Diversity and Micropropagation of Canna from West Bengal and Orissa

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By
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This work is dedicated to my Parents

Declaration

I declare that the thesis entitled “Diversity and Micropropagation of Canna from West Bengal and Orissa” has been prepared by me under the guidance of Dr. Arnab Sen, Associate Professor of Department of Botany, University of North Bengal. No part of the thesis has formed the basis for the award of any degree or fellowship previously.

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Abstract

Canna, the solitary genus of the family Cannaceae, is popularly known as an ornamental plant with flashy, brilliantly-colored flowers and large tropical foliage. Various morphological, cytological and taxonomical characteristics of the family Cannaceae is closely related to other members of Zingiberales like Musaceae, Strelitziaceae, Zingiberaceae, Marantaceae etc. The members of the Cannaceae possess a very diverse habitat. The genus *Canna* comprises of about 51 species of flowering plants. *Canna* species are native of tropical and subtropical humid neotropics (South and North America) and with the course of time they have been introduced in Asian paleotropics and subsequently evolved into semi-native varieties. The pantropical distribution of *Canna* species is most possibly the effect of human dispersal. Some of the wild species of Canna, namely *C. glauca*, *C. indica*, *C. iridiflora*, *C. warscwiczii* and *C. flaccid* etc. are popularly known as elemental species of Canna and are involved in producing natural as well as manmade hybrids. The entire cultivated garden ornamentals are included under two artificial hybrid species i.e. *Canna* x *orchiodes* L. H. Bailey and *Canna* x *generalis* L. H. Bailey.

However, there are number of problems related to the production of Canna. Seed germination is very difficult and not practiced very often leading to the problem in breeding of Canna for the production of improved varieties. Alternative methods like protoplast fusion for production of hybrid varieties has never been tried perhaps anywhere in the world. Till date no work has been done on the diversity of Canna found in west Bengal as well as in the adjacent state of Orissa. The taxonomy of Cannaceae is also disputed as the monogeneric family Canaceae has recently been formed and distinguishing features between Canna varieties are poor as they lack proper molecular documentation. Some Canna varieties like, *Canna edulis* and its cultivars are rich in edible starch content. It is now established as edible and medicinal

plant. However, nutritive and medicinal profiling for most of part of Canna is virtually untouched.

In the present study an attempt was made to access the genetic relationship among the 20 species and cultivars of Canna using RAPD, ISSR and PCR-RFLP (for TrnL-TrnF region of chloroplast genomes) techniques. A total of 159 major scorable bands ranging from 220 to 1757 bp were generated from 18 RAPD primers. The percentage of polymorphism was found to be 89.93%. The lowest similarity was observed between *Canna edulis* and *Canna* x *generalis* Cv. "Trinacria Variegata" (49.6%), while the highest value was recorded between *Canna* x *generalis* Cv. "Dwarf Red" and *Canna* x *generalis* Cv. "Dwarf Orange" and between *Canna* x *generalis* Cv. "Dwarf Orange" and *Canna* x *generalis* Cv. "Froken" (94.1%). In the dendrogram, both the cultivars of *Canna edulis* came together with a cluster sharing node at 89.1%. However, two varieties of *Canna indica* were separated out. Regarding the hybrid cultivars, all of them flocked together in a big cluster except *Canna* x *generalis* Cv. "Trinacria Variegata".

Ten ISSR primers resulted in 93 scorable bands showing 88.17% polymorphism among them. The band size ranged between 246 bp to 2017 bp. The highest correlation was found between *Canna edulis* and its green cultivar (96.4%), where as lowest was found between *Canna indica* and *Canna* x *generalis* Cv. "Orange Web" (56%). ISSR analysis results indicated that the elemental species *Canna indica* and its cultivar *Canna indica* Cv. "Purpurea" were close to each other, which is quite natural. Besides, there were three other distinct clades. One was with two elemental varieties, *Canna edulis* and *Canna edulis* green cultivar. Other 16 hybrid cultivars formed 2 different clades in the dendrogram.

The dendrogram based on the combined data sets of both RAPD and ISSR showed considerable similarity with that obtained from individual RAPD and ISSR analysis, except for the highest correlation, that was found to be between *Canna* x *generalis* Cv. "Tropical Red" and *Canna* x *generalis* Cv. "Orange Web" (0.936) while the lowest was found between *Canna* x *generalis* Cv. "Dwarf Red" and *Canna edulis* (0.601).

All the three dendrograms (RAPD, ISSR and combined) were similar to each other in many ways, such as

grouping of garden cultivars as a somaclone complex, separation of elemental species from the hybrid cultivars and the closeness of four dwarf cultivars etc. The only exception is the position of *C. indica* Cv. Purpurea. While in ISSR tree *C. indica* Cv. Purpurea went with *C. indica*, it positioned separately in RAPD tree and ISSR-RAPD combined tree.

All the bands generated through PCR amplification of Taberlet (TrnL-TrnF) region following restriction digestion were found to be monomorphic. Therefore, chloroplast genome may not be considered for studying polymorphism among various species and cultivars of Canna.

The phylogenetic tree constructed with Clustal Omega and Phylip 3.69 program of Taberlet region of various members of Zingiberales recovered all the major families intact. Families like Musaceae, Zingiberaceae, Cannaceae, Marantaceae, Strelitziaceae, Heliconiaceae and Lowiaceae were flocked together. The sequences of different Canna varieties done by us were perfectly placed with other sequences (Canna) retrieved from the public domain.

Canna is an important plant not only from the ornamental point of view but also it is an important plant for its medicinal values. In the present study, the antioxidant diversity among 20 different Canna accessions was determined on the basis of two parameters i.e. temperature changes and solvent types. The correlation of phytochemical characteristics and antioxidative properties of rhizome were taken up firstly through thermal changes of the aqueous extracts i.e. cold aqueous (CAE) and hot aqueous (HAE) extracts and secondly in two different solvent extracts i.e. in aqueous (HAE) and methanolic (ME) extracts. Total phenol and flavonoid contents of various Canna samples in different extracts were ranged between 22.67 to 158.22 mg GAE/g and 14.32 to 65.02 mg QE/g respectively. In both the cases methanolic extract was found to be better than aqueous extracts (ME>HAE>CAE). The DPPH radical scavenging activity was found to be highest in methanolic extract (84.96%). For ferric reducing power assay (FRP), methanol was proved to be the most effective solvent to assess the reductive potential for Canna rhizome. Both DPPH and FRP assay followed the same trend as that of phenols and flavonoids (ME>HAE>CAE). The Hydrogen peroxide scavenging activity

of various Canna samples in different extracts was quite different from other antioxidant properties. Cold aqueous extract had highest H_2O_2 scavenging activity (89.11%) followed by hot aqueous extract and methanolic extract. Separation and isolation of antioxidant molecules of *Canna edulis* (an edible Canna) was performed on the basis of their percolation in various polar and non polar solvents through silica gel column chromatography. Investigations were done to find out DPPH radical scavenging activity, total flavonols and total proanthocyanidin contents, nitric oxide scavenging activity, hydroxyl radical scavenging activity and anti lipid peroxidation activity of different solvent fractions *of C. edulis* rhizome. Out of 29 fractions studied, 6 fractions showed inhibition above 75%, and were used for further phytochemical screening. Diethyl ether : ethyl acetate (1:3) fraction showed the maximum inhibition percent (93.08%). Highest amount of total flavonol (i.e.37.12 mg/ml quercetin equivalent per 100 mg rhizome extract) and total proanthocyanidins (i.e. 0.012 mg/ catechin/g dry weight) were recorded in diethyl ether : ethyl acetate (1:3) fraction. Maximum NO scavenging activity (78.41%) and hydroxyl radical inhibition activity (39.04%) were observed in bioactive diethyl ether : ethyl acetate (1:1) fraction. Inhibition of lipid peroxides was maximum in ethyl acetate fraction (67.89%). Further, thin layer chromatography, confirms the distribution of phenolic compounds in the above bioactive fractions. Thus it can be concluded that the antiradical scavenging activity of *Canna* rhizome may be due to the presence of polyphenolic compouns like phenols, flavonoids, proanthocynidins etc.

GC-MS (Gas chromatography-Mass spectroscopy) analysis of *C. edulis* leaf extract identified the presence of 3 major compounds namely 22,23-Dibromostigmasterol acetate $$ 22,23-Dibromostigmast-5-en-3-yl acetate, N-Heptyl-N'-{9-[2-(heptyl-methyl-carbamoyl)-acetylamino]-nonyl}-N-methyl-malonamide and Cholest-5-ene, 3.beta.-chloro- $$ Cholesteryl chloride $$ Cholest-5-ene, 3-chloro-, (3.beta.)- $$ Cholesterol chloride $$ 3.beta.-Chlorocholest-5-ene, which have antibacterial, antifungal and anticoccidal activities.

In order to produce genetically modified and improved varieties of Canna, various *in vitro* techniques had

been tried. Banana micropropagation medium (BM) supplemented with 3% sucrose, 0.7% agar, and 0.17% NH_4NO_3 and different plant growth regulators like BAP (2 mg/l) and NAA (0.5 mg/l)) was found to be effective in inducing callus. Callus culture medium with BAP (2 mg/l) is ideal for somatic embryogenesis and plantlet regeneration in Canna tissue culture. After a period of 3 months, tissue cultured plants were successfully transferred to the field conditions.

To study the clonal fidelity among the *in vitro* regenerated plantlets, different molecular markers (RAPD and ISSR) were employed. Ten RAPD primers produced 60 amplicons while 7 ISSR primers generated 45 bands in both *in vitro* plantlets and mother plant. RAPD and ISSR analysis showed no evidence of polymorphism between parent plant and the regenerated plants as all the amplified products were found to be monomorphic.

Protoplast fusion technique was tried to generate genetically hybrid varieties. *In vitro* generated leaves and shoots of *Canna indica* and *Canna edulis* were used as the source for isolation of protoplasts. Viable protoplasts (viability range 60-75%) were generated in enzymatic combination of cellulase (1%) and pectinase (0.5%) with an incubation temperature of 24±3ºC for 16-18 hrs in dark. Isolated protoplasts were cultured in a medium consisted of BM medium, along with 2 mg/l BAP, 0.5 mg/l NAA, 2.8 mM glucose, 278 mM maltose, 116 mM saccharose, 2.5 mM myo inositol (pH 5.6±0.2) and 0.4% agar and were maintained at 25ºC in the dark. In the present experiment, protoplast fusion in Canna was standardized using Polyethylene glycol (PEG). Fused protoplasts were clearly seen under microscope. The fused protoplasts were cultured in the same media as described above.

Preface

The present endeavour is an outcome of my five years of research experience that has been carried out from 2009, since I was enrolled as a PhD student in the Molecular Genetics Laboratory, Department of Botany, University of North Bengal. I would like to express my heartiest thanks to Dr. Arnab Sen, Associate Professor, Department of Botany, University of North Bengal with a deepest sense of gratitude, for his excellent guidance, valuable suggestions and constant encouragement during my entire research. His thorough scientific knowledge, sincerity and genuine help guided me a lot despite of his busy academic and professional commitments. This thesis would not have been completed without his valuable input. I sincerely owe my work to him.

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[Tanmayee Mishra]

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Chapter 1
Introduction

"Flowers are the sweetest things God ever made, and forgot to put a soul into."
– Henry Ward Beecher, *Life Thoughts*, 1858

Ornamental plants transform a monochromatic landscape into a visual delight because of their attractiveness. They are mostly grown for displaying their aesthetic value. Examples of ornamental plants include roses, lilies, aster, lavender etc. Though these plants are commonly believed to be nonfunctional, but in reality there are some palatable varieties like Canna, Strawberry and Swiss Chard etc. At present the production of ornamental plants contribute a lot to the global economy. The flowering plant industry is comprehensive and dynamic, as the consumption of flowers has grown significantly over the last few decades. Ornamental plants are also aesthetically valuable to any landscape not only for their attractiveness but also for maintaining ecological balance. These plants come in varieties of shape, size and colors depending upon the climatic and gardening necessities. Like other plants, these plants also need regular fertilization, trimming, watering, mulching and pest control to keep them healthy and attractive. It is also important to select proper place and planting method, failing which

plants become vulnerable to insect and pest attacks.

Canna, the solitary genus of the family Cannaceae, is popularly known as an ornamental plant with beautiful flowers. It is cultivated extensively around homes and public parks for its decorative and widely varying flower colors (Nakornthap, 1965). Various morphological, cytological and taxonomical characteristics of family Cannaceae is closely related to other members of Zingiberales like Musaceae, Strelitziaceae, Lowiaceae, Heliconiaceae, Zingiberaceae, Costaceae and Marantaceae (Cronquist, 1981). It has long been recognized as a distinct, monophyletic group closely related to the family Marantaceae based on results of phylogenetic analyses of molecular and morphological data (Prince, 2010). Thus, Cannaceae is accepted by all taxonomists as a distinct entity at the family level within Zingiberales, with all types of proposed taxonomic classification (Kress, 1990).

Cannaceae is a monogeneric family with its solitary genus Canna. It covers the vast array of new world tropics from United States of America to West Indies and Northern Argentina and a large altitudinal range covering 3000 meter from the sea levels in the Andes, Peru and Bolivia. The members of the Cannaceae possess a very diverse habitat. *Canna* species are native of tropical and subtropical humid neotropics (South and North America) and with the course of time they have been introduced in Asian paleotropics and subsequently evolved into native varieties. The pantropical distribution of *Canna* species is most possibly the effect of human dispersal (Prince, 2010).The transportation of Canna from their native place may have been the reason for occurrence of beautiful ornamental plant in Europe, Asia and Africa (Maas and Maas, 2008).

The genus *Canna*, separated from the family Scitamineae to a monogeneric family Cannaceae by Winkler, 1930, which was further supported by various taxonomists on the basis of morphological characters (Tomlinson, 1961 and Hutchinson, 1959). The specific characteristics that differentiate Cannaceae from all other families included free sepals, single chambered stamen, adnate to lateral petaloid staminodium, four petaloid staminodia, 3-celled ovary with many ovules, flattened style, terminal stigma and straight embryo. The staminodia of the flowers give visual beauty to the

plant. The members of Cannaceae are further characterized by perennial rhizomatous root stock, pinnately arranged simple leaves with parallel venation, terminal raceme inflorescence, bisexual and asymmetric flowers with epigynous ovary, capsulated fruit with numerous hard seeds (Khoshoo and Mukherjee, 1970a; Maas and Maas, 2008; Tanaka, 2007 and Rogers, 1984).

The genus *Canna* comprises of about 51 species of flowering plants having rhizomatous root stock (Kranzlin, 1912). The flower of Canna is mainly red, orange, yellow, purple or white and cultivated as an ornamental plant around the globe. Although Cannas were previously considered as simple foliage plants, during the last two centuries, cultivation and improvement transformed them into attractive ornamental flowering plants with reduction in plant height, increase in hardiness, variability in flower color and such other positive attributes. Cannas are valued mostly for their large tropical foliage and flashy, brilliantly-colored flowers. The foliage is as ornamental as the flowers. Canna foliage may be of various colors for example, pure green, ruby, coppery to purplish and green with white stripes (Tjia and Black, 1991). In addition to that, it is one of the world's richest starch sources. Some of the species like *Canna edulis* Ker Gawler and its cultivar varieties are described as edible and have been selectively grown for their root stock, as a source of starch (Tanaka, 1998). It is distributed across the world and is known by a variety of local names such as 'Tous les mois' in West Indies, 'Imbirg' in Brazil, Chisgua' in Columbia, 'Capacho' in Venezuela, 'Queensland arrowroot' in Australia, 'Zembu' in Philippines, 'Lotus tuber' in Taiwan and 'Sagu' in Thailand (Tonwitowat, 1994). The leaves and young seeds of these plants are also edible.

Botanically, Canna is classified as follows (APG-III, Arthur Cronquist, 2002):

KINGDOM: Plantae
DIVISION: Magnoliophyta
CLASS: Liliopsida
SUB-CLASS: Zingiberidae
ORDER: Zingiberales
FAMILY: Cannaceae
GENUS: Canna

The transformation of Canna from wild to cultivated condition is prevailed historically. Canna is native to West Indies and America, however, it is well adapted to different environment of Europe and Asia. Thousands of Canna hybrids are being used as cultivated garden ornamentals around the world. Some of the wild species of Canna, namely *C. glauca*, *C. indica*, *C. iridiflora*, *C. warscwiczii* and *C. flaccid* etc. are involved in producing natural as well as man made hybrids. The above five species are popularly known as elemental species of Canna. The entire cultivated garden Cannas are included under two artificial hybrid species i.e. *Canna* x *orchiodes* L. H. Bailey and *Canna* x *generalis* L. H. Bailey (Hannay, 1936; Khoshoo and Mukherjee, 1970a). All the hybrid cultivars share some common features and bind themselves under the same horticultural species. Some phenotypic transformations were also taken place when they were shifted from wild to cultivated condition. The chief morphological factors involved are reduction in plant height i.e. from tall to dwarf plant, variation in shape, size and color of leaves, increase in flower size and diversity in flower color, durability of flowers and self-shedding pattern of flowers etc. Along with these characters, physiological alteration like increase in cold hardiness and stress resistance has also been involved as the foliage plants transform into colorful ornamental plants (Khoshoo and Mukherjee, 1970b).

In tropical and subtropical condition, Canna can grow under full sunlight or semi-shaded areas with loamy soils to reach an average height of 200-250 cm (Hermann *et al.*, 1997). The life cycle of different species of *Canna* is of approximately 9 months. In the tropical countries of South America (from where the lilies may have been originated), Cannas begin their life just after the end of winter. The lifecycle begins by producing leaves from the buds of underground rhizomes. During the early days, these plants require an average temperature of 15-20˚C, average relative humidity of 79%, and rainfall of 54 mm with approximately 12 hours of solar radiation. These ornamental plants continue to grow in the above mentioned climate to reach their maximum length. Blooming begins in their adult stage and continues till late autumn. During summer, Cannas reach their maximum size with abundant fruits and seeds. Germination of seeds rarely occurs because of the hard seed

coat. The aerial parts of Canna start drying during the summer. The dried plant remains underground throughout the winter season and again starts its life cycle in the next season (de las Mercedes Ciciarelli). Further, Canna is known to be highly drought tolerant and shade tolerant plant with high nitrogen and water use efficiency (Hermann *et al.*, 1997).

Cannas are perennial rhizomatus plants. They require adequate water and fertilizer for their optimum growth. These ornamental plants flourish in a well-drained loamy soil which is rich in organic nutrients. So cultivation of Canna require good fertilization and regular irrigation practices (Tjia and Black, 1991). The cultivation of edible Canna in fertile soil is an usual practice in different parts of Vietnam. Common people often use animal excretions as organic fertilizer for Canna cultivation (Tanaka, 2004). The yield of rhizome increases remarkably, when cultivated under nitrogen (N) fertilization (Hermann *et al.*, 1997).

Canna is a hermaphrodite plant. Seeds are produced by sexual reproduction. The seeds of Canna seldom germinate and the problem is related to extremely hard seed coat. For germination of Canna seeds one has to go through tedious scarification process (Joshi and Pant, 2010) and careful temperature controlling (Souza *et al.*, 2011). The pollination mechanism also is conspicuously specialized due to its typical flower structure. They are self-pollinating but most cultivars require an external pollinator.

Canna propagation is largely done through rhizomes. Cana, including starch producing edible Canna i.e. *Canna edulis* is long been cultivated through this process in Peru and asian countries like Thailand, Japan, Malaysia, Vietnam and Tibet. It takes about 2.5 months to produce flower after the initiation of shoot. However, the method is slow and vulnerable to viral (Canna specific virus) infection during multiplication (Momol *et al.*, 2004; Monger *et al.*, 2007 and Marino *et al.*, 2008).

Ornamental Canna is also cultivated as a commercial source of starch. Rhizome of edible Canna i.e. *Canna edulis* Ker Gawler is produced at a large scale to fulfill the demand of starch in various countries. Edible Canna or Achira is a tuber crop and is used as staple food by the native people. This crop is generally cultivated in subtropical highland, at an altitude range of about 1000-2500 meter of northern China

and Vietnam. This crop is largely produced in the Southeastern Asian countries to fulfill the industrial raw material of starch production. The starch of edible Canna is characterized by large granules, high amylase content, clear paste, higher viscosity, lower swelling power, high retrogradation, highly resistance to hydrolysis by α-amylase, higher acid and enzymatic hydrolysis (Piyachomkwan *et al.* 2002; Thitipraphunkul *et al.*, 2003 and Hung and Morita, 2005). Canna starch can be seen in the naked eye because of large granular size. It is easily digested and soluble in water. For this reason, Canna starch is an excellent food material for infants (Burkill, 1985). In Vietnam, rhizome of *Canna edulis* is used for producing noodles for commercial purposes. In China, people in rural area use fermented boiled rhizome of *C. discolor* to make alcohol. The people also use the remaining rhizome pieces and other parts of the plants to feed their livestock. Further, in the sub Himalayan region of India, starchy rhizome of *C. edulis* gains economic importance as it is very much used by the tribal people for their staple food (Tanaka, 2004). Thus, the cultivation of edible Canna may fulfill the production of potential source of food for human consumption.

The ornamental plants gained its economic importance in terms of herbal medicine. From the primordial period, common people use different parts of the plant as traditional medicines. In the south Asian countries, the village people commonly use Canna as an herbal medicine in their daily dealings. In some African countries, the whole plant is very popular as a folk medicine for malaria and local birth control. Canna is also very much popular among the Indian tribes for its healing capacities. Especially, the tribal communities of north eastern India use Canna rhizome for treatment of fever. In northern India, the village people prefer to use the rhizome extract as an antiinflammatory agent. It is also used as an ethno-medicine to reduce blood pressure (Choudhury *et al.*, 2010 and Singh *et al.*, 2010). Besides, *Canna indica* is known as a potential resource of antibiotics in animal husbandry (Mahady, 2002). Thus, Canna is widely accepted as an indispensable resource for the people in rural areas. Due to its medicinal uses in India, it is included under the database of "Ethno-medicinal plants of Western Ghats".

Environmental pollution is one of the biggest problems in the modern world.

Among all, heavy metal contamination causes severe damage to environmental quality. Now a days plants are exploited as phytoremediating agent to degrade, metabolize and accumulate wide range of hazardous heavy metals. *Canna indica* is a good phytoremediating agent, accumulating various environmental hazards. All parts of the plant i.e. root, stem and leaves are capable of absorbing heavy metal pollutants like Cadmium, Chromium, Lead, Nickel and Zinc etc. Canna can be designated as a good accumulator of heavy metals as it absorbs considerable amount of hazardous metal per KG of biomass (Subhashini *et al.*, 2013). *C. indica* is well-adapted in industrial sludge amended soil because, it has significant power in fractionation and translocation of heavy metals like iron, chromium, manganese, zinc, nickel, copper, cadmium and lead etc. Further, *C. indica* and *C. generalis* are known to be the potential species for phytoremediation of heavy metal contaminated soil and water. The activities of phytochelatin and antioxidant molecules are accountable for tolerance of *C. indica* to oxidative stress caused by some of the heavy metals like cadmium and copper (Bose *et al.*, 2008; Lingqiong *et al.*, 2007 and Shuangtao, 2005).

The beautiful landscape gardening plant is often used for home decoration. Besides, these plants have some folk users. During some specific occasion, the bright flowers of Canna are used for worshiping the idols in Hinduism (Maria *et al.*, 2010; Williams, 1876 and Mehra *et al.*, 1975). Globally, Canna meets many necessities of the common people. Ecologically Canna is significant by preventing soil erosion. Canna cultivation makes good sense of soil conservation because of their large leaves which completely covers the ground surface. The rhizomes, leaves and stems are used by the rural people to feed their livestock. Starchy rhizome of Canna is also utilized in making paper and cakes. Moreover the seeds of Canna are used in making jewellery as well as in producing purple dye. The leaves of the plants are insecticidal in nature. The red flowers of Canna, have a color strength, showing higher stability at room temperature even at acidic condition (pH of 4.0). For this, red Canna extract is suggested as a natural food dye (Tanaka, 2004 and Vankar and Srivastava, 2008). Furthermore, the presence of different types of anthocyanin pigments in the red Canna flowers are good nutraceuticals and thus, a promising pigment source of

food industries (Srivastava and Vankar, 2010). The tuber biomass is significantly used for the large scale production of ethanol, thus extending the economic value of Canna lilies (Huang *et al.*, 2013).

Canna is an important plant not only from the ornamental point of view but also it is an important plant for starch production as well as its medicinal values. In Asia the plant becomes overexploited. Edible Canna is reported to be a promising resource plant for future agricultural development (Imai *et al.*, 1993 and 1994). However, there are number of problems related to the production of Canna and breeding of Canna for the production of improved varieties. As mentioned above, seed germination is very difficult and not practiced very often. Therefore, production of new variety with mating and cross pollination is limited. I also found that standardization of tissue culture technique in Canna, particularly, varieties found in India have not at all been done. Besides asexual reproduction, conventional breeding is not very popular in Canna, alternative methods like protoplast fusion for production of hybrid varieties has never been tried perhaps anywhere in the world. Therefore, tissue culture is playing a major role in understanding this objective to meet the demand in a short span. Micropropagation technique may be employed for rapid growth in a sterile environment, producing variants and exploring the resultant variations decontaminated by virus. Therefore, in the present study a stable and efficient tissue culture system is established for the benefit of the larger farming community of the world. Though Canna is an introduced variety in India, it is now escaped and semi-naturalized. As a result new varieties have been evolved in due course of time. However, virtually no work has been done on the diversity of Canna found in west Bengal as well as in the adjacent state of Orissa. Further study of molecular diversity among the regenerated plantlets can provide suitable tool for crop improvement. The taxonomy of Cannaceae is also disputed as the monogeneric family Canaceae has recently been formed and distinguishing features between Canna varieties are poor and most of the Indian varieties lack proper molecular documentation. The diversity profile of different species and cultivars of *Canna* can be attempted with the help of various molecular markers to provide useful information for future researchers. Canna is now established

as edible and medicinal plant. However, nutritive and medicinal profiling for most of the parts of Canna is virtually untouched. Antioxidant diversity profiling of all the Canna varieties found in the particular region will give a scientific evidence of the presence of natural antioxidants. Studies on separation and isolation of the compounds, responsible for antioxidant activity would provide additional useful data on biological activities of these plants. Keeping all these in mind, following objectives have been chosen for the present work.

- Selection of species and cultivars of Canna from different parts of West Bengal and Orissa.
- Collection and maintenance of germplasm.
- Detection of genetic variability and phylogenetic relationship among the cultivars of Canna by different PCR based DNA fingerprinting methods like RAPD and ISSR.
- To study the trnL-trnF region of chloroplast genome of Canna using PCR-RFLP.
- Assessment of antioxidant activities of Canna rhizome of all the available cultivars.
- To separate the compounds of edible Canna (*Canna edulis*), meant for high antioxidant activities.
- Study of regeneration of Canna through tissue culture techniques.
- Detection of somaclonal variation among the *in vitro* regenerated plantlets.
- Protoplast fusion of two different species of *Canna* to create a new hybrid variety.

Chapter 2
Review of Literature

2.1 Survey of traditional taxonomy of Canna

From the primitive time, Canna was known to be a native of new world tropics and sub-tropics. It is the only genus in the family of Cannaceae, comprising of about 9 to 50 wild species. In the course of time it was developed as an ornamental plant in the completely different temperate environment of Europe specifically in France and Italy (Segeren and Maas, 1971). Out of all these wild species, five species namely *Canna indica*, *Canna glauca*, *Canna iridiflora*, *Canna warscwiczii*, *Canna flaccida* were considered to be elemental or basal species which are responsible for the origin of ornamental garden Cannas. The garden cultivars were included under an artificial hybrid species *Canna generalis*. The above changes from wild to cultivated conditions result some progressive increase or decrease in various morphological characters. This phenotypic transformation in Canna may be a culminated effect of hybridization, polyploidy and physiological variation (Khoshoo and Mukherjee, 1970a). Basically taxonomy of Canna is based on the morphological characters like height of the plant, shape and size of leaves, color, size, structure and blooming period of flower, type of inflorescence etc. It was studied from the literature that there are thousands of hybrid Cannas found in various parts of the world. Thus, there is a great diversity of morphological features among all the species, variety and cultivars of Canna. But these may not be considered as distinct morphologic entity to explain taxonomic differentiation among them, because these ornamental cultivars were produced by the mere crosses between two or more elemental species of Canna (Khoshoo and Mukherjee, 1970a). The traditional taxonomy of Canna that relies on floral morphology is very complex and disputed which

needs some molecular basis to solve the problem. Thus, phylogenetic study using molecular markers can be useful for genetical classification of cultivars or varieties of a species.

Canna is a diploid plant and it maintains the uniformity of somatic chromosome number i.e. 2n = 18 except for few species like *Canna discolor* Lindl. and *Canna edulis* Ker Gawler. *Canna discolor* Lindl. is a triploid plant having 2n = 27 chromosome number (Tanaka *et al.*, 2009). The edible Canna i.e. *Canna edulis* Ker Gawler, which is cultivated as a starch producing plant, was also reported to be triploid by Venkatasubban (1946). Canna was first hybridized for ornamental purposes in mid 19th century and became very popular as a hybrid plant (Khoshoo and Mukherjee, 1970b). Hundreds of hybrids have been evolved from multiple cross breeding between one or more wild Canna (Patra *et al.*, 2008). For this reason, hybrid cultivars are believed to be either diploid or triploid in nature having different somatic chromosome numbers (2n = 18 or 2n = 27). It was further analyzed by Khoshoo and Mukherjee (1970b) that the progressive increase or decrease in various morphological and physiological characters from elemental to diploid or triploid species may be a result of polyploidy. For example, plant height gradually decreases from elemental species to diploid and triploid hybrids. That means elemental species are the tallest plants and the triploid hybrid is the shortest one. Similarly the size of the flower is largest in triploid hybrid and smallest in the elemental species i.e. (flower size in triploid hybrid > diploid hybrid > elemental species).

2.2 Molecular taxonomy of Canna

The presence of thousands of hybrids within a single genus indicates the occurrence of complex crosses between genetically related members of Canna. The distinction between these hybrid groups have been uncleared by further interbreeding involving the parents from one or more than one species, varieties and hybrids (Patra *et al.*, 2008). However, these cultivars have some unique traits like variegated leaves, dwarf height, cold hardiness etc. contributing to the genetic pool of Canna. Though basic knowledge in the biology and genetics of *Canna* is lacking due to typical taxonomic status, but several molecular strategies could be employed to assess the genetic fidelity by detecting variations among

the individuals between and within the species.

2.2.1 Molecular taxonomy

The taxonomy of Canna is disputed and is very much complex as it relies on morphological markers. Molecular marker studies will help in overcoming the problem of classification of species and hybrid cultivars of the same genus. With the advent of molecular biology, the taxonomy of Canna has been reformed. Now a day, molecular markers have been increasingly used in each and every sphere of biology. The application of molecular marker in classifying Canna can prove to be a milestone. In Canna, these markers have several advantages, firstly for proper identification of Canna genotypes and secondly assessment of genetic variation among the species and cultivars irrespective of the geographic location or any other factors responsible to phenotypic variability. Though very little work has been reported in the literature, the present study aimed to classify some of the Canna species and cultivars on the basis of various molecular tools.

In recent years, a number of techniques have been developed by various researchers to detect DNA polymorphism. The methods involve the use of polymerase chain reaction (PCR) or restriction enzymes or combination of both.

Use of random amplified polymorphic DNA (RAPD) markers, detected by PCR amplification of small inverted repeats, 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 fingerprinting, where single arbitrary primer was used for amplification. Nanogram amounts of total genomic DNA are subjected to PCR using short synthetic oligonucleotides of random sequence. The technique essentially scans a genome for these small inverted repeats and amplifies intervening DNA segments of variable length. RAPD markers are user friendly and are most preferred among all the molecular markers. However not much work has been reported on the studies of genetic diversity of Canna cultivars in India and worldwide, except for Piyachomkwan *et al.*, 2002 and Patra *et al.*, 2008, who had done some experiments in a very limited way. Using ten base RAPD primers,

Piyachomkwan and his coworkers in 2002, found some genetic variation among four indigenous Thai Canna varieties. Patra *et al.* in 2008, used RAPD technique to study the genetic characterization of 10 cultivars of Canna lilies, including nine different horticultural plants and one elemental species. They found considerable genetic distance between each and every cultivar. Separate clusters in the dendrogram, gave an idea about genetic differences among themselves. Though very little work has been documented regarding the molecular characterization of different species and cultivars of Canna, RAPD technique was well studied in some close zingiberales like banana, heliconia, turmeric and zinger. It was reported that DNA fingerprinting study was well described for various banana and plantain germplasm. Genetic diversity and phylogenetic relationship of different cultivars of banana were analyzed by various authors using molecular marker likes RAPD (Howell *et al.*, 1994; Bhat *et al.*, 1995; Pillay *et al.*, 2001; Jain *et al.*, 2007 and Venkatachalam *et al.*, 2008). Kumar and his coworkers in 1998 studied the genetic analyses of Heliconia species and cultivars with randomly amplified polymorphic DNA (RAPD) markers (Kumar *et al.*, 1998). Further, Syamkumar and his coworkers in 2003 performed the PCR amplification of different varieties of turmeric and zinger DNA through RAPD technique to describe their genetic diversity (Syamkumar *et al.*, 2003). Using RAPD markers, genetic variability and phylogenetic relationship among different cultivar varieties of *Curcuma* and *Zingiber* were successfully studied by various authors (Jan *et al.*, 2011; Nayak *et al.*, 2005 and Kizhakkayil *et al.*, 2010).

