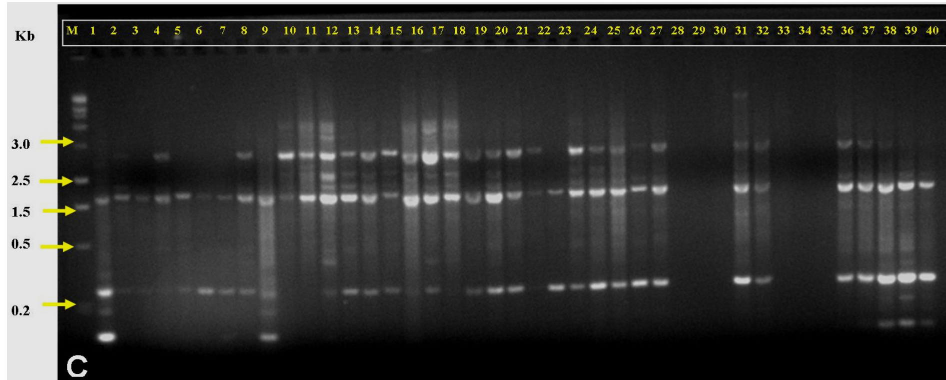


Figure 48 C Representative profiles amplified with CL 21 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1- 40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya**.**

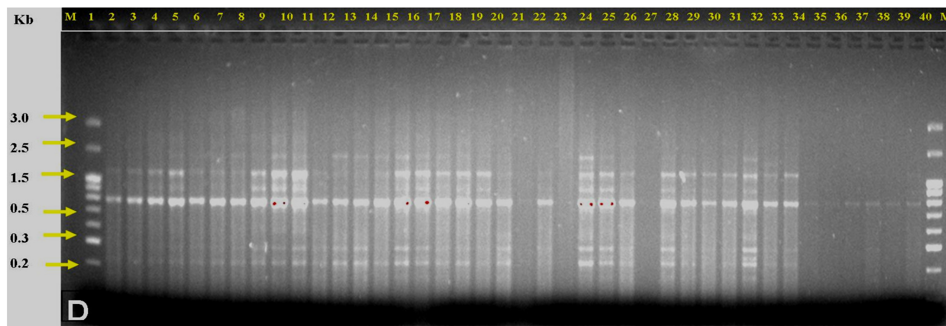


Figure 48 D Representative profiles amplified with CL 197 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1- 40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya.

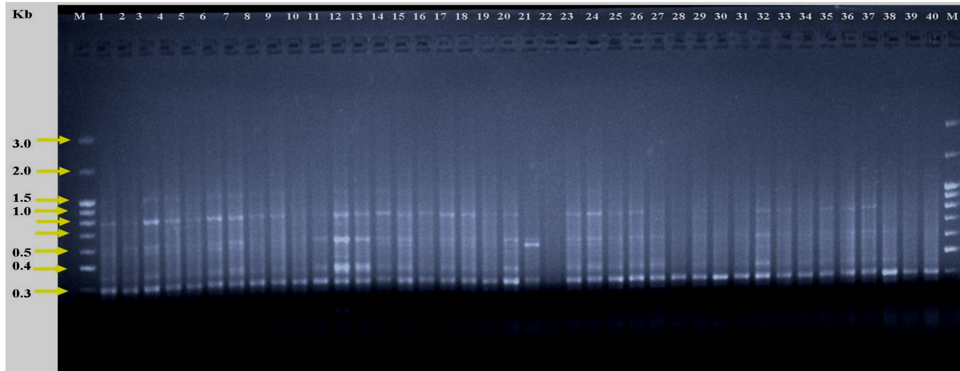


Figure 49 Representative profiles amplified with CL 25 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1-40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya.

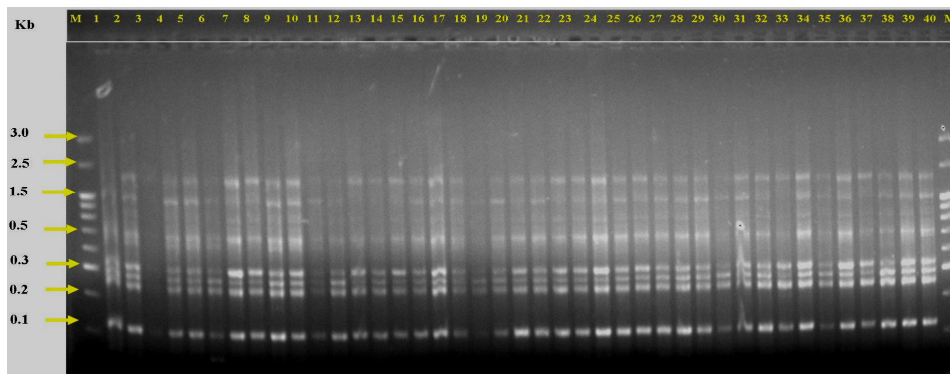


Figure 50 Representative profiles amplified with CL 53 RAPD primer. M is the 100 bp molecular marker (Fermentas, Life Science, Germany with size in bp indicated on the left). 1- 40 indicate the fourty different genotypes of *Zingiber officinale* of Sikkim Himalaya.

(0.2120 ±0.2849), while *Majhauley* cultivars recorded maximum (0.2120 ±0.2849) with a mean value (0.3329 ± 0.2849) per cultivar (Table 4.23).

Genetic differentiation within cultivars were observed based on Nei's original measures of genetic identity and genetic distance, in which genetic identity values measure genetic similarity between cultivars sample pairs. High degree of variability in genetic identity and genetic distance values were observed among the cultivars of gingers (Table 4.23 and Figure 51).

In pair wise comparisons of Nei's original measures of genetic identity within cultivars, smaller values were observed between SKG 2 (*Gorubthangey*) and SKG 5 (*Bhaisay*) cultivars exhibited genetically most diverse cultivars (0.8978), whereas SKG 2 (*Gorubthangey*) and SKG 3 (*Majhauley*) cultivars recorded genetically most similar (0.9815).

4.4.2 Genetic relationships and the germplasm conservation

Genetic distance refers to the genetic deviation between species or between populations within a species. It is measured by a variety of parameters like Nei's standard genetic distance. This measure assumes that genetic differences arise due to mutations and genetic drift, Nei's D_A distance. This distance assumes that genetic differences arise due to mutations and genetic drift, but this distance measure is known to give more reliable population trees than other distances particularly for DNA data. Similarity indices measure the amount of closeness between two individuals, the larger the value the more similar are the two individuals. There is a variety of alternative measures for expressing similarity, like Jaccard's coefficient of similarity which can be used for binary data and often is applied in RAPD-based studies. This coefficient is based on

number of positive matches between two individuals whereas joint absences are excluded. Dissimilarity coefficients instead estimate the distance or unlikeness of two individuals, the larger the value the more different are the two individuals. Smaller genetic distances indicate a close genetic relationship whereas large genetic distances indicate a more distant genetic relationship. Genetic distance can be used to compare the genetic similarity between different species. Genetic diversity studies helps in formulating proper conservation, preservation and selection planning which helps in interpreting the present status and future prognosis, particularly of the endangered and rare species.

The dendrogram (Figure 51) of the genetic relationships among the cultivars of *Zingiber officinale* of Sikkim was constructed based on the Nei's genetic distances and the UPGMA method (Figure 52). All samples showed three main clusters, viz., (1) Cluster I representing *Charinangrey*, *Gorubthangey* and *Majhauley*, Cluster II representing *Jorethangey* and Cluster III representing *Bhaisay*. The clusters revealed the level of relatedness (0.74994 – 6.26042) between the 5 cultivars studied (Figure 51).

The first cluster further divided into two sub-groups. Sub-group one (1) showed only one cultivar "*Charinangrey*" and the sub-group two (2) clubbed two cultivars *Gorubthangey* and *Majhauley*, while other two Clusters II and III showed separate cultivars *Jorethangey* and *Bhaisay* (Figure 51).

In the first cluster, cultivar *Charinangrey* separated distinctly with cultivars *Gorubthangey* and *Majhauley*, which may be due to the fact that traditionally ginger is classified on the basis of morphological features; for example, *Charinangrey* (bird's nail) itself is small in size. These morphological features are subjected to substantial

environment changes coupled with extensive cryptic mutation led to similarity in genetic makeup of the cultivars. The other two cultivars *Gorubthangey* (name of place)

Table 4.23 Estimation of genetic diversity parameter for the different cultivars of *Zingiber officinale* from Sikkim Himalaya based on RAPD markers

Population	n	np*	%p*	na*	ne*	h*	I*
Charinangrey	6	54	51.92	1.5192 ± 0.5020	1.3416 ± 0.3943	0.1947± 0.2082	0.2878 ± 0.2966
Gorubthangey	12	76	73.08	1.7308 ±0.4457	1.4010 ± 0.3857	0.2329 ± 0.1979	0.3518 ± 0.2735
Majhauley	10	86	82.69	1.8269 ± 0.3801	1.3717± 0.3250	0.2322 ± 0.1681	0.3638 ± 0.2310
Jorethangey	7	70	67.31	1.6731 ±0.4714	1.3721± 0.3739	0.2195 ± 0.1943	0.3329 ±0.2727
Bhaisay	5	40	38.46	1.3846 ±0.4889	1.2532 ± 0.3757	0.1434 ± 0.1992	0.2120 ±0.2849

(n = sample size; np* = no of polymorphic loci; p* = percentage of polymorphic loci; na* = observed number of alleles; ne* = effective number of alleles; h* = Nei's gene diversity; I* = Shannon's Information index.)

and *Majhauley* (medium in size) showed close similarity, which may be due to the reason that traditionally *Majhauley* is a subgroup of *Gorubthangey* and named according to the size of ginger. These cultivars possessed a high genetic identity (0.9815) and some degree of genetic differentiation (0.0488). *Jorethangey* and *Bhaisay* formed two distance clusters of their own and possessed a high genetic identity (0.9116) with some degree of genetic differentiation (0.0845) as recorded in (Table 4.21). The above information can play a very important role in conservation, selection and preservation of different cultivars of ginger in Sikkim Himalaya.

The same clusters were obtained in the dendrogram when the data were analyzed using NTSYSpc (Figure 52), which is in complete agreement with the earlier reports of ginger taxonomy. Dendrogram based on NTSYSpc analysis differentiated into five cultivars into four clusters with a Jaccard's coefficient of 0.35-0.87 (Figure 52). Cluster I represented the cultivar *Charinangrey*, which was further divided into two sub-groups. Subgroup I (one) included *Gorubthangey* and sub-group II (two) included *Majhauley*.

4.4.3 Principal component analysis of five cultivars of gingers

As the individuals plants are collected from different region of Sikkim. The relationship among the individual plants was further assessed by Principal Component Analysis (PCA). PCA was performed to confirm similarity and diversity among the individual clones. The present finding of PCA (Figure 53) were similar to the cluster analysis showing comparable topology. High genetic diversity was recorded among the clones from Sikkim India. Grouping pattern did not show any considerable dissimilarity except the clones SKG 13 and SKG 3 were classified as separate groups.

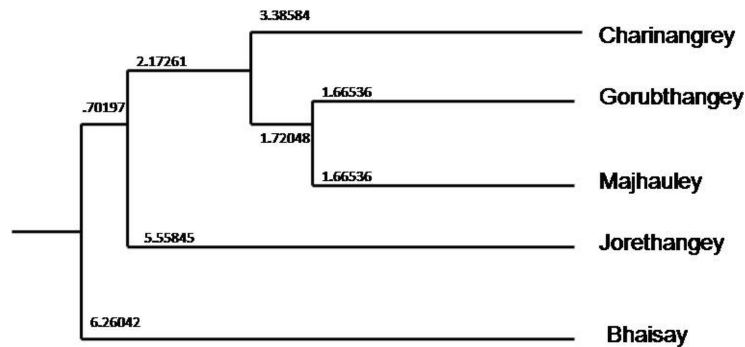


Figure 51 UPGMA dendrogram based on RAPD data showing relationships among different cultivars of the *Zingiber officinale*. Numbers at branch points represent bootstrap values with 1000 replications.

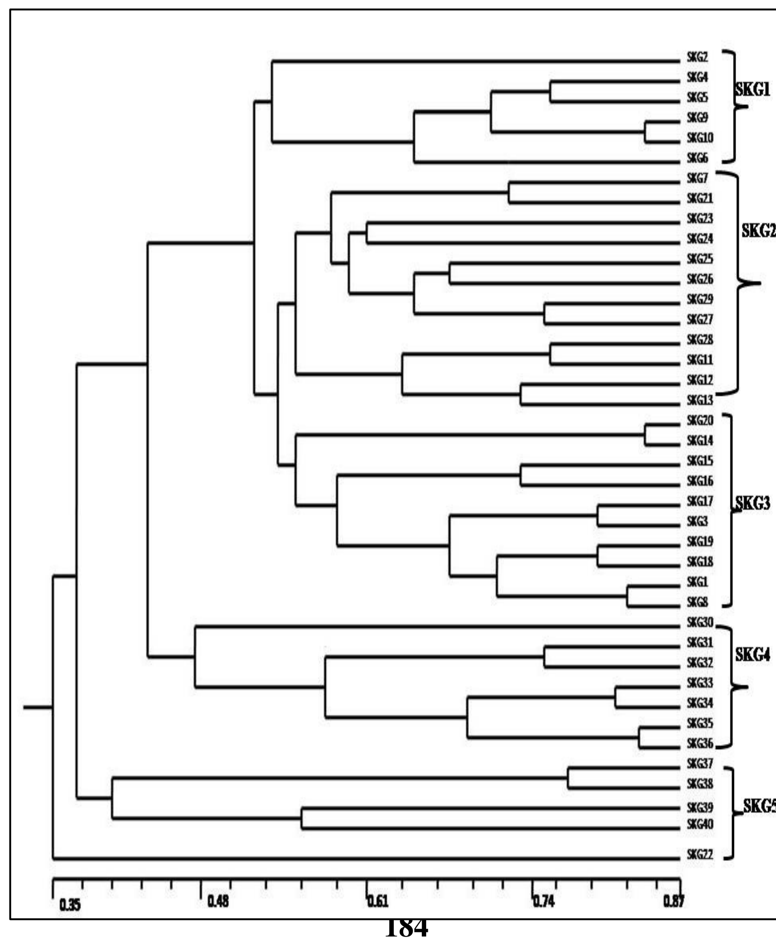


Figure 52 Dendrogram based on RAPD markers, indicating the genetic diversity between five cultivars of ginger (*Zingiber officinale*) of Sikkim. The horizontal axis represent genetic similarity coefficient (Jaccard 1908). The codes (SKG 1– SKG 40) indicate an individual genotype and corresponds to those listed in Table 4. SKG1, Charinangrey; SKG2, Gorubthangey; SKG3 Majhauley; SKG4 Jorethangey; SKG4 Bhaisay)