Inter-simple sequence repeat (ISSR) arises in genomes by the repetition of single sequence. This marker has been used for identification of genetic diversity of many plants (Akkaya *et al.*, 1992). The tandem repeats like ISSR are densely interspersed in eukaryotic genomes (Hamada *et al.*, 1982; Tautz and Renz 1984 and Weber and May, 1989) which can reveal high degree of allelic diversity by polymerase chain reaction. PCR analysis using inter simple sequence repeat primers have gained attention recently as an alternative means of characterizing complex genomes. The Inter simple sequence repeat-PCR (ISSR-PCR) approach is an attractive one because it

avoids the need to carry out costly cloning and sequencing inherent in the original microsatellite based approach. ISSR-PCR has been successfully used for genetic characterization of various plant species. Though many reports were documented regarding the genetic variability and phylogenetic analysis of several Zingiberales, single report was found for PCR amplification in Canna using ISSR markers. Patra and his coworkers in 2008, carried out molecular characterization of different cultivars of Canna using locus specific ISSR markers. They explained the presence of considerable of genetic variability among the closely related garden cultivars of Canna and the elemental species from which have been originated (Patra *et al.*, 2008). Inter simple sequence repeat have been used for PCR amplification and genetic diversity study of different subspecies, varieties and cultivars of banana (Pillay *et al.*, 2003; Racharak *et al.*, 2007 and Venkatachalam *et al.*, 2008). Various authors have also evaluated the genetic variability of *Curcuma* and *Zingiber* germplasm by exploiting ISSR as the molecular marker (Singh *et al.*, 2012; Kizhakkayil *et al.*, 2010 and Sajeev *et al.*, 2011).

Restriction fragment length polymorphisms (RFLP) technique is based on the differences in the restriction enzymes recognition site between genome sequences, which is effective in detecting intraspecific genetic diversity among the cultivars. The application of chloroplast DNA (Cp DNA) analysis provided valuable supplemental information about the classification and relation in the species, subspecies, varieties and cultivar level. RFLPs have been used as genetic markers for characterization of germplasm of various crops including some Zingiberales like banana and turmeric, which are closely related to ornamental Canna. On the basis of cytoplasmic differences, Gawel and Jarret in 1991, evaluated the diversity and evolution of various clones of banana and plantain (Gawel and Jarret, 1991). Genome composition and genetic diversity of *Musa* germplasm from China was revealed by using molecular marker like RFLP (Ning *et al.*, 2007). Similar works on molecular characterization of banana using RFLP technique, were reported by various authors (Gawel *et al.*, 1992 and Jarret *et al.*, 1992). By using RFLP marker, Hayakawa *et al.* in 2011, studied the difference between various hybrids of *Curcuma longa* on the basis

of curcumin content (Hayakawa *et al.*, 2011). However, till date single report was found for Canna, in which, Tanaka supported several segregated species of *Canna indica* by using RFLP marker (Tanaka, 2001). Though RFLP is known to be one of the best molecular markers, the regular use of RFLP in plant genotyping has been limited mainly due to the requirements of large amount of DNA along with the use of radioactive isotopes.

Amplified fragment length polymorphism (AFLP) is a molecular method developed by Vos *et al.*, 1995, which is described as a combination of RFLP and PCR. AFLP marker provides multilocus genome wise DNA profiling, suitable for molecular characterization of all the collected germplasm (Azhaguvel *et al.*, 2006). AFLPs are dominant markers, forms cluster in chromosomal regions and represent predominantly repeated DNA. Very recently AFLP method was tried for the first time to study genetic relationship and population analysis of ornamental Canna (Gupta *et al.*, 2013). They investigated AFLP fingerprinting technique to evaluate molecular diversity and genetic relationship among different species and hybrid cultivars of Canna. Molecular diversity of some other ornamental plants like rose and lotus have also been studied by using AFLP marker (Koopman *et al.*, 2008 and Hu *et al.*, 2012). Besides, AFLP markers are also used for genetic characterization of many close Zingiberales e.g. banana, zinger etc. (Loh *et al.*, 2000; Wong *et al.*, 2001; Wang *et al.*, 2007 and Das *et al.*, 2013). However, AFLP requires large amounts of template DNA as well as high input costs (Gresshoff *et al.*, 1998).

SCAR (Sequence characterized amplified regions) markers were identified and developed by Paran and Michelmore in 1993. These are PCR based molecular markers, representing DNA fragments at genetically defined loci that are identified by PCR amplification using sequence specific oligonucleotide primers. SCARs can be used both as genetic markers and physical landmarkers in the genome. To date there is no information of the use of Scars markers in the molecular characterization of Canna. However, some species specific SCAR markers were developed in *Musa* and *Curcuma*. SCARs have been used for the successful characterization of dwarf *Musa* varieties (Ramage *et al.*, 2004 and Suprasanna *et al.*, 2008). Various

authors were successful in developing specific SCARs to evaluate the quality control by detecting adulterant in some market samples of turmeric powder (Sasikumar *et al.*, 2005 and Dhanya *et al.*, 2011). SCAR markers were also studied in flowering plant like *Fragaria vesca* to get insight into the nature of polymorphism generated by the inter-simple sequence repeat system (Albani *et al.*, 2004).

Though very little work have been done in Canna, but DNA sequence based molecular phylogeny is well studied in Zingiberales. The chloroplast genome has been used to assess the phylogenetics of various plants including the members of Zingiberales since the origin of molecular systematic. Various authors used chloplast DNA for the molecular genetic study of Musaceae family. To resolve the infrageneric dispute, molecular phylogeny and systematics of genus banana was investigated from chloplast DNA fragment like trnL-F and rbcl gene (Liu *et al.*, 2010; Li *et al.*, 2010; Wan *et al.*, 2005 and Retnoningsih, 2009). In addition to the above experiment, Jiang *et al.*, in 2006 and Techaprasan *et al.*, in 2011 performed some valuable work on molecular phylogeny of zinger and turmeric using chloroplast DNA fragments. But till date, infrageneric study was not found in case of Canna.

In the course of time, Internal transcribed spacer (ITS) region of the nuclear ribosomal cistron became valuable in providing phylogenetic inferences of plants in the generic and infrageneric levels. This marker is now extensively used in every sphere of biological science to analyse the evolutionary history of various eukaryotic plants. It was found in the literature that ITS and Chloroplast rpL16 intron DNA sequence were used in Canna to infer the evolutionary relationship among different species of Canna. Internal transcribed spacer region were exploited to solve the conflict of origin and natural distribution of different species of Canna (Prince, 2010). Recently in 2012, diversity analysis to interpret the origin and distribution of banana cultivars have been studied by means of nucler ITS region (Burg *et al.*, 2012). Phylogeny of different species of *Curcuma* was also analyzed through nucler ITS and plastid DNA (Zaveska *et al.*, 2012). Further, ITS markers have also been used to explore the evolutionary relationship among the families of flowering plants. The ITS

based phylogeny of nuclear ribosomal DNA was studied to solve the controversial relationship within the flowering plant family of Apiaceae (Downie *et al.*, 2000).

Transposable elements are mobile genetic elements occupying considerable proportions of many eukaryotic genomes (SanMiguel and Bennetzen, 1998). These are capable of changing their location in the genome. Based on their mechanism of transpositions, the genetic elements were broadly classified into two classes namely, Retrotransposons or Class I and DNA Transposons or Class II (Feschotte *et al*., 2002). These are known to be the advanced molecular marker techniques for the detection and analysis of genetic variation in plants. Some reports of use of transposable elements based molecular technique were found in banana. These mobile elements helped in explaining the synthetic relationship between the species of *Musa* (Lescot *et al.*, 2008). However, till date there is no report of the use of such molecular markers is found among the cultivars of Canna.

Very few published data on systematic study and genetic diversity of different species and cultivars of Canna is available in the literature. Thus, the taxonomy and classification of Canna happens to be complex and confusing and needs some molecular approach.

The present review showed precisely how the molecular marker helps in sorting out the problems related to the species, subspecies and cultivar identification in Zingiberales with special reference to Canna. It provides a clear picture of the application of various molecular techniques at the cultivar level of Canna to detect the genetic variability among themselves. The above discussed markers are definitely useful tools to predict the population genetics, taxonomic and phylogenetic analysis but they must be used with caution to avoid any ambiguous result. Thus, molecular genetics study will help in resolving the problem of origin, taxonomic position and classification of different species and cultivars of Canna.

2.3 Canna Antioxidant Diversity

In our planet, human plant intimate relation begins from the prehistoric period. With the development of social and cultural sense in primitive men, their dependence on plant resources for food, fodder, fuel, drug and shelter also increased. In this early part of twenty first century, with increased

globalization and modernization, our society has a great concern for human health. So there is a demand for healthy food habit. Plants have been considered to be the major source of natural food containing large quantities of antioxidants (Larson, 1988 and Middha *et al.*, 2009). Uptake of phytochemicals has increased significantly to maintain sufficient level of natural antioxidants. Therefore plants have gained immense importance for their efficiency to produce high quality of herbal food. Among all, Canna is one of such plants, producing large quantities of starch and natural antioxidants.

The beautiful ornamental Canna has gained its economic importance in terms of food and herbal medicine. From the earlier days Canna has been used as traditional medicine in different parts of Asia, more specifically in Southeastern Asian countries like Thailand, Malaysia, Japan, Vietnam etc. In India, the Manipuri community of Barak valley of Assam used crushed root to treat fever (Choudhury *et al.*, 2010). In northern India, the rhizome is used by the rural people to cure hypertension, menstrual disorders. Canna root is also popular as an anti-inflammatory agent (Singh *et al.*, 2010). In Thailand, Canna root has also been used as a medicine for treating diabetes (Purintrapiban *et al.*, 2006). In Iraqi traditional medicine the whole plant is used as diuretic, demulcent and sudoforic agent (Al-Douri and Al-Essa, 2010). Not only in Asia but also in other continent like Africa, ornamental Canna is used traditionally by the local people for curing various ailments. Canna plays a very important role in Nigeria as its extract is locally used to cure malaria. Further, Canna is an excellent folk medicine to control birth rate (Lawal *et al.*, 2010). Though Canna is used as traditional medicine throughout the world but the scientific evidence is restricted. So it's the time of great concern for the researchers to explore this area scientifically.

Oxidation is a necessary evil, which is essential for biological energy production in most of the living organisms, but excessive reactive oxygen species produced in different oxidative reactions are accountable for various health hazards to human life causing the development of degenerative diseases (Chang and Slikker (Eds.), 1995). Antioxidant can slow down or terminate the activity of the oxidative chain reactions by way of removing free radical intermediates by

being oxidized themselves. These processes usually occur in human body and are constantly inhibited by an efficient network of antioxidant (Bagul *et al.*, 2005). Plants often contains good amount of antioxidants. Different bioactive phytochemicals like phenols, flavonoids, carotenoids, proanthocyanidins, flavonols and ascorbic acid can be utilized to scavenge the excess free radicals from human body (Pratt, 1992). Keeping these views in mind, uptake of phytochemicals has increased significantly since it might drift the balance towards a sufficient antioxidant status (Goyal *et al.*, 2010).

Various experiments have been carried out to isolate and identify different compounds of ornamental and edible Canna. Srivastava and Vankar in 2010 isolated six methylated anthocyanin glycosides from red *Canna indica* flower. These compounds are malvidin 3-0-(6-0-acetyl-b-D-glucopyranoside)-5-0-b-D-glucopyranoside, malvidin 3,5-0-b-D-diglucopyranoside, Cyanidin-3-0-(600-0-a-rhamnopyranosyl)-b-glucopyranoside, Cyanidin-3-0-(600-0-a-rhamnopyranosyl)-b-galactopyranoside, Cyanidin-3-0-b-glucopyranoside, Cyanidin-0-b-galactopyranoside. From the bright red flower of *Canna indica*, Srivastava and Vankar in 2010 further isolated and identify four different compounds of anthocyanin pigments other than quercetin and lycopene. These compounds are Cyanidin-3-0-(6”-0-α-rhamnopyranosyl)-β-glucopyranoside, Cyanidin-3-0-(6”-0-α-rhamnopyranosyl)-β-galactopyranoside, Cyanidin-3-0-β-glucopyranoside, Cyanidin-0-β-galactopyranoside. In 2004 Sook and his coworkers isolated two phenylpropanoid sucrose esters namely 3-0-p-coumaroyl-6-0-feruloyl-b-D-fructofuranosyl 6-0-acetyl-a-D-glucopyranoside and 3,6-di-0-p-coumaroyl-b-D-fructofuranosyl 6-0-acetyl-a-D-glucopyranoside from dry rhizome of *Canna edulis* Ker Gawler (Sook *et al.*, 2004). Along with the above compounds one phenylpropanoid sucrose ester, four phenylpropanoids i.e. caffeic acid, rosmarinic acid, caffeoyl-4’-hydroxyphenyllactic acid and salvianolic acid and a sucrose ester derivative were also isolated (Sook *et al.*, 2004). A number of different terpenes such as monoterpenes, sesquiterpene, diterpene and some fatty acid along with their ester derivatives from the essential oil of *Canna indica*

rhizome were identified by Indrayan and his group in 2011 (Indrayan *et al.*, 2011). Zhang and his coworkers isolated a novel compound i.e. 4-(3-(3,4-dihydroxyphenyl)acryloyl)-6-hydroxy-1-methoxy-1,2,3,4-tetrahydronaphthalene-2-carboxylic acid from the aqueous extract of *Canna edulis*. Some phenolic compounds were also isolated from the same plant by Zhang and his coworkers. These compounds were identified as ferulic acid, 1-caffeoylquinic acid, 3-caffeoylquinic acid, 4-caffeoylquinic acid, 5-caffeoylquinic acid, salicylic acid and gallic acid (Zhang *et al.*, 2011). Subsequently, Zhang confirmed the antioxidant activities of the isolated compound of *Canna edulis* rhizome. So due to the presence of above mentioned antioxidative compounds in Canna rhizome, it might be considered as a ideal plant having great pharmaceutical potential. Further, antioxidant compounds in banana, turmeric and ginger were also studied. Various phytochemicals were found in commercial banana (*Musa cavendis*) and was proved to be a good source of natural antioxidants (Lewis *et al.*, 1999; Someya *et al.*, 2002 and Veneziano *et al.*, 2004). Chandraju and his coworkers in 2011found considerable amount of sugar compounds like glucose, fructose, sucrose and maltose from banana peel (Chandraju *et al.*, 2011). Further research proved banana as a natural food colorant due to the presence of different anthocyanin pigments (Alexandra *et al.*, 2001). Some bioactive compounds like curcuminoids (curcumin, desmethoxycurcumin and bisdesmethoxycurcumin) and gingerols (6-gingerol, 8-gingerol, 10-gingerol) were isolated from turmeric and ginger through high performance liquid chromatography. All the compounds were proved as therapeutical significant molecules (Wichitnithad *et al.*, 2009; Jayaprakasha *et al.*, 2002; Hiserodt *et al.*, 1998; Jitoe *et al.*, 1992 and Schwertner and Rios, 2007).

Silica gel column chromatography is a traditional method used to purify individual chemical compound from a mixture of compounds. It is basically meant for the separation and purification of organic compounds on the basis of their polarity level. Low cost and disposability of silica gel attract the researchers to use as stationary phase in column chromatography. Though silica gel column chromatography is suitable for

compound separation, it is a time consuming process and sometimes gives poor result due to band tailing (Clark *et al.*, 1978). Though separation and purification of antioxidant compounds was not studied in Canna, Bhattacharya and his coworkers separated the mixture of organic molecules in zinger, on the basis of their polarity level. They further investigated the presence of phytochemical constituents and *in vitro* antioxidant activities of these solvent fractions and concluded ginger to be a therapeutic agent (Bhattacharya *et al.*, 2009).

Although Canna rhizome has long been used as a commercial source of starch, production of high quality food items has not yet received attention from researchers. So much interest is to be given in the direction of prospective food research in Canna. Recently this line of research starts by Atrooz. In 2007, Atrooz investigated the antioxidants like polyphenols and flavonoid contents of methanolic extracts of plant seeds of *Canna indica* (Atrooz, 2007). Vankar and his co-worker performed the phytochemical constituents and antioxidant activity of methanolic extracts of red and yellow varieties of *Canna indica.* They carried out the comparative study of total phenol, flavonoid and antioxidant activity by 1,1-Diphenyl-2-picryle-hydrazyl (DPPH) assay and Trolox equivalent antioxidant capacity (TEAC) assay. (Vankar and Srivastava, 2008). Some pharmacological studies such as laxative, cardiotonic activities, for investigation of total polyphenolic contents and antioxidant activities of edible flower extracts has been done. *In vitro* Antioxidant Activity of methanolic extract of aerial parts of *Canna indica* was studied by Joshi and his coworkers in 2009 (Joshi *et al.*, 2009). The antioxidant activity of the seed extract of *Canna indica* was evaluated for their antioxidant activity by DPPH radical scavenging activity, reducing power, RBC's hemolysis and linoleic acid oxidation along with the determination of the phenolic and flavonoid contents. All these experiments showed strong antioxidant activity and high content of phenolics and flvonoids (Atrooz, 2009). Indrayan and his co-workers studied the antibacterial activity of the essential oil of *Canna indica* rhizome showing inhibitory effect against some important gram positive and negative bacteria (Indrayan *et al.*, 2011). Despite the uses of Canna as food and

folk medicine for human, it is also utilized as an alternative for conventional antibiotics in animal husbandry.

Till date very little reports have been published on the phytochemical constituents of Canna, but it has been well explored in close Zingiberales. Research on some members of zingiberales showed that they have considerable amount of antioxidants. Someya and his coworkers had studied the presence of total phenolics and antioxidant activities in banana. They found that both phenolics and antioxidant activities were more abundant in peel than in pulp. High antioxidant activity in banana is attributed to its phenolic content (Someya *et al.*, 2002). Presence of different phytochemicals, high antioxidant activities and antibacterial activity in banana was further investigated by various scientists (Kanazawa and Sakakibara, 2000; Mokbel and Hashinaga, 2005; Vijayakumar *et al.*, 2008 and González -Montelongo *et al.*, 2010). Some other Zingiberales like turmeric, zinger is also proved to have some antioxidant activities. Turmeric, basically a spice is well known for its medicinal properties. In India and China, it is long been used as a herbal medicine for various ailments like cough, anorexia, diabetic wounds, hepatic disorders and rheumatism etc. High antioxidant and antiradical activity found in turmeric was because of the presence of higher amount of polyyphenolic compounds. Curcumin, a major component of *Curcuma longa* is a natural antioxidant having antimutagenic, antitumor, anti-inflammatory, antiparacitic, antispasmotic and antibacterial activities. It was investigated that turmeric extract reduces lipid peroxidation by enhancing the antioxidant enzymes. Turmeric leaf was also studied to have higher antioxidant activities (Jayaprakasha *et al*., 2002; Ruby *et al.*, 1995; Chattopadhyay *et al.*, 2004; Araujo and Leon, 2001; Pulla Reddy and Lokesh, 1994 and Yan and Asmah, 2010). Ginger was studied for high antioxidant activity and was established as a therapeutically significant food material (Ahmad *et al.*, 2008 and Nagendra chari *et al.*, 2013).

A series of work have been carried out by different scientists on different members of Zingiberales showed their efficiency as potential medicine. As a member of Zingiberales, we must think that Canna may have some therapeutic

uses. Though Canna is popular as an ornamental plant, the Indian tribes like Lepchas, Bhutias and Nepalis consume the starchy rhizome as their regular food (Sekar and Mariappan, 2007). Since decade it has fascinated the attention of researchers, worldwide due to its nutritional and medicinal values. Some work on dietary uses and medicical values of Canna has been carried out but review of literature did not provide any substantial work on nutritional values and antioxidative properties of different species and cultivars found in eastern India more specifically in West Bengal and Orissa. So scientific experiments and validation are essential to ensure Canna as a safe food additive and have significant role in the pharmaceutical and nutraceutical industries. Thus, a lot of research needs to be explored on detailed antioxidative properties of different cultivars of Canna found in the eastern part of India.

2.4: In vitro propagation in Canna

Canna is a perennial rhizomatus herb. It is a horticultural plant but its agricultural importance can't be undervalued. It is a self pollinating plant and require outside pollinator due to its typical flower structure. Sexual method of propagation using seeds is not considered to be reliable because Cannas have a slow tendency for seed setting (Cochrane, 2008). On the other hand if seeds were produced, they are either sterile or have extremely hard seed coat which contributes to their dormancy. This could be overcome by scarification of seeds before sowing. Scarification enhances germination but reduces the viability of seed (Joshi and Pant, 2010). Traditionally Canna is a vegetatively propagated root crop. The cultivation of this crop occurs through the division of rhizome. In Canna, vegetative propagation occurs through out the year but has some limitations. The conventional method of propagation is slow and susceptible to viral infection during multiplication (Hosoki and Sasaki, 1991; Momol *et al.*, 2004; Monger *et al.*, 2007 and Marino *et al.*, 2008). These limitations might be overcome by modern propagation techniques. Micropropagation technique can be employed for rapid growth producing superior variety, which will be free from any contamination.

Propagation using in vitro culture techniques

Plant tissue culture basically means the culture of plant tissue or cell in a sterile environment. *In vitro* cultured cell

usually retains its potentiality to grow and establish into a whole plantlet. Regeneration of an organism from a single cell or a group of cells raises the importance of tissue culture. The basis of culture of vegetative cell having potentialities for the generation of an elementary organism clearly establishes the concept of totipotency. Thus, the phenomenon of totipotency indicates the techniques of cultivating isolated plant cells in nutrient solution to produce the whole organism. Later, it was studied that totipotency of a plant cell retained for a longer period, but still the property is not stable and usually lost some time after the isolation of the cells (Reinert and Backs, 1968). As a pioneer, Haberlandt in 1902, justified the totipotency of plant cell. Subsequently, artificial culture through meristemetic tissues (White, 1934), embryo culture (Monnier, 1995) and successful rescue of embryos from seed culture (Laibach, 1929) were performed in completely defined nutrient medium. This was followed by the establishment of phenomenon of precocious germination (LaRue, 1936), thus, providing one of the earliest applications of *in vitro* culture.

With the development of techniques, two major events that revolutionized plant tissue culture were the discovery of plant growth regulators like auxins and cytokinins and the formulation of nutrient media i.e. Murashige and Skoog or MS media (Murashige and Skoog 1962). MS media consists of macro and micro nutrients, carbon source, vitamins, salt and growth regulators. The MS salt formulation is now the most widely used nutrient medium in plant tissue culture. Again in 1974, Murashige described the possible outcomes of micropropagation namely the formation of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Nevertheless, these findings set the stage for the dramatic increase in the use of *in vitro* cultures in the subsequent decades.

The culture of single cells by shaking callus culture in a conditioned medium gave rise to well established nurse cells (Reinert, 1959 and Steward *et al.*, 1958). Further research on single cell culture produced well defined cell colonies (Bergmann, 1959). The above technique was widely used for cloning of cells, culture of protoplasts and induction of somatic embryos from the callus (Kohlenbach, 1966). Later on *in vitro* culture of floral and seed parts

was successfully established (LaRue, 1942). Protoplast isolation and fusion technology was developed for the first time during 1970s because of the commercially available cell wall degrading enzymes. This led to the isolation and fusion plant protoplasts. In 1985, plant protoplasts were isolated mechanically from the plasmolysed tissues (Gautheret, 1985). The changes in the structure and physiology of cells in developing callus, cultured cells, and protoplasts had been carried out under light and electron microscope (Earle, 1978) The fusion of isolated protoplasts was achieved in 1909. For the first time, Takebe and his coworkers revolutionized the whole world by exploring the totipotency of protoplasts. In tobacco, the fused protoplasts were regenerated and subsequently produce interspecific hybrid plant (Takebe *et al.*, 1971).

Gradually, *in vitro* methods were increasingly used as an addition to traditional breeding methods for the modification and improvement of plants. Production of variants is one of the important roles played by cell culture. In case of callus mediated organogenesis and somatic embryogenesis there is a possibility of producing variants and aberrant plants. Thus *in vitro* somatic embryogenesis, tends to be the most effective and rapid method of plant regeneration (Evans *et al.,* 1981). For the first time during 1970s, somaclonal variants have been utilized for plant improvement. Somaclonal variation in tissue culture is dependent on the variation in a population of cells either natural or induced in the artificial culture (Larkin and Scowcroft, 1981), or may be genetic or epigenetic and is usually observed in regenerated plantlets (Larking *et al.*, 1985 and Scowcroft *et al.*, 1986). The variations in the regenerated plantlets have agricultural and horticultural significance and have been adopted for a number of economically and medicinally important plant species.

In the history of plant tissue culture, the growth and regeneration of isolated protoplasts to produce the whole angiospermic plantlets was initiated (Binding *et al.*, 1936). Subsequently, the fusion of protoplasts was standardized to generate superior plant through somatic hybrid formation. Protoplasts were fused mainly by two methods, one is physical method by using electric current i.e. electrofusion and other is chemical method by using polyethylene glycol i.e PEG method.

Both the methods were employed to produce somatic hybrid plants. But the major problem is the ability of hybrid cells to regenerate whole plantlet (Evans *et al.*,1984); Schieder and Kohn, 1986). Protoplast fusion has been used to produce unique nuclear-cytoplasmic combinations which generally results in hybrid seed production, but till date the success is limited to a few species

2.4.1. Histological study

Somatic embryogenesis involves control of three consecutive steps:

- Induction of embryogenic lines from sporophytic cells
- Maintenance and multiplication of embryogenic lines
- Maturation of somatic embryos and conversion into viable plantlets (Williams and Maheswaran 1986).

Induction of embryogenic lines and their subsequent conversion into plantlets have been studied by many workers (Sharp *et al.*, 1980; Tisserat *et al.*, 1979 and Wann, 1988). The steps involved in the multiplication of somatic embryo have been comparatively less studied although it directly contributes to the ability of the *in vitro* embryos for the germination and development of the complete plantlets (Zegzouti *et al.*, 2001). Two major problems have been reported concerning the multiplication steps. The first one is the difficulty in obtaining stable and subculture-suitable lines that will produce embryos for a longer period of time (Tisserat *et al.*, 1979 and Wann, 1988). The second problem is the lack of synchrony in embryo development and the risk of morphological abnormalities such as pluricotyledony, multiple apex formation and fused cotyledons etc.

Multiplication of embryogenic lines in angiospermic species can be achieved either by regular sub culturing of explants taken from compact or friable embryogenic calli (Tisserat *et al.*, 1979), or by the formation of new embryos from the previously developed somatic embryos (Bornman, 1991; Wann, 1988 and Williams and Maheswaran, 1986)). This second case is referred to as secondary embryogenesis.

In Quercus initiation of somatic embryogenesis has been described from a variety of sporophytic explants, namely stem segments, leaves and zygotic embryos. The multiplication of the embrogenic lines was first achieved from calli ageing on the same culture medium (Feraud-Keller and Espagnac,

1989 and Gingas and Lineberger. 1989) or via successive transfers into fresh culture media with different growth regulator supplements (Feraud-Keller and Espagnac, 1989 and Fernandez-Guijjaro *et al.*, 1995). Embryogenic response from anthers and ovary tissues was also obtained with similar procedures (Jorgensen, 1993).

Researchers have noted that

- Within one embryogenic line the somatic embryos could occur from different histological origin, as observed for example in *Theobromo cacao* (Adu-Ampomah *et al.*, 1988).
- The growth regulator composition of the culture medium influenced the histological origin of the somatic embryos in *Hevea brasiliensis* (Michaux-Ferriere *et al.*, 1992 and Michaux-Ferriere and Schwendiman, 1993) and *Elaeis guineesis* (Schwendiman *et al.*, 1988).

Somatic embryos development and their histological studies were well discussed among the members of monocot (Conger *et al.*, 1987 and Krishnaraj and Vasil 1995). Further, histology of *in vitro* somatic embryos was also studied in some close relatives of Canna. Novak and his coworkers described that the initiation of somatic embryo in banana occurred when basal leaf sheath and rhizome tissue were taken as explants for *in vitro* culture. (Novak *et al.*, 1989). In most of the reports of embryogenesis, emphasis has been given to manipulate the nutrient composition, growth regulators in culture medium, physical conditions of incubation and other stress treatments to induce somatic embryos. Histology of some other Zingiberales like *Zingiber officinale*, Curcuma *mangga*, *Heliconia psittacorum* was also discussed (Kackar *et al.*, 1993; Sundram *et al.*, 2012 and Nathan *et al.*, 1993). However, till date no report was published on the development and histology of *in vitro* grown somatic embryos of Canna. The investigation of the histological origin and structural organization of the *in vitro* somatic embryos of Canna is yet to be done.

In plant tissue culture, the type of explant plays an important role in the regeneration process. In Canna, different plant parts were used as explant for *in vitro* culture. The most common explants used for Canna micropropagation were meristematic shoot tip, rhizome, terminal bud etc. The list of explants used for *in vitro* culture of some members of

Zingiberales is given in table 2.1.

In tissue culture, surface sterilization of the explants has a great importance as it is the first step to be taken to check exogenous contamination. The main objective of surface sterilization of the explant is to get rid of the fungal and bacterial contamination without hampering the biological activity of the explants. The commonly used disinfectants are ethanol, sodium hypochlorite, mercuric chloride etc. The type and concentration of the chemical to be used for sterilization and the incubation time of explant in the particular sterilant depends on the nature of explant (Razdan, 2003). The list of various disinfectants used in the tissue culture of Canna and some close Zingiberales is given in table 2.2. Chemicals like extran, ethanol, sodium hypochlorite (NaClO), calcium hypochlorite [$Ca(ClO)_2$], mercuric chloride ($HgCl_2$), streptomycin sulphate [$(C_{21}H_{39}N_7O_{12})_2 \cdot 3H_2SO_4$] etc. were used in the *in vitro* culture of Canna and some close Zingiberales like banana, heliconia, turmeric and ginger (Table 2.2).

The artificially prepared nutrient medium plays an important role in the successful growth and differentiation of excised plant tissues. The culture media is composed of several components like inorganic salts, macro and micro nutrients, vitamins, aminoacids, sugars, growth regulators (phytohormones), agar or gelrite. The minerals present in the plant tissue culture medium are used by the plant cell for the synthesis of organic molecules. The ions of different salts play an important role in transportation or osmotic regulation and in maintaining the electrochemical potential of the plant.

The requirement of nutrient varies not only among different plants but also for

Table 2.1: List of various explants and synthetic media used for the *in vitro* culture of Canna species and some close Zingiberales

Plant species	Explants	Effective culture medium	References
Canna edulis	Shoot tip	MS	Hosoki and Sasaki, 1991
Canna edulis	Terminal bud	B5, ½ MS, MS	Sakai and Imai, 2007
Canna indica	Meristem	Liquid MS, ½ MS, MS (agar solidified)	Kromer and Ku-kulczanka, 1984
Canna indica	Rhizome	MS	Kromer, 1979
Musa paradisiaca	Shoot tip	Liquid MS	Ganapathi *et al.*, 1992
Heliconia psitta-corum	Shoot and bud	MS	Goh *et al.*, 1995
Curcuma longa	Sprout	MS	Salvi *et al.*, 2001
Zingiber officinale	Young rhizome sprout	MS	Bhattacharya and Sen, 2006

Table 2.2: List of surface sterilizing agents used in the culture of Cannas and some other Zingiberales

Plant species	Sterilant used	References
Canna edulis	Detergent and 0.6% sodium hypochloride for 10 min	Hosoki and Sasaki, 1991
Canna edulis	70% ethanol for 30s followed by 1% sodium hypochloride for 5 min	Sakai and Imai, 2007
Musa paradisiaca	0.1% $HgCl_2$ for 5 min	Ganapathi *et al.*, 1992
Heliconia bihai	70% (v/v) alcohol for 1 min followed by calcium hypochlorite solution.	Ulisses *et al.*, 2010
Curcuma longa	70% ethanol for 30s, 0.1% mercuric chloride for 15 min and 750 mg/l Streptomycin sulphate	Salvi *et al.*, 2001
Zingiber officinale	0.2% (w/v) $HgCl_2$ for 10 min followed by 70% ethyl alcohol for 10 min	Bhattacharya and Sen, 2006

different parts of the same plants. Therefore, a multiple media may be required for optimal growth of all plant tissues. To overcome this, different nutrient solutions were proposed by different authors from time to time like MS medium (Murashige and Skoog, 1962), B5 medium (Gamborg *et al.*, 1968), Banna micropropagation media (Readymade medium marketed by Himedia) Nitsch medium (Nitsch and Nitsch, 1969), White's medium (White, 1943), Woody plant medium (Lloyd and McCown, 1980) etc. Consequently, the most suitable medium for optimal growth of a particular tissue could be determined by trial and error method.

Though a little work has been done in the area of *in vitro* culture of Canna, MS was considered as the best medium for the optimal growth and regeneration of Cannas. From the literature it was found that some authors used different strength and phases (e.g. solid, liquid phases) of MS medium. Nutrient media other than MS medium were also used. For the culture of terminal buds, Sakai and Imai used B5 medium, ½ MS and MS medium to establish a tissue culture system for *Canna edulis* (Sakai and Imai, 2007). Kromer and his co-worker used liquid MS, ½ MS and agar solidified MS media for rapid multiplication in *Canna indica* (Kromer and Kukulczanka, 1984). In case of Sakai and his co-worker the survival rate of the explant was highest in B5 medium where as in the *in vitro* study of Kromer and his co-worker liquid MS help in the optimal growth of explants. In both the above case solidified agar medium became least effective for the optimal regeneration of plants. A list of different explants and the regenerating medium for different Zingiberales is given in table 2.1.

Sucrose is always supplied with the culture media as a source of sugar.

Usually it is used in the form of carbon source at a concentration of 3% (w/v) in almost all tissue culture experiments. In various *in vitro* culture studies of Canna, sucrose was added at a concentration of 3% (w/v) except for the shoot tip culture of *Canna edulis* by Hosoki and Sasaki, 1991. As a source of sugar they used 2% sucrose for the development of shoot and root. Similar report was also found in other Zingiberales like banana, where 2% sucrose was suggested for the growth and regeneration of banana fruits (Ram and Steward, 1964).

In tissue culture, the quality of regenerated plantlets is dependent on the range of acidity or alkalinity of the culture media. The optimum pH for regeneration of plant varies with the type of explant used. Generally in various tissue culture experiments pH is maintained within 5.6-5.8. In case of *in vitro* culture of Canna, pH - 5.6 has been considered for the successful regeneration of explants (Hosoki and Sasaki, 1991 and Sakai and Imai, 2007).

In plant tissue culture, there are three types of media are used namely solid, semisolid and liquid. A media becomes solid or semisolid depending upon the concentration of the solidifying agents used. Agar-agar which is obtained from algae like Gelladium or Gracilaria and gelrite, a naturally derived gelling polymer are most commonly used as soldifying agents. The media was solidified with agar 0.8% (w/v) in some of the cultures of Canna (Hosoki and Sasaki, 1991), where as in some other cases, lower concentration of agar i.e. 0.4% was used for better growth (Kromer and Kukulczanka, 1984). Gellan gum at a concentration of 2.5g/l was used as a gelling agent for *Canna edulis* (Sakai and Imai, 2007). Further, liquid media with filter paper bridge was also used in some of the Canna tissue culture experiments (Sakai and Imai, 2007 and Kromer and Kukulczanka, 1984).

Plant hormones or plant growth regulators play a vital role in the optimum growth and regeneration of plants. Phytohormones are added to synthetic culture media in a very minute quantity and subsequently they tend to increase the level within the tissue. Usually, only a little amount of the synthetic hormones remain in the free form because most of the plant hormones are rapidly inactivated after uptake into the living tissue. It has been found that, in case of auxins, less than 1% of the hormone being present in the

free form and rest exist in equilibrium between the free and conjugated form. The effect of hormones on the explant depends on the following factors

- rate of the uptake of hormone from the synthetic medium
- stability of hormone in the medium

sensitivity of the explant tissue towards the hormone

The discovery and use of growth regulators like auxins, gibberlins, cytokinins and abscicins along with other organic additives created new dimensions in plant tissue culture. The role of growth regulators and their optimum concentration should be carefully chosen for obtaining desired responses in tissue culture. The major growth regulators used in plant tissue culture are auxins [indole-3-acetic acid (IAA), 1-napthaleneacetic acid (NAA), indole-3-buturic acid (IBA), 2, 4-dichlorophenoxyacetic acid (2,4-D), piloram etc], cytokinins [6-benzylaminopurine (BA), zeatin, kinetin, thidiazuron etc], gibberellins (GA_3, GA_4, GA_1, GA_7 etc), abscisic acid, ethylene etc. The list of plant growth regulators used in the tissue culture of Canna for the formation of callus, somatic embryo, shooting and rooting are provided in table 2.3. The table also gives an idea about the effect of various phytohormones on the growth and regeneration of some of the Zingiberales like banana, heliconia, ginger and turmeric etc. The frequently

Table 2.3: Various plant growth regulators and organic additives used for the regeneration of different Canna species and some members of Zingiberales

Plant species	Plant growth regulator	Additives	References
Canna indica	Kinetin and IAA	No	Kromer, 1979
Canna indica	Kinetin, Adenine sulphate, NAA and Ascorbic acid	Yes	Kromer and Ku-kulczanka, 1984
Canna edulis	BA and IBA	No	Hosoki and Sasaki, 1991
Canna edulis	BA, NAA and TIBA*	No	Sakai and Imai, 2007
Musa paradisiaca (Cv. -Lacatan and Robusta)	2,4-D, 2,3,6-TPA*, BTOA*, Cou-marin, Adenine sulphate and IAA	Yes (Coconut milk)	Ram and Steward, 1964
Musa paradisiaca (Cv.- Prata)	BAP and IBA	No	Lameira *et al.*, 1990
Musa spp.	2,4-D, Biotin, NAA	No	Assani *et al.*, 2002
Heliconia psitta-corum	2,4-D	No	Goh *et al.*, 1995
Zingiber officinale	BAP, Kinetin and Zeatin	No	Bhattacharya and Sen, 2006
Curcuma longa	Picloram, NAA, BA and TIBA, 2,4-D	No	Salvi *et al.*, 2001

NB*: TIBA- 2,3,5- triiodobenzoic acid, 2,3,6-TPA- 2,3,6- trichlorophenylacetic acid, BTOA – Benzothiazole-2-oxyacetic acid benzothiazole-2-oxyacetic acid

used plant growth hormones in the regeneration of Canna are BA, IBA, NAA, KN, 2ip, IAA, 2,4-D etc. From the literature it was found that organic additives were not required for the growth of Canna except for Kromer and Kukulczanka, 1984, who used some organic supplements for the culture of *Canna indica* meristem. Further, coconut water is used as a supplement for the substantial growth in banana (Ram and Steward, 1964).

During aseptic culture of plant explant, various conditions for incubation play an important role for subsequent growth and development. In artificial culture, high temperature may lead to the disruption of the culture media and low temperature restricts the growth of tissue explant. Further, some tissue prefers to grow in light condition, while some other grows in dark. The intensity of light has a significant role in tissue regeneration. So an optimum temperature and light condition is required for the substantial growth of selected tissue explant. The incubation conditions followed by various researchers for regeneration of *in vitro* Canna and some other Zingiberales are shown in table 2.4.

Callus is defined as an unorganized and undifferentiated mass of parenchyma cells formed from isolated plant cells or tissues under aseptic conditions. It is formed as a result of continuous division and growth of the explant tissue. Since meristematic cells have a capacity for vigorous growth and division, these tissues are used for the

Table 2.4: Incubation condition required by Canna species and some members of Zingiberales

Plant species	Temperature	Light	References
Canna indica	6ºC (for 4 winter months)		Kromer and Kukulczanka, 1984
Canna edulis	28ºC	16 h illumination of 52 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of cool white fluorescent light	Hosoki and Sasaki, 1991
Canna edulis	28ºC in 90% RH*	16/8h light/dark cycle with photosynthetic photon flux densities of 100 $\mu mol\ m^{-2}\ s^{-1}$	Sakai and Imai, 2007
Musa spp.	27 ºC	16 h photoperiod (65 $\mu mol\ m^{-2}\ s^{-1}$)	Assani *et al.*, 2002
Heliconia psittacorum	26±2ºC	Dark condition	Goh *et al.*, 1995
Curcuma longa	25±2ºC	12.1 µm photons $m^{-2}\ s^{-1}$ white fluorescent illuminated for 12 h	Salvi *et al.*, 2001
Zingiber officinale	25±2ºC	16 h photoperiod with a light intensity of 2000-2500 lux in cool white fluorescent tubular lamps	Bhattacharya and Sen, 2006

NB*: RH- Relative humidity

initiation of callus. So in plant tissue culture, young and immature parts of plant like leaf, stem, root, nodes and seeds are used for callus initiation. In the culture media the explant absorbs exogenously supplied nutrients and hormones and divides to form an unorganized mass of cells and tissues, which become enlarge and swell to rupture. This indicates the initiation of callus formation in the particular cells or tissues. As the cells rupture, the endogenous growth regulators along with the exogenously supplied hormone and nutrients stimulate further division of the cells. Thus the unorganized mass of callus tissue gradually increases its size.

In *Canna edulis*, embryogenic callus like structure was obtained by supplementing 1.5 - 2 mg/l BA in two different nutrient media (i.e. B5 and MS media). Those callus like tissues were capable of growth and division, but failed to differentiate further and died soon thereafter. Where as, protocorm like structure was achieved by using 1 mg/l NAA in the same medium regenerating the complete plant by taking *in vitro* shoot tip as explant (Sakai and Imai, 2007).

As literature of review did not find any reliable data regarding the initiation of callus in different species or cultivars of Canna, various protocol for induction of callus or similar structures in some close Zingiberales may be discussed. While studying two cultivars (Cv. Lacatan and Cv. Robusta) of *Musa paradisiaca*, Ram and his coworker reported the formation of fluffy calli from *in vitro* pulp of banana in white medium containing adenine sulphate and IAA. Unfortunately these calli didn't differentiate to produce organ (Ram and Steward, 1964).

A protocol for producing both friable and compact calli in *Musa* sp. from *in vitro* male flower was reported by Assani and his coworkers in 2002. They found white friable calli and yellow compact calli after 5-6 months culture of male flower in MS medium containing 4.1 µM biotin, 18 µM 2,4-D and 5.7 µM NAA. Both the calli were capable of vigorous division and finally regenerated into complete plantlet (Assani *et al.*, 2002). In case of turmeric, Salvi and his coworkers observed initiation of embryogenic callus from *in vitro* leaf base when cultured in MS medium supplemented with 2 mg/l picloram or 5 mg/l NAA in combination with 0.5 mg/l BA. The callus thus formed had potency for

further growth, initiation of shoot and root and regeneration of whole plantlet (Salvi *et al.*, 2001).

In the later part of 1970s, Kromer studied that the addition of 2 ppm IAA and 1 ppm kinetin in MS medium was the best condition for the formation of shoot buds and regeneration of complete plant in *Canna indica* (Kromer, 1979). In the further study of *Canna indica*, Kromer and his coworker reported that supplementation of kinetin (2 mg/dm^3), adenine sulphate (100 mg/dm^3) and NAA (0.2 mg/dm^3) in MS medium was suitable for the initiation of auxiliary bud and subsequent formation shoot and root to produce *in vitro* plant in a shorter period (Kromer and Kukulczanka, 1984).

To promote the growth of shoot tip in *Canna edulis in vitro* culture, Hosoki & Sasaki (1991) observed that addition of 0.1 mg/l of BA in MS medium was optimum for shoot multiplication. Splitting of shoot after tip culture in the above mentioned concentration of BA increased the number of shoots. *In vitro* rooting initiated at a concentration of 0.1 mg/l IAA (Hosoki and Sasaki, 1991). Another work on *in vitro* shoot tip culture of *Canna edulis*, Sakai & Imai reported that the survival rate of shoot was higher in a optimum concentration of IBA and BA (0.5 mg/l each) in B5 medium. Lateral shoot initiation was shown by supplementing BA and TIBA (0.5 mg/l each) to the culture medium. The rate of rooting was highest, when the media was supplemented with NAA (0.1 mg/l) (Sakai and Imai, 2007).

The initiation of shooting and rooting was studied by various authors among different members of Zingiberales. Lameira and his coworkers in 1990 carried out *in vitro* culture of *Musa paradisiaca* (Cultivar- Prata) taking lateral suckers as explants. They noticed shoots were obtained with 2.5 mg/l BAP where as rooting was initiated by supplementing 5mg/l IBA in MS medium (Lameira *et al.*, 1990).

Salvi and his coworkers reported that shoot multiplication in *Curcuma longa* was started after 2 months of inoculation of sprouts with BA (1 mg/l) and NAA (0.1 mg/l). Auxiliary buds were initiated from the cultured shoot bud. Further elongation of shoot bud and development of root was observed from the excised shoot bud in MS medium without any phytohormone. *In vitro* generated callus was shown to produce shoot primordia by supplementing BA (5 mg/l) in

combination with TIBA (0.1 mg/l) or 2,4-D (0.1 mg/l). Callus produced by the combination of BA (0.5 mg/l) and NAA (5 mg/l) showed highest percentage of response for shoot primordia when those excised callus were cultured in a medium containing BA and TIBA (90%) than that of BA (≤5%) alone or in combination with 2,4 -D (75%). Thus TIBA, an antiauxin was proved to be beneficial for regeneration of *in vitro* turmeric plant. Further development of shoot was reported when subcultured in ½ MS with 2% sucrose in combination with kinetin (1 mg/l) (Salvi *et al.*, 2001). In case of *Zingiber officinale,* Bhattacharya and Sen (2006) used *in vitro* rhizome as explant and achieved maximum rate of shooting and rooting by the addition of BAP (4 mg/l) in MS medium than that of B5 medium. Maximum number of plantlets and their maximum height was observed in presence of BA, where as kinetin and zeatin showed moderate effect on number and height of plantlets (Bhattacharya and Sen, 2006).

Somatic hybridization seems to be a promising complement to classical breeding since protoplasts are amenable to complete plant regeneration. Further, protoplast fusion parameter for establishment of somatic fusion technology is one of the strategies for genetic improvements in plant tissue culture. Literature of review did not give any document on protoplast isolation and their fusion technology among different species of *Canna*. Till date, somatic hybrid formation and fusion process is also not so common among other members of Zingiberales except in banana. Protoplasts were isolated from young leaves, friable calli and cell suspensions of different diploid and triploid cultivars of Canna. Protoplasts isolated from cell suspension culture developed into complete plants where as mesophyll protoplasts and callus derived protoplasts were incapable of regeneration. The yield ($27.5*10^6$ protoplasts per ml of cell volume) and the viability of cell suspension protoplast (71-91%) were much higher than that of mesophyll and calli protoplasts (yield approx. $2.8*10^6$ and viability approx. 27-40%). Somatic embryo was produced after 8-10 weeks of protoplast culture and plants were observed after 11-12 weeks after the subculture of somatic embryo (Assani *et al.*, 2002). However, it was Papadakis and his coworkers who found that isolated plant protoplasts in

tobacco and grapevine had lower regenerating capacity because of suppression of totipotency. Reduced viability and cell division potential in isolated protoplasts was because of the presence of higher contents of reactive oxygen species and oxidized form of some of the antioxidant enzymes like ascorbate and glutathione etc. They proved that the reduced cellular antioxidant mechanism was significantly correlated with the suppression of expression of isolated protoplasts (Papadakis *et al.*, 2001). Yasuda and his coworkers observed similar result while isolating Brassica napus leaf protoplasts. The increase in the intracellular reactive oxygen species during the isolation of protoplasts will result in the apoptosis like cell death of the cultured protoplasts (Yasuda *et al.*, 2007).

Further study of protoplast fusion in banana, Assani and his coworkers in 2005 studied the fusion of banana protoplasts in two different methods, namely electrofusion method (by alternating current field) and chemical method (by poly ethylene glycol). The fusion efficiency was found to be higher in chemical procedure (17%) than that of electric method (10%). Optimum concentration of poly ethylene glycol (PEG) for banana protoplast fusion was also studied. According to them, more than 50% of PEG leads to severe damage of the protoplsts and finally hamper the process of fusion of cells. Further, its application longer than 30 min also hampers the fusion of protoplsts. Again it was observed that the cell division rate was higher in electric method (35%) than that of chemical method (24%). Some other aspects of banana protoplast fusion like rate and duration of somatic embryogenesis and plantlet regeneration were also higher in electric fusion technology (Assani *et al.*, 2005).

Tissue culture is extensively employed in the production, conservation and improvement of plant resources. In tissue culture, somaclonal variation is known to be an inherent variation in a population of cells usually observed in regenerated plants (Larkin and Scowcroft, 1981). Somaclones produced in plant tissue culture seems to have two contrasting aspects. On one hand, it affects the use of tissue culture negatively by hampering the conservation of plant resources and on the other hand it is a source of new desirable clones or variants with better agronomic traits. To date somaclonal

variation in Canna is not reported. However this process was well studied among the close Zingiberales like banana, turmeric and ginger. Various RAPD primers were used to detect somaclonal variation by comparing the DNA profiling of parent plant with *in vitro* regenerated plantlets. Increase in the time period of the sub-culturing increase the possibility of occurrence of more genetic variations. The occurrence of specific bands or loci in the regenerated plants of different sub-cultures may be used in the genetic identification of the somaclones (Sheidai *et al.*, 2008 and Sheidai *et al.*, 2010). Somaclonal variation among *in vitro* regenerated plants of turmeric was also reported (Salvi *et al.*, 2001). In contrast, genetic uniformity among *in vitro* regenerated cultivars of banana was observed using various ISSR markers (Rout *et al.*, 2009). Genetic fidelity was also observed among other Zingiberales like ginger and turmeric (Rout *et al.*, 1998, Panda *et al.*, 2007).

The successful transfer of *in vitro* generated plants from laboratory to field condition is an important part of the entire tissue culture experiment. (Wardle *et al.*, 1983). Drastic environmental changes occurred when the *in vitro* plants are transferred to open field condition. *In vitro* condition provides low light intensity, high humidity and poor root growth, where as under field or green house conditions there is higher light intensity, low humidity with various soil microflora (Desjardins *et al.*, 1987).). Several protocols have been suggested by different tissue culturist to overcome some of these obstacles.

In hardening of *in vitro* regenerated plantlets of the Canna and some related Zingiberales, different hardening materials like porous sand, sterilized potting soil, vermiculite, commercial soil, organic material, nutrient and manure (NPK) etc. have been used

Table 2.5: Hardening materials used and survival rate of the regenerated Cannas and some of the close Zingiberales

Plant species	Potting mixture	Survival rate	References
Canna edulis	Porous sandy soil and 15N -8P-17K (2.5 g/l)	92%	Hosoki, and Sasaki, 1991
Canna edulis	Sterile vermiculite:Commercial soil (1:1)	80-90%	Sakai and Imai, 2007
Musa paradisiaca L. (Cultivar- Prata)	Organic material, vermiculite and nutrient	Not known	Lameira *et al.*, 1990.
Curcuma longa	Sterile soil	90%	Salvi *et al.*, 2001
Zingiber officinale	Soil with potting mixture	94-100%	Bhattacharya, and Sen, 2006

(Table 2.5). The success rate of hardening depends upon the hardening material and the condition of the regenerated plantlets. High rate of survival of regenerated plantlets have been achieved in field condition.

Canna is popularly known as an ornamental plant; still its agricultural importance can't be undervalued. The garden plant is also cultivated as a commercial source of starch. Rhizome of edible Canna is produced at a large scale to fulfill the demand of starch in various parts of the world. Traditional breeding has been greatly hampered by the contamination of virus and insects. Literature of review does not provide the exact techniques of *in vitro* propagation of Canna, still the published information of Canna and other Zingiberales gives an idea about various important parameters of micropropagation. Thus, through micropropagation new plants can be regenerated in a sterile environment within a short period of time which will be free from any virus or insect infestation. Further research will result in genetic improvement in *Canna* by establishing new variety, development of efficient methods for mass production of superior quality planting stock and conservation of the genetic resources.

Chapter 3
Materials and Methods

3.1 The study area

The province of India, West Bengal and Orissa (presently named as 'Odisha') is known to be a biodiversity rich zone. The study area for the collection of germplasm covered large parts of West Bengal and Orissa.

3.1.1 Collection of germplasm

In the present study, germplasm had been collected from different places of six districts of West Bengal and four districts of Orissa (for detail please refer table 3.1). The field work was completed in ten months. The collected plant materials were planted in the experimental garden of Molecular Genetics Laboratory, Department of Botany, North Bengal University, for further study after authentication by the plant taxonomists.

3.2 Molecular diversity studies

Different PCR based molecular methods namely, RAPD, PCR-RFLP and Microsatellite (ISSR) markers were used for the genetic diversity study of Canna.

3.2.1 DNA extraction from leaf

The protocol developed by Doyle and Doyle (1987) was standardized for isolation of genomic DNA of Canna with the following modifications:

- Approximately 5 grams (gm) of fresh young leaves were grounded with liquid nitrogen in a mortar and pestle. Pulverized materials were quickly transferred to 30 milliliters (ml) Oakridge tube (Tarsons, Cat#541040) containing 15 ml of prewarmed CTAB extraction buffer (Appendix-B for composition) and mixed gently.
- The solution was incubated in a water bath (Rivotek, Cat#50121002) for couple of hours (hrs) at 65ºC with occasional

Table 3.1: Germplasm collection sites of West Bengal and Orissa

Sl #	State	District	Latitude	Longitude
1.	West Bengal	Darjeeling	26° 31' to 27° 13' N	87° 59' to 88° 53' E
2.		Jalpaiguri	26° 16' to 27° 0' N	88° 4' to 89° 53' E
3.		Coochbehar	25° 57' to 26° 36' N	89° 54'35" to 88° 47' E
4.		N. Dinajpur	25° 11' to 26° 49' N	87° 49' to 90° 00' E
5.		S. Dinajpur	26° 35' to 25° 10' N	89° 00'30" to 87° 48' E
6.		Malda	24° 40' to 25° 32' N	87° 45' to 88° 28' E
7.	Orissa	Mayurbhanj	21° 16' to 22° 34' N	85° 40' to 87° 11' E
8.		Balasore	21° 3' to 21° 59' N	86° 20' to 87° 29' E
9.		Bhadrak	20° 43' to 22° 11' N	82° 39' to 85° 13' E
10.		Keonjhar	21° 1' to 22° 10' N	85° 11' to 86° 22' E

mixing by gentle swirling.

- Following incubation, an equal volume of chloroform (Merck India, Cat#822265): isoamyl alcohol (Merck India, Cat#8.18969.1000) (24:1) was added, followed by gentle mixing.
- The mixture was then centrifuged (REMI make, Model No.C-24) for 15 minutes (min) about 6,500 rpm (5,000Xg) at room temperature (25ºC). Three layers were formed in this process, upper aqueous layer, middle layer with cell debris and lower chloroformic layer. The upper aqueous phase was carefully transferred to a fresh tube, without disturbing the middle layer, which was followed by the addition of approximately 0.6 volume of ice cold isopropanol (Merck India, Cat#17813) and mixed gently.
- The mixture was then incubated overnight at -20ºC.
- In the following day, the mixture was centrifuged at about 6,500 rpm (5,000Xg) for 30 minutes at 4ºC.
- In this process, DNA-CTAB complex was precipitated along with some whitish mass of starch.
- Upper aqueous layer was removed and the pellet was washed with chilled 70% alcohol and air dried.
- The pellet was dissolved in 500 microlitre (µl) of 1X TE buffer (pH 7.4) (Refer Appendix-B for composition).
- The solution was extracted with an equal volume of equilibrated phenol (pH 8.0) (Sigma, Cat#P4557-400ML), mixed properly and centrifuged at 13,000 rpm (16,000Xg) for 15 minutes.
- The upper aqueous phase was then taken in a fresh tube and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and then centrifuged at 10,000 rpm

(10,000Xg) for 10 minutes at room temperature.

- The upper aqueous phase was transferred to a fresh tube, 0.1 volume of 3M sodium acetate (pH 5.2) (SIGMA, Cat#S-9513) was added followed by the addition of 2 volume of ice cold absolute ethyl alcohol (BDH, Cat#10107).
- The above mixture was incubated for 1 hr in -20ºC and centrifuged at 4ºC for 30 minutes (REMI make, Model No.C-24) at 13,000 rpm (16,000Xg).
- The pellet thus obtained was washed with chilled 70% ethyl alcohol, air dried and dissolved in 1000µl of 1X TE buffer (pH 7.4).

3.2.2 Purification of Canna DNA

Major contaminants in crude DNA preparation are RNA, protein and polysaccharides and it is essential to remove them as these will hamper further downstream processing. Besides, I found that Canna DNA always remain contaminated with starch when isolated through conventional procedure described above. To get rid of this starch, I tried several methods, however, polyethyleneglycol (PEG) purification of Canna DNA was found to be the best.

3.2.2.1 PEG purification of isolated DNA

- One ml of DNA solution in TE was mixed with 400µl 30% weight/ volume (w/v) PEG solution (pH 7.4) (SIGMA, Cat#P5413) [Refer Appendix-B for composition] and incubated for 14 hrs at 0ºC.
- The incubated mixture was centrifuged at 12,000 rpm (13,500Xg) for 30 min at 4ºC.
- The pellet obtained was resuspended in 500µl of 1X TE buffer.
- To this 0.1 volume of 3M sodium acetate (pH 5.5) and 2.5 volume of ice cold absolute ethanol was added for DNA precipitation.
- The mixture of solution was incubated overnight at -20ºC and after incubation it was centrifuged at 13,000 rpm (16,000Xg) for 30 min at 4ºC.
- Transparent DNA pellet was obtained which was washed with 70% ethyl alcohol, air dried and finally dissolved in 500µl of 1XTE (pH 7.4) buffer.

3.2.2.2 RNaseA treatment

- RNaseA (50µg/ml) (SIGMA,

Cat#R-4875) [Refer Appendix-B for composition] was added into the genomic DNA dissolved in 500µl of 1X TE buffer (pH 7.4) and it was incubated at 37ºC for 1 hr in a Dry water bath (GeNei™ make, Cat#107173).

- An equal volume of chloroform:isoamyl alcohol (24:1) was added and mixed properly and centrifuged at 10,000 rpm (10,000Xg) for 15 minutes at room temperature.
- The aqueous phase was then transferred to a fresh micro centrifuge tube (Tarsons, Cat#500010).
- To the aqueous phase 0.1 volume of 3M sodium acetate (pH 5.2) and double volume of absolute ethyl alcohol was then added for DNA precipitation. It was then centrifuged at 13,000 rpm (16,000Xg) for 30 minutes at 4ºC.
- The DNA pellet obtained was washed with chilled 70% ethyl alcohol, air dried and finally dissolved in 100µl of 1XTE (pH 7.4) buffer.

3.2.3 Quantification of DNA

Reliable measurement of DNA concentration is important for applications in molecular biology including amplification of target DNA by polymerase chain reaction and complete digestion of DNA by restriction enzymes. DNA quantification is generally carried out by spectrophotometric measurements or by agarose gel analysis. Both the methods were used in the present study.

3.2.3.1 Spectrophotometric measurement

- Spectrophotometer (Thermo UV1 spectrophotometer, Thermo Electron Corporation, England, UK) was calibrated at 260nm as well as 280 nano meter (nm) by taking 600µl 1X TE buffer in a cuvette.
- DNA (6µl diluted in 594µl of 1X TE) was taken in the cuvette, mixed properly and the optical density (OD) was recorded at both 260nm and 280nm.
- DNA concentration was estimated by employing the following formula: Amount of DNA (ng/µl) $= \frac{OD_{260} \times 50 \times Dilution\ factor}{1000}$
- The quality of DNA was considered from the OD values recorded at 260nm and 280nm. The

DNA showing A_{260}/A_{280} ratio around 1.8 was chosen for further PCR amplification by using RAPD and ISSR markers and PCR-RFLP techniques.

3.2.3.2 Gel analysis

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

The pure DNA thus obtained was used for various fingerprinting studies.

3.2.4 Gel Photography

Photographs were taken for each gel by using an indigenously built gel documentation system fitted with Cannon SLR camera (EOS350D) and Marumi orange filter (58 mm YA2, Marumi, Japan). EOS utility software was used for this purpose.

3.2.5 RAPD (Random Amplified Polymorphic DNA) analysis

A total of 30 random 10mer primers (GeNei™) were screened for 20 different species and cultivars of Canna (table 3.2).

3.2.5.1 RAPD-PCR amplification

In a sterile 0.2ml thin wall PCR tube

Table 3.2: List of primers used for RAPD analysis

Primer ID	Sequence (5'-3')
OPA01	CAGGCCCTTC
OPA02	TGCCGAGCTG
OPA03	AGTCAGCCAC
OPA04	AATCGGGCTG
OPA05	ATTTTGCTTG
OPA06	GGTCCCTGAC
OPA07	GAAACGGGTG
OPA08	GTGACGTAGG
OPA09	GGGTAACGCC
OPA10	GTGATCGCAG
OPA11	CAATCGCCGT
OPA17	GACCGCTTGT
OPA18	AGGTGACCGT
OPA19	CAAACGTCGG
OPA20	GTTGCGATCC
OPB01	GTTTCGCTCC
OPB02	TGATCCCTGG
OPB03	CATCCCCCTG
OPB04	GGACTGGAGT
OPB05	TGCGCCCTTC
OPB06	TGCTCTGCCC
OPB07	GGTGACGCAG
OPB08	GTCCACACGG
OPF09	CCAAGCTTCC
OPG19	GTCAGGGCAA
OPH04	GGAAGTCGCC
OPN04	GACCGACCCA
OPN05	ACTGAACGCC
OPN13	AGCGTCACTC
OPN19	GTCCGTACTG

(Tarsons, Cat#500050), 25µl of PCR mixture was taken. For reaction mixture, the components were added in the following order:

- Pyrogen free water- To a final volume of 25µl
- PCR master Mix 2X (GeNei™, Cat#610602200031730 Pl. No. MME22) - 12.5µl
- Primer -1.25µl (0.25 µM)
- Template DNA -2µl (25ng)
- One negative control (PCR mix without DNA) tube was prepared.
- The PCR reactions were performed on a Perkin-Elmer Thermocycler 2400. The amplification cycle is consisting of the following specifications:
- Cycle 1: Denaturation at 94ºC for 4 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 2-44: Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 2 min.
- Cycle 45: Denaturation at 94ºC for 1 min, Primer annealing at 37ºC for 1 min, Primer extension at 72ºC for 10 min.
- The PCR products were separated on 1.5% (w/v) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer (pH-8).
- PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix-B for composition).
- The samples were loaded and electrophoresis was carried out at 50V for 2.4 hrs.

The gels were visualized with a UV

Table 3.3: List of primers used for ISSR analysis

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

transilluminator (GeNei™, Cat# 107161) and photographed with Gel Documentation System as stated above. A DNA ladder (λ DNA/*Eco*RI/*Hin*dIII double digest) (GeNei™, Cat#106000) and 100 base pair (bp) DNA ladder (GeNei™, Cat#612652670501730) were 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).

3.2.6 ISSR analysis

A total of 15 ISSR primers were screened for 20 different species and cultivars of Canna which is listed in table 3.3.

3.2.6.1 ISSR-PCR Amplification

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

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

The PCR reactions were performed on a Perkin-Elmer Thermocycler 2400. The amplification cycle is consisting of the following specifications:

- Cycle 1: Denaturation at 94ºC for 5 min, Primer annealing at 52ºC for 1 min, Primer extension at 72ºC for 1 min.
- Cycle 2-34: Denaturation at 94ºC for 45second (s), Primer annealing at 52ºC for 1 min, Primer extension at 72ºC for 1 min.
- Cycle 35: Denaturation at 94ºC for 45s, Primer annealing at 52ºC for 1 min, Primer extension at 72ºC for 7 min.
- The PCR products were separated on 1.5% (w/v) agarose gel containing Ethidium bromide

solution (0.5µg/ml) run in 0.5X TBE buffer.

- PCR product (12µl) was mixed with 4µl of 6X Gel loading dye (Refer Appendix-B for composition).
- The samples were loaded and electrophoresis was carried out at 50V for 2.4 hrs.

The gels were visualized with a UV transilluminator (GeNei™, Cat#107161) and photographed with Gel Documentation System as mentioned in section 3.2.4. A DNA ladder (λ DNA/*Eco*RI/*Hin*dIII double digest) (GeNei™, Cat#106000) and 100 bp DNA ladder (GeNei™, Cat#612652670501730) were used to find out the band size. All PCR reactions were run at least thrice and only reproducible and clear bands were scored and aligned by diversity data base software (NTSYSpc).

3.2.7 PCR-RFLP analysis

3.2.7.1 Primers for "Taberlet region" of Canna

Tab c-f in *trn*L-*trn*F (Taberlet *et al*., 1991) region of the Canna genome was amplified. The primer sequence Tab c 5'-CGAAATCGGTAGACGCTACG-3' and Tab f 5'-ATTTGAACTGGTGACACGAG-3' was used on the basis of the known sequence (Taberlet *et al*., 1991) from the Taberlet region of the other plant species. A schematic representation of the primer location is shown in figure 3.1. The primers were synthesized by GeNei™.