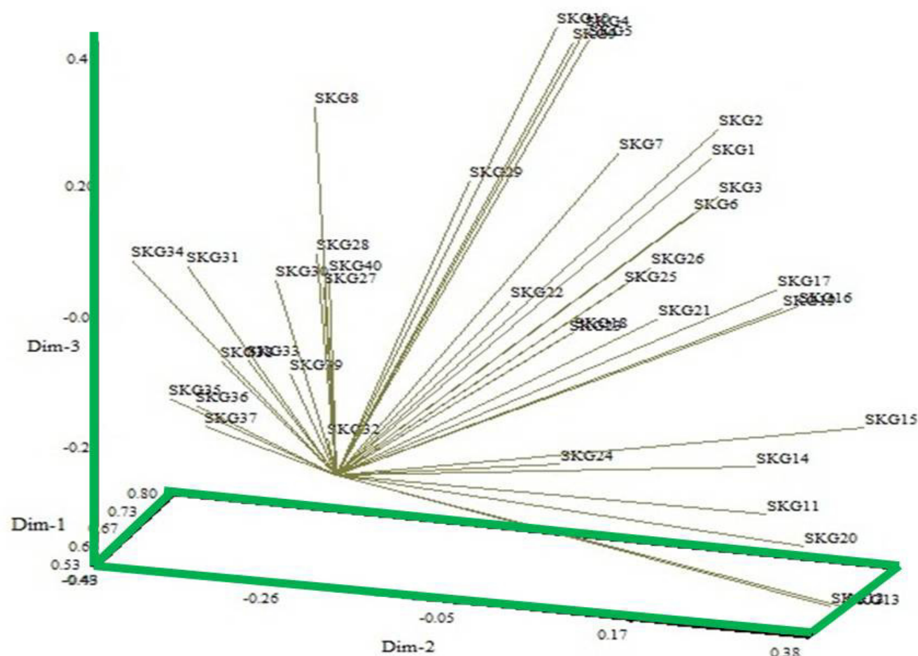


Figure 53 Principal Component Analysis showing grouping of ginger scatter plants individual on a three dimensional scatter plot**.**

4.4.4 Genetic fidelity analysis of micropropagated clones of *Zingiber officinale* (*Majhauley*)

4.15.1 Standardization of amplification (PCR)

In the present study, PCR amplification protocol for amplification of ginger DNA was standardized using a gradient PCR. RAPD PCR reactions were generated using 15 different decamers (Clonitech Technologies) as primer. Out of that, 10 primers were selected as visualized in electrophoresis bands for further analysis based on their ability to detect distinct band and showing monomorphic bands (Table 4.24). Total 50

different plantlets were used for this study using 10 selected primers to examine the genetic fidelity of *in-vitro* culture of *Zingiber officinale* over a period of more than 1.5 years. RAPD analysis in micropropagated plants of *Zingiber officinale cv Majhauley* was performed with total of 38 amplified reproducible bands produced from 10 random decamers primers ranging from 150-950 base pair. The number of bands per primer ranged from 1 in CLT 112 (575 bp) and CLT 192 (650 bp) to 7 bands in CLT 53 (150-875 bp). The size of amplified products varied from 150-950 base pair in size with an average of 3.8 bands per RAPD primer. All the primers were found to be monomorphic and amplified bands were exhibited monomorphism within all the *in-vitro* propagules and similar those to control plants. Shown in (Figure 54, A, B, C). Numerous analyses of soma clonal variations have been done using PCR- based techniques. No genetic variations or polymorphic amplified DNA bands were obtained after amplification by

Table 4.24 RAPD banding pattern of micropropagated plants of *Zingiber officinale* cv *Majhauley*

Sl. No	Primers	Sequences	Range of amplicons (in bp)	Total bands
1.	CLT 182	5′-GTTCTCGTGT-3′	250-900	4
2.	CLT 76	5′-GAGCACCAGT-3′	450-950	6
3.	CLT 78	5′-GAGCACTAGC-3′	150-750	5
4.	CLT 53	5′-CTCCCTGAGC-3′	150-875	7
5.	CLT 112	5′-GCTTGTGAAC-3′	575	1

6.	CLT 192	5′-GCAAGTCACT-3′	650	1
7.	CLT 25	5′-ACAGGGCTCA-3′	200-600	3
8.	CLT 54	5′-GTCCCAGAGC-3′	300-900	5
9.	CLT 105	5′-CTCGGGTGGG-3′	150-300	2
10.	CLT 30	5′-CCGGCCTTAG-3′	175-500	4
			Total	**38**

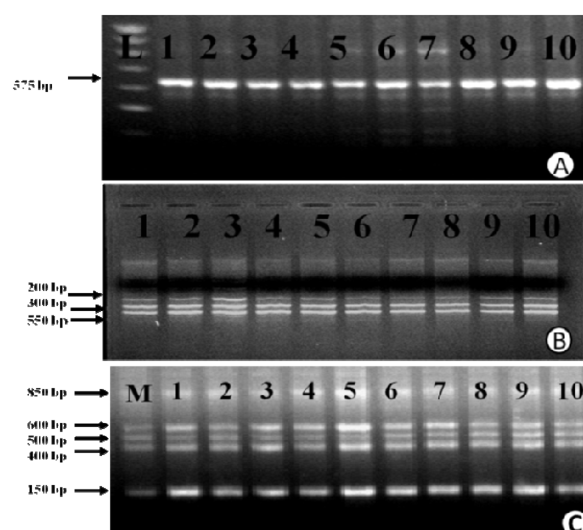


Figure 54 A-C RAPD banding pattern showing monomorphism A) primer CLT 112, L represent ladder (marker) and 1-10 represent micropropagated ginger plants. B) Primer CLT 25, 1-10 represent micropropagated plants. C) Primer CLT78, M represent mother plants and 1-10 micropropagated plants. Arrow represents the size of the bands with markers.

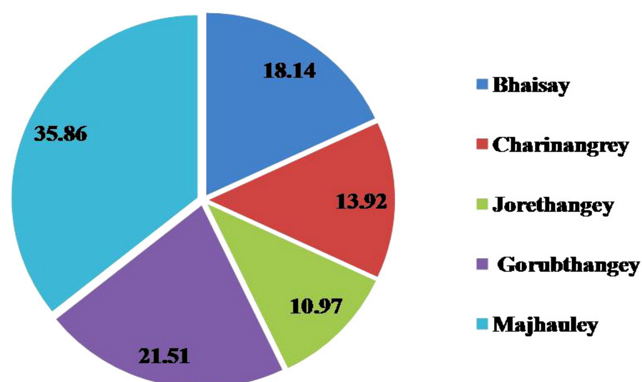


Figure 55 Showing percentage of five different gingers cultivars on the basis of total areas surveyed in Sikkim Himalaya.

Figure 54 A-C RAPD banding pattern showing monomorphism A) primer CLT 112, L represent ladder (marker) and 1-10 represent micropropagated ginger plants. B) Primer CLT 25, 1-10 represent micropropagated plants. C) Primer CLT78, M represent mother plants and 1-10 micropropagated plants. Arrow represents the size of the bands with markers.

Chapter 5

Discussion

5.1 Ginger cultivation and genetic resources in Sikkim

Sikkim is one of the important ginger cultivation areas in India and about five major cultivars have been used by the local farmers. The principal cultivar is the "*Majhauley*' which occupy 35.86 % of total production followed by the cultivars "*Gorubthangey*" (21.51 %), "*Bhaisay*" (18.14 %), "*Charinangrey*" (13.92 %) and "*Jorethangey*" (10.97 %) (Figure 55).

In the present study, ginger cultivation was recorded at an elevation of 1019-5784 ft in Sikkim which is contrary to earlier work that reported ginger from sea level up to an altitude 4921.26 ft (Ravindran *et. al.,* 2005; Utpala *et al*., 2006). The present differences may be due to adaptive nature of monocot species.

It was recorded that *Majhauley* cultivar is preferred by most of the ginger farmers and the possible reasons is the higher percentage of production of *Majhauley* cultivar in Sikkim. This cultivar has commercial importance because of its higher content of zingiberene (16.6 %) followed by e-citral (12.0 %), z-citral (8.8 %), camphene (7.6 %) and ocimene (6.5 %) Raina *et al.,* (2005). It is also adaptive in nature and resistant against diseases. Moreover, size long and thickness 7.5 cm of the rhizomes of this cultivars are considered as optimum.

5.1.1 Spatial distribution of five ginger cultivars of Sikkim

Present ecological studies of ginger in Sikkim with the help of GIS technology showed that cultivation of ginger depends on individual cultivars, altitudinal climate and soil type. This result is concurred with that of Peter *et al.,* (2005). A map was drawn showing the total number of cultivars cultivated in different districts of Sikkim (Figure 152). Similar studies was reported by Utpala *et al.,* (2008) based on total numbers of varieties cultivated and the quality varieties cultivated in the different states of India.

Geographic Information System can provide information about the geographic location and fundamental characteristics of ginger. Utpala *et al.,* (2006), studied site suitability of ginger in India and predicted that suitability direct impact on ginger production. Kris Sunarto (2011) also studied land suitability of ginger in Indonesia using GIS technology. Information generated by GIS can then be applied to the development of conventional breeding technology and biotechnology, such as gene transfer or cell fusion, to increase the applicability of ginger. It is also useful for the conservation and sustainable application of crop genetic resources.

5.1.2 Morphological characterization of five ginger cultivars of Sikkim

Morphological characterization of five cultivars of *Zingiber officinale,* namely, *Majhauley, Bhaisay, Gorubthangey, Jorethangey* and *Charinangrey* as shown in showed distinct morphological characters between them. Knowing clear information about morphological/genetic variations among cultivars is essential for breeding purpose reported by Ravindran *et. al.,* (2005).

The significant differences in morphological characters of the given cultivars considered in the present investigation reveals that the occurrence of each ginger cultivar in different region could have played a major role in contributing to evolve distinct morphological characters in ginger cultivation of Sikkim.

In my present investigation, ANOVA revealed high variations in a number of morphological characters, viz., plant height, number of shoots, canopy, leaf density, leaf length, spike length, numbers of tillers, rhizome thickness and weight of rhizome among the five cultivars ginger of Sikkim**.** Several studies have reported that ginger cultivars differs significantly in a number of morphological characters (Pandey and Dobhal, 1993; Manmohandas *et al.,* 2000).

Furthermore, a researcher reported a high genotypic coefficient of variation in characters such as length and weight of secondary rhizomes, weight of primary rhizomes, number of secondary and primary rhizomes and rhizome yield per plant. The author has further reported that some varieties/cultivars can be distinguished to a certain extent based on rhizome characters Yadav (1999).

The significant linear positive and negative relationships between different morphological characters of the ginger cultivars in the present investigation indicate that the aerial morphological character were directly or indirectly connects with underground morphological character like rhizome yield, weight of rhizome, and thickness of rhizome. There are several contributing characters which are directly associated with rhizome yield, weight of rhizome, and thickness of rhizome and it simultaneously affects a number of other correlated traits.

Therefore, the clear knowledge regarding association of characters with rhizome characters and among different characters provides a guideline to the plant breeder for making crop improvement through selection. In my present investigation results reveals that significant linear correlation between mean weight of rhizome and mean plant height per plant, mean canopy per plant, mean number of shoots per plant, mean number of leaf density per plant, mean leaf length per plant, mean spike length per plant. The analysis results demonstrated that these traits had certain inherent relationships with rhizome characters and it is recommended that assortment of these traits would directly improve rhizome character and yield in ginger.

Similar studies made on 100 accessions of ginger reports positive relationship of different morphological characters between rhizome yield and yield attributes in ginger (Sasikumar *et al.,* 1992; Singh, 2001). Further, these authors have reported that plant height shows a significantly positive association between leaf and the tiller number and between length and the width of leaf. They found that rhizome yield was positively correlated with plant height, tiller and leaf number, and leaf length and width. Few researchers also reported on positively correlation between plant heights was positively and significantly correlated with number of leaves, leaf length, rhizome length, rhizome breadth, and yield per plot. Rattan *et. al.,* (1988), Pandey and Dobhal (1993), Islam *et al,* (2008).

Furthermore, investigation of principal component analysis reveals 51.78 % total variance of differing five cultivars based on three qualitative and ten quantitative morphological characters. Similar studies reported moderate variability on 100

accession ginger germplasm based on morphological, yield and quality factor, and found highest variations in tiller numbers and rhizome yield plant^{-1} Ravindran *et al.,* (1994).

Researchers also reported that any vegetatively propagated species such as ginger, the extent of genetic variability will be limited unless samples are drawn from distinctly different agro-ecological situations Ravindran *et al.,* (1994). In my present study reveals that the following morphological characters Plant height, Canopy, Numbers of shoots, Leaf Density, Spike Length and Shoot Diameter, Rhizome Shape, Spike Length and Leaf Density and Rhizome thickness size played a significant role in differentiating character to discriminate between the five cultivars of *Zingiber officinale*.

Furthermore, ginger cultivars have been given their names according to the size of rhizome, viz., *Bhaisay* (big/large size), *Majhauley* (medium size), *Charinangrey* (nail of bird- smaller in size) (Pradhan *et al.,* 2011) and *Gorubthangey* and *Jorethangey* have been named according to name of place originated or cultivated. Some researchers conducted similar studies and reported that a high genotypic coefficient of variation for length and weight of secondary rhizomes, weight of primary rhizomes, number of secondary and primary rhizomes, and rhizome yield/plant (Yadav, 1999). Manmohandas *et. al.,* (2000) found that all the cultivars differed significantly in tiller number and leaf number.

5.2 Micropropagation of *Zingiber officinale* of Sikkim Himalaya

In the present investigation, the shoot tip was found as the most suitable explants for *in-vitro* micropropagation of ginger. Maximum number of multiple shoot regeneration was recorded in shoot tip explants cultures. The present results are in accordance with previous report of Malmug *et.al.* (1991), Mukund, (1998), Rout *et al.,* (2005) Sharma and Singh, (1997), Khatun *et al.,* (2003). Furthermore, ginger root tip and leaf explants were failed to initiate the growth.

In the present investigation, contamination free regeneration could be achieved after surface sterilization of the explants by treating them with 1% solution of tween 20 for 15 min followed by 0.1 % mercuric chloride for 10-15 min. The explants were then dipped in 6% sodium hypochlorite for 5-8 min and finally washed in double distilled sterile water. Earlier, Berger *et. al.,* (1994) had reported surface sterilization of explants using 1% sodium hypochlorite or saturated calcium hypochlorite that resulted in the decrease of microbial contamination up to 62 % to 90 %. Sushma *et al.,* (2005) developed a sterilization protocol for *Hedychium spicatum*; the explants were treated with tween 20 for 10 min followed by the treatment with 1 % bavistin and 1 % $HgCl_2$ for 3 min. Rahaman *et. al.,* (2004) reported that rhizome buds were treated with solution of antiseptic savlon 5% (v/v) for 10 min. Then explants were washed with distilled water and finally treated with $HgCl_2$ (0.1%) for 14 min gave good results.