3.2.7.2 PCR amplification of trnL-trnF region

The polymerase chain reaction was carried out in a 25µl volume containing the following components:

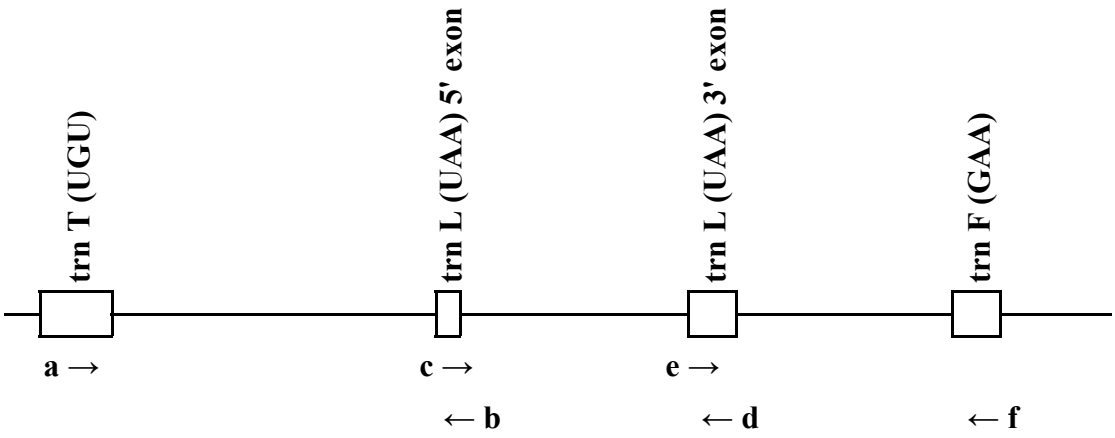

Figure 3.1: Schematic representation of Tab c-f primer location

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

The PCR reactions were performed on a Perkin-Elmer Thermocycler 2400. The amplification cycle is consisting of the following specifications:

- Cycle 1: Denaturation at 95ºC for 5 min, Primer annealing at 54ºC for 45s, Primer extension at 72ºC for 2 min.
- Cycle 2-34: Denaturation at 95ºC for 45s, Primer annealing at 54ºC for 45s, Primer extension at 72ºC for 2 min.
- Cycle 35: Denaturation at 95ºC for 45s, Primer annealing at 54ºC for 45s, Primer extension at 72ºC for 7 min.
- The PCR products were separated on 1.5% (w/v) agarose gel containing Ethidium bromide solution (0.5µg/ml) run in 0.5X TBE buffer.
- PCR product (12µl) was mixed with 4µl of 6X Gel loading dye.
- The samples were loaded and electrophoresis was carried out at 50V for 2.4 hrs.

The gels were visualized with a UV transilluminator (GeNei™, Cat#107161) and photographed with Gel Documentation System as mentioned in section 3.2.4. A DNA ladder (λ DNA/*Eco*RI/*Hin*dIII double digest) and 100 bp DNA ladder (GeNei™, Cat#612652670501730) were used as a molecular size marker.

3.2.7.3 PCR product Restriction digestion

The PCR products were subjected to restriction digestion with 5 different restriction endonucleases, namely *Taq*I (GeNei™, Cat# 610100700041730 PI. No. MBE7S), *Alu*I (GeNei™, Cat# 610101700041730 PI. No. MBE17L), *Hinf*I (GeNei™, Cat# 610102100021730 PI. No. MBE21S),

Table 3.4: List of restriction enzymes along with their cutting site and optimum temperature

Restriction Enzyme	Cutting Site	Temp.
*Alu*I	AG↓CT	37 ºC
*Hinf*I	G↓ANTC	37 ºC
HaeIII	GG↓CC	37 ºC
*Msp*I	C↓CGG	37 ºC
*Taq*I	T↓CGA	65 ºC

*Hae*III (GeNei™, Cat# 610101000041730 PI. No. MBE10L) and *Msp*I (GeNei™, Cat# 610103100021730 PI. No. MBE31S), all were 4-base cutters. The list of the enzymes along with their cutting sites and other information are given in a tabular form (table 3.4).

Protocol for PCR-RFLP restriction digestion:

- The restriction digestion reaction was done in total of 20µl volume containing the following components for each sample:
- Restriction Enzyme buffer 2µl.
- Pyrogen free water to make up the final volume.
- Enzyme 5 units.
- PCR product @ 5 µl.
- The restriction digestion mix was spinned for a moment.
- The mix was then incubated at specific temperature for each enzyme for a period of 1 hr in a Dry bath (GeNei™ make, Cat#107173).
- The digested product was separated on 2% (w/v) agarose gel containing Ethidium bromide solution (0.5µg/ ml) run in 0.5X TBE buffer as mentioned above.

The gels were visualized with a UV transilluminator (GeNei™, Cat#107161) and photographed with Gel Documentation System as mentioned in section 3.2.4. A DNA ladder (λ DNA/*Eco*RI/*Hin*dIII double digest) and 100 bp DNA ladder were used to find out the band size.

Note: In case of enzyme *Taq*I BSA (0.2 µl) was also added separately in the reaction mixture.

3.2.8 Fingerprinting Data Analysis

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

Correspondence analysis (2D and 3D plot) of right vectors from the binary data was performed on the basis of graphically summarized associations within the varieties. This analysis was performed through a batch file by using

the software package NTSYSpc.

3.2.9 Sequencing of PCR products

I have used 20 species/cultivars of Canna for PCR amplification and DNA fingerprinting studies. Out of these, there are two elemental species namely *Canna edulis* and *Canna indica* and there are two special varieties of cultivars, one was called *Canna indica* Cv. "Purpurea" and another one was *Canna* x *generalis* Cv. "Italia". I would like to study the taberlet region of the chloroplast of these specimens in further details. Therefore, the PCR products of the taberlet region of chloroplast genome of these species were subjected to DNA sequencing. Sequencing of both strands of taberlet region was done using the primer Tab c and Tab f. The sequencing was carried out through outsourcing to a private company [Chromous Biotech Pvt. Ltd., #842, II, Floor, Shankar Bhawan, A Block, Shankar Nagar, Bangalore-5600092 (www.chromous.com)].

3.2.9.1 Sequence submission

The raw sequences of 4 different species/cultivars of Canna were documented with the help of Sequin Application Version 12.30 Standard Release [Nov 13, 2012] for Database submission to GenBank for providing necessary informations like, definition of the sequence (i.e. the specific region of the genome), source of the sequence (chloroplast DNA in this case; name of the plant species along with its taxonomic position, date and place of collection, tissue type etc.).

3.2.9.2 Sequence analysis and costruction of phylogenetic tree

To compare my sequences with the global sequences of Zingiberales and to construct a phylogenetic tree of Zingiberales, DNA sequences of taberlet region of other Zingiberales were downloaded from NCBI (http://www.ncbi.nlm.nih.gov). This consisted of 8 varieties of Zingiberaceae, 2 varieties of Musaceae, 3 varieties of Marantaceae, 3 varieties of Strelitziaceae, 1 each variety of Lowiaceae and Heliconiaceae and 2 varieties of Cannaceae, other than varieties sequenced by us. GenBank accession number and the details of the sequences are listed in table 3.5. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) online server was used to align the above said sequences. The aligned sequences were subjected to Phylip-3.69 (http://www.softpedia.com/get/Science-CAD/PHYLIP.shtml) package programme to construct the phylogenetic tree using

Table 3.5: Details of the sequences for construction of phylogenetic tree

Sl. No.	Plant name	GenBank Accn.	Reference
1	*Curcuma longa*	DQ471971.1	Ardiyani *et al.*, 2006*
2	*Kaempferia elegans*	AY424790.1	Ngamriabsakul *et al.,* 2003
3	*Zingiber junceum*	AY424800.1	Ngamriabsakul *et al.*, 2003
4	*Roscoea bhutanica*	AY424794.1	Ngamriabsakul *et al.*, 2003
5	*Alpinia galanga*	AY424775.1	Ngamriabsakul *et al.*, 2003
6	*Caulokaempferia thailandica*	AY424781.1	Ngamriabsakul *et al.*, 2003
7	*Cornukaempferia longipetiolata*	AY424783.1	Ngamriabsakul *et al.*, 2003
8	*Paracautleya bhatii*	AY424792.1	Ngamriabsakul *et al.*, 2003
9	*Musa acuminata*	FJ621283.1	Liu *et al.*, 2010
10	*Ensete ventricosum*	FJ621288.1	Liu *et al.*, 2010
11	*Heliconia stricta*	FJ621299.1	Liu *et al.*, 2010
12	*Strelitzia reginae*	FJ621298.1	Liu *et al.*, 2010
13	*Ravenala madagascariensis*	FJ621296.1	Liu *et al.*, 2010
14	*Phenakospermum guyannense*	FJ621297.1	Liu *et al.*, 2010
15	*Orchidantha fimbriata*	FJ621300.1	Liu *et al.*, 2010
16	*Maranta bicolor*	AY140385.1	Prince and Kress, 2006
17	*Maranta leuconeura*	AY140386.1	Prince and Kress, 2006
18	*Maranta arundinacea*	JQ341208.1	Borchsenius *et al.*, 2012
19	*Canna peniculata*	AY140423.1	Prince and Kress, 2006
20	*Canna indica*#	AM113702.1	Asmussen *et al.*, 2006
21	*Canna edulis*	KC404813	Mishra *et al*., 2012*
22	*Canna indica*	KC404800	Mishra *et al.*, 2012*
23	*Canna indica* Cv. Purpurea	KC404802	Mishra *et al.*, 2012*
24	*Canna* x *generalis* Cv. Italia	KC404805	Mishra *et al.*, 2012*

Sequence retrieved from public domain and variety not known; * not published in any journal

neighbor-joining method. Fig tree-1.4.0 (http://tree.bio.ed.ac.uk/software/figtree) was used to view the constructed phylogenetic tree.

3.3 Antioxidant diversity study

3.3.1 Plant material and extraction

3.3.1.1 Crude Rhizome extracts

Crude rhizome extract will be prepared by using solvents like water (H_2O) and methanol. Young rhizome was washed properly and shade dried. These were then crushed in double distilled water (DDW) and methanol using mechanical grinder to get the required concentration of 1:4 (biomass:H_2O or biomass:methanol), w/v.

3.3.1.1.1 Cold aqueous extract (CAE)

The crude aqueous extract, was kept overnight in refrigerator for cold percolation. Next day, the sample was filtered using Whatman-2 filter paper. The filtrate was subjected to lyophilization (Eyela Freeze Dryer FDU- 506) to form powdery mass. Then required amount of double distilled water was added to those powdery extract to make a concentration of the stock solution to 1miligram/milliliter (mg/ml) and stored at -20ºC for further use.

3.3.1.1.2 Hot aqueous extract (HAE)

The same aqueous extract was

subjected to exhaustive distillation with soxhlet apparatus at boiling temperature for a period of 3 hours. The extract was then lyophilized and stored as described previously.

3.3.1.1.3 Methanolic extract (ME)

The crude methanolic extract, prepared as per the above mentioned method was subjected to exhaustive distillation with soxhlet apparatus at boiling temperature for a period of 3 hours. The extract was then lyophilized and stored as described previously.

3.3.1.1.4 Rhizome extract for silica gel column chromatography

The crude rhizome extract of *Canna edulis* Ker Gawler was soxhleted exhaustively at boiling temperature for a period of 12 hours. The aqueous extract thus obtained was evaporated at 45°C to make a final volume of 3 ml.

3.3.2 Animal material

Goat liver, which was used for anti lipid peroxidation assay, was collected from slaughter house immediately after slay. Experiment was conducted within one hour after collection.

3.3.3 Determination of total phenolic content

Total phenolic content of cold aqueous (CAE), hot aqueous (HAE) and methanolic extract (ME) were determined using Folin-Ciocalteu (FC) reagent method of Singleton and Rossi (1965) with slight modification. The rhizome extracts (0.5 ml) was mixed with 0.5 ml of FC reagent (previously diluted 1:1 with double distilled water) and incubated for 5 min at room temperature (RT). Then 1ml of 20% sodium carbonate (Na_2CO_3) solution was added and incubated at RT for 10 min. Finally the absorbance was measured at 730 nm (with Themo UV1 spectrophotometer). Gallic acid monohydrate was used as standard. The total phenolic content was quantified from the Gallic acid equivalent curve per 1gm of plant extract. All tests were performed in triplicates.

3.3.4 Determination of total flavonoid content

The total flavonoid content was determined with aluminium chloride ($AlCl_3$) method of Zhishen *et al*. (1999) with some modification. In this experiment the plant extract (0.25 ml) was mixed with 1.25 ml DDW which was followed by the addition of 75µl of 5% sodium nitrite ($NaNO_2$). The above mixture was incubated for 5 min at RT and to it; 0.15 ml of 10% $AlCl_3$ was added. After a further incubation for 6

min at RT, the reaction mixture was treated with 0.5 ml of 1 mM sodium hydroxide (NaOH). Finally, the reaction mixture was diluted with 275µl of DDW followed by an incubation of 30 min at RT. The absorbance was recorded at 510 nm and all the experiments were carried out in triplicates. The flavonoid content was quantified from a quercetin standard curve.

3.3.5 DPPH radical scavenging activity

The free radical scavenging capacity of the rhizome extracts was determined using 2,2-diphenyl-1-picryl hydrazyl (DPPH) by minor modification of Hasan *et al*., 2006. DPPH solution (0.006% w/v) was prepared in 95% methanol. Freshly prepared DPPH solution was taken in test tubes and rhizome extracts was added in each test tube, to make a final volume of 2 ml. Discoloration of these extracts was measured at 517 nm after incubation for 30 min in dark at 37ºC. Ascorbic acid was used as a reference standard and dissolved in DDW to make the stock solution with the same concentration (1 mg/ml). Control sample was prepared containing the same volume without any extract. Methanol was used as blank. DPPH scavenging activity (%) was measured using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample (rhizome extracts of Canna). The actual decrease in absorption induced by the test compounds was compared with the positive controls.

3.3.6 Reducing power Assay (FRP)

The reducing power of rhizome extracts was determined according to the method described by Oyaizu (1986). Rhizome extract (1 ml each) was taken in different tubes to which 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] were added and incubated at 50°C for 20 min. Following incubation 2.5 ml of 10% trichloroacetic acid (TCA) was added to the mixture. Since no precipitation occurred, the upper layer (2.5 ml) of the solution was mixed with DDW (2.5 ml) and ferric chloride ($FeCl_3$) (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as a reference standard. Phosphate buffer (pH 6.6)

was used as blank solution. The absorbance of the final reaction mixture of two parallel experiments were taken and expressed as mean ± standard deviation.

3.3.7 Scavenging of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) scavenging activity was determined as per Ruch *et al*., 1989. All the rhizome samples were mixed with 2 mM solution of H_2O_2 in the ratio of 1:0.6 volume/ volume (v/v) and incubated for 10 min at RT. After incubation, absorbance of hydrogen peroxide at 230 nm was determined against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for background subtraction. The percentage scavenging activity of hydrogen peroxide by rhizome extracts was calculated as follows,

$$\%\,SA = \frac{Abs\,(control) - Abs\,(standard)}{Abs\,(control)} \times 100$$

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

3.3.8 Linear correlation between CAE, HAE and ME

The correlation co-efficient between various parameters of CAE, HAE and ME was determined by using SPSS (15.0) software.

3.3.9 Column chromatography

The rhizome extract (prepared as described above in section 3.3.1.1.4) was then subjected to Silica gel (SD fine chem Limited, 200-300 mesh size) column chromatography. Different solvents (based on their polarity level) like hexane, benzene, chloroform, diethyl ether, ethyl acetate, acetone, methanol and water were eluted in the column to separate different phytochemicals in order of their affinity towards these solvents. The above series of less polar to highly polar solvents were passed through the silica column in various combinations of volumes like 25, 50, 75 and 100% to get different solvent fractions. The fractions were then air dried and the air dried fractions were dissolved in 5 ml of methanol and stored in amber glass bottle at 4°C until further use.

3.3.10 Phytochemical screening

Different solvent extracts as prepared through silica gel column chromatography, were tested for various phytochemicals and antioxidant properties, which is represented as follows.

3.3.11 Free Radical Scavenging Activity (DPPH method)

DPPH radical scavenging activity of 29 different solvent fractions of *Canna edulis rhizome* was determined as per the protocol described in section 3.3.5. The scavenging activity of all the solvent fractions was recorded by using the following formula:

$$I = \frac{A_0 - A_1}{A_0} \times 100 \quad \ldots\ldots\ldots\ldots (1)$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample (different fractions of Canna), whereas 'I' stood for %age inhibition of DPPH.

The fraction showing maximum antiradical responses was diluted to different concentrations (100 µg to 1000 µg) and their antiradical activities were observed. IC_{50} value was calculated from graphical presentation of concentration verses radical scavenging activity (Bhattacharya *et al.*, 2009).

3.3.12 Determination of total flavonol content

The protocol developed by Kumaran and Karunakaran, 2007 was used to estimate the total flavonols using quercetin as a standard. Briefly, the bioactive fractions (2 ml each) were mixed with equal volume of 2% ethanolic solution of $AlCl_3$ followed by the addition of 3 ml of sodium acetate (CH_3COONa) (50gm/l). The mixture was incubated at 20°C for 2.5 hours. The absorbance was measured at 440 nm. Total flavonol content was calculated from a quercetin standard curve.

3.3.13 Determination of total proanthocyanidins

Total proanthocyanidins was determined according to Sun *et al.*, 1998, using catechin as a standard. The reaction mixture contains 0.5 ml of extract, 3 ml of 4% methanolic solution of vanillin and 1.5 ml of conc. Hydrochloric acid (HCl) and incubated for 15 min at RT. The absorbance was measured at 500 nm. The proanthocyanidin content was calculated from catechin standard curve.

3.3.14 Inhibition of nitric oxide production

Nitric oxide (NO) scavenging activity of bioactive fractions of *C. edulis* rhizomes was determined as per standard protocol of Marcocci *et al.*, 1994 and Sreejayan and Rao, 1997 with slight modification. Briefly, sodium nitroprusside solution (1 ml of

10mM) was mixed with 1 ml of different concentrations of bioactive fractions in PO_4 buffer (pH-7.4). The mixture was incubated at 25°C for 150 min. To 0.5 ml of incubated solution, 1 ml of Griess reagent (1 ml of 1% sulphanilamide, 0.5 ml of 2% orthophosphoric acid and 1 ml of 0.1% naphthyl ethylene diamine dihydrochloride) was added and the reaction mixture was incubated at 25°C for 30 min. The absorbance of the pink chromophore formed by the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 540 nm. Control sample was prepared containing the same volume without any extract. Nitric oxide scavenging activity (%) was calculated using formula 1, where 'I' stood for % age inhibition of NO.

3.3.15 Hydroxyl radical scavenging

Hydroxyl radical (OH^-) scavenging activity was measured according to Kunchandy and Rao, 1990 with some modifications, by studying the competition between test extract and deoxyribose for hydroxyl radical generated by Fenton's reaction. The reaction mixture containing 0.2 ml of 20mM phosphate buffer (pH-7.4), 0.2 ml $FeCl_3$ (10 mM), 0.1 ml ascorbic acid (0.1 mM), 0.1 ml EDTA (1 mM), 0.1 ml H_2O_2 (10 mM), 0.2 ml of 2-deoxy-D-ribose (10 mM) and different concentration of bioactive fractions (1ml each) was incubated at room temperature for 60 min. To this, 1ml each of 1% Thiobarbituric acid (TBA) and 2.8% TCA were added. The final mixture was kept in boiling water bath for 30 min to get pink chromophore. The damage imposed due to free radicals was determined colorimetrically by measuring the thiobarbituric acid reactive substances (TBARS) at 532 nm. Control sample was prepared containing the same volume without any extract. Hydroxyl radical scavenging activity (%) was measured using formula 1, where 'I' stood for %age inhibition of OH^-.

3.3.16 Inhibition of lipid peroxidation

Lipid peroxide inhibition activity was determined *in vitro* according Kumar *et al.*, 2000. Freshly collected goat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of hepatocyte protective activity. Goat liver (5%) was homogenized with 40 mM tris HCl buffer and centrifuged at 3000 rpm (1000Xg) for 10 min to get a clear supernatant. Reaction mixture

containing 0.5 ml supernatant, different concentrations of bioactive fractions (1 ml each), 100 µl of .15 M potassium chloride (KCl), 1 ml of 15 mM ferrous sulphate ($FeSO_4$), 1ml of 6 mM ascorbic acid was incubated at 37°C for 1 hr. TCA (1 ml ; 10%) was added to the mixture and the samples were centrifuged at 6000 rpm (4000Xg) for 20 min at 4°C to remove insoluble proteins. Supernatant was removed and 1 ml of TBA (0.8 %) was added to this fraction followed by heating at 90°C for 20 min in a water bath. A pink chromophore was produced after cooling which was extracted with organic solvent (2 ml ice cold butanol) and absorbance was measured at 532 nm. Control sample was prepared containing the same volume without any extract. Inhibition of lipid peroxidation activity (%) was calculated from formula 1, where 'I' stood for %age inhibition of lipid peroxide.

3.3.17 Thin layer chromatography (TLC)

The bioactive fractions of rhizome extracts of *C. edulis* were analyzed through thin layer chromatography (TLC) using the method of Wagner and Bladt, 1996. About 10 µl of extract (2 mg/ml) of all the bioactive fractions were loaded on TLC plates (Merck, India 10X10 cm^2). The plate was air dried and developed in Hexane: Diethyl ether (2:3) for 30 min. The plate was dried in a hot oven (at 80ºC for 10 min) and detected under UV light (365 nm) and ammonia vapor. For spraying iodine solution, Natural Product/Polyethylene Glycol reagent (5% NP/PEG in ethanolic solution), ferric chloride (1% solution in 50% aqueous methanol) were used in the experiment.

All the tests were performed in triplets.

3.3.18 Statistical Analysis

Results were expressed as mean ± S.E.M. of triplets. The groups were compared by two-way ANOVA using Graph Pad Prism, Version 5.0 (Graph Pad Software, San Diego, CA, USA). P -values < 0.001 were considered to be statistically significant.

3.4 GC-MS analysis

GC-MS (Gas chromatography-Mass spectroscopy) is a combined analytical method that separate and identify components of different unknown samples. This technique has gained much attention, because of its applications like identification of bio-active phyto chemicals, drug detection, analysis of

environmental pollutants, investigation of explosive etc.

3.4.1 Extract for GC-MS analysis

The crude methanolic leaf extract of *C. edulis* [biomass:methanol (1: 10) w/v] was subjected to exhaustive distillation with soxhlet apparatus at boiling temperature for a period of 6 hours. The extract was then lyophilized and stored at -20°C for further use. Two µl of the methanolic leaf extract of *C. edulis* was provided for separation and identification of the components. GC-MS analysis was carried at Dr. P. S. Ramanathan Advanced Instrumentation Center.

3.5 *In vitro* regeneration studies of Canna

In vitro culture studies of Canna was done to standardize the protocol for regenerating the whole plantlet by using the explant and different hormone combinations in synthetic medium. *Canna indica* Linn. and *Canna edulis* Ker Gawler were chosen for various *in vitro* culture studies.

3.5.1 Sterilization of plant explant

- Leaves, rhizome and immatured healthy fruits were collected from greenhouse for aseptic culture.
- The collected leaves (pieces without midrib), rhizome (having eye) and fruits were scraped and were washed carefully by tap water.
- Only the rhizome tissue was treated with 1% PVP (Polyvinylpyrrolindole) for 1 hr to remove excess of phenol that obstructs regeneration.
- All the three types of explants were washed in 1% tween 20 (Sd fine Chem. Cat#76368) for 30 minutes and then rinsed several times with double distilled water (DDW).
- Different plant tissue was then surface sterilized with 70% alcohol for 45 sec and then rinsed several times with sterile DDW under the laminar air flow cabinet.
- Explants were finally treated with 0.1% $HgCl_2$ (Merck India Cat#17524) for 1.5 min and washed several times with sterile DDW to remove the traces of $HgCl_2$.
- Then the leaves were scraped on both the sides, cut into small pieces, blot dried with sterile tissue paper and inoculated in the media.
- The rhizome tissue was blot dried and kept ready for inoculation.
- Surface sterilized fruits were blot dried, followed by fruit coat removal to expose the seeds.

- Finally the seeds having single cotyledon were cut to pieces (usually 2 pieces) on a sterile glass plate and conserved for further *in vitro* procedure.

3.5.2 Establishment of aseptic culture media

- Pieces of leaves, rhizomes and cotyledons were aseptically inoculated in culture tubes containing MS medium (Murashige and Skoog, 1962), BM medium (Banana micropropagation medium) (Readymade medium procured from Hi-media) and B5 medium (Gamborg *et al.*, 1968) supplemented with 3% sucrose (Hi-media, Cat#RM1158) and Calcium chloride (Merck India Cat#17644).
- In case of banana micropropagation medium, ammonium nitrate (Merck India Cat#17804) was added replacing Calcium chloride.
- The pH of the medium was adjusted to 5.6±0.2 with 1N NaOH or 1N HCl.
- After adjusting to the desired pH (5.6±0.2) of the culture media, 0.7% agar was supplemented as a solidifying agent.
- This was followed by autoclaving the media at 121ºC for 20 min at 15 psi.
- Different concentrations of cytokinins like BAP (6-benzyl amino purine), auxins like NAA (1-napthaleneacetic acid) and 2, 4-D (2,4 dichloro-phenoxy acetic acid) were used in this study.
- The growth regulators were filter sterilized and supplemented to the culture media in desired concentration.
- The media was then poured in sterile test tubes (to form slants) as well as in jam bottles under laminar air flow cabinet.
- Slants were also prepared without any growth hormone to mark the differences of growth and regeneration.
- After the preparation of slants, inoculation of explants was done as per the standard method.

Table 3.6: Different combinations of growth hormone (mg/ml) for callus culture

Media	BAP	NAA	2,4,D
	1	–	–
	2	–	–
	2.5	–	–
	3	–	–
MS	1	0.5	–
BM	2	0.5	–
B5	2.5	0.5	–
	3	0.5	–
	–	–	1
	–	–	2
	–	–	2.5
	–	–	3

Murashige and Skoog medium (MS); Banana micropropagation medium (BM); Gamborg medium (B5)

- Cultures were incubated at 25°C±2°C with a photoperiod of 16 hrs at 2000-3000 lux light intensity of cool white fluorescent light.
- Subculturing was done at regular interval of 2 weeks in the same media having the same composition. BAP, NAA and 2,4-D at the rate of 1, 2, 3 and 4 mg/l were applied to observe various *in vitro* growth and plantlet regeneration.

3.5.3 In vitro germination of seed

For *in vitro* germination studies, cotyledons were aseptically inoculated in all the three above mentioned medium [i.e.MS (Himedia, Cat#PT018), B5 (Himedia, Cat#TS1014) and BM (Himedia, Cat#PT076)] without any growth regulators. In contrast, some cotyledons were also cultured in different concentration of 2,4-D (Himedia, Cat# RM515), BAP (SIGMA, Cat# B-3408) alone and in combination with NAA (SIGMA, Cat# N0640). The rate of *in vitro* germination and their survival was recorded in every 2 weeks up to 3rd subculture.

3.5.4 In vitro *shooting*

The same aseptic media (discussed above in section- 3.4.2) with (different combinations of BAP, BAP+NAA and 2,4-D) and without any phytohormone were considered for formation of *in vitro* shooting. The response of explants to the inoculating media and phytohormone for shooting was observed up to 4th subculture.

3.5.5 Induction of callus tissue

Three different plant explants i.e. leaves, rhizomes and seeds were aseptically cultured in different callus induction medium (Table 3.6) containing 1% agar (Hi-media Cat#RM201), 30gm/l sucrose (Hi-media, Cat#RM1158) and 0.33mg/l $CaCl_2$ (Merck India Cat#17644) with pH of 5.6. Cultures were also prepared in a hormone free medium. In case of BM medium, $CaCl_2$ was replaced by 0.17% NH_4NO_3. Data related to induction of callus and their survival was recorded up to the regeneration of plants or formation of somatic embryo.

3.5.6 Somatic embryogenesis and plant regeneration

- Somatic embryos were produced after 5-6 weeks, by culturing the callus tissues in BM medium with slight modification of adding 2 mg/l BAP.
- The embryo like structure was then subcultured into the same medium

and histological studies were performed on the 15-30 days old somatic embryos which were maintained by regular transfer into fresh medium.

- Roots were produced simultaneously along with the embryogenic calli.

After 1 month of somatic embryo formation, tissues were subcultured for the formation of shoot.

The shoots were appeared after a week of sub culturing the somatic embryos in BM medium, which contain BAP, 3% sucrose and 0.7% agar with pH of 5.6.

- After 6 weeks, in a multiple shooting and rooting condition the plantlets were ready for acclimatization.

3.5.7 Histological observations of the cultured somatic embryo

Somatic embryo cultured for 15 days and 1month respectively, were taken for preparation of both transverse and longitudinal sections.

- Sections were dehydrated in graded Ethanol solutions (30 to 95%) and stained with safranin (Hi-media, Cat#RM1315) and light green (Hi-media, Cat#RM386) (Santra *et al*., 1989).
- The tissues were at first dipped into 30% of alcohol and then of 50% alcohol for duration of 3 minutes each.
- Again the tissues were dipped into 1% solution of safranin (in 50% alcohol) for 5 minutes.
- After 5 min in safranin, the tissues were washed in 80% alcohol.
- The embryonic tissues were then dehydrated in 70%, 80%, and 90% alcohol, keeping 5 minutes in each solution.
- Then these were counterstained in 1% solution of light green (in 95% alcohol) for 30 sec.
- After staining with light green, the tissues were washed in 90% alcohol and then dehydrated in 95% alcohol for 3 minutes.
- These tissues were then kept in absolute alcohol (BDH, Cat#10107) for 5 min for further dehydration.
- The dehydrated tissues were passed through alcohol:xylol (1:1) mixture for 5 minutes.
- The tissues were cleared in xylol (Hi-media, Cat#RM1877), for 10 minutes.
- Finally, the tissues were mounted in DPX (Hi-media, Cat#RM655) and dried.

Sections both transversely as well as longitudinally cut were than observed under microscope and photographed (Motic Digital Microscope- 31007749).

3.5.8 Hardening and transfer of plant to soil

After 6 weeks of rooting and shooting *in vitro*, the whole plantlet was taken out of the culture bottle carefully so that the root system is not damaged. The roots were washed gently under running tap water to remove the medium completely. The plantlets were acclimatized in the substrates having autoclaved mixture of soil and sand. Then the plantlets were transferred to a mixture of sandy soil and farm yard manure with a ratio of 1:1 (by volume) for 30 days in hardening in a green house condition and finally to the field conditions. The survival percentage of the acclimatized plantlet was recorded.

3.5.9 Somaclonal variations detection

3.4.9.1 DNA isolation from the in vitro *regenerated plantlets*

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

- Approximately 100 mg *in vitro* callus regenerated leaves were ground into fine powder `in a small mortar and pestle using liquid nitrogen.
- The ground plant tissue was then lysed using 350µl of lysis solution (Part A) and 50µl of lysis solution (Part B) and mixed thoroughly by inversion and incubated at 65ºC for 10 min.
- Following incubation 130µl precipitation solution was added, mixed by inversion and incubated on ice for 5 min to pellet debris. The supernatant was transferred to blue filtration column and centrifuged at 12,000 rpm (13,500Xg) for 1 min.
- To the filtrate 700µl of binding solution was added and mixed thoroughly by inversion. The mixture was then transferred to binding column (prepared by adding 500µl of column preparation solution to the binding column and spinning for 1 min and discarding the flow-through) and centrifuged for 1 min at 12,000 rpm (13,500Xg) and the flow through was discarded. The process was repeated with the remaining mixture. The column was then transferred to a new collection tube.
- Then 500µl of wash solution (containing ethanol) was added to the column and centrifuge at 12,000 rpm (13,500Xg) for 1 min. The column

was then transferred to a new collection tube. The process was repeated where the spinning time was increased to 3 minutes.

- Finally 100µl of elution solution (pre -warmed at 65ºC) was added to the column and centrifuged at 12,000 rpm (13,500Xg) for 1 min. The filtrate contained the pure genomic DNA.

3.5.9.2 RAPD analysis

Please refer to section 3.2.5 for details of the analysis.

3.5.9.3 ISSR analysis

Please refer to section 3.2.6 for details of the analysis.