To reduce bacterial contamination, Wondyfraw and Surawit, (2004) reported that buds of *Korarima* were rinsed with 70 % ethanol for 1 min followed by 6 % sodium hypochlorite washed with sterile distilled water resulted in lowest degree of

contamination. Kobza and Vachunova, (1991) reported that chlorinated lime at 10 % concentration and $HgCl_2$ at 0.1 % for 10 min were the best sterilants for *Dracena* explants. Vuylsteke and De Langhe, (1985) used ethanol (95 %) for 15 seconds followed by 15 min treatment in a hypochlorite solution (1.5 %) to achieve surface sterilization of the explants. To control microbial contamination, Kambasaka and Santilata (2009) has washed ginger explants with 5 % ethanol for 10 min followed by Bavistin 0.3 % and final rinsing in streptomycin 0.2 % for ten min. Malmug *et al.* (1991) were able to establish contaminant free *in-vitro* ginger by sterilized explants with tween 80 followed by a 10 min treatment in a sodium hypochlorite solution (active chlorine 0.5 %).

Most of the *in-vitro* micropropagation work on ginger was seen to carried out in MS medium (Sharma and Singh, 2007; Rout *et al.,* 2001; Khatun *et al.,* 2003 etc.). Pandey *et al.,* (1997) reported *in-vitro* propagation of *Zingiber officinale* using MS medium fortified with different concentration of growth regulator. Jamil *et al.,* (2007) investigated regeneration of ginger plant from callus culture using MS medium fortified with different growth regulator. Nasirujjaman *et. al.,* (2005) grew turmeric rhizome bud on MS medium containing different concentrations of BA and NAA. Balchandran *et al.* (1990) reported that rhizome buds excised from *Curcuma longa* was inoculated on MS medium with different combinations of BAP and Kinetin. For best shoot multiplication BA 3 mg /l was found to be good. Malmug *et al.* (1991) reported that shoot proliferation of the regenerated shoots was induced with the addition of NAA 1 mg + BA 5 mg /l in MS.

In the present investigation best medium for induction of shoot tip was recorded GM 23 which were found to be superior than the Khatun *et al.,* (2003), where the author recorded 20 days for shoot induction in MS medium fortified with BAP 1.0 mg/l +0.5 Kn. The maximum time (40-45 days) required for shoots initiation was recorded in the medium GM 5 mg/l and GM 19 (MS + Kinetin 5.0 mg/l). It was recorded that shoot initiation was better in MS medium fortified with both growth regulators (cytokinin and auxin) than MS medium fortified with only single growth regulator. The present result was concurred with Rout *et al.,* (2001), that medium having IAA or NAA alone had no effect on shoot multiplication or growth in ginger.

The present investigation revealed that more multiple shoot formation was higher in BAP as compared to Kinetin. This result was in concurred with the results of Wong, (1986) and Zamora *et al.* (1986) who observed that BAP is the cytokinin of choice for induction of shoot bud proliferation *in-vitro* and BAP has been found to be superior to Kinetin in banana.

The results revealed that MS supplemented with BAP 2.5 mg/l gave highest mean number of shoots. This present result was accordance with previous report of Dipti *et al.* (2005), who reported that the highest number of multiple shoots in media supplemented with BAP 2 mg/l in shoot tip and BAP 3 mg/l in rhizome bud in turmeric, proved its superiority over Kinetin and NAA by producing more number of multiple shoots. BAP at 3 mg/l was most beneficial for proliferation in turmeric (Balachandran *et al.,* 1990). Similarly, Winnar and Winnar, (1981) reported that BAP 1 mg/l was most

useful for development of multiple shoots. These findings are in conformity with the Keshavachandran and Khader (1989) and Shetty *et. al.,* (1982).

In the present experiment, *Majhauley*, *Bhaisay* and *Charinangrey, Jorethangey* and *Gorubthangey* cultivars showed considerable response in the culture medium having combination of BAP 2.5 mg/l and NAA 0.5 mg/l with addition of 2 mg/l activated charcoal. This combination of growth hormones in the culture medium was able to produce dark leaf, optimum height and a good number of roots**.**

The number of shoots on the best combination of GM 23 (MS + BAP 2.5 mg/l + NAA 0.5 mg/l) and 24 (MS + BAP 3.0 mg /l + NAA 0.5 mg/l) ranged from 17-19 numbers of shoots per explants after incubation for 50-60 days of incubation, which was significantly higher than reported by (Inden *et. al.,* 1990), who found 4 shoots per explants in modified MS medium. Kambaska *et. al.,* (2009) reported that maximum number of shoots (7.7) was obtained on the medium containing BAP 2 mg/l and NAA 0.5 mg/l shoots per explants of cultivars Suprava and Suruchi. Balchandran *et al*., (1990) also reported 4 shoots per bud on MS medium with BA 3 mg/l after 4 weeks of incubation.

After sub-culturing the ginger propogules on same fresh medium, adventitious buds were initiated after 10–15 days of culture and complete plantlets developed within 40 days which were maintained for 18 months. Sharma and Inden (1988) have reported that *in-vitro* culture of ginger propagules can maintain up to 28 months and 1 year.

When a single growth regulator was used in the culture medium the maximum number of shoots was observed for GM 14 (9.25 ± 0.4 in BAP 2.5 mg/l), followed by

GM 4 (6.65 ± 0.2 in Kinetin 2 mg/l) with lower number of leaf and roots as compared with GM 23 and GM 24, a comparatively lower response was recorded when BAP and Kinetin was added alone in the medium this shows that the combination of cytokinins and auxin in the culture medium enhanced the response in a number of species in terms of shoot growth. Similar result was concur with *Spathiphyllum floribundam* when cultured on media with BAP alone, a limited 1.5 shoots per culture was examined; while addition of IAA produced an average number of 11.6 shoots per explants (Malamug *et al*, 1991) Similar observation was reported in *Hovenia dulcis* nodal culture (Echeverrigaray *et al.,* 1998). Same result was significantly coordinated with the work done by (Kambasaka *et al.,* 2009) after addition of auxin produced more numbers of shoots.

In the present investigation, good number of roots produced in the same multiple shoots hormonal concentration. The present results concur with the reports published by Khatun *et al.,* (2003) and Kavyashree (2009) against ginger species. Similar results have been reported by Meenakshi *et al,* (2001), where maximum rooting observed in NAA 0.3 mg/l with maximum root length. These results were in accordance with the findings of Dogra *et al,.* (1994) in ginger and Raju *et al.,* (2005) in turmeric.

The highest mean shoot length was recorded in (7.01 cm) in MS medium with BAP 2 mg/l plus NAA 0.5 mg/l and lowest mean shoot length was recorded (2.98 cm) in MS fortified with BAP 3 mg/l. Similar results were observed as earlier in mean shoots number from that single growth regulator have less efficiency in formation of

mean root numbers and mean root length. But the potential of regenerated propogules increases after incorporation of both growth regulators (cytokinin and auxin).

The present investigation on five cultivars of ginger showed that the shoot tip as explants in all five samples of *Majhauley, Bhaisay, Gorubthangey, Jorethangey* and *Charinangrey* responded well and found to be the best explants for the production of disease free planting materials. A total of 37 different hormone concentrations with MS medium had been tried in tissue culture experiments. The best medium was MS fortified with sucrose 30 g/l, BAP 2.5 mg/l, NAA 0.5 mg/l and activated charcoal 2 mg/l.

5.3 Antimicrobial activities

The rhizome of five different cultivars of *Zingiber officinale* were extracted using five different solvent like petroleum ether, ethanol, chloroform methanol and acetone for extraction of bioactive compounds based on polarity of solvents for various studies. Similar solvents were used by different authors for extraction of polar and non-polar compounds. Balladin and Headley (1999) had isolated less polar compounds from ginger rhizome using petroleum ether and diethyl ether by liquid chromatography. More polar compounds such as aliphatic acids, sesquiterpene hydrocarbons, ar-curcumene, bisabolene, farnesene and zingiberene was isolated from more polar solvent pentane and ethanol using column chromatography (Herout *et al.,* 1953). Sane *et al.,* (1998) also used methanol for extraction of gingerol during study of geographical variation of the content of gingerols for different areas of India and no such variations were observed.

Ginger rhizome extracts were tested for antimicrobial activity towards human pathogenic microorganisms (*Staphylococcus aureus* (MTCC 96), *Bacillus subtilis*

(MTCC 441), *Klebsiella pneumoniae* (MTCC 432), and *Pseudomonas aeruginosa* (MTCC 424). They showed good antimicrobial activity. The antimicrobial activity of ginger is depended on the bioactive compounds, namely gingerone and gingerol (Chen *et al.,* 2001; Hirasa and Takemasa, 1998). However, no activity was recorded against *Escherichia coli* (MTCC 739). The present results were concurred with the previous work on ginger (Nguanpaug *et al.,* 2011; Adeshina *et al.,* 2011).

Among five solvents, the most responsible for the susceptible action were recorded in chloroform and ethanol extracts. The reasons for chloroform and ethanol extract may be accredited to the presence of active components responsible for antimicrobial activity. Earlier, Hasan *et al.,* (2009) reported that antibacterial activities of chloroform extract against some human pathogenic bacteria. Ayse *et. al.,* (2008) used ethanol and chloroform extracts of ginger for antimicrobial and cytotoxic activities on human cervical cancer cell lines. Anjan *et al.,* (2012) studied antimicrobial activities of 10 % ethanol ginger extracts against microorganisms that cause oral infections. Nader *et. al.,* (2009) used ethanolic extracts for antimicrobial studies against gram negative bacteria. Pedgee *et al.,* (2012) reported high antimicrobial activity against gram genitive bacteria. Auta *et al.,* (2011) recorded ethanol extracts of ginger showed high antimicrobial activity against pathogen for gastrointestinal tracts.

Among gram positive and gram negative bacteria, the greater susceptibility of gram positive bacteria has been previously reported for South American (Paz *et al*., 1995), African (Kudi *et al*., 1999; Vlietinck *et al*., 1995) and Australian (Palombo and Semple, 2001) plant extracts. The reason accountable for the high susceptibility of the

test organism to ginger are not exactly known but may be attributed the secondary metabolite and phytochemicals (gingerol and shogaol, flavonoids) (Stewart *et al*., 1991). Barasch *et al.,* (2004) reported that responsible for antifungal and antibacterial activities of ginger were presence of saponin, alkaloids and flavonoids. This outcome is comparable with other investigation which has shown that ginger has broad antibacterial activity (Chen *et al.,* 2008, Atai *et al.,* 2007, Ficker *et al.,* 2003). The results obtained in this study are in accordance with that of Ficker *et al*. (2003), Grange and Davey (1990) and Zahra *et al*. (2009). Least susceptibility towards gram-negative bacteria was observed, may be due to bacterial cell wall outer membrane appears to act as a barrier to many substances including antibiotics (Tortora *et al*., 2001). Other extracts also obtained modest sensitivity against *P. aerugi*nosa. Yusha**,** *et al.,* (2008) and Auta *et al.,* (2011) also reported highly sensitivity against *P. aerugi*nosa using ethanol ginger extracts. This study was directly concurred with that of (Yusha**,** *et al.,* 2008; Indu and Menon, 2010; Jha and Barman, 2013).

The significant inversely correlation between total phenol content and antimicrobial activity and total flavonoid content and antimicrobial activity of the given cultivars reveals the responsible for antimicrobial activity. Based on statistically differences in antimicrobial activity of the given cultivars considered in the present investigation, it is demonstrated that due to the presence of bioactive compound like gingerol and shogaol. As several studies have also specified that the antimicrobial sensitivity of ginger is attributed to the presence of oxygenated mono-and sesquiterpene,

phenolic compounds like shogaol and gingerol (Michielin *et al.,* 2009; Singh, 2008; Hiserodd *et al.,* 1998; Hasan *et al.,* 2012; Wang *et al.,* 2009; and, Liu *et al.,* 2011).

Bajpai *et al.,* (2009) and Hasan *et al.,* (2012) reported that bioactive compounds have different ways of effect since these compounds not only attack cell walls and cell membranes i.e., affecting their permeability and release of intracellular constituents (e.g. ribose, glutamate) but they also interfere with membrane functions (electron transport, nutrient uptake, protein, nucleic acid synthesis and enzyme activity). Thus, these compounds might have several invasive targets which could lead to the inhibition of bacterial pathogens.

Thus it can be inferred from the above results that all the ginger extracts irrespective of the variety or the solvent type are more effective against the gram-positive bacteria compared to the results for the gram-negative ones. The higher resistance of the gram-negative bacteria could be due to the complexity of the cell wall of this group of microorganisms. Indeed, the external membrane of gram-negative bacteria renders highly hydrophilic surfaces whereas the negative charge of the surface of the gram-positive wall may reduce their resistance to antibacterial compounds (Michielin *et al.*, 2009).

5.4 Antioxidant study

Phenolic compounds is considered support on group of chemicals that possess antioxidant activities as well as known powerful chain breaking antioxidant (Shahidi et. al., 1992) and have mainly due to their redox properties (Zheng and Wang, 2001) which play an important role in neutralizing free radicals. In present investigation, total

phenol content in case of ginger (*Zingiber officinale*) ranged in between 99.00 to 228.557 mg/ml GAE (gallic acid equivalent) per gm. of plant extract irrespective of the variety or the solvent type were recorded.

The present results concurred with the work done by various researchers that the rhizome of *Z. officinale* contains high antioxidant activity and 6-gingerol is the polyphenol compound that presented in the extracts responsible for antioxidant activity (Stoilova *et al.,* 2007). Furthermore, Surch (2002) showed that polyphenol phytochemicals presented in methanolic extracts of ginger and generally possess strong anti-inflammatory and anti-oxidative properties and exert substantial anti-carcinogenic and anti-mutagenic activities. Ali *et al.,* (2010) reported that young ginger rhizome and leaf showed positive relationship between total phenolic content and high antioxidant activities in *Z. officinale* (Halia Bara).

Flavonoids are regarded as one of the most widespread groups of natural constituents found in plants (Patel *et. al.,* 2010). Ferguson *et al.,* 2001 also reported that flavonoids too serve as health-promoting compounds due to their anion radicals. The values of flavonoid content varied from (621.52 mg/ml QE) chloroform extract of *Charinangrey* and *Bhaisay* (45.12 mg/ml QE). The totals contain of flavonoids and phenolic are influenced by the interaction between varieties and plant parts (Ali *et al.,* 2010). The higher amounts of total flavonoids in the petroleum ether extract have also been reported in some of the medicinal plants (Kaneria *et. al.,* 2009) and *Murraya paniculata* (Gautam *et al.,* 2012).

In this study, results showed that DPPH radical scavenging abilities of the extracts showed activity ranged from 84.81% to 94.18%. The standard ascorbic acid showed the scavenging activity of 64.76%. Kikuzaki and Nakatani (1993) reported that ginger solvent extracts may include phenolic and hydrox-phenolic compounds responsible for higher antioxidant activity. Shobana and Naidu (2000) reported that ethanol extracts of *Z. officinale* recorded potent antioxidant activity and prevent lipid peroxydation. Similarly, Stoilova *et al.,* (2007) recorded ginger rhizomes extracts displayed higher free radical scavenging ability than butylated hydroxytoluene (BHT) and Quercetin.

The reducing ability of different cultivar of ginger extracts was in the range from the highest reductive capability noted in GCC (OD 700 nm = 1.177) while the lowest reducing ability was noted in GCB (OD 700 nm = 0.176), however the standard ascorbic acid (OD 700 nm = 0.041) showed much lower than GCB. The ferric reducing ability (FRAP assay) is widely used in the evaluation of the antioxidant component in dietary polyphenols (Luximon *et al.,* 2005). Among the five different solvents chloroform was found to be more effective in transforming Fe^{3+} to Fe^{2+} in most of the plant varieties and this might be due the enormous amount of phenolic content in the extracts (Oktay *et al.,* 2003; Ghasemzadeh *et al.,* 2010).

The scavenging ability of different solvents extracts of *Zingiber officinale* on hydrogen peroxide is shown in (Figure 47) and compared with that of ascorbic acid. Different extracts of *Zingiber officina*le were capable of scavenging hydrogen peroxide in a concentration-dependent manner. These results showed that all extracts of *Zingiber*

officinale L. had effective hydrogen peroxide scavenging activity ranged from 21.68 % to 58.31 %, 36.64 % to 70.99 %, 21.07 % to 58.90 %, 17.90 to 48.57 % and 38.18 to 67.92 in case of ginger varieties *viz. Majhauley*, *Bhaisay*, *Gorubthangey*, *Jorethangey* and *Charinangrey* respectively. Scavenging of H_2O_2 by the plant extracts may be ascribed to their phenolic, which donate electron to H_2O_2, thus reducing it to water (Goyal *et al.,* 2011b). The considerable potential of the ginger extract to scavenge H_2O_2 peroxide has also been reported earlier (Yesiloglu *et al.,* 2013).

5.5 RAPD analysis of five ginger cultivars of Sikkim

Genetic diversity is the product of long term evolution and it signifies the evolutionary potential of a species. It provides species the ability to acclimatize to changing environments, including new pests and diseases and new climatic environment. Genetic adaptation and the rate of evolutionary response to selective forces depend on inherent levels of genetic diversity present at the time a species experiences a threat to its survival (Li *et al.,* 1999). The genetic variations using RAPD markers has been studied in a number of economically important plants (Rout *et al.,* 1998; Sheng *et al.,* 2006; Rout 2006; Pattanayak *et al.,* 2011).

The present investigation comprises the classification and genetic differentiation of five cultivars of *Zingiber officinale* of Sikkim Himalaya. Based on the RAPD profile, measures of Shannon's index of diversity were from 0.21 to 0.36 with an average value of 0.33 (Table 4.23). The results show the prevalence of a relatively high level of polymorphism in the cultivars of ginger from Sikkim Himalaya. This is a fact that sample size and the total amount of bands can influence the percentage of polymorphic

bands, other parameters, such as, Shannon's index of diversity and Simpson's index of diversity are more suitable in estimating genetic variability (Cruzan, 1998). The average of Shannon's index of diversity in the cultivars is 0.33 (Table 4.3) indicating a relatively high degree of diversity among the ginger population. The result is in agreement with the high percentage of polymorphism as recorded by Nei's genetic diversity index. The genetic relation using RAPD markers provides reliable methods for identification of varieties than morphological characters (Paterson *et al.,* 1991). Rout *et al.,* (2007) analyzed genetic fingerprinting among eight varieties of *Zingiber officinale* using RAPD markers, the investigation showed the distant variation within the varieties, similar result was obtained by (Harisaranraj *et al.,* 2009) within the eight varieties of ginger of Orissa. Similarly, Pattanayak *et al.,* (2010) assayed forty nine ginger clones cultivated in North East India using RAPD markers reported high polymorphism detected in a cultivated species. Nayak *et al.,* (2005) reported significant genetic variations among 16 elite cultivars of gingers using cytological and RAPD markers.

Based on NTSYSpc analysis differentiated into five cultivars into four clusters with a Jaccard's coefficient of 0.35-0.87 (Figure 52) and occurrence of three cultivars in the same group indicating that habitat homogeneity between three cultivars (*Charinangrey*, *Majhauley* and *Gorubthangey*), which is in agreement with the reports of Pattanayak *et al.,* (2011). Cluster II and III comprised of *Jorethangey* and *Bhaisay*. But in the case of Cluster IV included single cultivar *Bhaisay* (SGK22). The possible for such separate group of the sample SKG 22 may be due to geographical bias or cryptic mutation. Other studies are also in agreement with the present results

(Kizhakkayil and Sasikumar, 2010). Cultivars/genotypes that form different clusters are potential germplasm that may be exploited to broaden the genetic base (Figure 52).

Principal Component Analysis (PCA) was further assessed to study relationship among individual's plants. Results of PCA showed high genetic diversity were recorded among the clones from Sikkim India. Similar investigation also reported by Jatoi *et al.,* (2008) against different *Zingiberaceae* genus and observed higher diversity within *Z. officinale* and other genus. Sanjeev *et al.,* (2011) also reported high diversity among the clones collected from India. Similar study was carried out by Watanabe *et al.,* (2006) using rice SSR marker as RAPD marker for genetic diversity analysis in *Zingiberaceae.* They reported that high variation was found among ginger, turmeric and galangal species. Jaing *et al.,* (2006) reported high genetic diversity using phylogenetic analysis and metabolic profiling among and within ginger species and result found that gingers variant from different geographical origins were indistinguishable.

5.5.1 Genetic fidelity analysis of micropropagated clones

Genetic fidelity analysis of micropropagated plants have been reported by various authors on different important plants species using PCR-RAPD technique. Such reports are available on *Zingiber officinale* (Rout *et al* 1998), *Pinus thunbergii* (Goto *et al,* 1998); *Plumbagao zeylanica* (Rout and Das, 2002), Almond (Martins *et al,* 2004), *Chestnut hybrids* (Carvalho *et al*, 2004) and *Cucurma olonga* (Panda *et al,* 2007). In the present investigation, merismetic axillary rhizome buds were used as explants for rapid multiplication of *Zingiber officinale* because it lowers the risk of genetic stability and

after sub cultured for 18 months, no genetic variations could be obtained using RAPD analysis. Similarly, Sheony and Vasil (1992) also reported that the micropropagated plants through axillary explants meristem showed with low risk of genetic stability because they are generally more resistant to genetic changes that might occur during differentiation under *in-vitro* condition. Similar results were coincided with same species V_3S_8. by Rout *et al,* (2004) reported monomorphic stability in micropropagated plantlets. Pandey *et al,* (2007) in *Cucurma longa* who sub cultured for two years and did not find genetic variations. Same results were concurred with Martins *et al.,* (2004), Angel *et al.,* (1996), in almond and cassava plantlets with no genetic variations. Few authors reported that the time in *in-vitro* give support to soma clonal variations and suggested that this variability was due to accumulating mutations during long term clonal growth (Major *et.al,* 1998,; Rani *et. al.,* 1995). Gould (1986) reported that culture period does not give the impression to be the only parameter affecting genetic stability.

During the time of *in-vitro* micropropagation, the plants have been exposed to high doses of plant growth regulator, which leads to alteration of genetic makeup of the plants for the duration of many sub cultured, ultimately high frequency of soma clonal variations results. Many researchers recorded soma clonal variation in micropropagated plants (Larkin and Scowcroft 1981, Earl and Demarly1982, Rani *et al.,* 1995, Hasmi *et al.,* 1997). This somaclonal variation is frequently heritable (Larkin *et al.,* 1981, Breiman *et al.,* 1987) and is unwanted for true to type propagation. Thus it is very important to detect this somaclonal variation early in the *in-vitro* reproduction succession of the plant, to save from great economic loss in future. Various researchers

have tried to assess these variations in different plants species through morphological, biochemical and molecular analyses (Renfroe and Berlyn 1984,; Mo *et al.,* 1989,; Shenoy and Vasil 1992). However these analytical methods have their own limitations. Molecular markers are widely used to detect and characterize somaclonal variation at the molecular level (Ford-Lloyd *et al.,* 1992.; Barrett *et al.,* 1997). Of the available methods, RAPD is among the easiest, inexpensive and useful ones (Rani *et al.,* 1995, Taylor *et al.,* 1995, Rout *et al.,* 1998).

Chapter 6

Summary

Zingiber officinale (Family- Zingiberaceae), commonly called ‘ginger’, is an herbaceous rhizomatous perennial plant commonly cultivated in India. Plants are erect, semi erect, aerial shoots (pseudo-stems) with leaves, have numerous fibrous and fleshy roots that emerge from the branched rhizomes. Since time immemorial, ginger has been regarded to have numerous medicinal properties. Ginger has been cultivated since ancient times. Because of its long history of cultivation, many varieties/cultivars have been made or developed till date. Identification and characterization of ginger germplasm are very important for the conservation and utilization of plant genetic resource. Selection and breeding for the production of desirable traits in this crop have resulted in severe erosion of its genetic base over the time. Lack of proper conservation programmes have caused major reduction in its gene pool, as most of the breeding and conservation programs are still based on conventional morphological and agronomical descriptors, which are dependent on environmental and developmental factors. However, like any other crop, the main objective of ginger breeding is to improve the quantity and quality of the product. This apart, the prediction of the performance of mature ginger based on their evaluation in the early years has not been perfected.

In ginger research the previous era held major emphasis on standardizing parameters of *in-vitro* protocol, such as using a suitable explant, overcoming microbial

contamination, and optimizing media composition combined with growth regulation for better proliferation. Presently, attention is increasingly focused on evaluating field performance of the transformed *in-vitro* grown whole plantlets. However, there is no stable technique available for *in-vitro* multiplication of ginger in Sikkim.

In order to stop further reduction of ginger gene pool and to breed for new ginger types with more productiveness; less prone to natural calamities, diseases, as well as higher contain of ginger oil, a thorough knowledge of the existing genetic diversity, *in-vitro* culture studies and improvement of the existing varieties through various molecular biology, tissue culture and biotechnological techniques is a pre-requisite in ginger research.

Sikkim is known for its ginger production and different varieties. At present, many ethnic tribes inhabit in rural areas of Sikkim, and possibly these different tribes have transferred ginger cultivars to this place during the period of their settlement. Till date, rural people of Sikkim only know the ginger cultivars but lack scientific information and reports on it. However, systematic studies are not available particularly in the area of characterization of ginger germplasm, micropropagation and genetic fidelity study of tissue culture plants. Considering the importance of this cash crop, the present study was designed to address and resolve many problems that have cropped up in ginger cultivation.

6.1 Ecological studies

In order to assess the requirement of specific habitats, ecological studies of ginger in Sikkim Himalaya has been done using Geographic Information System (GIS)

with an overlay technique. The study revealed that the maximum ginger farmers of Sikkim preferred to cultivate *Majhauley* cultivar (35.86 %) followed by *Gorubthangey* (21.51 %), *Bhaisay* (18.14 %), *Charinangrey* (13.92 %) and *Jorethangey* cultivars (10.97 %) in all altitudinal ranges. The least cultivated *Charinangrey* in Sikkim at an altitude range from 4080 to 4790 ft. reflects that this cultivar prefers to specific habitats.

From 237 plots of ginger growing areas studied, 125 plots showed no diseases and 112 plots infected by fungal and bacterial diseases. According to district-wise, only north district of Sikkim showed no infection of diseases and resistant/less susceptible against pathogenic microorganism especially for *Charinangrey* and *Majhauley* cultivars.

Morphological characters on five cultivars of *Zingiber officinale* based on quantitative traits found that plant height, numbers of shoots, shoot diameter, canopy, leafs density, leaf length, numbers of tillers rhizome thickness and weight of rhizome, and qualitative traits like spike bract tip colour and shape of rhizome were important traits for differentiating among five ginger cultivars. Among all the morphological traits, characters, such as, rhizome thickness, no. of shoots and shoot diameter showed important contribution in differentiating different ginger cultivars. Further, many morphological characters are positively correlated, for examples, mean weight of the plant with the mean plant height per plant, mean canopy per plant, mean number of shoots per plant, mean shoot diameter per plant and mean number of leaf density per plant. Other morphological characters that have shown positive correlations are between mean no shoots vs. number of leaf density per plant, mean leaf length per plant vs. mean

spike length per plant. This information would play important role to directly improve rhizome character and yield in ginger.

Among the five cultivars of *Zingiber officinale,* based on ten quantitative and three qualitative morphological characters, cultivar *Charinangrey* differentiates distinctly from other four ginger cultivars. PCA analysis suggests that characters, such as plant height, canopy, numbers of shots, leaf density, spike length and shoot diameter, rhizome shape, spike length and leaf density and rhizome thickness size are important for differentiating five cultivars of *Zingiber officinale*.

6.2 Tissue culture

In the present tissue culture experiment, full and half strength MS Medium (Murashige and Skoog, 1962), Gambrog B5, SH (Schenk and Hildebrandt, 1972) and White (White, 1963) medium containing BAP in various concentrations was used for selection of the medium. Out of the five media tested, MS medium gave the best result.

Among the various explants tried, shoot tips explants gave the suitable response for initial growth and the highest number of multiple shoots as compared with root tip and leaf explants of ginger. Various concentrations of mercuric chloride ($HgCl_2$) and sodium hypochlorite (NaOCl) were attempt for aseptic sterilization. Among that concentration 0.1 % $HgCl_2$ and 6 % NaOCl emerged as the best treatment for sterilization

Shoot initiation was good in MS medium fortified with both growth regulators (cytokinins and auxin) than MS medium fortified with only single growth regulator.

Early initiation of shoot was observed in MS medium containing BAP 2.5 mg/l and NAA 0.5 mg/l which took minimum time 8-10 days. When single growth regulator were used in MS medium the highest number of shoots were recorded in BAP showed as compared to kinetin.

In MS medium, *Majhauley*, *Bhaisay* and *Charinangrey, Jorethangey* and *Gorubthangey* cultivars showed maximum number of shoots, medium having combination of BAP 2.5 mg/l and NAA 0.5 mg/l with addition of 2 mg/l activated charcoal. This combination of growth hormones in the culture medium was able to produce dark leaf, optimum height and a good number of roots**.**

Various growth regulators were used in different concentration in the MS medium. Out of that BAP 2.5 mg/l plus NAA 0.5 mg/l gave the maximum number of shoots (19.98) per explant, as compared to Kinetin 2.0 mg/l plus NAA 0.5 mg/l gave (14.15) number of shoots per explant.

In MS medium, with the same hormonal concentration, BAP 2.5 mg/l and NAA 0.5 mg/l gave a good number of roots, this protocol eradicate a step of *in-vitro* rooting.

Three different combination were tried for best hardening media (A) perlite, soil and farmyard manure with ratio of 1:1: B) soil, farmyard manure, sand, perlite at the ratio 1:1:1:1 (C) soil, farmyard manure and sand 1:2:1. Media B gave the maximum survival percentage with better growth resulting as a suitable medium for hardening.

In t-test analysis, single growth regulator medium gave significance difference between and within treatments (P (T<=t) two tail = 0.01356997), whereas combination

of growth regulator showed no significance (*P* (*T*<=t) two tail = 0.321765267) but the number of shoots were recorded highest in this condition only.

Genetic fidelity of *in-vitro* micropropagated clones of PCR-RAPD examination showed no variations in the micropropagated plants.

6.3 Antimicrobial activity

Total twenty five extracts were examined for antibacterial activity towards five microorganisms (*Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 441), *Klebsiella pneumoniae* (MTCC 432), and *Pseudomonas aeruginosa* (MTCC 424). However, inhibitory activity was not recorded against *Escherichia coli* (MTCC 739).