3.5.10 Protoplast isolation and fusion

3.5.10.1 Isolation and purification of protoplast

Isolation and purification of protoplasts were standardized as per Assani *et al.*, 2002 and De, 1997 with some modifications. As described earlier (section- 3.4.3 and 3.4.4), 4 to 5 weeks old *in vitro* cultured leaves of *Canna indica* and *Canna edulis* were considered for isolation of protoplast. One gram of leaves of both the species were peeled and cut into pieces of 1 × 0.5 mm size. Cut pieces of leaves were placed lower surface down in sterile petriplates in a cell and protoplast washing medium i.e. solution-I (represented in appendix- B). After 5 minutes, solution-I was replaced by solution-II i.e. enzymatic solution of cellulase (Sigma Cat# C1184) and pectinase (Sigma Cat# P2401). Different combinations of both cellulase (0.5%, 1%, 1.5%, 2%) and pectinase (0.15%, 0.3%, 0.5%, 0.75%) were tried to observe the best result. Peeled leaves were incubated at 24±3ºC for 16-18 hrs in dark. After the incubation for enzymatic digestion, solution-II was gently replaced by solution-I without disturbing the leaf pieces. The digested leaf pieces were gently agitated and squeezed with fine sterile forcep to facilitate the release of protoplasts. Purification of isolated protoplast was done by passing the digestion mixture through 100µm sterile metallic mesh to remove the debries and large cell colonies. The filtrate was transferred to centrifuge tube and spinned at 900 rpm (100Xg) for 5 minutes at RT. The protoplasts were settled as pellet which were resuspended in second cell and protoplast washing medium i.e. solution-III (described in appendix- B) and centrifuged at 1,300 rpm (200Xg) for 7 minutes. The viable protoplasts

forming dark green band (De, 1997) were collected from the surface of the mixture of solution-III. Protoplasts were washed by suspending in solution -I and centrifuged at 900 rpm (100Xg) for 5 minutes for 2-3 times to remove the sucrose. Protoplast viability was checked by using phenosafranin according to the method described by De, (1997). Phenosafranin (0.1%) was mixed with protoplast preparation to view the staining.

3.5.10.2 Culture of protoplast

Medium was prepared couple of days before protoplast isolation. The isolated protoplasts were sieved through 250 µm sterile metallic mesh to select the small cell aggregates for their culture. The culture medium consisted of BM media, 2 mg/l BAP, 0.5 mg/l NAA, 2.8 mM glucose, 278 mM maltose, 116 mM saccharose, 2.5 mM myo inositol (pH 5.6±0.2) and 0.4% agar. The media was autoclaved for sterilization. The mixture was carefully poured into sterilized bottles. Then the sieved mesophyll protoplasts were gently plated on the agar medium. The cultures were maintained at 25ºC in the dark to observe further development of mesophyll cells.

3.5.10.3 Fusion of protoplasts

Fusion of protoplast was done as per Assani *et al.*, (2005) with desired modification. Viable protoplasts (as described above in section 3.4.10.1) of both the species were mixed in equal proportion in a fusion solution containing 0.5 M mannitol and 0.5 mM $CaCl_2$. Protoplast mixture was slowly dropped down to the sterile petriplate followed by the addition of PEG solution (50% PEG, 0.5 M mannitol and 0.5 mM $CaCl_2$). After 30 minutes of incubation in RT, the PEG solution was replaced by liquied culture media. Further the protoplasts were studied under microscope for possible fusion.

3.5.10.4 Culture of fused protoplast

Fused protoplasts were cultured in the same medium as described above in section 3.4.10.2 to induce their cell division. The cultures were maintained at 25 ºC in the dark to observe further development of fused mesophyll cells.

Chapter 4
Results and Discussion

4.1 Collection of germplasm

Twenty different Canna species and cultivars were collected from different places of West Bengal and Orissa like Siliguri, Darjeeling, Kalimpong, Jalpaiguri, Lataguri, Pundibari, Madarihat, Pankhabari, Coochbehar, Malda, Baripada, Balasore, Soro, Bhadrak, Karanjia and Keonjhar and were authenticated by plant taxonomists (figure 4.1). The collected germplasm were planted in the experimental garden of Molecular Genetics Lab., North Bengal University for further study. List of different species and cultivars of Canna along with their morphological characters are given in table 4.1.

4.2 Molecular diversity studies

4.2.1 DNA isolation, purification and quantification

4.2.1.1 DNA isolation

Canna DNA was isolated using the standard protocol of Doyle and Doyle, 1987 with minor modifications. The DNA-CTAB complex gave a whitish network of nucleic acid and starch, which was subjected to purification process for complete elimination of polysaccharide. The pure DNA was used for further downstream processing. The presence of DNA bands were shown through agarose gel analysis.

4.2.1.2 DNA purification

The presence of various contaminants like polysaccharides, polyphenols and other secondary metabolites can interfere with the successful enzymatic reaction with DNA. Specifically, the polysaccharides in DNA inhibit Taq DNA polymerase activity (Fang *et al.*, 1992). The extraction of pure DNA is very essential for further downstream processes like PCR amplification, DNA restriction and gene cloning. The impurities like RNA and protein in crude DNA also hamper the amplification process, thus, DNA needs to be further purified. Addition of CTAB in the DNA extraction buffer helped a lot in the elimination of polysaccharides from DNA

Canna edulis Ker Gawler

C. edulis (green cultivar)

C. indica Linn.

C. x *generalis* L.H. Bailey Cv. "Italia"

C. x *generalis* Cv. "Tropical red"

C. x *generalis* Cv. "Orange web"

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Figure 4.1: Flowers and foliages of selected species and cultivars of Canna used in the present study

Table 4.1: Morphological features of different species and cultivars of Canna collected from West Bengal and Orissa

Sample ID	Name of the Species/Cultivars	Phenotype of Species/Cultivars
C_1	*Canna edulis* Ker Gawler*	Plants tall; leaves broad, green with deep violet sheds; flowers small, red
C_2	*Canna edulis* Ker Gawler (green cultivar)*	Plant tall; leaves broad, green without any sheds; flowers small, reddish orange with yellow patches
C_3	*Canna indica* Linn.	Plant tall; leaves green; flowers small, red
C_4	*Canna* x *generalis* L.H. Bailey Cv. "Italia"	Plant tall; leaves green; flowers big, yellow with orange spots, sheds on the petals
C_5	*Canna* x *generalis* L.H. Bailey Cv. "Tropical Red"	Plant tall; leaves broad, green with red margin; flowers big, red
C_6	*Canna* x *generalis* L.H. Bailey Cv. "Orange web"	Plant tall; leaves broad, green with red margin; flowers big, orange
C_7	*Canna* x *generalis* L.H. Bailey Cv. "Austria"	Plant tall; leaves green; flowers big, yellow with red spots in the middle
C_8	*Canna* x *generalis* L.H. Bailey Cv. "President"	Plants short; leaves green with white boarder; medium sized, cherry red flowers
C_9	*Canna* x *generalis* L.H. Bailey Cv. "Pink silk"	Plants short; leaves green; big, light pink flowers
C_{10}	*Canna* x *generalis* L.H. Bailey Cv. "City of Portland"	Plants short; leaves green; flowers deep pink
C_{11}	*Canna indica* Linn. Cv. "Purpurea"	Plants tall; leaves green with violet sheds; flowers small, orange
C_{12}	*Canna* x *generalis* L.H. Bailey Cv. "Roi King Humbert"	Plants tall; leaves bronze; flowers dark red
C_{13}	*Canna* x *generalis* L.H. Bailey Cv. "Biercee"	Plants short; leaves green; flower creamish yellow with red throat
C_{14}	"*Canna* 21" (Crozy cultivar) [Unidentified]	Plants short; leaves green; flowers orange
C_{15}	*Canna* x *generalis* L.H. Bailey Cv. "Dwarf Yellow"#	Plants dwarf; leaves green; flowers yellow
C_{16}	*Canna* x *generalis* L.H. Bailey Cv. "Dwarf Red"#	Plants dwarf; leaves green with red margin; flowers dark red
C_{17}	*Canna* x *generalis* L.H. Bailey Cv. "Dwarf Orange"#	Plants dwarf; leaves green with red margin; flowers dark orange
C_{18}	*Canna* x *generalis* L.H. Bailey Cv. "Froken"#	Plants dwarf; leaves narrow; green; flowers yellow, spotted red
C_{19}	*Canna* x *generalis* L.H. Bailey Cv. "Jessica"	Plants short; leaves green; flowers pinkish orange
C_{20}	*Canna* x *generalis* L.H. Bailey Cv. "Trinacria Variegata"	Plants short; leaves variegated; flowers yellow

NB-* represents edible Canna, # represents dwarf cultivars, Sample ID "C1-C20" were used for further downstream processing

precipitations. In case of Canna, whitish viscous mass of polysaccharide was co-precipitated with DNA during the early stage of DNA extraction. Similar problem was also reported in *Entelea arborescens*, where isolated DNA was contaminated with highly viscous mass of polysaccharides (Shepherd and McLay, 2011). So it was obvious that addition of CTAB in the DNA extraction buffer failed to eliminate the starch compound completely from the DNA. Thus, CTAB method of DNA extraction was

Table 4.2: List of Canna samples showing their purity

Sample ID	A_{260}/A_{280} ratio for purity
C_1	1.86
C_2	1.88
C_3	1.82
C_4	1.79
C_5	1.8
C_6	1.81
C_7	1.77
C_8	1.87
C_9	1.8
C_{10}	1.78
C_{11}	1.84
C_{12}	1.8
C_{13}	1.82
C_{14}	1.88
C_{15}	1.8
C_{16}	1.76
C_{17}	1.8
C_{18}	1.8
C_{19}	1.8
C_{20}	1.78

modified for complete elimination of polysaccharides. So the DNA isolated through CTAB method was further extracted once with phenol:chloroform:isoamyl alcohol, followed by purification in polyethyleneglycol (PEG) for removal of polysaccharides. The RNAase A enzyme was found to be effective in eliminating RNA. After the above purification steps, the DNA pellet were completely soluble in TE.

4.2.1.3 DNA quantification and quality check

Two different methods were followed for quantification of Canna DNA, one was spectophotometric and the other with agarose gel electrophoresis. The DNA was quantified in a UV spectrophotometer with 260 nm and 280 nm filters. Results were scored and the ratio of A_{260}/A_{280} was calculated. All the experiments were performed in three or more replicates and the samples showing a ratio of around 1.8

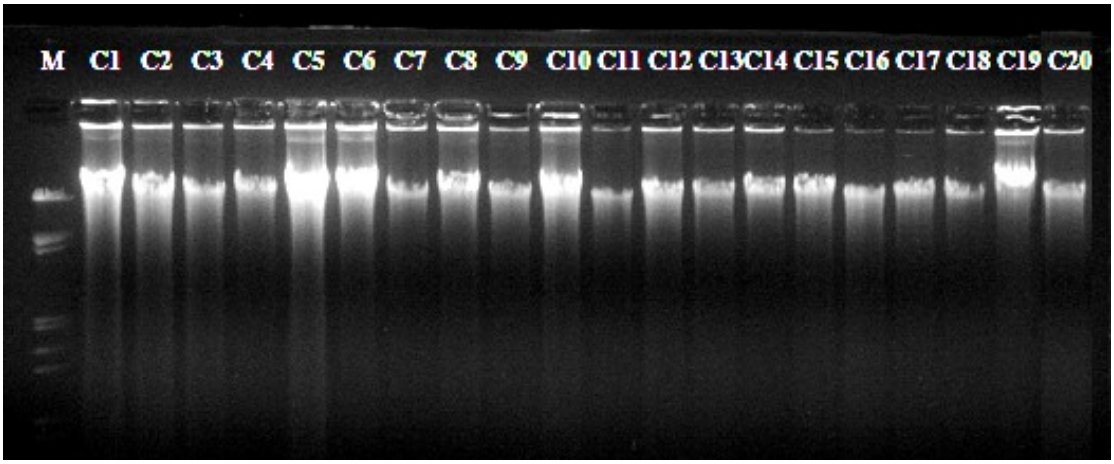


Figure 4.2: Crude DNA of all the Canna samples (Lane C1- C2O: different samples of Canna under study, Please refer table 4.1 for the name of species and cultivars); Lane M: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

(table 4.2) were chosen for further studies. The intactness of the DNA was determined with the help of 0.8% Agarose gel electrophoresis using λ DNA-EcoRI+*Hin*dIII double digest. The size of the DNA was found to be approximately 25 kb (figure 4.2). Samples with relatively larger bands were chosen for downstream reactions.

4.2.2 RAPD analysis

In the present study, genetic diversity has been studied among different species and cultivars of Canna. So far the molecular diversity of Canna was concerned, not much work has been done worldwide and there is only one report on the diversity of handful of garden varieties of Canna (Patra *et al.*, 2008). In the present study, I have used 30 different oligomer RAPD primers to determine the diversity of 20 Canna species/cultivars of West Bengal and Orissa. Results are summarized in table 4.3. Out of 30 RAPD primers 18 yielded reproducible RAPD bands in

Table 4.3: Total number and size of amplified bands, number of polymorphic and monomorphic bands and percentage of polymorphism generated by the RAPD primers

Primer	Seq. (5'-3')	Band No.	MB	PB	Pol%	Band size
OPA01	CAGGCCCTTC	14	1	13	92.85%	400-1700
OPA02	TGCCGAGCTG	10	1	9	90%	348-1757
OPA03	AGTCAGCCAC	12	0	12	100%	305-1650
OPA04	AATCGGGCTG	6	1	5	83.33%	220-1200
*OPA05	ATTTTGCTTG	—	—	—	—	—
*OPA06	GGTCCCTGAC	—	—	—	—	—
OPA07	GAAACGGGTG	11	1	10	90.9%	432-1600
*OPA08	GTGACGTAGG	—	—	—	—	—
*OPA09	GGGTAACGCC	—	—	—	—	—
OPA10	GTGATCGCAG	7	1	6	75%	550-1600
OPA11	CAATCGCCGT	11	1	10	90.9%	250-1170
OPA17	GACCGCTTGT	14	1	13	92.85%	230-1214
OPA18	AGGTGACCGT	7	0	7	100%	570-1385
*OPA19	CAAACGTCGG	—	—	—	—	—
OPA20	GTTGCGATCC	5	4	1	20%	400-1142
OPB01	GTTTCGCTCC	10	0	10	100%	533-1463
*OPB02	TGATCCCTGG	—	—	—	—	—
*OPB03	CATCCCCCTG	—	—	—	—	—
*OPB04	GGACTGGAGT	—	—	—	—	—
*OPB05	TGCGCCCTTC	—	—	—	—	—
*OPB06	TGCTCTGCCC	—	—	—	—	—
*OPB07	GGTGACGCAG	—	—	—	—	—
*OPB08	GTCCACACGG	—	—	—	—	—
OPF09	CCAAGCTTCC	6	1	5	83.33%	359-1099
OPG19	GTCAGGGCAA	8	0	8	100%	461-1422
OPH04	GGAAGTCGCC	9	0	9	100%	335-1134
OPN04	GACCGACCCA	10	1	9	90%	320-1115
OPN05	ACTGAACGCC	9	0	9	100%	490-1612
OPN13	AGCGTCACTC	3	2	1	33.33%	614-986
OPN19	GTCCGTACTG	7	1	6	85.71%	410-1205
Total		**159**	**16**	**143**		

*Not amplified; MB=Monomorphic bands; PB=Polymorphic bands; Pol%=%age of polymorphism

different varieties. A total of 159 major scorable bands ranging from 220 base pair (bp) to 1757 bp were generated from these 18 primers, out of which only 143 fragments were polymorphic and the rest were monomorphic (table 4.3). The percentage of polymorphism was found to be 89.93%. The number of polymorphic bands generated by each decamer primers ranged in between 1 (OPA20 and OPN13) and 13 (OPA01 and OPA17). The RAPD profile of the 20 accessions of Canna generated using primers OPA03 and OPH04 are depicted in figure 4.3. Some primers like OPA03, OPA18,

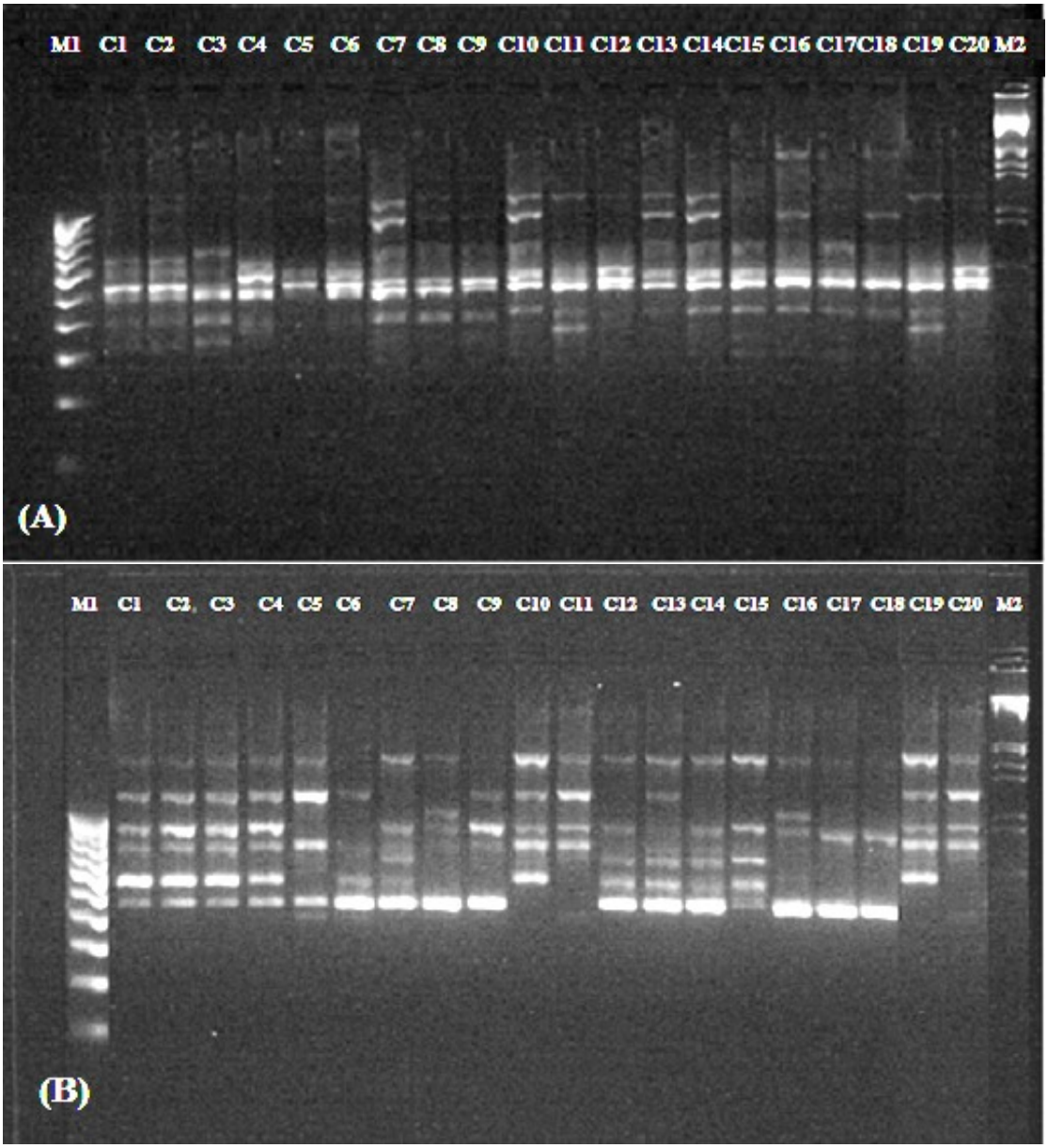


Figure 4.3: A representative of RAPD profile of 20 accessions of Canna amplified with (A) OPA03 primer and (B) OPH04 primer. Lane M1: 100 bp molecular marker; Lane C1- C2O: different accessions of Canna under study (Please refer table 4.1 for the name of the species and cultivars); Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

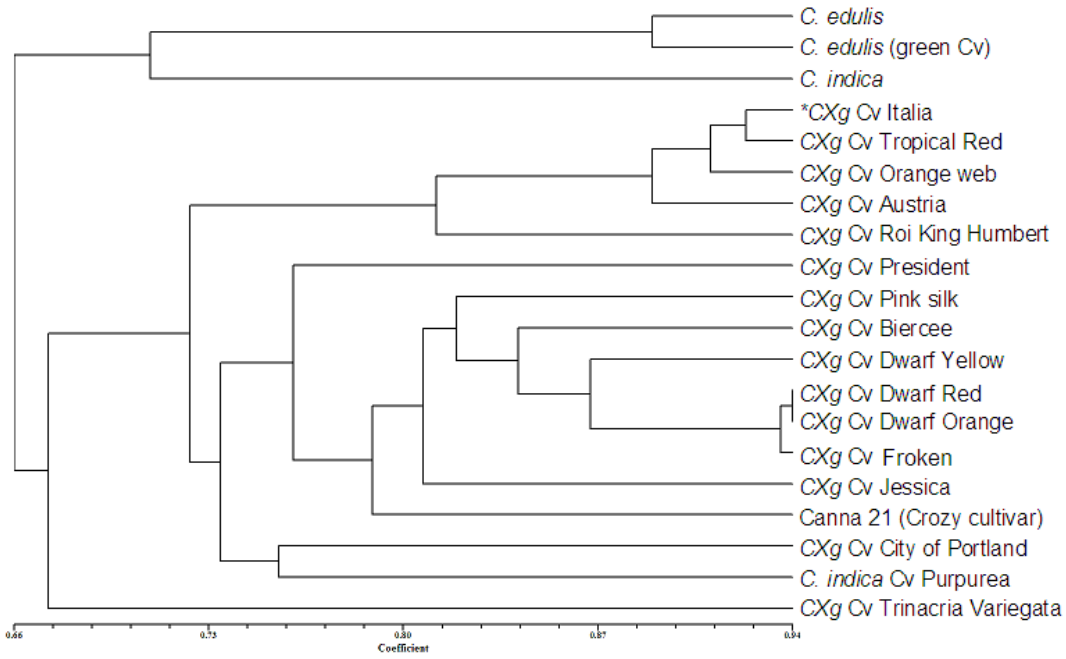
OPB01, OPG19, OPH04 and OPN05 showed 100% polymorphism among the cultivars owing to their self incompatibility. This makes them highly heterogeneous and consequently shows broad genetic variation among them. All the other primers showed more than 75% polymorphism except for OPA20 and OPN13, where polymorphism was 20% and 33% respectively. Selection of alleles was carried out in a very careful manner and only the clear and distinct bands were scored and used in further statistical analysis. The similarity matrix obtained using the Dice coefficient of similarity (Nei and Li, 1979) is depicted in table 4.4. Similarity coefficient among the 20 accessions ranged from 0.496- 0.941. The lowest similarity was observed between C_1 (*Canna edulis* Ker Gawler) and C_{20} (*Canna* x *generalis* Cv. "Trinacria Variegata"), while the highest value was recorded between C_{16} (*Canna* x *generalis* Cv. "Dwarf Red") and C_{17} (*Canna* x *generalis* Cv. "Dwarf Orange") and between C_{17} (*Canna* x *generalis* Cv. "Dwarf Orange") and C_{18} (*Canna* x *generalis* Cv. "Froken").

The dendrogram constructed on the basis of the data obtained from RAPD analysis using NTSYSpc is represented in figure 4.4. Among the 20 different accessions, we had 2 different elemental species having two cultivars each and 16 hybrid cultivars. In the dendrogram, both the cultivars of *Canna edulis* came together. However, two varieties of *Canna indica* were separated out. This may be due to geographical barrier between the two cultivars and thus, they evolved independently. Even morphologically also, these two cultivars were quite different. *Canna indica* Cv. "Purpurea" have purpulish green leaves with orange flowers, whereas the other variety had green leaves with red flowers. Regarding the hybrid cultivars, all of them flocked together in a big cluster except *Canna* x *generalis* Cv. "Trinacria Variegata". In this big cluster, I could recover all four dwarf varieties, namely *Canna* x *generalis* Cv. "Dwarf Yellow", *Canna* x *generalis* Cv. "Dwarf Red", *Canna* x *generalis* Cv. "Dwarf Orange" and *Canna* x *generalis* Cv. "Froken" which grouped together. The dwarf Red and dwarf Orange cultivars showing a node at 94.1% proximity level, were found to be the closest among all the hybrid plants. Thus, the dendrogram was broadly divided into two clusters, the

Table 4.4: The similarity matrix obtained using Dice coefficient of similarity among the 20 accessions of Canna based on RAPD profiling

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
C1	1.000																			
C2	0.891	1.000																		
C3	0.681	0.739	1.000																	
C4	0.647	0.655	0.697	1.000																
C5	0.655	0.681	0.672	0.924	1.000															
C6	0.630	0.672	0.664	0.899	0.924	1.000														
C7	0.630	0.672	0.681	0.882	0.891	0.899	1.000													
C8	0.689	0.731	0.723	0.756	0.765	0.790	0.739	1.000												
C9	0.597	0.672	0.714	0.714	0.739	0.765	0.731	0.807	1.000											
C10	0.664	0.739	0.681	0.681	0.706	0.697	0.664	0.756	0.748	1.000										
C11	0.622	0.714	0.706	0.689	0.697	0.723	0.672	0.714	0.756	0.756	1.000									
C12	0.613	0.672	0.664	0.832	0.807	0.798	0.815	0.739	0.748	0.697	0.739	1.000								
C13	0.613	0.689	0.664	0.697	0.689	0.731	0.697	0.773	0.798	0.782	0.723	0.697	1.000							
C14	0.622	0.697	0.706	0.723	0.697	0.739	0.723	0.782	0.756	0.773	0.748	0.773	0.840	1.000						
C15	0.622	0.697	0.655	0.756	0.731	0.773	0.756	0.765	0.807	0.723	0.714	0.739	0.857	0.782	1.000					
C16	0.588	0.681	0.672	0.706	0.697	0.723	0.689	0.731	0.824	0.756	0.731	0.723	0.840	0.798	0.882	1.000				
C17	0.613	0.706	0.697	0.731	0.723	0.748	0.714	0.756	0.832	0.731	0.739	0.731	0.832	0.790	0.874	**0.941**	1.000			
C18	0.622	0.714	0.689	0.739	0.748	0.773	0.739	0.765	0.840	0.739	0.731	0.739	0.840	0.815	0.849	0.933	**0.941**	1.000		
C19	0.588	0.664	0.655	0.706	0.714	0.723	0.689	0.714	0.790	0.672	0.697	0.706	0.756	0.748	0.832	0.832	0.824	0.815	1.000	
C20	**0.496**	0.571	0.630	0.731	0.689	0.681	0.714	0.672	0.664	0.563	0.588	0.714	0.647	0.655	0.706	0.655	0.664	0.655	0.773	1.000

For details on sample ID C1 to C20 please refer table 4.1



*CXg denotes *Canna* x *generalis*

Figure 4.4: Dendrogram derived from UPGMA cluster analysis of RAPD markers illustrating the genetic relationship among 20 accessions of Canna

smaller one contained two different elemental species and the larger cluster contained all the available hybrid cultivars of Canna. On the basis of similarity indices, first sub-cluster of dendrogram showed two cultivars of same species i.e. *Canna edulis* and *Canna edulis* green cultivar sharing a node at 89.1%. Similar result was reported by Piyachomkwan *et al.*, 2002, where high level of genetic similarity was found between indigenous Thai cultivars of *Canna edulis*. Further, *Canna indica* was placed in the first cluster with *Canna edulis and Canna edulis green cultivar*, sharing the similarity level of 68.1% and 73.9% respectively. All the 17 ornamental cultivars were hybrid plants and grouped together to form the larger cluster. It was hypothesized that the group of garden plants were clustered together to form a somaclone complex, which might have originated from somatic mutation of single cultivar at different times and different geographical locations (Ude *et al.*, 2003). Though morphological diversity was seen among the members of somaclone complex, they may share some common characters which bound them under single horticultural species i.e. *Canna generalis* Bailey and distinguished them from the elemental species which were responsible for their origin (Khoshoo and Mukherjee,

1970). As the variegated Canna i.e. *Canna* x *generalis* Cv. "Trinacria Variegata" was separated out from the somaclone complex of the hybrid plants, rest of the 16 garden cultivars were grouped in 3 different sub-clusters on the basis of various morphological and physicochemical characters namely plant height, flower color, flower size, blooming period etc. Among the hybrids, *Canna* x *generalis* Cv. "Roi King Humbert" was grouped together with *Canna* x *generalis* Cv. "Austria", "Orange Web", "Tropical Red" and "Italia" to form the first sub-cluster. The above 5 cultivars were tall plants having large and bright flowers. On the basis of their phenotypic characters like plant height and flower pattern, they resemble each other and grouped together to form the sub-cluster. It was observed that 9 garden cultivars such as *Canna* x *generalis* Cv. "President", "Pink silk", "Biercee", "Jessica", "*Canna* 21" (an unidentified Crozy cultivar) and 4 dwarf varieties were placed together to form a bigger sub-cluster. The above plants were assembled together because of their short height with narrow and green foliage. Further *Canna* x *generalis* Cv. "City of Portland" and *Canna indica* Cv. "Purpurea" were clustered together to form the smallest sub-cluster as they had medium height and same blooming period (about 15-20 days in mid summer). In addition to this, the above two hybrid plants shared the same growth habitat as both were collected from the same geographical region. The above findings were in accordance with Patra *et al.*, 2008, where morphologic characters were focused in discussing the molecular variability of different cultivars of Canna. In the present study, though morphological character was considered for analyzing the genetic variability, it was observed that the big pink flowered cultivars like *Canna* x *generalis* Cv. "Pink silk", "Jessica" and "City of Portland" were distantly placed in the dendrogram. Loh and his coworkers in 1999, studied the AFLP analysis of 2 species and 7 cultivars of *Caladium* to establish genetic differentiation of closely related cultivars and they found two cultivars having different foliage type i.e. fancy and strap shaped foliage were placed together in a single group. The differences in the morphology of the leaf might be due to some allelic differences and couldn't be considered to be very strong taxonomic character (Loh *et al.*, 1999). This result was in close correspondence with the

Phylogenetic evaluation of Patra *et al.* 2008, where two Canna cultivars having deep red flowers were placed wide apart under two different clades. Thus it could be inferred that some allelic characters may be responsible for placing different pink flowered plants at a distance from each other. The separation of *Canna* x *generalis* Cv. "Trinacria Variegata from all other hybrid cultivars might be due to their variegated leaves that have been appeared as a result of mutation, probably caused by the changes in extreme geographic and climatic condition i.e. from South America to an Asian country like Thailand. It was Dr. Khoshoo, a renowned Indian botanist who explained the appearance of variegated leaves in the above described Canna cultivar and established it to be a hybrid of Canna and not a chimeral plant [Percy-Lancaster, Sydney (1927)]. The principal coordinate analysis which is based on the similarity coefficients or variance-covariance among the traits validated the dendrogram (Akond *et al*., 2007) (figure 4.5 and 4.6). Both the principal coordinate analysis and the dendrogram showed the similar type of clusters.

This study demonstrates that RAPD offers a suitable means for detecting genetic diversity in Canna.

4.2.3 ISSR (Inter Simple Sequence repeat) analysis

Fifteen ISSR primers were initially screened to generate polymorphic bands in studied Canna samples, out of which only 10 primers were able to produce distinct, scorable bands (table 4.5) and were selected for further study. A total of 93 bands were produced using these 10 primers. The band size was ranging between 246 bp to 2017 bp. Highest number of bands (15 bands) were generated by UBC873, whereas UBC841 produced the lowest number of bands (6 bands). Of all, 11 amplified fragments were found to be monomorphic in nature. The frequency of polymorphism was 88.17%. Two primers (UBC815 and UBC824) showed 100% polymorphism among the cultivars, where as all other primers produced more than 75% polymorphism except for UBC841 (40%). A representative of ISSR profile of the 20 accessions of Canna generated with primers UBC873 and UBC815 is depicted in figure 4.7. Nei's genetic similarity between each pair of species ranged between 0.560 - 0.964 (table 4.6). The highest correlation was found between *Canna*

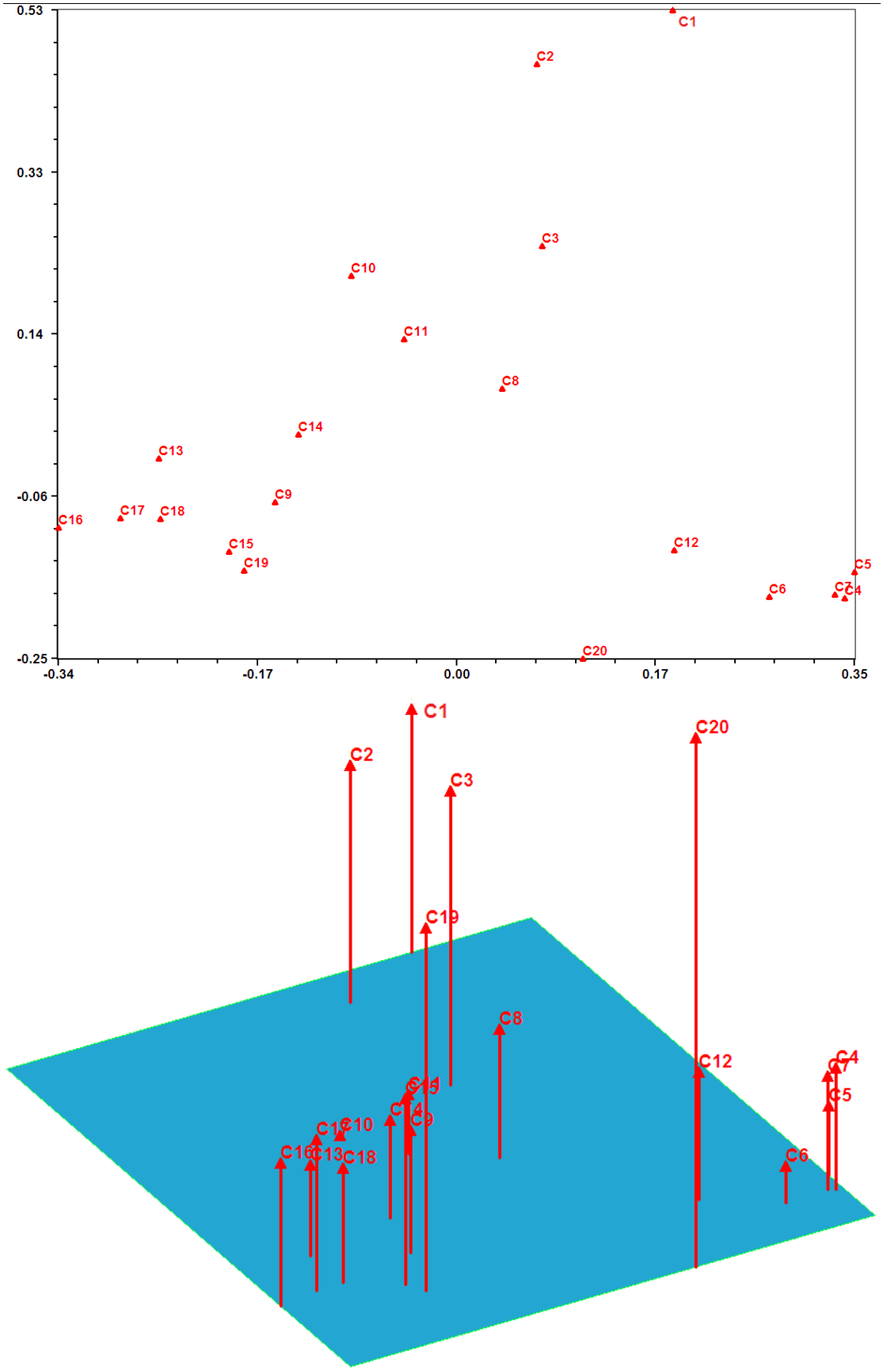


Figure 4.5 & 4.6: Principal coordinate analysis of 20 species and cultivars of Canna based on RAPD analysis data. Figure 4.5- 2-dimensional plot and Figure 4.6- 3-dimensional plot. C1- C2O: different accessions of Canna under study (Please refer table 4.1 for name of the species and cultivars)

edulis and it's green cultivar, where as lowest was found between *Canna indica* Linn. and *Canna* x *generalis* Cv. "Orange Web".