Chloroform extracts of all the cultivars showed inhibition against the growth of *Staphylococcus aureus and Bacillus subtilis.* The chloroform extract of cultivar *Majhauley* showed widest zone of inhibition of 26 mm and 24 mm.

Various solvents extracts of cultivars of ginger were examined against two gram negative bacteria *Klebsiella pneumonia* and *Pseudomonas aeruginosa.* The chloroform extracts of *Majhauley* showed high inhibition against both the gram negative bacteria with 14 mm and 18 mm inhibition zone, respectively.

The statistically significant difference of one way ANOVA was achieved among all microorganism (P < *0.01790*), gram positive bacteria (P < 7.69778E-15) and gram negative bacteria (P < 1.06101E-09). The maximum solvent significant found in acetone with significant value of *(*P < *1.44E*-13), followed by methanol (P < *2.04231E-*

11), ethanol ($P < 2.92E$-11), chloroform ($P < 3E$-11) and petroleum ether ($P < 6.21E$-11).

Among various solvents extracts, acetone extracts was found as the highest significant, followed by methanol, ethanol, chloroform, and petroleum ether extracts. The present investigation showed that there was significant direct correlation between total phenol and total flavonoid content against antimicrobial activity towards gram positive and gram negative bacteria.

6.4 Antioxidant activity

Various solvents were used for estimation of total phenols and flavonoid content of five different cultivars of ginger. Chloroform proved to be the best solvent for extraction of phenol and flavonoid. The highest total flavonoid and total phenol content was found in the chloroform extract of *Charinangrey* and *Majhauley* cultivars. The differences in total phenolic content among five cultivars of gingers used were statistically significant ($P < 0.0000661$) whereas flavonoid contents were not significant ($P < 0.348$). Among all 25 extracts the highest DPPH scavenging activity found in acetone extracts of *Majhauley* cultivar, the highest reductive capability of the transformation of Fe^{3+} to Fe^{2+} in presence of the extract found in Chloroform extracts of *Charinangrey*, and highest H_2O_2 scavenging activity found in methanol extracts of *Bhaisay*.

6.5 Genetic diversity study of five cultivar gingers using RAPD

The results showed the prevalence of a relatively high level of polymorphism in the cultivars of ginger found in Sikkim Himalaya. A total of 104 clear, reproducible and scorable RAPD fragments ranging from 150–13000 bp were generated from 21 primers. Of the 104 scorable RAPD bands, 99 were found polymorphic. Among five cultivars the highest percentage of polymorphic loci, gene diversity and Shannon's diversity index observed in *Majhauley*, *Gorubthangey* and *Bhaisay* cultivar respectively. Out of the five cultivars of ginger, *Gorubthangey* and *Bhaisay* found more diverse while *Gorubthangey* and *Majhauley* showed similarity. The cultivars *Jorethangey* and *Bhaisay* also showed similarity.

BIBLIOGRAPHY

Addeo, G., Guastadisegni, G., and Pisante, M. L. (2001) Land Water quality for sustainable and precision farming. *In: Procs 1st World Congress on Sustainable Agriculture, Madrid*, 1-4.

Adeshina, G. O., Jibo, S., Agu, V. E., Ehinmidu, J. O. (2011) Antibacterial activity of fresh juices of *Allium cepa* and *Zingiber officinale* against multidrug resistant bacteria. *In International Journal of Pharma and Bio Sciences*, **2**:289-295.

Altman, R. D., and Marcussen, K.C. (2001) Effects of a ginger extract on knee pain in patients with osteoarthritis. *Arthritis and Rheum,* **44** (11): 2531-2538.

Al-Zahim, M.A., Ford-Lloyd, B. V., and Newbury, H. J. (1999) Detection of somaclonal variation in garlic (*Allium sativum L.*) using RAPD and cytological analysis. *Plant Cell Rep*, **18:** 473-477.

Anamika, P., G.Thapa., Adreeja, B., Purabi, M., Mohan C. K., Lingaraj, S. (2010) Rapid plant regeneration, analysis of genetic fidelity and essential aromatic oil content of micropropagated plants of *Patchouli, Pogostemon cablin* (Blanco) Benth–An industrially important aromatic plant. *Industrial Crops and Products***,** **32:** 366-374.

Angel (1996) Stability of cassava plants at the DNA level after from 10 years of *in-vitro* storage. *Euphytica*, **90**: 307-313.

Anushri, V., Lakshmikumaran, M., and Srivastava, P. S. (2001) Establishment of genetic fidelity of *in-vitro* raised *Lillium* bulblets rough RAPD markers. *In-vitro cell.dev.biol.plant,* **37**:227-231.

Archana, M., Mathur, A. K., P. Verma., S. Yadav., M. L. Gupta., and Mahendra, P. D. (2008) Biological hardening and genetic fidelity testing of microcloned progeny of *Chlorophytum borivilianum* Sant. *et* Fernand. *African Journal of Biotechnology*, **7** (8):1046-1053.

Arteca, R.N. (1995) Brassino steroids. In: Davies PJ, editor. Plant hormones: physiology, biochemistry and molecular biology. York, NY: *Kluwer Academic Publishers*, 206–213.

Atai, Z. (2009) Inhibitory Effect of Ginger Extract on *Candida albicans*. *American Journal of Applied Sciences*, **6**(6): 67-69.

Auta, K.I., Galatia, B., Olowoniyi, M., and Yak. (2011) Antimicrobial properties of the ethanolic extracts of *Zingiber officinale. Annals of Biological Research*, **2** (3): 307-311.

Ayse, N., Duygu, A. T. I., Hakk, A., Tansel, O. Y., Ismet, D. G., Ismail, K. (2008) Antimicrobial and Cytotoxic Activities of *Zingiber officinale* extracts. *Fabad J. Pharm. Sci*, **33**: 77-86,

Babu, K. N., Samasudeen, K., and Ratnambal, M.J. (1992) *In-vitro* plant regeneration from leaf derived callus in ginger (*Zingiber officinale* Rosc.). *Plant Cell Tissue Organ Culture,* **29**: 71-74.

Bajpai, V.K., Al-Reza, S.M., Choi, U.K., Lee, J.H., and Kang, S.C. (2009) Chemical composition, antibacterial and antioxidant activities of leaf essential oil and extracts of *Metasequioa glyptostroboides* Miki ex Hu. *Food Chem Toxicol*, **47**: 1876-1883.

Baker, (2006) An endangered medicinal herb. *Plant Cell Rep,* **25**: 499-506.

Baker, J. G. (1882) Scitamineae. In: Hooker, J.D., *The Flora of British India*, Vol. VI, Bishen Singh Mahendrapal Singh, Dehra Dun, Rep. 1978; 198-264.

Balchandran, S.M., Bhatt, S.R., and Chandel, K.P.S. (1990) *In-vitro* clonal multiplication of turmeric (*Curcuma* spp.) and Ginger (*Zingiber officinale* Roscoe). *Plant Cell Rep,* **8**: 521-524.

Balladin, D.A., and Headley, O. (1999) Liquid chromatographic analysis of the main pungent principles of solar dried West Indian ginger (*Zingiber officinale* Rosc.). *Renewable Energy,* **18** (2): 257-261.

Barasch, A., Safford, M.M., Dapkute-Marcus, I., and Fine, D.H. (2004) Efficacy of chlorhexidine luconate rinse for treatment and prevention of oral candidiasis in HIV-infected children: a pilot study. Oral Surg. Oral Med. Oral Pathol. *Oral Radiol. Endod.* **97** (2): 204-207.

Bardakei, F. (2001) Random Amplified Polymorphic DNA (RAPD) Markers. *Turk .J. Biology,* 78-79.

Barman, K.L., and Jha, D. K. (2013) Comparative chemical constituents and antimicrobial activity of normal and organic ginger oils (*Zingiber officinale* Rosc.). *International journal of applied biology and pharmaceutical technology,* **4** (1): 259-266.

Bhagyalakshmi, N.S. (1998) Meristem culture and micropropagation of a variety of ginger (*Zingiber officinale* Rosc.) with a high yield of oleoresin. *J Hortic Science,* **63**:321-327.

Bhatia, R.K., Singh, P., Jhang T., and Sharma, T.R. (2009) Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers. *Sci.Horti,* **119**: 208-211.

Bordia, A. Verma, S. and Srivastava, K. (1997) Effect of ginger and fenugreek on blood lipids, blood sugar and platelet aggregation in patients with coronary artery disease. *Prostaglandins Leukat. Essent. Fatty Acids,* **56**: 379–384.

Breiman, A. D., Rotem, K. A., and Shaskin, H. (1987) Heritable somaclonal variation in wild barley (*Hordeum spontaneum*). *Theor. Appl. Genet*, **71**: 637-643.

Breiman, A. D., Rotem-Abarbanell, K. A., and Shaskin, H. (1987) Heritable somaclonal variation in wild barley (*Hordeum spontaneum*). *Theor. Appl. Genet*, **71**: 637-643.

Buchanan, F. (1807) A Journey from Madras Through the Countries of Mysore, Canara and Malabar. Directors of East India Co., London (Reprint).

Burtt, B.L., and Smith, R.M. (1972) Tentative keys to the subfamilies, tribes and genera of Zingiberaceae. *Notes Roy. Bot. Gard. Edinb,* **31:**171-176**.**

Butler, E. J. (1907) An account of genus *Pythium* and some Chytridiaceae. *Mem. Dep. Agric. India*, **1** (5): 70.

Buyukokuroglu, M.E., Gulcin, I., Oktay, M., and Kufrevioglu, O.I. (2001) *In-vitro* antioxidant properties of dantrolene sodium. *Pharmacol. Res*, **44**: 491-494.

Carvalho, L.C., Goul, L., Oliveria, C., Gonalves, J. C., and Amncio, S. (2004) RAPD assessment for identification and genetic stability of *in-vitro* propagated chestnut hybrids. *Plant Cell, Tissue and Organ Culture*, **77**: 23-27.

Chen, J.C., Huang, L.J., Shih, W., Sheng, C. K., Tin, Y. H., and Chien, Y. H. (2007) Ginger and Its Bioactive Component Inhibit Entero toxigenic *Escherichia coli* Heat-Labile Enterotoxin-Induced Diarrhoea in Mice. *Journal of Agricultural and Food Chemistry,* **55** (21): 8390-8397.

Chen, Y., Zhou, X., Li, S., and Cai, T. (2001) Antimicrobial activity of ginger oleoresins. *Shipin Yu Fajiao Gongye,* **27**(4): 30-34.

Chen, Z. and Chen S.J. (1988) The significance of Chromosome number of Zingiberaceae in taxonomy, unpublished manuscript. Chromosome number and cytotaxonomy. *Proceeding of Indian Academy of Sciences*, **17**(1943): 118-132.

Chongtham, T., Chatterjee, R., Hnamte, V., Chattopadhyay, P. K., and Khan, S. A. (2013) Ginger (*Zingiber officinale* Rosc.) germplasm evaluation for yield and quality in southern West Bengal. *Journal of Spices and Aromatic Crop,* **22** (1): 88-90.

Chopra, R.N., Chopra, I. C., Handa, K. L., and Kapoor, L. D. (1958) *Indigenous Drugs of India.* Academic Publishers, Calcutta.

Chwann, Theodor and Schleyden, M. J, Microscopical researches into the accordance in the structure and growth of animals and plants. Printed for the Sydenham Society, 1847. Read ore: http://www.answers.com/topic/matthias-jakob schleidenixzz 2 OpJ5FrpX.

Daubenmire, R.F. (1943) Vegetation zonation in the Rocky Mountains. *Botanical Review,* **9** (6): 325-393.

Dekkers, A.J., Rao, A.N., and Goh, C.J. (1991) *In-vitro* storage of multiple shoot culture of ginger at ambient temperature of 24-29 degree-C. *Sci Hortic,* **47**: 157-167.

Dodds (1991) *In-vitro* methods of Conservation of Plant Genetic Resources. Chapman and Hall, London.

Dogra, S. P., Koria, B. N., and Sharma, P. P. (1994) *In-vitro* clonal propagation of ginger (*Zingiber officinale* Rosc.). *Horticultural Journal*, **7**: 45-50.

Dohroo, N. P. (1989) Seed transmissions of pre-emergence rot and yellow in ginger. *Plant Dis Res*, **4**:73-74.

Dohroo, N.P. (1997) Global status on diseases of ginger (*Zingiber officinale* Roscoe). Directorate of Extension Education, UHF, Solan (H.P.), India.

Doyle, J. J., and Doyle, J. L. (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**: 11-15.

Easter ling, W. E., Crosson, P. R., Rosenberg, N. J., Mc Kenny, M.S., Katz, L. A. and Lemon, K. M. (1993) Agricultural impacts of and responses to climate-changes in the Missouri-Iowas-Nebraska- Kansas (MINK) region. *Climate Change*. **24**: 23-61.

Echeverrigaray, S., Mossi, A.J., and Munari, F. (1988) Micropropagation of resin tree (*Hovenia dulcis* Thunb.) through axillary bud culture. *J. Plant Biochem. Biotechnol,* **7**: 99-102.

El-Ghorab, A. H., Nauman, M., Anjum, F. M., Hussain, S., and Nadeem, M. (2010) A comparative study on chemical composition and antioxidant activity of ginger (*Zingiber officinale*) and cumin (*Cuminum cyminum*). *Journal of agricultural and food chemistry*, **58**(14): 8231-8237.

Farber, J.L., (1994) Mechanisms of cell injury by activated oxygen. *Environmental Health Perspectives*, **102**:17-24.

Ficker, C., Smith, M.L., Akpagana, K., Gbeassor, M., Zhang, J., Durst, T., Assabgui, and A., Arnason, J.T. (2003) Bioassay guided isolation and identification of antifungal compounds from ginger. *Phytoher Res*, **17**: 897-902 .

Frahm, J. P., and Gradstein, S. R. (Nov. 1991) An Altitudinal Zonation of Tropical Rain Forests Using Bryophytes. *Journal of Biogeography,* **18** (6): 669-678.

Gamborg, L., Murashige, T., Thorpe, T.A. and Vasil, I.K. (1976) Plant Tissue Culture Media. *In Vitro*, **12**: 473- 478.

Gautam, M. K., Gangwar, M., Nath, G., Rao, C. V., and Goel, R. K. (2012) *In-vitro* antibacterial activity on human pathogens and total phenolic, flavonoid contents of *Murraya paniculata* Linn. leaves. *Asian Pacific Journal of Tropical Biomedicine*, **2** (3): 1660-1663.

Ghasemzadeh, A., Jaafar, H. Z., and Rahmat, A. (2010). Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinale* Roscoe). *Molecules*, **15**(6): 4324-4333.

Goto, S., Thakur, R. C., and Ishi, K. (1998) Determination of genetic stability in long term micropropagated shoots of *Pinus thunbergii* Parl using RAPD markers. *Plant Cell Reports*, **18**:193-197.

Goulao, L., and Oliveira, C. M. (2001) Molecular characterisation of cultivars of apple (*Malus* × *domestica* Borkh.) using microsatellite (SSR and ISSR) markers. *Euphytica,* **122**: 81-89.

Gould, A. R. (1986) Factors controlling generation of variability *in vitro*. In: Vashil IK (ed) Cell culture and somatic cell genetics in plants 3. *Plant regeneration and genetic varability. Academic*, *Orlando*, 549-567.