The dendrogram (figure 4.8) prepared out of ISSR analysis results indicated that *Canna indica* is the out-group in the tree and therefore, it became considered as the root. *Canna indica* Cv. "Purpurea" is closest to the root which is natural. Both the varieties of *Canna indica* were elemental species and known to be involved in the creation of hybrid cultivars (Khoshoo and Mukherjee, 1970 and Patra *et al.*, 2008). This result was in correspondence with the phylogenetic analysis study of Canna by Patra and his coworkers in 2008. They observed *Canna indica* and it's hybrid *Canna indica* "Tropicanna" segregated out from the rest of the cultivars of *Canna* x *generalis* complex (Patra *et al.*, 2008). Then there were three distinct clades. One was with two elemental varieties, *Canna edulis* and *Canna edulis* green cultivar. Being the cultivars of the same species, they had a cluster sharing node at 96.4%, showing the highest similarity coefficient in the dendrogram. High level of similarity between these two cultivars was reported by Piyachomkwan and his coworkers (Piyachomkwan *et al.*, 2002). The next clade consisted of 7 hybrid cultivated varieties of *Canna* x *generalis* Cv. "Italia", "Austria", "Tropical Red", "Orange Web", "Roi King Humbert", "Jessica", "Trinacria Variegata". These 7 hybrids of *Canna* x *generalis* were assembled together as that of RAPD, but the only difference was that *Canna* x *generalis* Cv. "Trinacria variegata" was included within the group. From the above result it could be assumed that variegation in the leaf structure may not be considered as a distinct morphologic character for segregation of a cultivar from a particular group (Percy-Lancaster, Sydney, 1927). Whereas, the other clade consisted of 9 hybrid cultivated varieties namely, *Canna* x *generalis* Cv. "President", "Pink silk", "City of Portland", "Biercee", "*Canna* 21" and all the 4 dwarf varieties. These 9 ornamental cultivars clustered together as a somaclone complex as described in case of RAPD primers (Ude *et al.*, 2003). The dwarf varieties, which were clustered together in RAPD tree, also clustered here except the *Canna* x *generalis* Cv. "Dwarf Yellow", which clustered with non dwarf varieties like Cv. "Biercee" and "*Canna* 21".

Table 4.5: Total number and size of amplified bands, number of polymorphic and monomorphic bands and percentage of polymorphism generated by the ISSR primers

Primer ID	Sequence (5'-3')	Total bands amplified	Monomorphic bands	Polymorphic bands	Percentage of polymorphism	Band size (bp)
*UBC807	(AG)8T	—	—	—	—	—
*UBC808	(AG)8C	—	—	—	—	—
UBC810	(GA)8T	13	1	12	92.30%	447-1517
*UBC811	(GA)8C	—	—	—	—	—
UBC813	(CT)8T	9	1	8	88.88%	310-1185
UBC815	(CT)8G	10	0	10	100%	348-1152
UBC818	(CA)8G	14	1	13	92.85%	280-1436
UBC822	(TC)8A	8	2	6	75%	574-1860
UBC824	(TC)8G	6	0	6	100%	481-1520
UBC825	(AC)8T	5	1	4	80%	351-1175
*UBC834	(AG)8YT	—	—	—	—	—
*UBC836	(AG)8YA	—	—	—	—	—
UBC841	(GA)8YC	5	3	2	40%	246-1225
UBC856	(AC)8YA	8	1	7	87.5%	265-866
UBC873	(GACA)4	15	1	14	93.33%	419-2017
Total		**93**	**11**	**82**		

*Not amplified

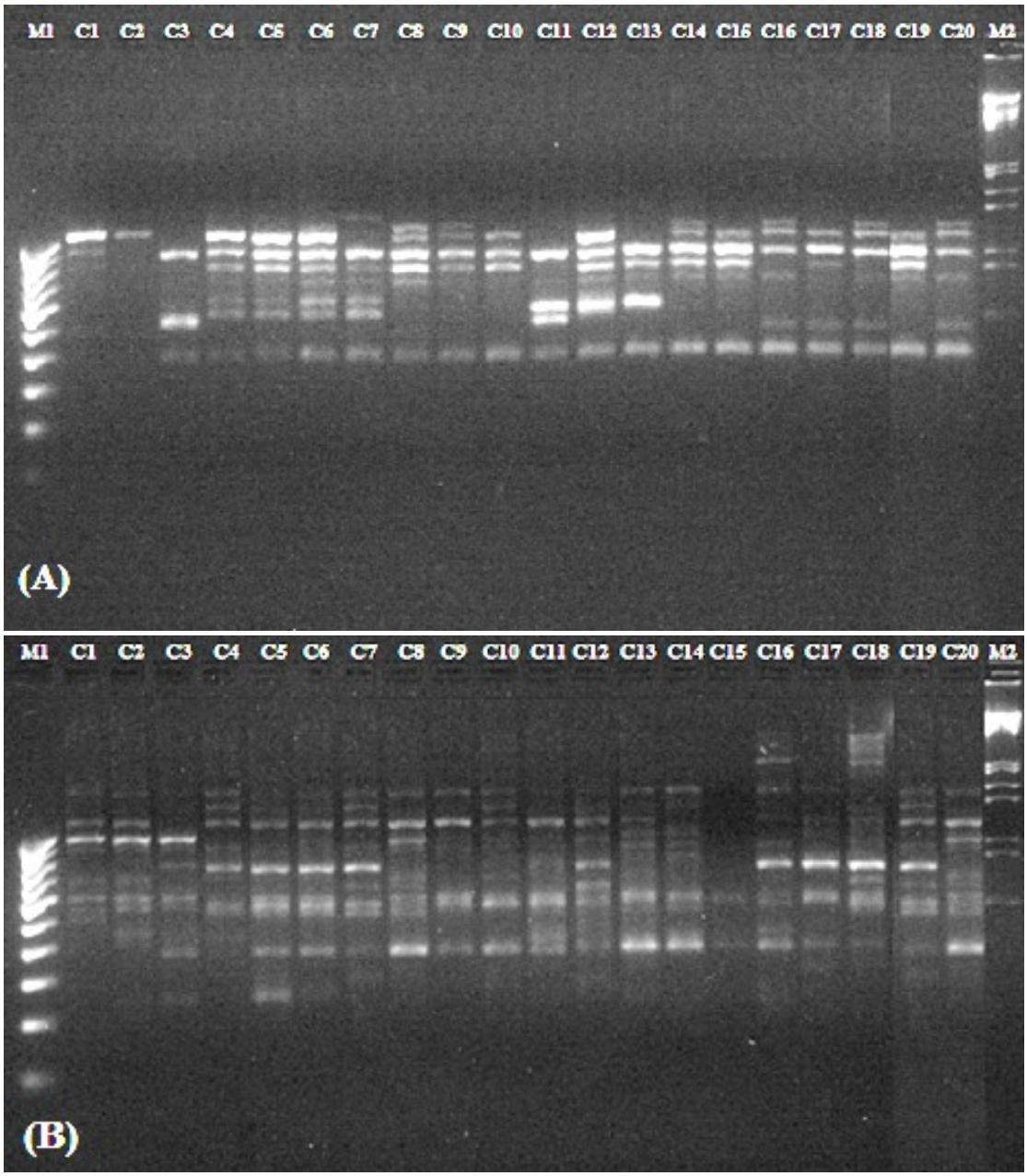


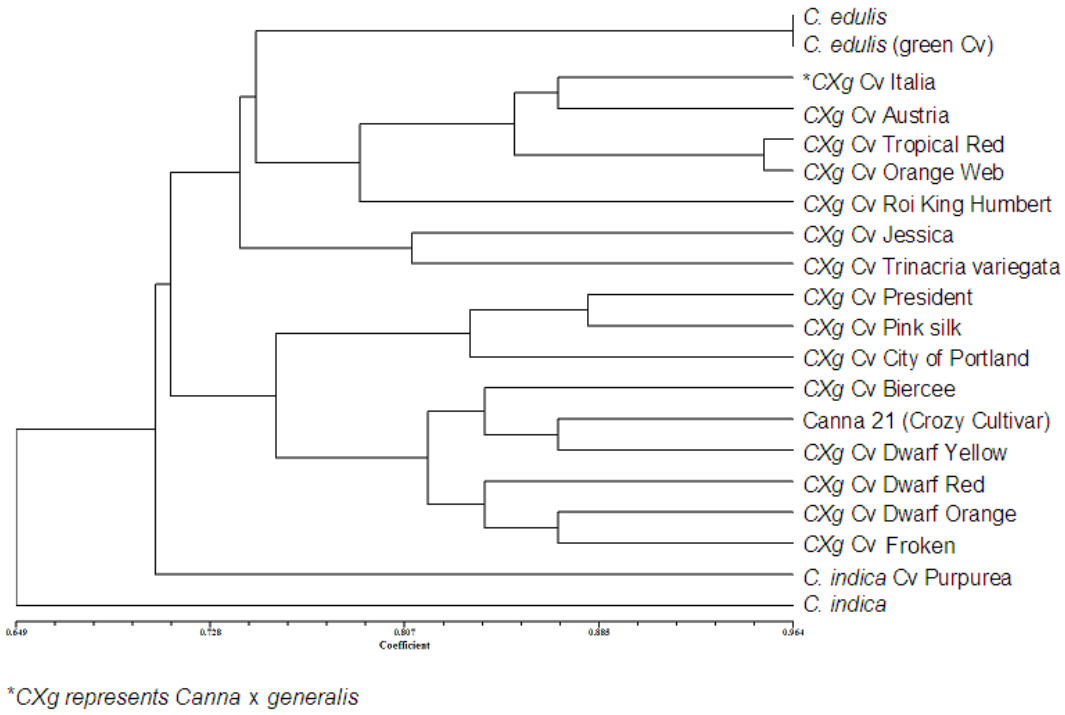
Figure 4.7: A representative of ISSR profile of 20 accessions of Canna generated by (A) UBC815 primer and (B) UBC873 primer. Lane M1: 100 bp molecular marker; Lane C1- C2O: different accessions of Canna under study (Please refer table 4.1 for the name of the species and cultivars); Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

However, in RAPD tree also, I found that out of 4 dwarf varieties, dwarf Yellow cultivar was relatively distant from rest of the three. This showed that the Yellow dwarf variety perhaps evolved differently than that of the other dwarf varieties. The principal coordinate analysis which is based on the similarity coefficients or variance-covariance among the traits validated the dendrogram (Akond *et al*., 2007) (figure 4.9 and 4.10). Both the dendrogram and the principal coordinate analysis showed the similar

Table 4.6: The similarity matrix obtained using Dice coefficient of similarity among the 20 accessions of Canna based on ISSR profiling

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
C1	1.000																			
C2	**0.964**	1.000																		
C3	0.702	0.714	1.000																	
C4	0.750	0.738	0.619	1.000																
C5	0.762	0.750	0.583	0.869	1.000															
C6	0.714	0.702	**0.560**	0.821	0.952	1.000														
C7	0.738	0.750	0.631	0.869	0.881	0.833	1.000													
C8	0.714	0.702	0.607	0.750	0.762	0.786	0.690	1.000												
C9	0.714	0.702	0.607	0.726	0.738	0.738	0.690	0.881	1.000											
C10	0.750	0.738	0.667	0.786	0.774	0.750	0.750	0.845	0.821	1.000										
C11	0.690	0.679	0.702	0.702	0.690	0.667	0.690	0.714	0.738	0.774	1.000									
C12	0.774	0.786	0.643	0.786	0.798	0.774	0.798	0.750	0.798	0.762	0.750	1.000								
C13	0.655	0.667	0.643	0.714	0.726	0.726	0.702	0.774	0.821	0.762	0.679	0.738	1.000							
C14	0.631	0.643	0.643	0.690	0.702	0.702	0.631	0.798	0.774	0.786	0.702	0.762	0.857	1.000						
C15	0.643	0.655	0.607	0.750	0.714	0.714	0.667	0.738	0.738	0.774	0.667	0.726	0.821	0.869	1.000					
C16	0.619	0.631	0.655	0.679	0.690	0.690	0.619	0.738	0.667	0.750	0.690	0.679	0.750	0.869	0.810	1.000				
C17	0.655	0.667	0.690	0.690	0.702	0.702	0.631	0.750	0.750	0.738	0.726	0.714	0.810	0.833	0.869	0.845	1.000			
C18	0.690	0.679	0.679	0.750	0.762	0.738	0.690	0.738	0.714	0.774	0.690	0.726	0.750	0.798	0.857	0.833	0.869	1.000		
C19	0.690	0.702	0.631	0.726	0.714	0.690	0.714	0.714	0.690	0.774	0.714	0.750	0.702	0.726	0.786	0.714	0.726	0.786	1.000	
C20	0.786	0.798	0.750	0.750	0.738	0.714	0.786	0.714	0.714	0.750	0.738	0.798	0.726	0.726	0.738	0.690	0.702	0.738	0.810	1.000

For details on sample ID C1 to C20 please refer table 4.1



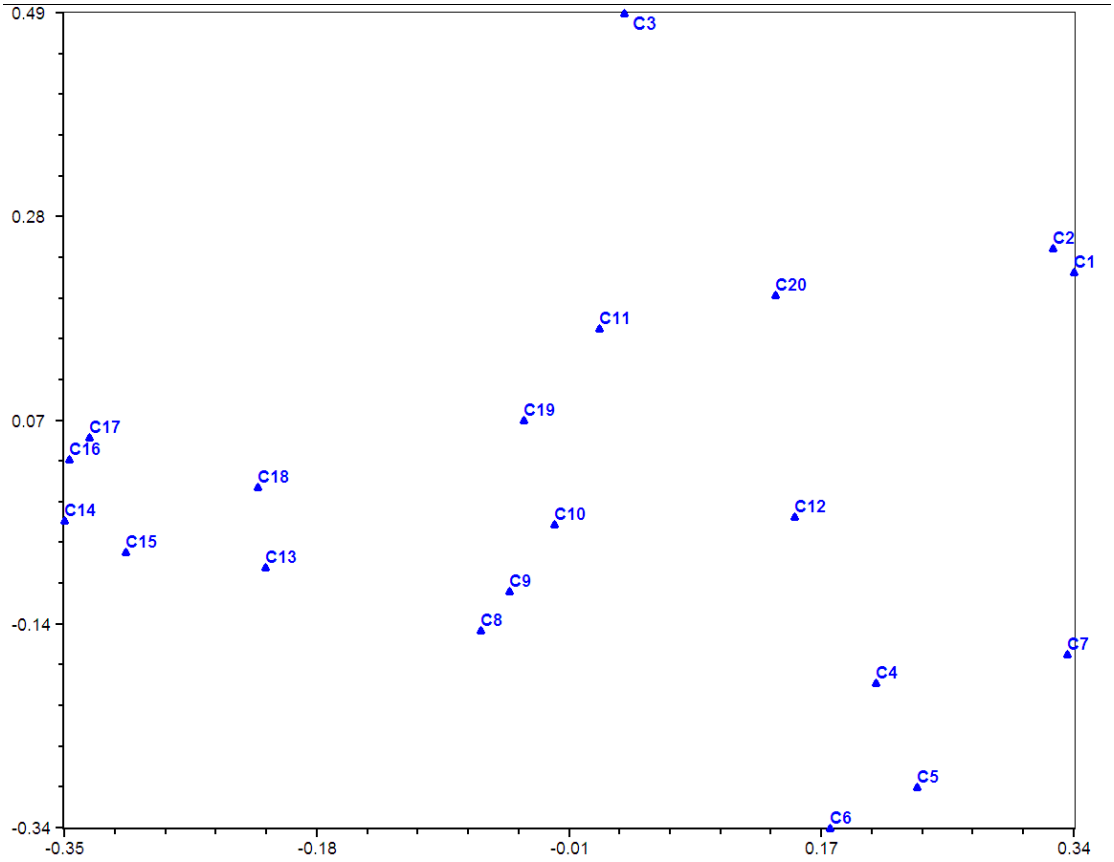
*CXg represents Canna x generalis

Figure 4.8: Dendrogram generated from the cluster analysis of ISSR markers illustrating the genetic relationship among 20 accessions of Canna

cluster. Thus it can be inferred from the ISSR marker study that along with morphological features, Phylogenetic origin of plants could be considered for the correct taxonomic differentiation.

4.2.4 Combined RAPD and ISSR based analysis

Both RAPD and ISSR markers were combined to construct the phylogenetic tree of 20 different accessions of Canna. The similarity coefficients based on 159 RAPD and 93 ISSR loci ranged in between 0.601 to 0.936 (table 4.7). Unlike RAPD and ISSR data, the highest correlation was found in between *Canna* x *generalis* Cv. "Tropical Red" and *Canna* x *generalis* Cv. "Orange Web" (0.936) while the lowest was found between *Canna* x *generalis* Cv. "Dwarf Red" and *Canna edulis* (0.601). Cluster analysis performed from the combined data of both RAPD and ISSR markers generated a dendrogram which is illustrated in figure 4.11. Like the other two trees, in the combined tree also, the elemental species were separated out from the hybrid varieties, giving enough indication that *Canna indica* could be the root. In this tree, the hybrid varieties were clustered in 3 distinct groups, the first group consisted of 2 hybrid varieties namely *Canna* x *generalis* Cv. "Jessica" and "Trinacria Variegata" followed by a cluster of 5cultivars such as *Canna* x



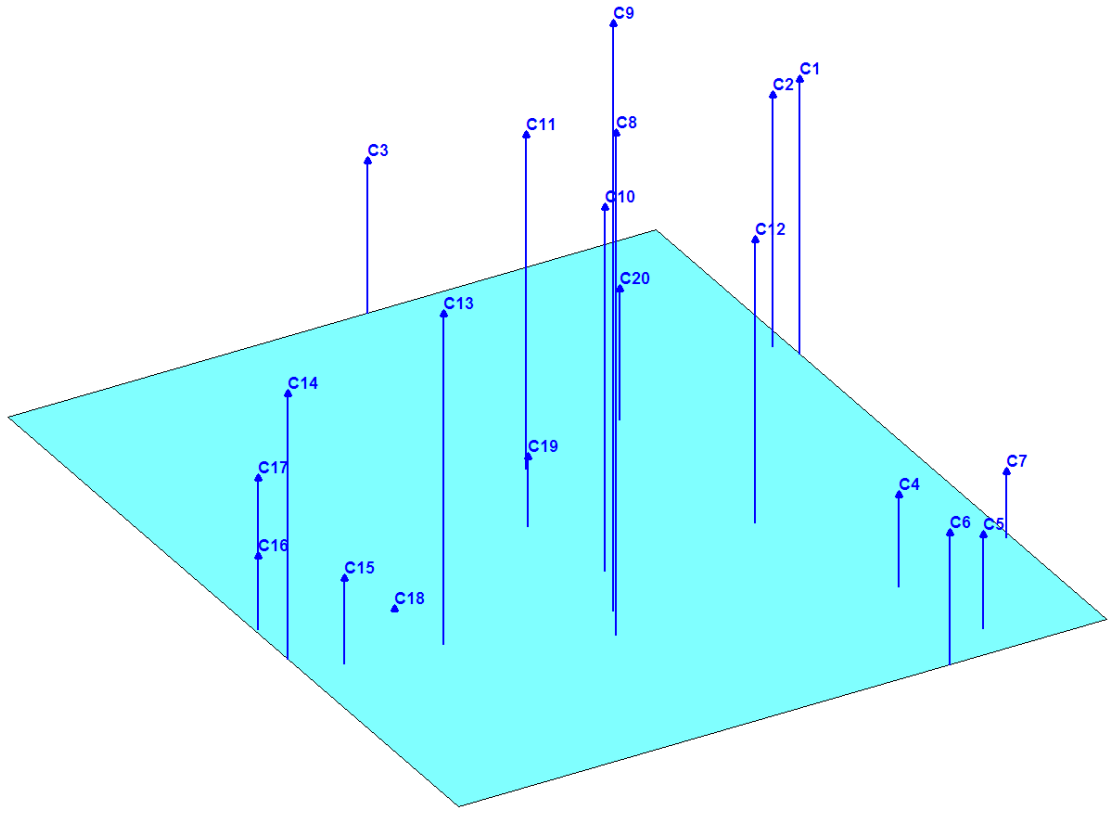


Figure 4.9 & Figure 4.10: Principal coordinate analysis of 20 species and cultivars of Canna based on ISSR analysis data. Figure 4.9- 2-dimensional plot and Figure 4.10- 3-dimensional plot. C1- C2O: different accessions of Canna under study (Please refer table 4.1 for name of the species and cultivars)

generalis Cv. "Italia", "Tropical Red", "Orange Web", "Austria" and "Roi King Humbert" as second cluster. The third and ultimate cluster was a big one with 2 distinct sub-groups consisted of pink or pinkish red variety like *Canna* x *generalis* Cv. "President", "Pink silk" and "City of Portland", where as the other sub-group had a chunk of 4 dwarf varieties flocking together and a sub-group of 2 varieties, of which "Canna 21" was an unknown Canna crozy group cultivar. The correspondence analysis of both 2D and 3D (figure 4.12 and 4.13) was in accordance with the cluster analysis results.

All the three dendrograms (RAPD, ISSR and combined) were similar to each other in many ways, such as grouping of garden cultivars of as a somaclone complex, separation of elemental species from the hybrid cultivars and the closeness of four dwarf cultivars etc. The only difference was that *Canna indica* Cv. "Purpurea" was separately from *Canna indica* in the RAPD and combined tree. However, the RAPD based tree was found to be more similar with combined tree than that of ISSR tree.

The results obtained from RAPD, ISSR and the combination of these two markers revealed that it is possible to separate the elemental species from the horticultural taxa and their cultivars through molecular markers. The phenotypic characters along with phylogenetic origin of plants should be preferred as suitable parameters to prevail over taxonomic complexities. The cultivars cannot be segregated from the somaclone complex just based on the color, height and parental origin, but some significant infra-specific genome relationship might also be considered. This indicates that considerable genetic diversity do exists among the cultivars of popular garden plants. Thus the present study supports the earlier findings that sufficient genetic variability exists within and among the species, cultivars and hybrids of the horticultural species, attributing to the origin, nature and genetic constitution of the parent plant coupled with climatic, edaphic and other environmental factors.

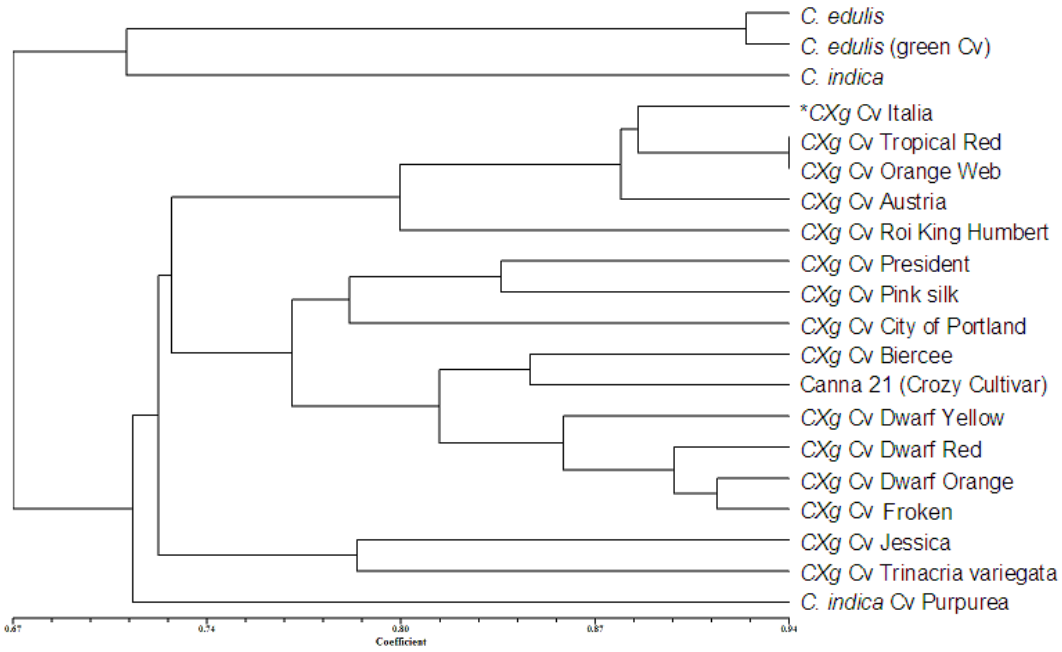
4.2.5 PCR-RFLP analysis

PCR-RFLP is a simple and inexpensive method that plays an important role in studying the genetic diversity of different plant species. This technique was applied in the present study to assess the fingerprinting of different species and cultivars of Canna.

Table 4.7: The similarity matrix obtained using Dice coefficient of similarity among the 20 accessions of Canna based on combined RAPD and ISSR profiling

	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
C1	1.000																			
C2	0.921	1.000																		
C3	0.690	0.729	1.000																	
C4	0.690	0.690	0.665	1.000																
C5	0.700	0.709	0.635	0.901	1.000															
C6	0.665	0.685	0.621	0.867	**0.936**	1.000														
C7	0.675	0.704	0.660	0.877	0.887	0.872	1.000													
C8	0.700	0.719	0.675	0.754	0.764	0.788	0.719	1.000												
C9	0.645	0.685	0.670	0.719	0.739	0.754	0.714	0.837	1.000											
C10	0.700	0.739	0.675	0.724	0.734	0.719	0.700	0.793	0.778	1.000										
C11	0.650	0.700	0.704	0.695	0.695	0.700	0.680	0.714	0.749	0.764	1.000									
C12	0.680	0.719	0.655	0.813	0.803	0.788	0.808	0.744	0.768	0.724	0.744	1.000								
C13	0.631	0.680	0.655	0.704	0.704	0.729	0.700	0.773	0.808	0.773	0.704	0.714	1.000							
C14	0.626	0.675	0.680	0.709	0.700	0.724	0.685	0.788	0.764	0.778	0.729	0.768	0.847	1.000						
C15	0.631	0.680	0.635	0.754	0.724	0.749	0.719	0.754	0.778	0.744	0.695	0.734	0.842	0.818	1.000					
C16	**0.601**	0.660	0.665	0.695	0.695	0.709	0.660	0.734	0.759	0.754	0.714	0.704	0.803	0.828	0.852	1.000				
C17	0.631	0.690	0.695	0.714	0.714	0.729	0.680	0.754	0.798	0.734	0.734	0.724	0.823	0.808	0.872	0.901	1.000			
C18	0.650	0.700	0.685	0.744	0.754	0.759	0.719	0.754	0.788	0.754	0.714	0.734	0.803	0.808	0.852	0.892	0.911	1.000		
C19	0.631	0.680	0.645	0.714	0.714	0.709	0.700	0.714	0.749	0.714	0.704	0.724	0.734	0.739	0.813	0.783	0.783	0.803	1.000	
C20	0.616	0.665	0.680	0.739	0.709	0.695	0.744	0.690	0.685	0.640	0.650	0.749	0.680	0.685	0.719	0.670	0.680	0.690	0.788	1.000

For details on sample ID C1 to C20 please refer table 4.1



*CXg represents Canna x generalis

Figure 4.11: Dendrogram constructed on the basis of data obtained from the combined RAPD and ISSR analysis

4.2.5.1 PCR amplification and agarose gel analysis

In the present study 20 accessions (species and ornamental cultivars) of Canna were subjected to PCR amplification with locus specific primer pair (Tab c 5'-CGAAATCGGTAGACGCTACG-3' and Tab f 5'-ATTTGAACTGGTGACACGAG-3') developed based on the Tab c-f in "Taberlet" (TrnL-TrnF) region of the chloroplast genome of *Canna* for which the nucleotide information was available with respect to other plant species in the public domain. The Tab c-f in "Taberlet" (TrnL-TrnF) region of the chloroplast genome of Canna was successfully amplified by the primer pair. Using the template DNA, the primer pair generated a single band following PCR of expected length of approximately 1060 bp. The amplified product is shown in figure 4.14.