Govindarajan, V. S., and Lewis, Y.S. (1972) Chemical composition of ginger Varieties and dehydration studies on ginger. *Journal of food Sciences and Technology*, **9**:220.

Govindaranjan, V. S. (1982). Ginger: Chemistry, technology and quality evaluation: Part-2. Crit. Rev. in Food. *Science and Nutrition,* **17**:189-258.

Goyal, A. K., Basistha, B. C., Sen, A., and Middha, S. K. (2011b). Antioxidant profiling of *Hippophae salicifolia* growing in sacred forests of Sikkim, India. *Functional Plant Biology*, **38** (9):697-701.

Goyal, A. K., Middha, S. K., and Sen, A. (2011a). *In-vitro* antioxidative profiling of different fractions of *Dendrocalamus strictus* (Roxb.) Nees leaf extracts. *Free Radicals and Antioxidants*, **1**(2): 42-48.

Goyal, A. K., Middha, S. K., and Sen, A. (2010) Evaluation of the DPPH radical scavenging activity, total phenols and antioxidant activities in Indian wild *Bambusa vulgaris*" Vittata" methanolic leaf extract. *Journal of Natural Pharmaceuticals*, **1**(1): 40.

Grange, J.M., and Davey, R.W. (1990) Antibacterial properties of *Propolis* (bee glue). *J R Soc Med*, **83**(3): 159-60.

Grant, K.L., and Lutz, R.B., (2000). Ginger, Am. J. *Health Systpharm*, **57**: 945-947.

Green (1971) Handbook on Genetically Standardized Jax Mice, (A. Green ed.) The Jackson Memorial Laboratory, Bar Harbor, ME. 4-15.

Guidelines for the Conduct of Test for Distinctiveness, Uniformity and Stability on Ginger (*Zingiber officinale* Rosc.) (2007) Protection of Plant Varieties & Farmers' Rights Authority (PPV & FRA) Government of India. The test guidelines developed by Indian Institute of Spices Research, Calicut was finalized by the Task Force (7/2007) constituted by PPV & FR Authority.

Haberlandt, G. (1902) Kulturversuche mitisolierten Pflanzenzellen. Sitzungsber. Akad. Wiss. Wien. Math. Naturwiss. Kl. *Abt. J*, **111**:69–92.

Haberlandt, G. (1913) Zur physiologie der zellteilung. Sitzungsber Akad Wiss Berlin. *Phys Math C*, **l**: 318–345.

Hackett, W. P. (1966) Application of tissue culture to plant propagation. *Proceedings of International Plant Propagation Society*, 88-92.

Hasan, H. A., Ayad, M. R. R., Basama, M. A. R. and Bassam, A. Hassan, R. (2012) Chemicals composition and antimicrobial activity of the crude extracts isolated from *Zingiber officinale* by different solvents. *Pharmaceut Anal Acta*, **3**: 9-14.

Hasan, M. S., Ahmed, M.I., Mondals, S., Uddin, S. J., Masud M.M., Sadhu, S. K., and Ishibashi, M. (2006) Antioxidant antinociceptive activity and general toxicity study of *Dendrophthoe falcate* and isolation of Quercetin as the major

component. *International journal Oriental Pharmacy and Experimental Medicine*, **6**: 355-360.

Hasim, H. M., Ibrahim. H. and Rahim, Z. (1988) Preliminary studies on some nutritional composition of the edible gingers. In advance in Aiochemistry and Biotechnology in Asia and Oceania. Proceeding of the 7th Federation of Asia and Oceania Biochemists Symposium (FAOB) EI-2.

Haung, J. H. (1995) *In-vitro* propagation and preservation of ginger germplasm resources. *Scientia Agricultura Sinica*, **28**: 24-30.

Haware, M. P., and Joshi, L. K. (1973) Basal rot of ginger (*Zingiber officinale*) caused by *Sclerotium rolfsii* from Madhya Pradesh. *Indian Phytopathol,* **26** : (3) 575-576.

Herout, V., Benesova, V., and Pliva, J. (1953) The sesquiterpene of ginger oil. *Coll. Czeck. Chem. Comm,* **18**: 248-256.

Hirasa, K., and Takemasa, M. (1998) *Spices—Science and Technology*. Marcel Dekker, New York.

Hofmann, N. E., Raja, A., Nelson, R. L. and Korban, S. S. (2004) Mutagenesis of embryogenic cultures of soybean and detecting polymorphism using RAPD markers. *Biol.Plant*, **48**:173-177.

Holttum, R. E. (1 950) Zingiberaceae of the Malay Peninsula. *Gard. Bull. Singapore*, **13**:1-249.

Hoque, M. I., Praveen, S., and Sarker, R.H. (1999) *In-vitro* propagation of Ginger (*Zingiber officinale* Rosc.) *Plant Tissue cult,* **9**:45-51.

Hosoki, T. and Sagawa, Y. (1977) Clonal propagation of ginger (*Zingiber officinale* Rosc.) through tissue culture. *Horti Science*, **12**:451-452.

Iahi, I. and Jabeen, M. (1987) Micropropagation of *Zingiber officinale* Roscoe. *Pakistan Journal of Botany*, **19**:61-65.

Inden H, Asahira, T. and Hirano, A. (1988) Micropropagation of ginger. *Acta Hortic,* **230**:177-184.

Indu, S., and Menon, A. N. (2010) Comparative chemical composition and antimicrobial activity fresh and dry ginger oils (*Zingiber officinale* roscoe). *International journal of current pharmaceutical research*, **2** (4): 40-43.

Isabel, N. L., Tremblay, M. M., Tremblay. F. M., and Bousquet, J. (1993) RAPD as an aid to evaluate the genetic integrity of somatic embryogenesis derived population of *Picea mariana* (Mill.) B.S.P. *Theor Appl Genet*, **86**: 81-87.

Ishi, M. and Aragaki (1963) Ginger wilt caused by *Psedomonas solacearum* E.F. Smith. *Plant Disease Reptt*, **47**: 710-713.

Jaleel, K. and Sasikumar (2010) Genetic Diversity analysis of ginger (*Zingiber officinale* Rosc.) Germplasm based on RAPD and ISSR markers. *Scientia Horticulturae*. **125:** 73-76.

Jatohi, S. A, Akira K., Makiko, S.S., and Kazuo, N. W. (2008) Relationship of Zingiber species, and genetic variability assessment in ginger (*Zingiber officinale*) accession from ex-situ gene bank, on farm and rural markets. *Breeding Science*, **58**:261-270.

Joshi, L. K., and Sharma, N. D. (1980) Diseases of ginger and turmeric. In Nair, M.K., Premkumar, T., Ravindran, P.N., and Sharma Y.R. (eds.) *Ginger and Turmeric*. CPCRI, Kasaragod, India, 104–119.

Kaekar, A., Bhatt, S.R., Chandel, K.P.S., and Malik, S.K. (1993) Plant regeneration via somatic embryogenesis in ginger. *Plant Cell Tissue Organ Cult,* **32**: 289-292.

Kaneria, M., Baravalia, Y., Vaghasiya, Y., and Chanda, S. (2009) Determination of antibacterial and antioxidant potential of some medicinal plants from Saurashtra region, India. *Indian journal of pharmaceutical sciences*, **71**(4): 406.

Kim, E. C., Min, J. K., Kim. T. Y., Lee, S. J., Yang, H. O., Kim, Y. M. and Kwon, Y. G. (2005) (6)-Gingerol a pungent ingredient of ginger inhibits angiogenesis *in-vitro and in-vivo*. *Biochemical and biophysical research communications*, **335**: 300-308.

Kirtikar, K.R., and Basu, B.D. (1935) *Indian Medicinal Plants.* Vol. 4, Bishen Singh Mahendrapal Singh, Dehradun, India (reprint).

Kris, S. (2011) GIS application for land suitability of ginger cultivation. *Asia Geospatial Forum*. Jakarta, Indonesia.

Kudi, A.C., Uhoh, J.U., Eduvie, L.O., and Gefu, J. (1999) Screening of some Nigerian medicinal plants for antibacterial activity. *Journal of ethno pharmacology*, **67**:225-228.

Kumar, S. and Raju D.C.S, Ginger and its allies in Sikkim Himalayas. Higher plants of Indian Subcontinent Vol II: 249-255, (Additional series of Indian journal of Forestry).

Kuruviashetty, M. S., Horidasan, P. and Lyer, R. D., M.K. (1982) Naik et. al procs. Natl. Seminar on ginger and turmeric CPCRI, Kasarogod, Kerala, India. 39-41.

Langner, E. (1998) Greifenberg S., Gruenwald O., *Ginger: history and. Adv. Ther,* **15**: 25-44.

Larkin, P., and Swkocroft, N. (1981) Somaclonal variation–a novel source of variability from cell culture for plant improvement. *Theor Appl Genet,* **60**: 197-214.

Larkin, P., and Swkocroft, N. (1981), Somaclonal variation–a novel source of variability from cell culture for plant improvement. *Theor Appl Genet*, **60**:197-214.

Lattoo, S. K., Bamotra, S. R., Dhar, S., Khan, S., and Dhar, A. K. (2006) Rapid plant regeneration and analysis of genetic fidelity of *in-vitro* derived plants of *Chlorophytum arundinaceum*. *Lilium* bulbets through RAPD markers. *In-vitro Cell. Dev. Biol. Plant,* **37**: 227-231.

LD Reynolds and NG Wilson, “Scribes and Scholars” 3rd Ed. Oxford: (1991). 193-4.

Leffring, L. (1971) Vegetative vermeerdering van gerbera vabel *Bioemist*, **26**: 9.

Linnaeus, C. (1753) Species plantarum, exhibentes plantas rite cognitas, ad genera relatas, cum differentiis specificis, nominibus trivialibus, synonymis selectis, locis natalibus, secundum systema sexuale digestas. Holmiae, Impensis Laurentii Salvii. (L. Salvius, Stockholm)Starting point for Spermatophyta, Pteridophyta, Sphagnaceae, Hepaticae, Fungi (incl. slime moulds and lichen-forming fungi) and Algae (pro parte).

Linschotten, J.H. (1596) Voyage of John Huygen van Linschotten in India, Vol. II. (Cited from Watt (1872).

Liu, Z. (2011) Diterpene Glycosides as Natural Solubilizers. United States Patent Application Publication. Patent Department Taylor, Porter, Brooks and Philips.

M. Modgil, K. Mahajan, S.K. Chakrabarti, D.R. Sharma, and R.C. Sobti. (2005) Molecular analysis of genetic stability in micropropagated apple rootstock MM106. *Scientia Horticulturae* **104**:151–160.

Mahaboob, K. R., Evan P. S., Segu, R., Ramya, P.P., Smita, P., Niharika, Punya., P. and Mishra, J.S. (2010) Hepatoprotective and Antioxidant Effects of Gallic Acid in Paracetamol-Induced Liver Damage in Mice. *Journal of Pharmacy and Pharmacology,* **62** (5): 638-643.

Mahindru, S.N. (1982) *Spices in Indian Life*. Sultan Chand & Sons, Delhi.

Major, A., Malvolti, M. E., and Cannata, F. (1998) Comparison of isozyme and RAPD variability of black locust (*Robinia pseudoacacia*) clones selected for silvicultural objectives. *J. Genet. Breed*, **52**:49-62.

Malamug, J.J.F., Inden, A., and Asahira, T. (1991) Plantlet regeneration and propagation from ginger callus. *Sci Hortic***, 48**:89-97.

Manicom, B.Q. (1998) This ginger disease is a threat to the industry. *Neltropika Bull*, **299**: 8.

Manju, V., and Nalini, N. (2009) Chemopreventive efficacy of ginger, a naturally occurring anti carcinogen during the initiation, post-initiation stages of 1, 2-dimethylhydrazine induced colon cancer. *Clinica Chimica Acta*, **358:** 60-67.

Manju, V., and Nalini, N. (2005) Chemopreventive efficacy of ginger, a naturally occurring anticarcinogen during the initiation, post-initiation stages of 1,2-dimethylhydrazine induced colon cancer. *Clinica Chimica Acta,* **358**: (1-2), 60-67.

Manmohandas, T.P., Pradeep Kumar, T., Mayadevi, P, Aipe, K.C., and Kumaran, K. (2000) Stability analysis in ginger (*Zingiber officinale* Rosc), genotypes. *J. Spices and Aromatic Crops,* **9**:165–167.

Martin, M., Sarmento, D., and Oliveria, M., (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers, *Plant Cell Reports*, **23**: 492-496.

Martins, M. D., Sarmento, M., and Oliveira, M. (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. *Plant Cell Rep*, **3**: 492-496.

Matthes, M., Singh, R., Cheah, S. C., and Karp, A. (2001) Variation in oil palm *Elaeis guineensis* (Jacq.) tissue culture-derived regenerants revealed by AFLPs with methylations sensitive enzymes. *Theoretical applied Genetics*, **102**: 971-979.

Michielin, E. M. Z., Salvador, A. A., Riehl, C. A. S., Smania-Jr. A., Smânia, E. F. A. and Ferreira, S. R. S. (2009) Chemical composition and antibacterial activity of *Cordia verbenacea* extracts obtained by different methods. *Bioresource Tech*nology, **100** (24): 6615-6623.

Miyazawa, M., and Kameoka, H. (1988) Volatile flavour components of Zingiberis Rhizoma (*Zingiber officinale* Rosc.). *Agricultural and Biological Chemistry,* **52**: (11) 2961-2963.

Munthali, M. T., Newbury, H. J., and Ford-Lloyd, B. V. (1996) The detection of somaclonal variants of beet using RAPD. *Plant Cell Rep*. **15**: 474-478.

Murashige, T. (1974) Plant propagation through tissue culture. *Plant Physiology*, **22**: 135165.

Murashige, T. (1978) Principles of rapid propagation, In Propagation of Higher Plants through Tissue Culture a Bridge between Research and Application (K.Huges,

R. Henke and M. Constantin, Eds.) Technology Information Centre, USDE Oak, Ridge, 14-24.

Murashige, T., and Skoog, F. A. (1962) Revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant*, **15**: 473-497.

Nada, A.L., Sharma, L.R., Dohroo, N.P. and Prashar, R.S. (1996) Status of ginger production in Sirmour district of Himachal Pradesh. UHF, Solan, (H.P.), India 26.

Nadgauda, R.S., Kulkarni, D.B., Mascarenlas, E.F., and Jaganathan, V. (1980) Developments of plantlets from tissue culture of ginger. *In proceeding of Annual symposium of Plantation Crops*, 143-147.

Nadkarni, K.M. (1954) *Indian Materia Medica.* Vol. 1. 3rd ed. Popular Prakasan. Bombay Ahmed, N. Katiyar, S.K., and Mukhtar, H. (2001) Antioxidants in chemoprevention of skin cancer, *Curr. Probl. Dermatol.* **29**: 128-39.

Narayan, M., Bhatt, P.P., Ranjan, R. K., and Subba, J.R. (2004) Managing rhizome rot on ginger in Sikkim. *Journal Indian Horticulture,* **9**: 12-18.