4.2.5.2 PCR product restriction digestion and agarose gel analysis

The PCR product obtained from the primer pair Tab c-f were subjected to restriction digestion using five different restriction enzymes like *Taq*I, *Alu*I, *Hinf*I, *Hae*III and *Msp*I to examine the degree of genetic variation among different species and hybrids of Canna. Three restriction enzymes i.e. *Taq*I, *Alu*I, *Hinf*I digested the PCR product

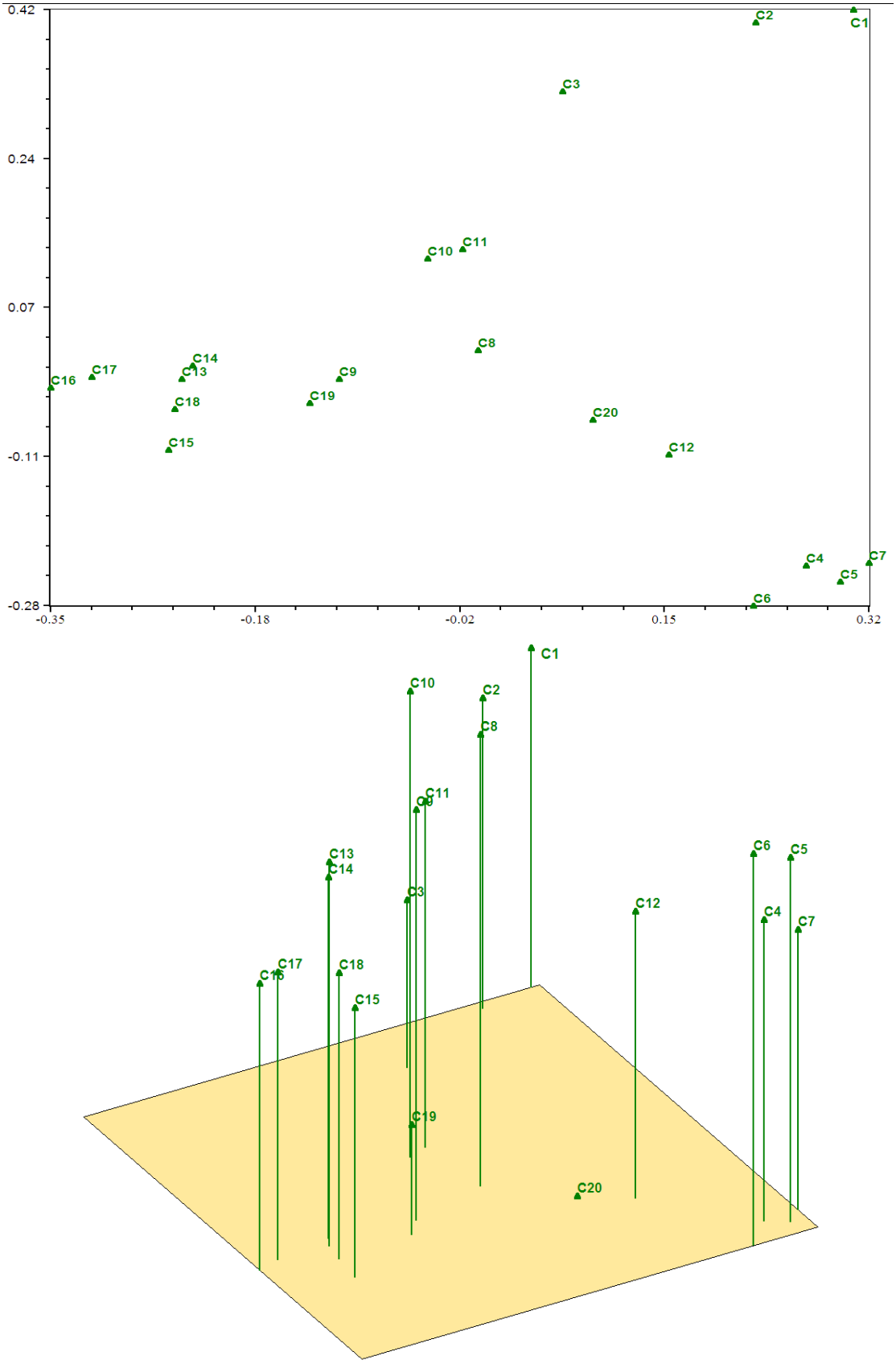


Figure 4.12 & Figure 4.13: Principal coordinate analysis of 20 accessions of Canna based on combined RAPD and ISSR analysis data. Figure 4.12- 2-dimensional plot and Figure 4.13- 3-dimensional plot. C1- C2O: different accessions of Canna under study (Please refer table 4.1 for name of the species and cultivars)

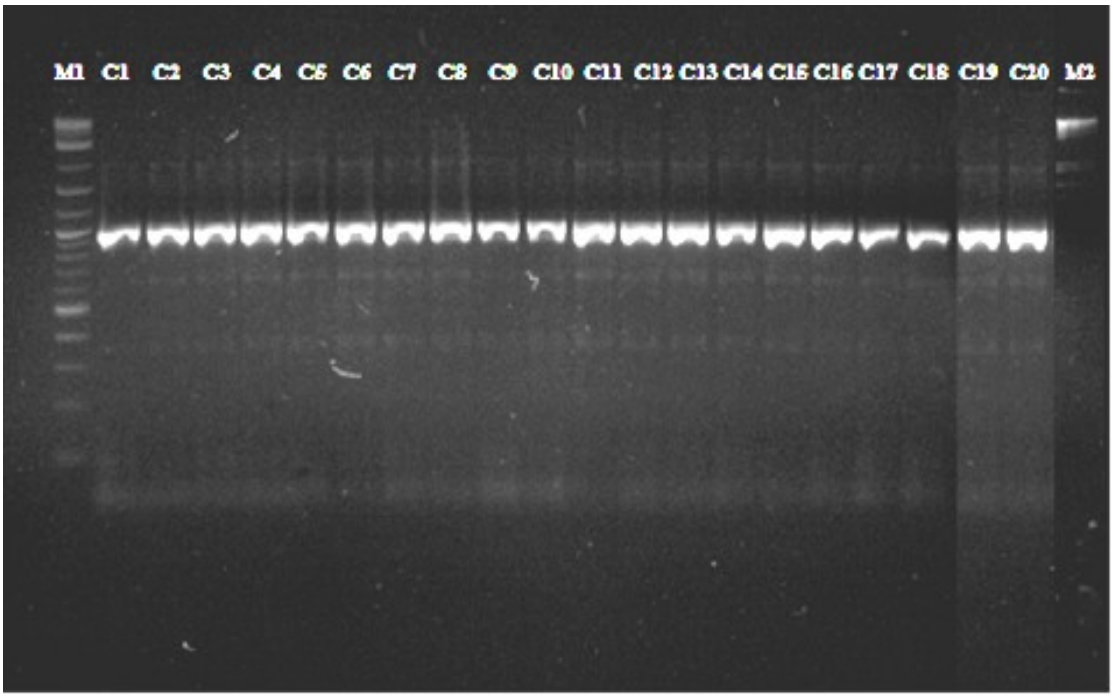


Figure 4.14: Amplification of different Canna accessions with Tab c-f (TrnL-TrnF) primer. Lane M1: 100 bp molecular marker; Lane C1- C2O: different accessions of Canna listed in table 4.1; Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

and generated specific banding pattern, while other two failed to digest the same product. All the bands produced by the above three restriction enzymes were monomorphic in nature. The restriction enzyme *Taq*I produced bands between 367-462 bp, *Alu*I 307-1060 bp and *Hinf*I 162-311 bp. The result obtained from restriction digestion with the enzyme *Taq*I, *Alu*I and *Hinf*I is depicted in figure 4.15. However the chloroplast DNA of the different samples analyzed showed no variation among them. Thus, it can be concluded that the chloroplast genome of different species and cultivars of Canna is highly conserved (Squirrell *et al.*, 2002). Therefore, further statistical analysis was not performed.

4.2.6 Taberlet region sequence anylysis

Four different species/ cultivars of Canna (two elemental species and two ornamental cultivars) were sequenced from Chromous Biotech Pvt. Ltd, Bangalore for both the forward and reverse primers individually. The sequencing resulted in an average of 790 bp for each reaction. In the present study the nucleotide BLAST was performed for each of the sequence obtained to find out the homology with the sequences already present in the GenBank. The nucleotide BLAST showed 96 to 100% identity with Canna sequence already available in

the GenBank. After authentication, sequences were submitted to the GenBank. The list of 4 different samples of Canna along with their GenBank accession number is given in table 4.8.

4.2.7 Comparative account of DNA fingerprinting studies

A detailed DNA fingerprinting study was conducted by using various molecular techniques like RAPD, PCR -RFLP (trnL-trnF gene) and ISSR markers. Of the above three techniques, RAPD and ISSR markers proved to be efficient in revealing polymorphism among 20 different species and cultivars of Canna under study. The eighteen RAPD primers (table 4.3) and ten ISSR primers (table 4.5) revealing 89.93% and 88.17% polymorphism respectively, may prove RAPD techniques to be slightly better molecular marker than that of ISSR. These two markers may provide a cheap, rapid and effective means to evaluate the genetic diversity among different Canna variety and helped in overcoming the taxonomic complexities in a simpler manner. PCR -RFLP technique using trnL-trnF region of the 20 accessions of Canna could not reveal any considerable polymorphism, thus it may be concluded that trnL-trnF region of the genome may not be a good candidate for the study of Canna diversity. However, PCR-RFLP of trnL-trnF region of chloroplast genome of Canna have provided us with a very good data i.e., sequences of 4 accessions of Canna (2 elemental species and 2 ornamental cultivars) under study can be helpful in the conservation of Canna

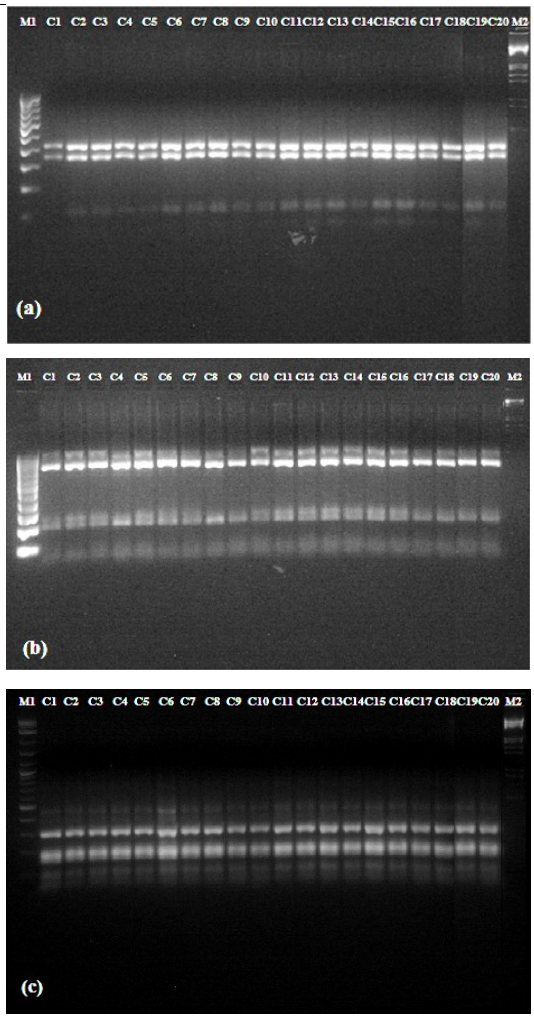


Figure 4.15: Restriction digestion products of Trn L-Trn F region of chloroplast genome. (a) *Taq*I; (b) *Alu*I and (c) *Hinf*I. Lane M1: 100 bp molecular marker; Lane C1- C2O: different accessions of Canna listed in table 4.1; Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

Table 4.8: Canna species/cultivars with GenBank accession numbers for trnL-trnF region

Sl. No.	Plant species	GenBank Accession number
1.	*Canna edulis* (TabC)	KC404813
2.	*Canna edulis* (TabF)	KC404816
3.	*Canna indica* (TabC)	KC404800
4.	*Canna indica* (TabF)	KC404806
5.	*Canna* x *generalis* Cv. "Italia" (TabC)	KC404805
6.	*Canna* x *generalis* Cv. "Italia" (TabF)	KC404820
7.	*Canna indica* Cv. "Purpurea" (TabC)	KC404802
8.	*Canna indica* Cv. "Purpurea" (TabF)	KC404824

germplasm and will also help other researchers working in this particular field. Thus, overall DNA fingerprinting study of Canna cultivars have generated lots of new markers for identification of Canna and can prove to be effective and promising in assessing genetic variations among the ornamental Cannas cultivars collected from various places of West Bengal and Orissa.

4.2.8 Sequence comparison and phylogenetic tree analysis

In the phylogenetic tree (figure 4.16) constructed with Clustal Omega and Phylip 3.69 programme of taberlet region of various members of Zingiberales, we kept *Musa acuminata* as the root of the tree, because *Musa* is the type genus of family Musaceae and Musaceae is one of the oldest families of Zingiberales. The next branch to emerge was that of the *Ensete ventricosum*, which is the another member of Musaceae in one hand, from which the members of Marantaceae appeared. On the other hand, another branch of Musaceae given rise to two distinct clades, one consisted of Strelitziaceae, Lowiaceae and Heliconiaceae and the other consisted of Zingiberaceae and Cannaceae. The salient feature of the tree was that it could recover all the major families intact. Members of families like Musaceae, Zingiberaceae, Cannaceae, Marantaceae, Strelitziaceae etc. were flocked together. The sequences of different Canna varieties done by us were perfectly placed with other sequences (Canna) retrieved from the public domain. In Zingiberaceae we took *Alpinia galangal*, which is a member of Alpinioideae sub family and seven other genera of sub family Zingiberoideae. All the seven genera of Zingiberoideae were appeared together, where as, it has been found that *Alpinia galangal* separated out from Zingiberoideae at a later stage of evolution, however the separation is distinct as evident from the boot strap

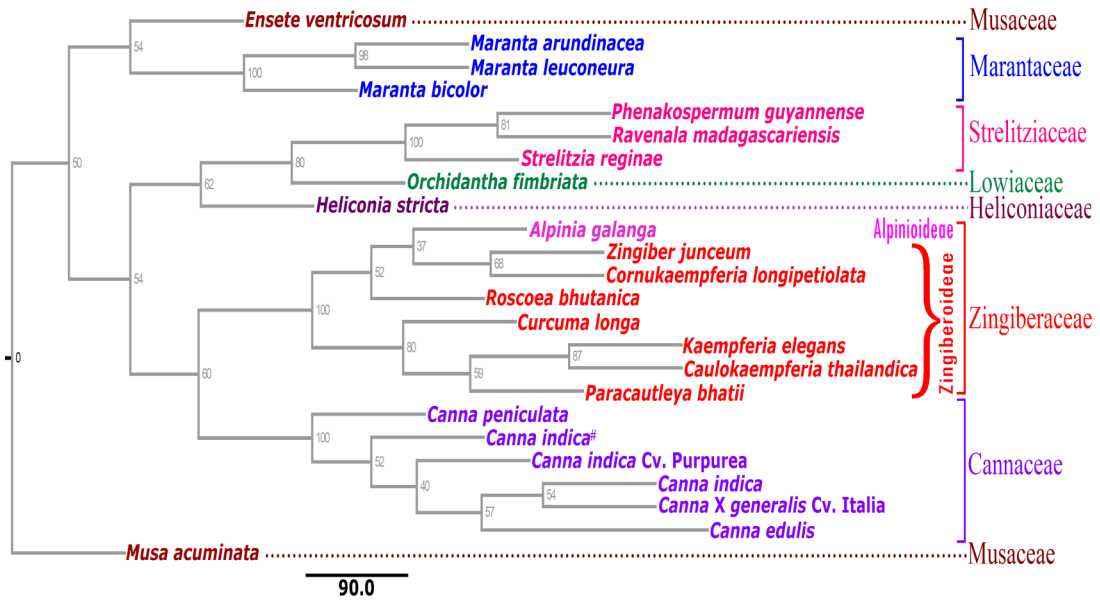


Figure 4.16: Most parsimonious tree (neighbour joining method) showing the relationship of Taberlet region of different Canna cultivars under study with GenBank published sequences of some members of Zingiberales. The tree indicates the phylogenetic evolution of different families of Zingiberales including Cannaceae. Numbers at nodes indicate the bootstrap values

value (37) of the branch.

The only deviation in our tree from the conventional concept of closeness of different families of Zingiberales, was the position of Marantaceae. In our tree the members of Marantaceae were very closely placed with Musaceae, with *Ensete ventricosum* to be précised than that of Cannaceae. Nevertheless the tree was constructed with only one locus of the chloroplast genome of various members of Zingiberales and further study was required with other loci to conclusively determine the position of Marantaceae.

4.3 Antioxidative diversity

Cannas are known to be ornamental plants having spectacular flowers. Besides, some of them tickles our test buds as they are known to be edible and mainly consumed by the people of South eastern countries of Asia (Tanaka, 2004) and Indian tribes like Lepchas, Bhutias and Nepalis (Sekar and Mariappan, 2007). Though different parts of the plants like rhizomes, leaves and seeds are used as medicines, there is still scarcity of information related to the antioxidative properties of different Cannas. So here is an attempt to assess the *in vitro* antioxidant activities of different species and cultivars of Canna to determine the antioxidative diversity among them. Further, the separation and isolation of antioxidant molecules of edible Canna variety was performed

to establish Canna as a medicinal plant.

In the present study, the antioxidant diversity among different Canna accessions was determined on the basis of two parameters i.e. temperature changes and solvent types. The correlation of phytochemical characteristics and antioxidative properties of rhizome were taken up firstly through thermal changes of the aqueous extracts i.e. cold aqueous (CAE) and hot aqueous (HAE) extracts and secondly in two different solvent extracts i.e. in aqueous (HAE) and methanolic (ME) extracts. Separation and isolation of antioxidant molecules of *Canna edulis* was performed on the basis of their percolation in various polar and non polar solvents through silica gel column chromatography.

4.3.1 Total phenol content

The total phenol content of cold aqueous, hot aqueous and methanolic extracts of different species and cultivars of Canna rhizome was obtained from the regression equation (R) for the calibration curve of gallic acid and expressed as gallic acid equivalent (GAE). The summary of the total phenol content in the tested extracts are depicted in figure 4.17 and 4.18. The concentration of phenols in the experimented samples (CAE and HAE) ranged between 22.67 to 98.33 mg GAE/g. The total phenol was found to be highest in hot rhizome extract of *Canna indica* Linn, while the lowest was recorded in *Canna edulis* Green cultivar. Interestingly, it was found that, hot extracts of all most all the cultivars had more phenolics than that of cold extracts except for *Canna* x *generalis* Cv. "Orange Web", where cold extract was proved to be better than hot extract. The release of low molecular weight phenolic compounds due to heat treatment may be responsible for increase in the total phenol in hot extract (Xu *et al.*, 2007).

Further on the basis of solvent types, total phenol was higher in methanolic extracts as compared to aqueous extracts. The concentration of phenol in the examined extracts (HAE and ME) was found between 31.33 mg GAE/g in *Canna edulis* Green cultivar to 158.22 mg GAE/g in *Canna indica*. It was observed that polarity level of the solvent played a key role in the solubility of the phenolic compounds. Higher polyphenols in methanolic extract might be due to the solubility of less polar phenolic compounds in the particular solvent used for extraction process (Lapornic *et al*., 2005 and

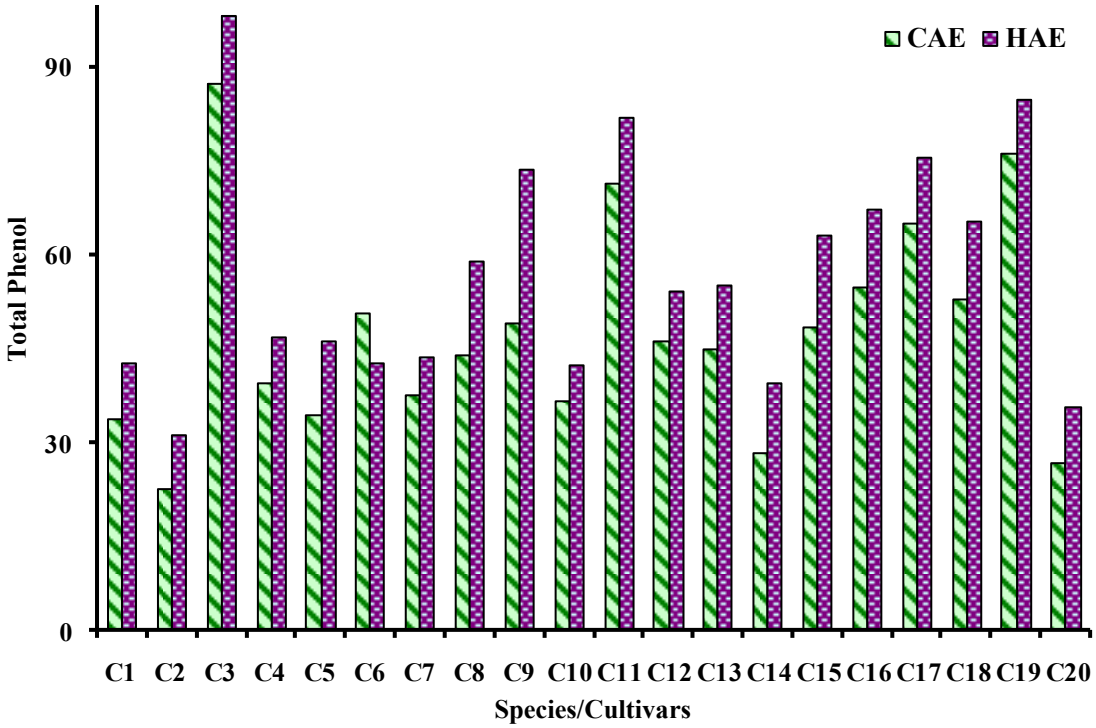


Figure 4.17: Total Phenols of Canna rhizome extract; CAE: Cold aqueous extract, HAE: Hot aqueous extract. C1- C2O: different accessions of Canna under study (Please refer table 4.1 for name of the species and cultivars)

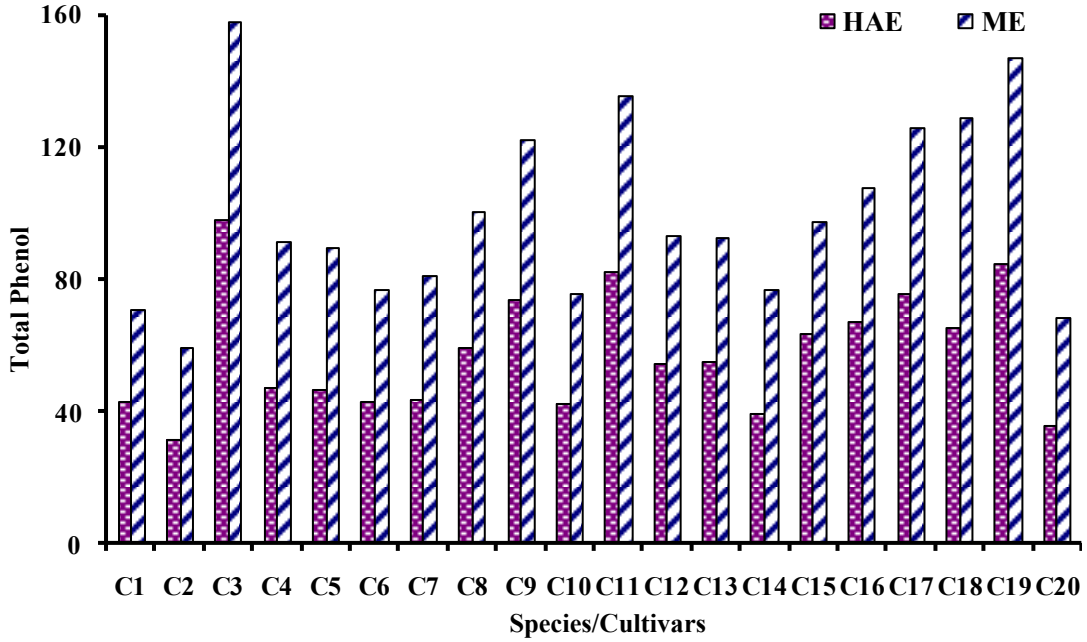


Figure 4.18: Total Phenols of Canna rhizome extract; HAE: Hot aqueous extract, ME: Methanolic extract. (Please refer table 4.1 for name of the species and cultivars)

Arabshahi-Delouee and Urooj, 2007).

It can be inferred that phenolic extraction depends on heat treatment and polarity level of the solvent. In the present study, the phenolic compounds have hydrophobic properties and thus, percolated best in methanol than other

aqueous solvents.

4.3.2 Total flavonoid content

The concentration of flavonoids of CAE, HAE and ME of different species and cultivars of Canna was expressed in terms of quercetin equivalent (QE). Total flavonoids in the tested samples (CAE and HAE) ranged in between 14.32 to 51.22 mg QE/g (figure 4.19). Here also we found that hot extracts exhibited higher flavonoids than cold extracts. But in case of *Canna* x *generalis* Cv. "Orange Web", it was seen that cold extract had higher flavonoids as compared to hot extract. The total flavonoid was found to be highest in hot rhizome extract of *Canna indica*, while the lowest was recorded in *Canna edulis* Green cultivar. The increase in total flavonoids of rhizome extracts after heat treatment may be due to disruption of cell wall, which helps in release of flavonoids from the cell matrix (Choi *et al.*, 2006)

The determination of total flavonoid contents of all the Canna accessions were also tested on the basis of polarity level of different solvent types which was ranging in between 19.84 to 65.02 mg QE/g (figure 4.20). It was found that ME had higher flavonoids than that of HAE. As of phenol, highest concentration of flavonoid was exhibited by *Canna indica* where as lowest was seen in *Canna edulis* Green cultivar. Higher level of flavonoids in methanolic extracts can be attributed to the fact that methanol is less polar than water and thus has the potential to dissolve the less polar flavonoids and polyphenols from the cell wall of the plant (Lapornic *et al*., 2005).

Thus it can be suggested that cold water was least preferable for flavonoid extraction, where as methanol was proved to be the best among the tested solvents.

4.3.3 Total Antioxidant Activity

4.3.3.1 DPPH (2,2-Diphenyl-1-picrylhydrazyl) Method

The rhizome extracts (CAE, HAE and ME) of all the Canna cultivars were tested for their antioxidant properties to determine their power to scavenge reactive oxygen species by quenching DPPH radical (figure 4.21 and 4.22). While testing DPPH radical scavenging activity by taking temperature as a parameter, it was found that the HAE exhibited higher antioxidant than the CAE. The highest scavenging activity of 73.04% was noted in the HAE of *Canna indica* followed by 71.45%,

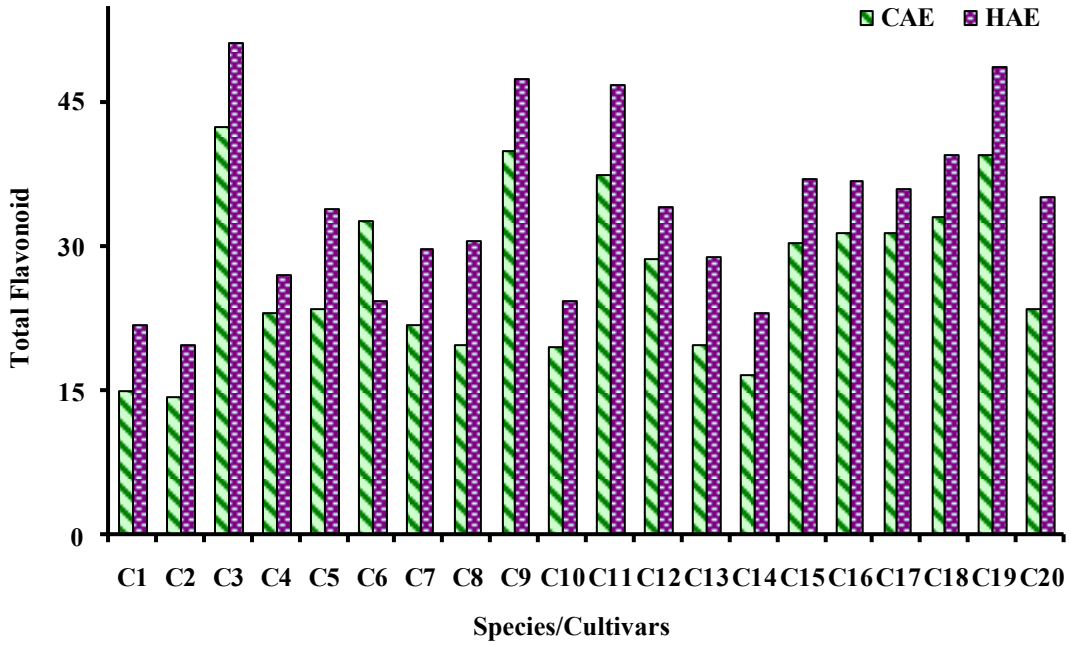


Figure 4.19: Total Flavonoids of Canna rhizome extract; CAE: Cold aqueous extract, HAE: Hot aqueous extract. (Please refer table 4.1 for name of the species and cultivars)

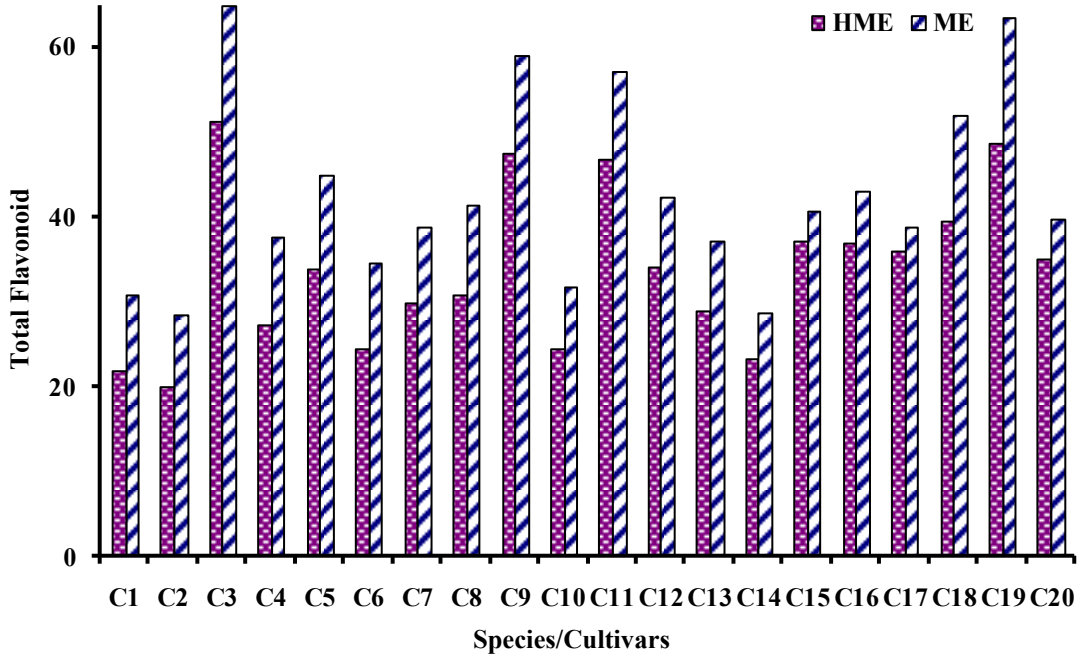


Figure 4.20: Total Flavonoids of Canna rhizome extract; HAE: Hot aqueous extract, ME: Methanolic extract. (Please refer table 4.1 for name of the species and cultivars)

70.14% and 70.04% in *Canna* x *generalis* Cv. "Jessica", *Canna* x *generalis* Cv. "Pink Silk" and *Canna indica* Cv. "Purpurea". Lowest activity was found in cold extract of *Canna* x *generalis* Cv. "Trinacria Variegata" (32.06%). The high level of polyphenolic constituents in Canna can

be attributed for its increased antioxidant activity during the DPPH assay. Specifically, hot rhizome extract having large amount of polyphenols is responsible for higher antioxidant activities (Rice-Evens *et al*., 1997).

On the basis of polarity level, methanolic extract was found to have higher DPPH scavenging activity than that of aqueous extract. DPPH antiradical activity of both HAE and ME was ranging in between 43.98% (*Canna* x *generalis* Cv. "Trinacria Variegata") to 84.96% (*Canna indica*). Elevated levels of the polyphenolic compounds in the methanolic extract may attribute to the higher antioxidant activity (Chueng *et al*., 2003; Arabshahi-Delouee and Urooj, 2007 and Alothman *et al.*, 2009).

Thus it can be concluded that methanol was the best solvent for assessing DPPH radical scavenging activity among different Canna cultivars.

4.3.3.2 Ferric reducing power (FRP) assay

The electron donation capacity or reducing power of bioactive compounds of plant extract is associated with antioxidant activity (Siddhuraju *et al.*, 2002, Yen *et al.*, 1993). The reducing power of Canna rhizome extract was determined from distinct color changes (i.e. from yellow to green and blue) at 700 nm, depending on the reducing power of the sample concentration. The high absorbance of the reaction mixture indicates high reducing power. The reducing capacity of the various samples is depicted in figure 4.23 and 4.24. FRP assay of the plant extract was determined by the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in presence of the extract and compared to that of ascorbic acid, which is a strong reducing agent (Akinpelu *et al*., 2010). HAE extracts possessed higher value than CAE. *Canna indica* exhibited highest reductive potential (OD_{700}= 0.988), where as lowest was found in cold extracts of *Canna edulis* Green cultivar (OD_{700}=0.112). As HAE contains higher amount of total phenolics, it is a better reducing agent than CAE (Arabshahi-Delouee and Urooj, 2007).