Nayak, S., and Nayak, P.K. (2006) Factors effecting *In-vitro* Microrhizome Formation and growth in *Cucurma longa* L. and Improved Field Performance of Micropropagated Plants. *Science Asia*, **32**: 31

Nei, M. (1972) Genetic distance between populations. *The American Naturalist,* **106**: 283-292.

Nei, M. and Li, W.H. (1979) Mathematical models for studying genetic variation in terms of restriction endonuclease. *Proc. Natl. Acad. Sci*ence, **76**:5269-5273.

Nei's (1973) Analysis of gene diversity in subdivided populations. Proc Nat. Acad. Sci, **70**:3321-3323.

Nepali, M.B., Prasad, R.B., and Sah, D.N. (2000) Survey of ginger growing areas in Syanja, Palpa, Gulni and Arghaknach districts with special emphasis on rhizome rot disease. *Bull., Lumle Agric. Res. Station,* Nepal.

Newman, M. (2001) Nomenclatural notes on Zingiberaceae *J. Bot*, **58**: 173-174.

Noguchi, Y. and Yamakawa, O. (1988) Rapid clonal propagation of ginger (*Zingiber officinale* Rosc.) by roller tube culture. *Japan J. Breed,* **38**: 437-442.

Nybe, E.V., Nair, P.C.S., and Mohanakumaran, N. (1982) Assessment of yield and quality components in ginger. In: Nair, M.K., Premkumar, T., Ravindran, P.N., and Sarma, Y.R. (1982) (eds*),* Ginger and Turmeric, CPCRI, Kasaragod, India, 24-29.

Okigbo, R.N., and Nmeka, I.A. (2005) Control of yam tuber rot with leaf extracts of *Xylopia aethiopica* and *Zingiber officinale. African Journal of Biotechnology,* **4** (8): 804-807.

Oktay, M., Gulcin, I., and Kufrevioglu, O. I. (2003). Determination of in-vitro antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *LWT-Food Science and Technology*, **36** (2): 263-271.

Oyaizu, M. (1986) Studies on products of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition*, **44:** 307-314.

Palai, S. K., Raut, G. R., Das, P., and Edison, S. (1997) Micropropagation of ginger (*Zingiber officinale Rosc.*) Interaction of growth regulators and culture conditions. *Proceedings of the National Seminar on Biotechnology of Species and Aromatic Plants*, **8**: 20-24.

Palombo, E.A., and Semple, S.J., (2001) Antibacterial activity of traditional Australian medicinal plants. *Journal of Ethnopharmacology*, **77:**151-157.

Panda, M. K., Mohanty, S., Subudhi, E., Acharaya, L., and Nayak, S. (2007) Assessment of genetic stability of micropropagated plants of *Cucurma longa* L. by cytophotmetry and RAPD analyses. *International Journal of Integrative Biology*, **1**:189-195.

Pandey, G., and Dobhal, V.K. (1993) Genetic variability, character association and path analysis for yield components in ginger (*Zingiber officinale* Rosc). *J. Spices & Aromatic Crops,* (2) 16-20.

Pandey, P. Y., Sagswansupyakom, C., Sahavachadria, O., Thaveechae, N. and Pandey, Y. (1997) *In-vitro* propagation of ginger (*Z. officinale*). Kastetsaet. *Journal of Natural Science*, **3**: 81-86.

Parry, T. W. (1969) Spices Chemical Publishing Company, Inc., New York, **2**: 79-80.

Paterson, A.H., Damon, S., Hewitt, J.D., Zamir, D. and Rabinowitch, H.D. (1991) Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, environments. *Genet*.**127**: 181-97.

Patiram, U. R.C., and Singh, L.N. (1995) An appraisal of ginger (*Zingiber officinale* Rosc.) production in Sikkim, India. *J. Spices and Aromatic Crops,* **4**:111–118.

Patiram, U. R.C., and Singh, L.N. (1995) An appraisal of ginger (*Zingiber officinale* Rosc.) production in Sikkim, India. *J. Spices and Aromatic Crops,* **4**: 111–118.

Paz, E.A., Cerdeiras, M.P., Fernandez, J., Ferreira, F., Moyna, P., Soubes, M., Vazquez, A., Vero, S., and Zunino, L. (1995) Screening of Uruguayan medicinal plants for antimicrobial activity. *Journal of Ethno pharmacology*, **45**: 67-70.

Pegg, K. G., Melda, L., Meffett, M.L., and Colbran, R.C. (1974) Disease of ginger in Queensland. *Queensland Agric. J,* **5**: 611–614.

Peter, K.V., Nybe, E.V., and Kurien, A. (2005) Yield gaps and constraints in ginger In Ravindran P N& Nirmal Babu K (Eds.) Ginger The genus Zingiber (527-532) CRC press, Bocaraton.

Peterson, O.G. (1889) Zingiberaceae. In: *Engler and Prantl's Naturlichen Pflanzenfamilien*, 2, 6, 10-30 (cited from Tomlinson, 1956).

Phillips, R. L., Kaeppler, S. M., and Olhof, P. (1994) Genetic instability of plant tissue cultures: Breakdown of normal controls. *Proc.nat. Acad. Sci. USA*, **91**: 5222-5226.

Pillai, S.K., and Kumar, K.B. (1982) Note on the clonal multiplication of ginger *in vitro*. *Indian Journal of Agric. Sci*, **52**:397-399.

Pradhan, S., Basistha, B. C., and Handique, P. J. (2012) Determination of genetic fidelity of Micropropagated plants of *Zingiber officinale cv-Majhauley* of Sikkim Himalaya using RAPD markers. *International Journal of Fundamental & Applied Sciences*, No. **2**: 21-24.

Pruthy, J.S. (1993) Major Spices of India: Crop Management, Post-Harvest Technology. *Indian Council of Agricultural Research*, New Delhi, 514.

Purseglove, J.W., Brown, E.G., Green, C.L., and Robbins, S.R.J. (1981) *Spices,* Vol. 2. Longman, London.

Rachel, F. G., Luiz, R. H., Georgia, P., Carlos, A., Oliveira, L. A. Carneiro, J., and Francisco, M. V. (2007) Elisabeth Mansur and Maria Lucia Carneiro Vieira. Assessment of Genetic Stability Among. *In-vitro* Plants of *Arachis retusa* Using RAPD and AFLP Markers for Germplasm Preservation. *Journal of Integrative Plant Biology,* **49** (3):307-312.

Raghavan, T.S., and Venkatasubban, K.R. (1943) Cytological studies in the family Zingibereaceae with special reference to chromosome number and cytotaxonomy. *Proceeding of Indian Academy of Sciences,* **17**:118-132.

Rahman, M.M., Amin, M.N., Jahan, H.S., and Ahmed, R. (2004) In-vitro regeneration of plantlets of *Cucurma longa* L.A valuable spice in Bangladesh, *Asian Journal Plant Science*, **3** (3):306-309.

Rai, S., and Anita, G. (1997) Mother rhizome extraction of ginger (*Zingiber officinale* Rosc.) an age old practice in Sikkim and Darjeeling hills. *Environment and Ecology,* **15**(4): 910–912.

Raina, V.K., Kumar, A., and Aggarwal, K.K. (2005) Essential oil composition of ginger (Zingiber officinale Roscoe) Rhizomes from different place in India. *Journal of Essential oil Bearing Plants,* 8 (2): 187-191.

Rajan, K.M., and Agnihotri, V.P. (1989) *Pythium* induced rhizome rot of ginger, problems and progress. In: Agnihotri V.P, Sing N, Chaube H.S, Sing V.S., and Dwivedi, T.S. (eds.) *Perspectives in Phytopathology*; Today and Tomorrow's Principles and Publishers, New Delhi 189-198.

Ramnirez, M.R., Borodanenko, A., Barrera-Gurra, J.L., and Ochoa, A. N. (2001) Shoot number and shoot size as affected by growth regulators in *In-vitro* cultures of *Spathyphyllum floribundum*. *Sci Horti,* **89**:227-236.

Rani, V. S. and Raina, N. (1998) Genetic analysis of enhanced-axillary-branching-derived *Eucalyptus tereticornis* Smith and *E. camaldulensis* Dehn. Plants. *Plant Cell Reports,* **17**: 236-242.

Rani, V., Parida, A., and Raina, S. N. (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoids* Marsh. *Plant Cell Rep*, **14**:459-462.

Rao, K. S., Munjuluri, P. R., and Keshar, N. K. (2011) *In-vitro* Antioxidant Activity and Total Phenolic Content of Mimusops elengi Bark. *Ind J Pharm Edu Res*, **45**(4): 317.

Rattan, R.S. (1988) Varietal performance of ginger. Proc. Ginger Symposium, Naban, Himachal Pradesh, Feb 1988.

Ravindran, P. N., and Babu K.N., (2005) Ginger-The genus *Zingiber* ISBN 0-415-32468-8.

Ravindran, P.N., Nirmal, B.K., and Shiva, K.N. (2005) Botany and crop improvement of ginger. In: Ravindran P.N. and Bimal Nirmal (ed) Ginger the genus Zingiber CRC Press New York, 15-85.

Ravindran, P.N., Sasikumar, B., George, J.K., Ratnambal, M.J., Normal Babu, K., and Zachariah, T.J.(1994) Genetic resources of ginger (*Zingiber officinale* Rosc.) and its conservation in India. *Plant Genetic Resources Newsletter,* No. **98**: 1–4.

Rohlf, F.J. (1998) NTSYSpc 21 Numerical taxonomy and multivariate analysis system. Exeter Software, Setauket, NY.

Rosengarten, Jr. (1969) The Book Of Spices. Jove Publ., Inc., New York.

Rout, G. R. (1998) Determination of genetic stability of micropropagated plants of ginger using Rapid Amplified Polymorphic DNA (RAPD) markers. *Botanical Bulletin of Academia Simica*, **39**:2327.

Rout, G. R., and Das, G. (2002) An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers. *Bilogia Plantarum,* **45**:27-32.

Rout, G.R., Palai, S.K., and P. Das. (1997) *In-vitro* micropropagation of ginger (*Zingiber officinale* Rosc) Interaction of growth regulators and culture conditions. *Indian J. Herbs Spice,* (In press).

Ruch, R.J., Cheng, S.J., Klaunig, J.E (1989) Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechin isolated from Chinese green tea. *Carcinogenesis*, **10**:1003-1008.

Sahavacharin, O. (1995) Tissue culture micropropagation technology. Department Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok.

Sakamura, F., Ogihara, K., Suga, T. Taniguchi. K., and Tanaka, R. (1996) Volatile constituents of (*Zingiber officinale* Rosc.) rhizomes produced by *in-vitro* shoot tip culture. *Phytochemistry,* **25**:1333-1335.

Salter, C., Joseph H., Jesse W., and Kostbade J. T. (2005) Essentials of World Regional Geography 2nd Edition. New York: *Harcourt Brace*. 464-465.

Salvi, N. D. (2002) Micropropagation and field evaluation of micropropagated plants of turmeric. *Plant Cell Tissue and Organ Culture*, **68**:143-151.

Samuel Rai, (2009) Management of Ginger (*Zingiber officinale* Rosc.) Rhizome Rot in Darjeeling and Sikkim Himalaya Region.

Sane, R.T., Phadke, M., Hijli, P.S., Shah, M., and Patel, P.H. (1998) Geographical variation study on gingerol (a pungent principle from *Zingiber officinale*) and ginger oil, using HPTLC technique and accelerated stability study on gingerol from *Zingiber officinale* using HPTLC method. *Indian Drugs,* **35** (1): 37-44.

Sang, H, K., Chang, D., Jun, K.S., Byung, J.C., Hyunjeung, L., Seunja, P., Seung, H. L., Hye, Y. S., Dae-Keun, K., and Tae-Yong, S. (2006) Gallic Acid Inhibits Histamine Release and Pro-inflammatory Cytokine Production in Mast Cells. *Toxicological Sciences*, **91**(1): 123-131.

Sanjeev, S., Roy, A.R., Iangrai, B., Pattanayak, A and Decka, B.C. (2011) Genetic diversity analysis in the traditional and improved ginger (*Zingiber officinale* Rosc.) clones cultivated in North east India. *Scientia Horticulturae*, **128**:182-188.

Sasikumar, B., Nirmal Babu, K., Abraham, J., and Ravindran, P.N. (1992) Variability, correlation and path analysis in ginger germplasm. *Indian J. Genet*, 52: 428-431.

Scheiden, M. J. (1938) Deitrage Zur phytogenesis, Muller, Arch, Ant. *Eiss Med*, 137-176.

Schenk, R.H. and Hildebrandt, A.C. (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany,* **50**:199-204.

Schumann, K: Zingiberaceae. (1904) In: *Engler's Pflanazenreich*, **4**:1-428.

Schwann, T. (1939) Mikraskapische untersuch ugen uber die ubereinstimmung in derstruktur and dem wach stume der tiere and pfanzen (Ostwards kallsiker) de exakten Wissenschaften No. 176, Engelmann-Leipzig 910.

Shadia, M. I. Maria Cotrim, Oliveira L. *E., Teràn, J., Lauro, V., Camargo, L., and Fernando, B.* (2006) Chemopreventive property of dietary ginger in rat urinary bladder chemical carcinogenesis. *World Journal Urology,* **24**: 591-596.

Shannon, T.R and Wyatt, R (1986) Reproductive biology of *Asclepias exaltata. American Journal of Botany,* **73**:11-20.

Sharma, H., Clark, M., and Christopher, M.M. (1998) *Contemporary Ayurveda: medicine and research in Maharishi Ayurved*, Medical Guides to Complementary and Alternative Medicine, Foreword by Gary Kaplan, New York: Churchill Livingstone, ISBN978-0-443-05594-2 Jansen.

Sharma, T.R., and Singh, B.M. (1994) *In-vitro* micro rhizome production in (*Zingiber officinale* Rosc.). *Plant Cell Rep*, **15**:274-277.

Sharma, T.R., and Singh, B.M. (1997) High frequency *in-vitro* multiplication of disease free *Zingiber officinale* Rosc. *Plant Cell Reports*, **17**: 68-72.

Sharma, T.R., and Singh, B.M., Chauhan, R.S. (1994) Production of disease free encapsulated buds of *Zingiber officinale* Rosc. *Plant Cell Rep,* **13**:300-307.

Shahidi, F. and P.D. Wanasundara, (1992) Phenolic antioxidants*. Cri. Rev. Food Sci. Nutr*, **32**: 67-103.

Sheony, V. B., and Vasil, I. K. (1992) Biochemical and molecular analysis of plant derived from embryogenic tissue cultures of Napiergrass (*Penisetum purpureum* K cultures of Schum). *Theoretical and Applied Genetics*, **83**:947-955.

Simmonds, J.H. (1955) Science Branch, Plant Pathology Section Rep. Dept. *Agric, Queensland*, (1953) **54**:55-56.

Singh, A., Negi, M. S. Moses, V. K., Venkateswarlu, B., Srivastava, P.S., and Lakshmikumaran, M. (2002) Molecular analysis of micropropagated neem plants using AFLP markers for ascertaining clonal fidelity. *In-vitro cell. dev. Biol. Plant*, **38**:519-521.

Singh, A.K. (2001) Correlation and path analysis for certain metric traits in ginger. *Ann. Agri. Res.,* **22**: 285-286.

Singh, G., Kapoor, I.S., Singh, P., Heluani, C.S., Lampasona, M.P., and Catalan, C.A.N. (2008) Chemistry, antioxidant and antimicrobial investigation on essential oil and oleoresin of *Zingiber officinale*. *Food Chem. Toxicol*, **46**: 3295-3302.

Singh, M., Saroop, J., and Dhiman, B. (2004) Detection of intra-clonal genetic variability in vegetatively propagated tea using RAPD markers. *Biol. Plant*, **48**:113-115.

Singleton, V. L., and Rossi, J. A. (1965) Colorimetry of total phenolic with phosphomolybdic phosphotungstic acid reagents. *American Journal of Enology and Viticulture,* **16**: 144–153.

Sinha, A.P., and Mukhopadhya, A.N. (1988) Foot rot of papaya and rhizome rot of ginger incited by *Pythium* species. In Agnihotri, V.P., Sing, N., Chaube, H.S, Sing, V.S, and Dwivedi, T.S. (eds.) *Perspectives Phytopathology*, Today and Tomorrow's Principles and Publishers, New Delhi 282-291.

Smith, M.K., and Hamil, S.D. (1996) Field Evaluation of Micropropagated and Conventionally propagated ginger in subtropical Queensland Australia. *J. Exp. Agricul,* **36**:347-354.

Srivastava, L.S., Gupta, S.R., Basnet, C.P., Mahato, U.P., and Neopani, B. (1998) Microorganisms associated with ginger in Sikkim. *J. Hill Res.*, **11** (1): 120-122.

Stewart, J.J., Wood, C.D., and Mims, M.E. (1991) Effects of ginger on motion sickness susceptibility and gastric function. *Pharmacology*, **42**:111–120.

Stoilova, I., Krastanov, A., Stoyanova, A., Denev, P., and Gargova, S. (2007) Antioxidant activity of a ginger extract (*Zingiber officinale*). *Food chemistry*, ***102***(3): 764-770.

Suagaya, A. (1991) Micropropagation of turmeric (*Curcuma domestica valvet*) and other Cucurma species. *Biotechnology in Agriculture and Forestry* **19**:277-294.

Subba, J. R. (1984) Agriculture in the Hills of Sikkim Science, *Sikkim Science Society*, 286 pages.

Subba, J. R. (1999) Limboos of the Eastern Himalayas with special reference to Sikkim, (SYMS, Gangtok, Sikkim).

Subba, J. R. (2008) Indigenous knowledge on bio resource management for livelihood of the people of Sikkim. *Indian Journal of Traditional Knowledge*. **8**: (1) 56-64.

Subba, J. R. (2008) The Evolution of man and Modern Society in Mountains Sikkim, (Gyan Publishing House, New Delhi).

Sunitibala, H.M., Damayanti, M., and Sharma, G.J. (2001) *In-vitro* propagation and rhizome formation in *Cucurma longa* Linn. *Cytobios*, **105**:71-82.

Syamkumar, S., Bosco, L. and Sasikumar, B. (2003) Isolation and amplification of DNA from rhizomes of turmeric and ginger. *Plant Mol. Biol. Reptr*, **21**:171-174.

Synder, W.C., Hansen, H.N. (1940) The species concept in *Fusarium. Am. J. Bot,* **27**: 64-67

Taylor, P. W. J., Geijskes, J. R., Fraser, H. L., Henry, T. A., Birch, R. J. (1995) Sensitivity of random amplified polymorphic DNA to detect genetic variation in sugarcane during tissue culture. *Theoririical Appl. Genet*, **90**:1169-1173.

Tortora, G.J., Funke, B.R., and Case, C.L., (2001), Microbiology: An Introduction, Benjamin Cummings, San Francisco

Trujillo, E.E. (1963) *Fusarium* yellows and rhizome rot of common ginger. *Phytopathology:* **53**: (11): 1370-1371.

Varshney A, Lakshmikumaran M., Srivastava P S, and Dhawan V, Establishment of genetic fidelity of in vitro-raised

Vasil, I.K., Henke, R., Hughes, K., Constanin, M., Hollaender, A. (eds) (1985) Tissue culture in forestry and agriculture. Plenum Press, New York, **6**: (796) 31-47.

Vlietinck, A.J., van Hoof, L., Totte, J., Lasure, A., Vanden, Berghe, D., Rwangabo, P.C., and Mvukiyumwani, J. (199) Screening of a hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *Journal of Ethnopharmacology*, **46**:31-47.

Wang, H. (1989) *In-vitro* clonal propagation of ginger sprouts. *Acta Bot Yunnaniva***,** **11**:231-233.

Wang, W., Zhang, L., Nan, L., and Yuangang, Z. (2012) Chemical composition and in-vitro antioxidant, cytotoxicity activities of *Zingiber officinale* Roscoe essential oil *African Journal of Biochemistry Research,* **6**(6):75-80.

Watt, G. (1872) *The Commercial Products of India.* Today & Tomorrows Pub. Delhi (Reprint, 1966).

White, P.R. (1963) The cultivation of animal and plant cells. Ronald Press Co, New York, 228.

Willdenow (1797) Linnaei species *Plantarum Curante* C.L. Willdenow, Beroline **10** vol.

Willey, R.W. (1979) Intercropping—its importance and research needs. Part 2. Agronomy and research approaches. *Field Crop Abst*. **32**:73-85.

Williams, J.G.K. (9901) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, **18:**65.

Yadav, R.K. (1999) Genetic variability in ginger (*Zingiber officinale* Rosc.). *J. Spices & Aromatic Crops*, **8**:81-83.

Yeh F., Yang, R. and Boyle, T. (1997) POPGENE 1.32. http:www.ualberta.ca/~fyeh/index.htm.

Yesim, Y., Hatice, A. and Ismail. K. (2013) *In-vitro* Antioxidant Activity of Various Extracts of Ginger (*Zingiber officinale* L.) Seed. *Asian Journal of Chemistry,* **25**(7): 3573-3578.

Yusha, M. L., Garba and Shamsuddeen, U. (2008) In-vitro inhibitory activity of garlic and ginger extracts on some respiratory tract isolates of gram-negative organisms. (Ginger) on *Escherichia coli* and *Pseudomonas aeruginosa*. *International Journal of Biomedical and Health Sciences,* **4** (2): 57-60.

Zahra, A., Manjieh, A., and Mohsen, M. (2009) Inhibitory effect of ginger extract on *Candida Albicans*. Am. *J. Appl. Sci,* **6**(6): 1067-1069.

Zakaria, M and Ibrahim H., (1986) Phytochemical screening of some Malaysian species of *Zingiberaceae. Malaysian J. Sci,* **8**:125-128.

APPENDIX I

Composition of plant tissue culture media used

Constituents	Media (amount in mg^{-1}) [a]			
	MS[b]	B_5[c]	Nitschs[d]	White's [e]
Inorganic				
KH_4NO_3	1650	-	720	
KNO_3	1900	2527.5	950	80
$CaCl_2.H_2O$	440	150	-	
$CaCl_2$	-	-	166	
$MgSO_4.7H_2O$	370	246.5	1233	720
KH_3PO_4	170	-	68	
$(NH_4)SO_4$	-	134	-	
$NaH_2PO_2H_2O$	-	150	-	16.5
KCL	-	-	-	65
KI	0.83	0.75	-	0.75
H_2BO_3	6.2	3	10	1.5
$MnSO_4.\ 4H_2O$	22.3	-	25	
$MnSO_4.\ H_2O$	-	10	-	7
$ZnSO_4.\ 7H_2O$	8.6	2	10	3
$NaMoO_4\ 2H_2O$	0.25	0.25	0.25	
$CuSO_4.\ 5H_2O$	0.025	0.025	0.025	0.001
$CoCl_26H_2O$	0.25	0.025	-	
Na_2SO_4				200
$Ca(NO_3)_24H_2O$				300
MoO_3				0.0001
$FeSO_4.7\ H_2O$	27.8	-	27.8	2.5
Na.EDTA $2H_2O$	37.3	-	37.3	
Sequestrene 330 Fe	-	28	-	
Organic				
Inositol	100	100	100	-
Nicotinic acid	0.5	1	5	-
Pyridoxine HCL	0.5	1	0.5	-
Thiamine HCL	0.1	10	0.5	-
Glycine	02	-	2	-
Folic acid	-	-	0.5	-
Biotin	-	-	0.05	-
[a] Sucrose	3%	2%	2%	3%

[a] Concentration of Sucrose is expressed in percentage
[b] Murashige and Skoog (1962)
[c] Gamborg et al (1968)
[d] Nitsch (1969)
[e] White (1963)

APPENDIX II

DNA Extraction Buffer (1 X concentration)

MATERIALS REQUIRED	PREPERATION
100 mM Trizma base[c] (pH – 8.0 at 27ºC) 20mM EDTA[b] (pH – 8.0) 1.4 M NaCl[a] 2% (W/V) CTAB[c] 2% (W/V) PVPP[c]	Take 54 g of molecular biology grade Trizma base [Sigma, USA. Cat No. T-1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$. (M.W. 12.11g) was dissolved in 800ml of sterile double distilled water; pH adjusted to 8.0 and was divided to into two parts. To one part 7.44g EDTA was added and in other part 81.82 g NaCl, 20 g CTAB (Hexadecyl trimethyl ammonium bromide, $C_{19}H_{42}NBr$) and 10g PVPP was added. Both the parts were then mixed.

[a] MERCK (India) Ltd. Mumbai, India.
[b] Qualigens Fine Chemicals, Mumbai, India.
[c] SIGMA – ALDRICH Chemicals Pvt. Ltd. USA
[d] SRL Chemicals, Mumbai, India

MATERIALS REQUIRED	PREPERATION
10 mM Tris (pH – 8) [a] 10 mM EDTA (pH – 8) [b]	Take 1.21 g of molecular biology grade Trizma base (Sigma, USA Cat No. 1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$, (MW – 12.1 g) was dissolved in 400 ml of double distilled water and pH

TE – Tris EDTA Buffer (pH – 8)

	adjusted to 8.0 with conc. HCl[1] and autoclaved. Similarly, 0.732 g Di-sodium EDTA[2] was dissolved in 400 ml of double distilled water. The solution was stirred properly and pH was adjusted to 8.0 with NaOH[1] pellets and sterilized by autoclaving. Both the solutions were then mixed and volume made up to 1 liter by adding sterilized double distilled water.

[a] E MERCK (India) Ltd. Mumbai, India.

[b] Qualigens Fine Chemicals, Mumbai, India.

TBE – Tris – Borate EDTA Buffer (5 X concentration)

MATERIALS REQUIRED	PREPERATION
0.045 M Tris – Borate [a] 0.001 M EDTA [b]	54 g of molecular biology grade Trizma base (Sigma, USA Cat No. 1503, Tris (hydroxymethyl) aminomethane, $C_4H_{11}NO_3$, (MW - 1.21 g) and 27.5 g Boric

Preparation of 5 X stock

	acid[1] were dissolved in 800 ml of sterile double distilled water. To it 20 ml of 0.5 M EDTA[2] (pH – 8.0) was added. pH was adjusted to 7.6 with conc HCl[1]. The solution was dispensed into aliquots and kept at room temperature. TBE was used in a final concentration of 1 X, by diluting 5 X stock to 1 X by adding Double Distilled H_2O.

[a] E MERCK (India) Ltd. Mumbai, India.

[b] Qualigens Fine Chemicals, Mumbai, India.

SL no.	Study area	P^H	Org.Carbon (%)	Nitrogen kg/ha	Phosphorous kg/ha	Potassium kg/ha
1.	East Sikkim	6.1H	0.60L	94.08L	87.10L	265.50M
2.	East Sikkim	6.0H	1.74H	72.03L	62.40H	369.00H
3.	East Sikkim	5.8M	1.86H	107.00L	135.20H	306.00M
4.	East Sikkim	7.4H	0.60L	103.48L	80.60H	351.00H
5.	East Sikkim	6.5M	0.60L	100.35L	96.20H	378.00H
6.	East Sikkim	5.7M	2.40H	97.21L	36.40H	364.50H
7.	East Sikkim	6.2H	1.20M	94.08L	106.60H	319.50H
8.	East Sikkim	6.2H	1.20M	107.00L	86.00H	279.00M
9.	East Sikkim	5.8M	1.20M	81.53L	45.50H	355.50H
10.	West Sikkim	5.4M	1.80H	97.21L	60.00H	423.00H
11.	West Sikkim	5.9M	1.14M	59.58L	64.00H	369.00H
12.	West Sikkim	8.2H	2.34H	78.40L	58.50H	427.50H
13.	West Sikkim	7.0H	1.20M	72.12L	129.00H	396.00H
14.	West Sikkim	6.4H	1.80H	97.21L	47.00H	333.00H
15.	West Sikkim	6.1H	1.80H	100.35L	71.50H	270.50M
16.	West Sikkim	6.5H	0.60L	75.26L	124.50H	382.50H
17.	West Sikkim	5.5H	1.80H	78.40L	71.50H	306.00M
18.	West Sikkim	6.5H	1.20M	72.12L	47.00H	427.50H
19.	West Sikkim	6.1H	1.80H	75.26L	67.60H	432.00H
20.	West Sikkim	7.0H	1.20M	65.85L	92.30H	364.50H
21.	South Sikkim	5.7M	0.90M	59.58L	83.20H	229.50M
22.	South Sikkim	6.1H	1.80H	62.72L	70.20H	333.00H
23.	South Sikkim	7.0H	1.80H	109.76L	53.30H	346.50H
24.	South Sikkim	6.8H	2.40H	72.12L	48.10L	252.00M
25.	South Sikkim	7.5H	3.00H	81.53L	51.00H	351.00H
26.	South Sikkim	7.5H	1.20M	97.21L	48.10H	333.00H
27.	South Sikkim	6.0M	1.80H	97.21L	82.00H	274.50M
28.	South Sikkim	6.5H	1.20M	97.21L	93.60H	270.00M
29.	South Sikkim	6.9H	1.68H	78.40L	91.00H	247.50M
30.	South Sikkim	8.5H	1.32M	94.08L	39.00H	220.50M
31.	West Sikkim	9.5H	1.80H	78.40L	54.60H	265.50M
32.	West Sikkim	8.0H	1.20M	68.99L	48.00H	261.00M
33.	West Sikkim	6.9H	1.20M	84.67L	51.00H	279.00M
34.	West Sikkim	7.2H	2.40H	88.00L	57.00H	261.00M
35.	West Sikkim	6.5H	1.80H	62.72L	73.00H	333.00H
36.	West Sikkim	6.8H	1.14M	62.72L	78.00H	319.00H
37.	West Sikkim	5.6M	1.26M	62.72L	86.00H	418.50H
38.	West Sikkim	6.8H	1.20M	84.67L	48.10H	378.00H
39.	West Sikkim	8.5H	1.80H	53.31L	88.40H	373.00H
40.	West Sikkim	6.8H	1.14M	62.72L	78.00H	319.00H
41.	West Sikkim	5.6M	1.26M	62.72L	86.00H	418.50H
42.	West Sikkim	6.8M	1.20M	84.67L	48.10H	378.00H
43.	West Sikkim	8.5H	1.80H	53.31L	88.40H	373.00H