Further on the basis of solvent types, methanolic extracts have higher reductive potential than that of aqueous extract. Highest and lowest reductive ability was found in *Canna indica* and *Canna edulis* Green cultivar respectively. FRP showed the same trend as that of DPPH. Methanolic

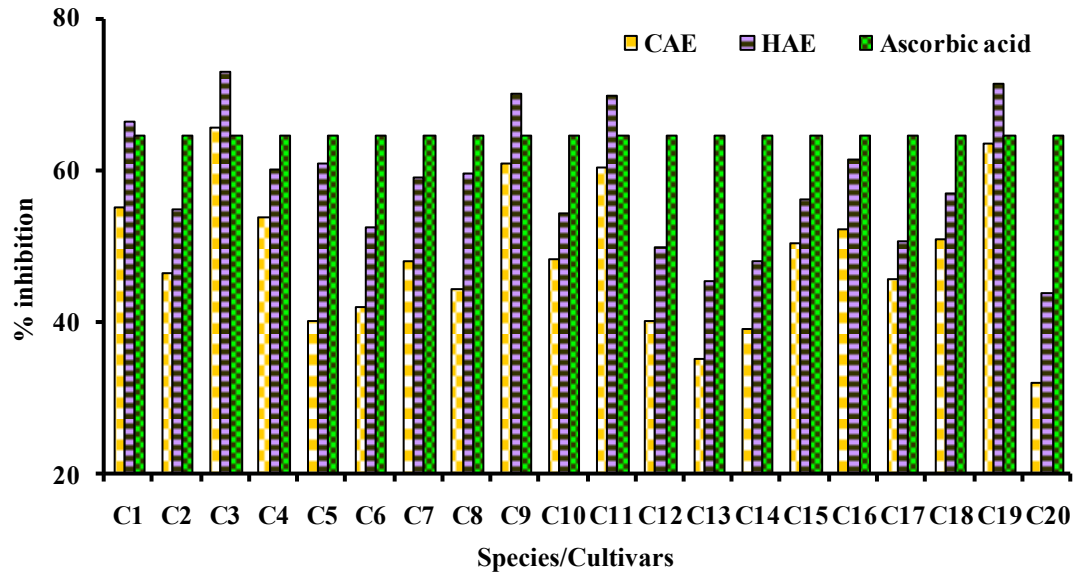


Figure 4.21: DPPH radical scavenging activity of Canna rhizome extract; CAE: Cold aqueous extract, HAE: Hot aqueous extract. (Please refer table 4.1 for name of the species and cultivars)

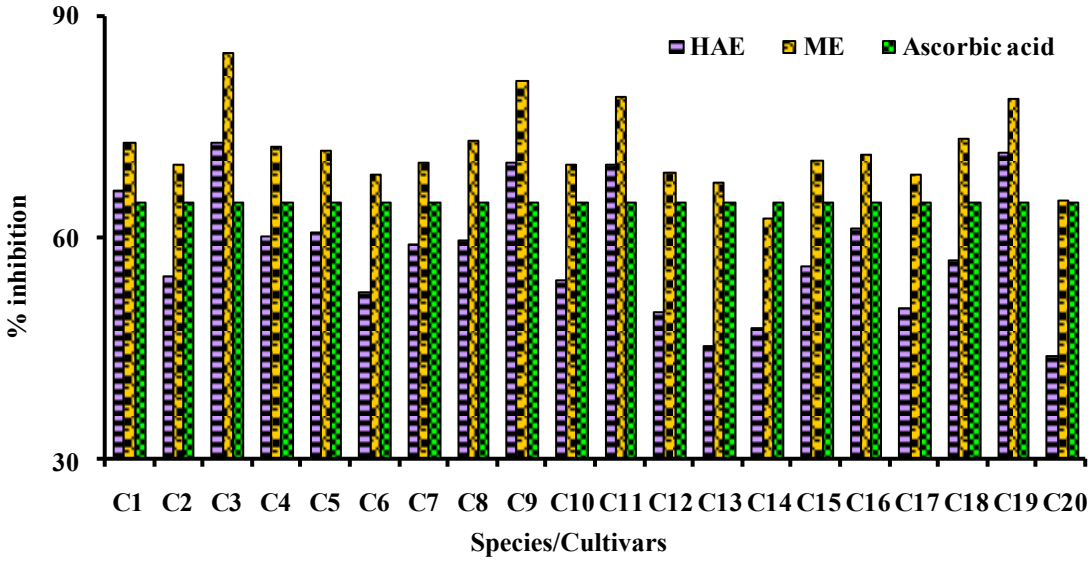


Figure 4.22: DPPH radical scavenging activity of Canna rhizome extract; HAE: Hot aqueous extract, ME: Methanolic extract. (Please refer table 4.1 for name of the species and cultivars)

extract containing highest amount of polyphenolic compounds, was considered as the most potent reducing agent. Similar results were obtained by various authors, where methanolic plant extract was proved as the most effective reducing agent (Arabshahi-Delouee and Urooj, 2007 and Alothman *et al.*, 2009)

Thus it can be concluded that amongst all the tested solvents, methanol is the most effective solvent to assess the reductive potential for Canna rhizome.

4.3.3.3 Hydrogen peroxide scavenging (H_2O_2) activity

Hydrogen peroxide is a reactive

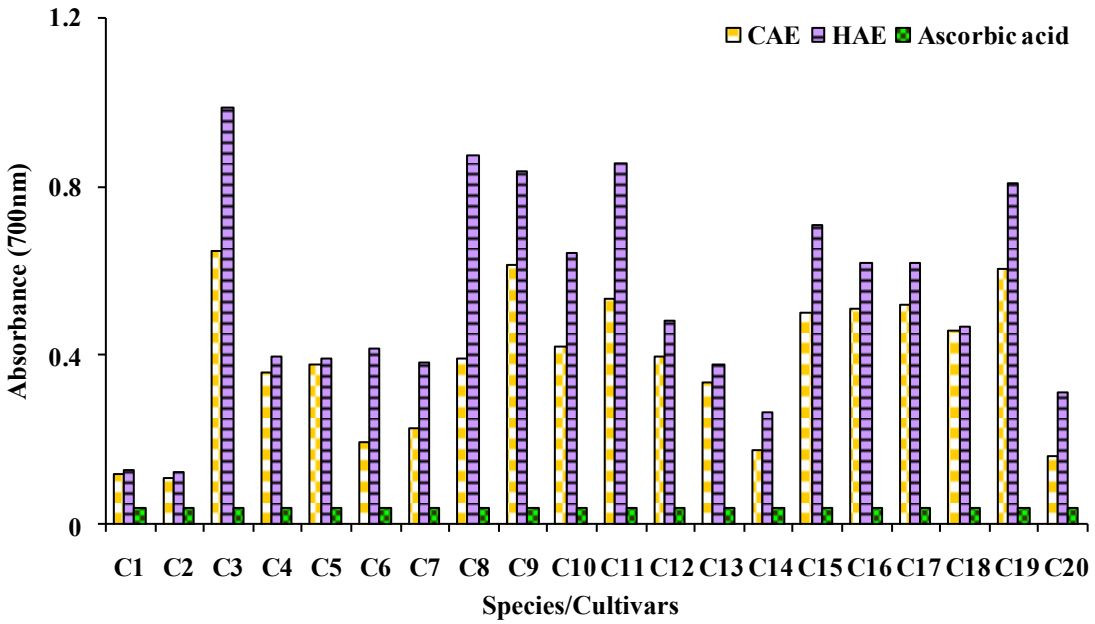


Figure 4.23: Reducing power assay of Canna rhizome extract; CAE: Cold aqueous extract, HAE: Hot aqueous extract. (Please refer table 4.1 for name of the species and cultivars)

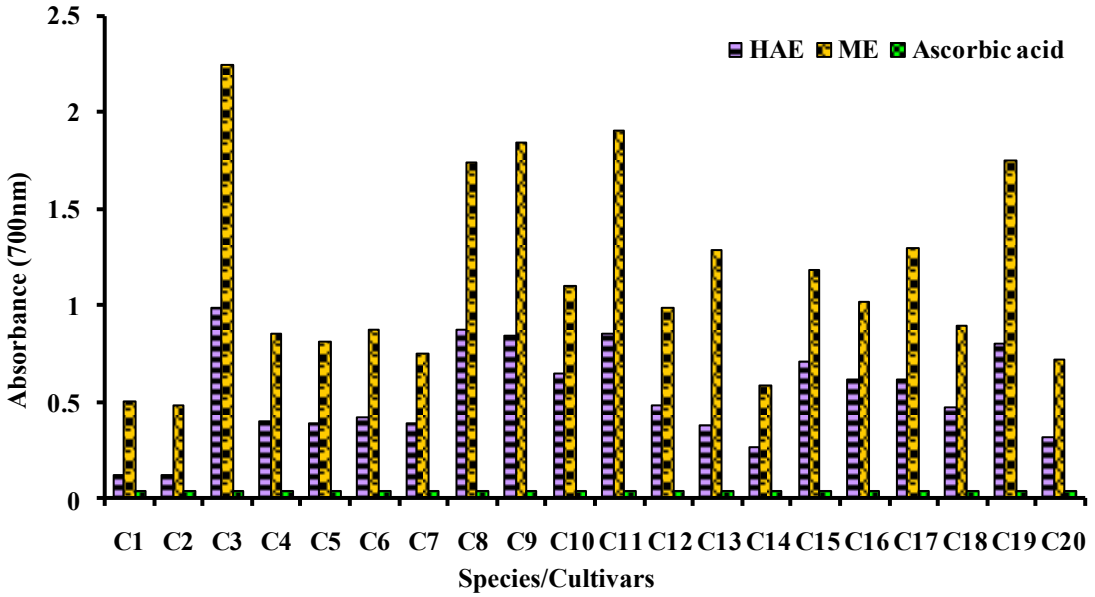


Figure 4.24: Reducing power assay of Canna rhizome extract; HAE: Hot aqueous extract, ME: Methanolic extract. (Please refer table 4.1 for name of the species and cultivars)

nonradical compound but it can sometimes be toxic to cell as it converts into more reactive species like singlet oxygen and hydroxyl radical (Halliwell, 1991). So, the removal of H_2O_2 is very important for antioxidant defense in cell and food system.

Hydrogen peroxide scavenging activity of various Canna samples in cold and hot aqueous extracts was found in a range of 56.52% to 89.11%, which is depicted in figure 4.25. The result was quite different from other antioxidant properties of CAE and HAE of

available Canna cultivars. Here CAE of all the samples had higher H_2O_2 scavenging activity than that of HAE, except for two edible varieties of Canna, where HAE had slightly higher activity than CAE. Highest activity was exhibited in CAE of *Canna* x *generalis* Cv. "Tropical Red", while HAE of *Canna* x *generalis* Cv. "Pink silk" showed lowest scavenging activity. Lower H_2O_2 scavenging activity in HAE may be due to the disruption of the electron donating compounds as a result of heat treatment.

Interestingly, on the basis of polarity level (figure 4.26), it was observed that ME had lower scavenging activity than HAE. However three samples i.e. *Canna* x *generalis* Cv. "Trinacria Variegata" and two dwarf cultivars of the horticultural species namely, "Dwarf red spotted yellow" and "Dwarf yellow" were deviating from the above trend. The percolation of more amounts of polar compounds in water might be the cause of higher H_2O_2 scavenging activity in HAE (Oktay *et al.*, 2003).

Therefore, it is obvious that the hydrogen peroxide scavenging compounds are heat sensitive and hydrophilic in nature. In case of Canna, cold aqueous extract can be suggested as the best solvent for H_2O_2 radical scavenging activity.

On an average methanol was found to be the best solvent for the extraction of antioxidants. Except for H_2O_2 radical scavenging activity, all the above results of *in vitro* antioxidant assay were in correspondence with Atrooz, 2009 and Joshi *et al.*, 2009, who had

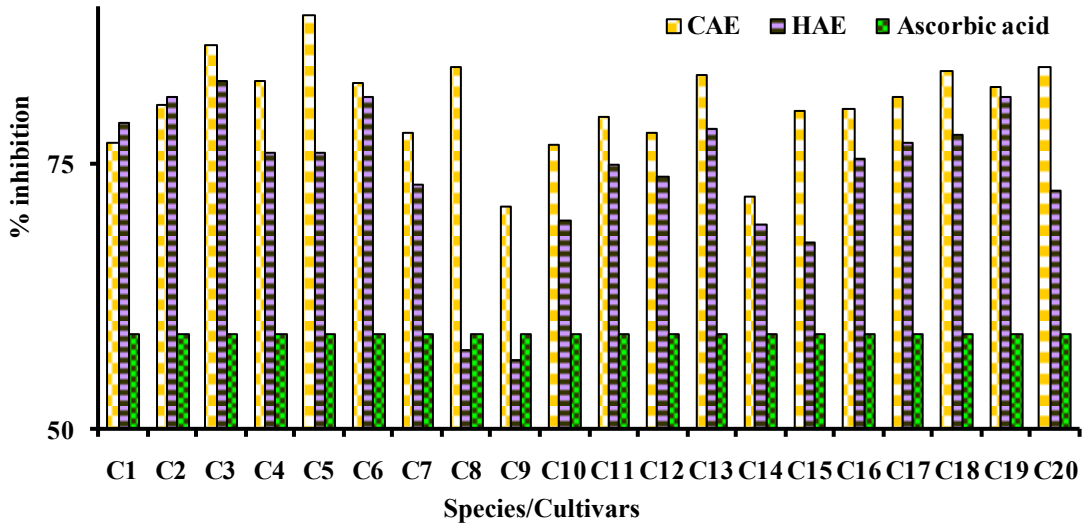


Figure 4.25: Hydrogen peroxide scavenging activity of Canna rhizome extract; CAE: Cold aqueous extract, HAE: Hot aqueous extract. (Please refer table 4.1 for name of the species and cultivars)

observed higher antioxidant activities in the methanolic extracts of different plant parts of Canna.

4.3.4 Correlation Co-efficient

The *in vitro* assay of all the twenty species and cultivars of Canna under study illustrated the presence of high levels of polyphenols and antioxidant activity irrespective of the thermal changes and the solvent type used for the preparation of the rhizome extract. On an average, among the three extract types, methanol was found to be more suitable compared to hot and cold percolated extracts. Oxidative stress can be effectively attenuated via its antioxidant property. Significant correlation was found between all the parameters of CAE, HAE and ME except for hydrogen peroxide scavenging activity (table 4.9). Correlation was highest for phenolic contents of HAE and ME and lowest for hydrogen peroxide scavenging activity of CAE and HAE. The values were 0.976 (significant at $p < 0.01$ level) and 0.457 (significant at $p < 0.05$ level) respectively. Higher correlation of FRP and DPPH scavenging activity between CAE, HAE and ME might be due to the presence of higher amount of total phenolic and flavonoid compounds. Thus, these correlations confirm the phenolic compounds are the major constituents contributing to the antioxidant activities of Canna rhizome.

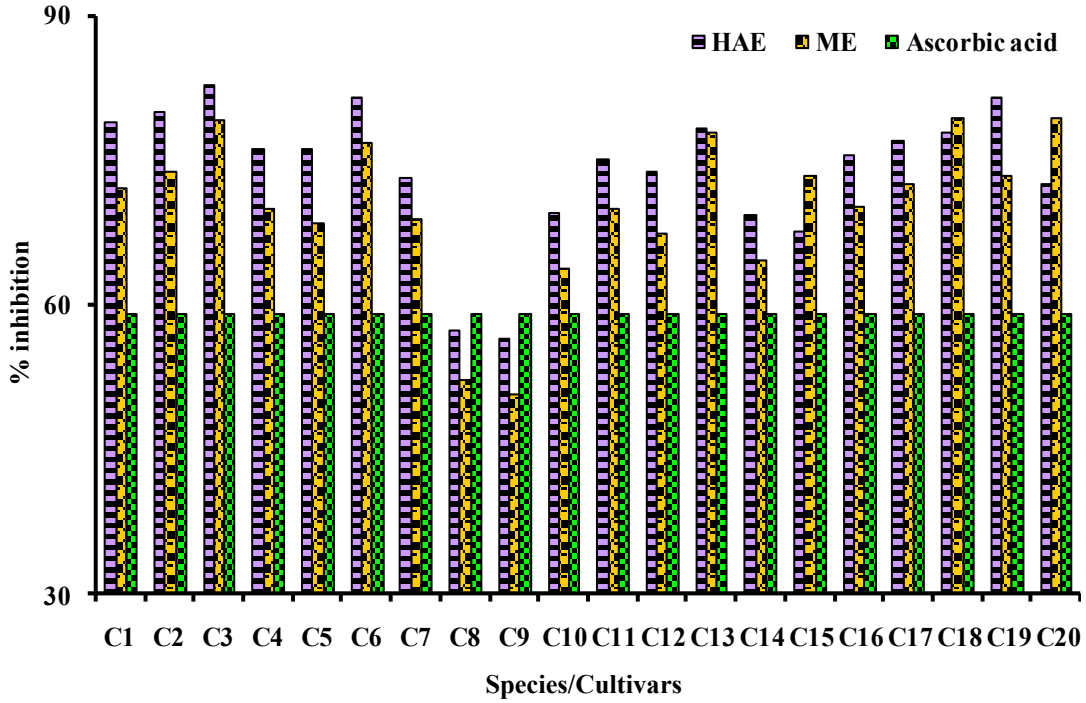


Figure 4.26: Hydrogen peroxide scavenging activity of Canna rhizome extract; HAE: Hot aqueous extract, ME: Methanolic extract. (Please refer table 4.1 for name of the species and cultivars)

4.4 Phytochemical Screening and *In Vitro* Antioxidant Activity

Though moderate antioxidant activity was observed in *Canna edulis* compared to other Canna cultivar varieties, column was run for the above species for extraction of compounds on the basis of polarity, since it is consumed as a staple food in various part of the world including some of the Indian tribes like Lepchas, Bhutias and Nepalis (Sekar and Mariappan, 2007).

4.4.1 Silica gel column chromatography

4.4.1.1 Identification of bioactive fraction

In the present study, 29 solvent fractions were generated. Out of which 6 fractions showed inhibition above 75%. Rest of the fractions showed inhibition less than 25%, except one fraction [ethyl acetate: acetone (3:1)] which showed activity little over 50% (figure 4.27). These six fractions are chloroform: diethyl ether (1:3) [Fr-A], diethyl ether [Fr-B], diethyl ether : ethyl acetate (3:1) [Fr-C], diethyl ether : ethyl acetate (1:1) [Fr-D], diethyl ether : ethyl acetate (1:3) [Fr-E], ethyl acetate [Fr-F] and identified as bioactive fractions of *C. edulis* rhizome. Interestingly these fractions are moderately polar and nonpolar. Six fractions which showed scavenging activity more than 75% were used for further phytochemical screening. Fr-E [Diethyl ether: ethyl acetate (1:3)] showed the maximum inhibition percent (93.08%) for DPPH scavenging activity. The above fraction was diluted to different concentration (100 µg to1000 µg) and subjected to DPPH assay. The most bioactive fraction showed a concentration dependent DPPH antiradical activity with IC_{50} of 658 µg/ml fresh weight basis.

4.4.1.2 Extractive yield of the bioactive fractions

Yield of these bioactive fractions were in the range of 5 mg/ml to 12 mg/ml. These fractions were diluted to 1 mg/ml for further use.

Table 4.9: Correlation co-efficient between the parameters of different rhizome extract of Canna

Parameters	Phenol	Flavonoid	DPPH	FRP	H_2O_2
CAE/HAE	0.949**	0.896**	0.921**	0.889**	0.457*
HAE/ME	0.976**	0.964**	0.92**	0.936**	0.856**

NB: **- Correlation is significant at the 0.01 level (2-tailed) , *- Correlation is significant at the 0.05 level (2-tailed)

4.4.2 Total flavonol and proanthocyanidin contents

The determination of total flavonol contents of the above six fractions showed flavonols ranging from 37.12 mg/ml to 9.92 mg/ml quercetin equivalent per 1000 mg rhizome extract (figure 4.28a). As of DPPH antiradical activity, Fr-E also showed highest flavonol contents (37.12 mg/ml quercetin equivalent per 1000 mg rhizome extract) followed by Fr-F (25.12 mg/ml quercetin equivalent per 1000 mg rhizome extract). We found that total flavonol was found to be decreased with decrease in concentration.

We also studied total proanthocyanidin contents of above six fractions of *Canna* rhizome extract (figure 4.28b). Similar to flavonol contents, Fr-E had highest total proanthocyanidins (i.e. 0.012 mg/catechin/g dry weight) among all the bioactive fractions. Total proanthocyanidins content showed a dose dependent increase with increase in concentration. Fr-F and Fr-A were recorded to contain least amount of total proanthocyanidins (0.0005 mg/ catechin/g dry weight) at 0.25 mg/ml.

In this study, we found, elevated amount of total flavonols and

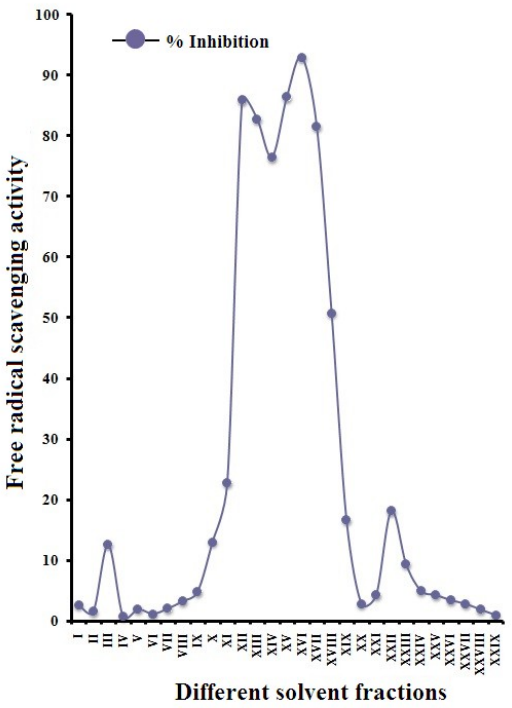


Figure 4.27: DPPH radical scavenging activities (%) of different solvent fractions of *C. edulis* : I- Hexane, II- Hexane : Benzene (3:1), III-Hexane : Benzene (1:1), IV- Hexane : Benzene (1:3), V- Benzene, VI- Benzene : Chloroform (3:1), VII- Benzene : Chloroform (1:1), VIII- Benzene : Chloroform (1:3), IX- Chloroform, X- Chloroform : Diethyl ether (3:1), XI- Chloroform : Diethyl ether (1:1), XII- Chloroform : Diethyl ether (1:3), XII- Diethyl ether, XIV- Diethyl ether : Ethyl acetate (3:1), XV- Diethyl ether : Ethyl acetate (1:1), XVI- Diethyl ether : Ethyl acetate (1:3), XVII- Ethyl acetate, XVII- Ethyl acetate : Acetone (3:1), XIX- Ethyl acetate : Acetone (1:1), XX- Ethyl acetate : Acetone (1:3), XXI- Acetone, XXII- Acetone : Methanol (3:1), XXIII- Acetone : Methanol (1:1), XXIV- Acetone : Methanol (3:1), XXV- Methanol, XXVI- Methanol : Water (3:1), XXVII- Methanol : Water (1:1), XXVIII- Methanol : Water (1:3), XXIX- Water

proanthocyanidin in bioactive Fr-E. The release of high extent of flavanols may be due to the affinity of free hydroxyl group of flavonoid compounds towards ethyl acetate and diethyl ether fractions while, the nucleophilic character of

proanthocyanidins, which have more attraction towards ethyl acetate and diethyl ether for carbocation (an ion with a positively-charged carbon atom) formation may be the cause of its elevation. The results are in line with Termentzi *et al.*, 2008 and Salas *et al.*, 2004.

4.4.3 Free radical inhibition activities

In the present study, we observe the bioactive fractions of *Canna* rhizome inhibit the generation of NO radicals. Figure 4.29a indicates that *Canna* rhizome has nitric oxide scavenging activity. Maximum scavenging activity observed in bioactive Fr-D was 78.41% at a concentration of 1 mg/ml. Here we found that NO scavenging activity decreased with decrease in concentration. Nitric oxide (NO) is an important chemical mediator which involves in the regulation of various biochemical and physiological processes. Excess concentration of NO is associated with several diseases (Mondal *et al.*, 2006). Oxygen reacts with excess amount of nitric oxide to generate nitrite and peroxynitrite anions, which act as free radicals (Cotran *et al.*, 1999). In this experiment, fraction containing diethyl ether and ethyl acetate showed strong scavenging and preventive capacity against NO because of the presence of higher amount of polyphenolic compounds in those fractions (Bahramikia and Yazdanparast, 2008 and Singh *et al.*, 2009).

Hydrogen peroxide reacts with ferrous salt to form hydroxyl radical via Fenton's reaction (Lloyd *et al.*, 1997). The hydroxyl radical (OH^{-}) thus produced may attack the sugar of DNA base causing sugar fragmentation, base loss and DNA strand breakage (Kaneko *et al.*, 1997). In the present study, this hydroxyl radical scavenging activity was shown by all the six bioactive factions (figure 4.29b). This radical scavenging activity showed a dose dependent increase with increase in concentration. The highest inhibition percent was observed in Fr-D (i.e. 39.04% at a concentration of 1 mg/ml). Maximum activity of inhibition of hydroxyl radicals in fractions containing diethyl ether and ethyl acetate may be because of their higher ability to bind iron ion and potential for direct scavenging activity on OH^{-}, which revealed the same results as Verma *et al.*, 2010.

Free radicals induce lipid peroxidation in polyunsaturated lipid rich area like brain and liver (Coyle and Puttfarcken, 1993). We analyzed that different

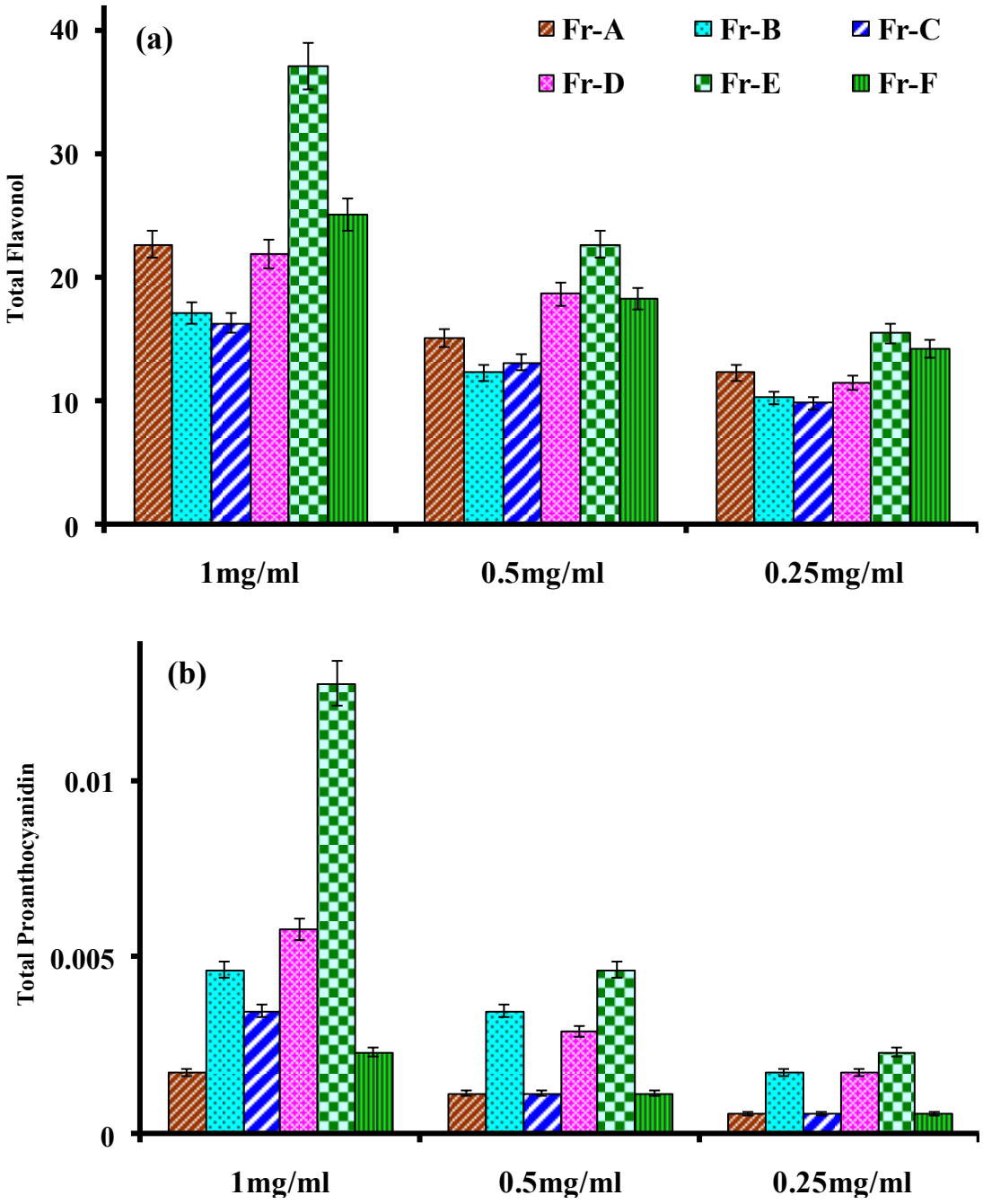


Figure 4.28: **(a)** Total flavonols of bioactive solvent fractions of *C. edulis* **(b)** Total proanthocyanidins of bioactive solvent fractions of *C. edulis*. Here X and Y axes represent Conc. of solvent fractions and amount of total flavonol and proanthocyanides respectively

Canna rhizome fractions showed protection against damage due to lipid peroxides (figure 4.29c). Maximum inhibition was recorded in Fr-F (67.89%) and lowest in Fr-A (8.27%). Here we observed hepatocyte protective activity increases with decrease in concentration. In our study *in vitro* hepatocyte protective activity was induced in goat liver by using $FeSO_4$ and ascorbic acid and it was observed that generation of lipid

peroxides were prevented by bioactive fractions of *Canna edulis* rhizome extract. Maximum inhibition in Fr-F i.e. 67.89% is same as the results obtained by Kang *et al.*, 2011. Lowest antilipid peroxidation activity was observed in Fr-A because of lowest level of polyphenolic compounds among the bioactive fraction (Kviecinski *et al.*, 2011). Thus among the bioactive solvent fractions of *Canna* rhizome lipid peroxidase activity was lower in the less polar chloroform fraction and higher in more polar ethyl acetate fraction.

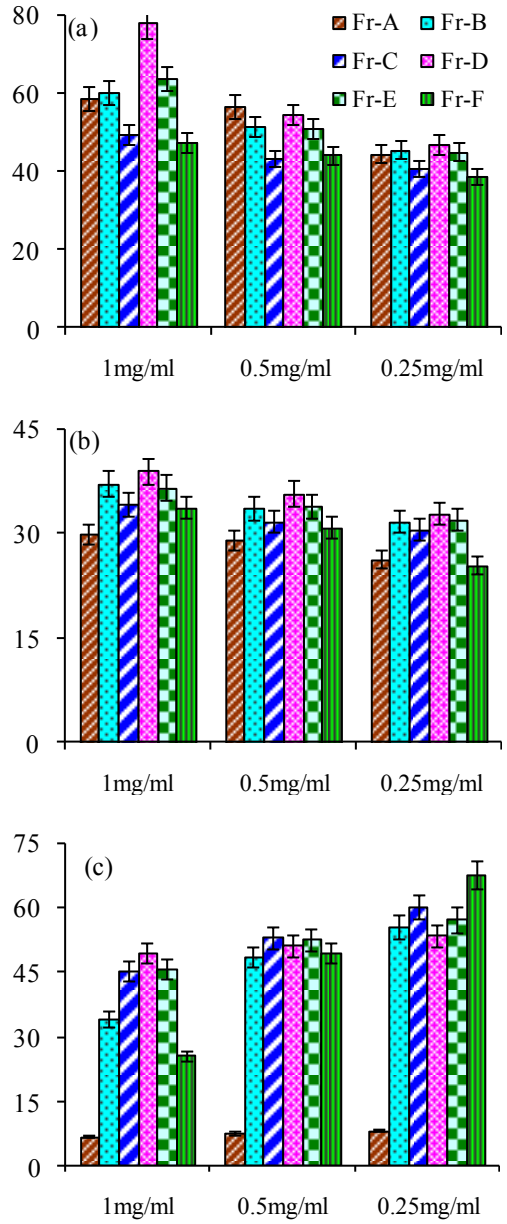


Figure 4.29: **(a)**Nitric oxide scavenging activity of bioactive solvent fractions of *C.edulis* **(b)** Hydroxyl radical scavenging activity of bioactive solvent fractions of *C.edulis* **(c)** Lipid peroxidase activity. Here X and Y axes represent Conc. of solvent fractions and Inhibition percent respectively

4.4.4 Compound detection through TLC

Highly fluorescent bands were viewed in three bioactive fractions like Fr-B, Fr-D and Fr-E under UV light (365 nm). The compounds were separarated in the TLC plate and viewed as described in table 4.10. We analyzed the distribution of polyphenols in different bioactive fractions. It was found that phenolic compounds were distributed from less polar to more polar solvent i.e. chloroform to ethyl acetate fraction where as mixture of flavonoid glycoside or probably triterpene glycoside related compounds were restricted to Fr-E and Fr-F as these bioactive fractions showed their sensitivity against NP/ PEG (Kumar *et al.*, 2000). Therefore, it may be concluded that the polyphenols present in *Canna* rhizome may be responsible for their antiradical scavenging activity. Phenols and flavonoids have

already been reported to act as potent antioxidant in *Alpinia nutants* (Habsah *et al.*, 2003), *Citrus sinensis* (Anagnostopoulou *et al.*, 2006) etc.

We undertook the present study with an aim to make a profile of antioxidant activity of an important edible ornamental plant of sub Himalayan West Bengal which is also used widely as medicinal plants among tribal. We also had an aim to establish this important plant as a medicinal one, scientifically. In conclusion, the result of the present work indicated that, among the different solvent fractions derived from *Canna edulis* rhizome, the more polar fractions like Fr-D, Fr-E and Fr-F possessed highest antioxidant activity and free radical scavenging activity. It was analyzed that high scavenging activity of *Canna* rhizome may be due to the presence of some polyphenolic compounds like phenols, flavonoids, proanthocyanides, glycosides etc. This result also indicated that the antioxidant rich fractions of *Canna* may probably be used in preventing the oxidative deterioration of food. The present findings appear useful in leading to further experiments on the isolation, identification, characterization and structural elucidation of the active constituents that are responsible for relatively high antioxidant activities.

4.5 GC-MS analysis

The identified compounds of the leaves of *C. edulis*, their retention time, Molecular formula, Molecular weight etc. are given in (table 4.11). The results showed the presence of 22,23-Dibromostigmasterol acetate $$ 22,23-Dibromostigmast-5-en-3-yl acetate (21.97%), N-Heptyl-N'-{9-[2-(heptyl-methyl-carbamoyl)-acetylamino]-nonyl}-N-methyl-malonamide (20.89%) and Cholest-5-ene, 3.beta.-chloro- $$ Cholesteryl chloride $$ Cholest-5-ene, 3-chloro-, (3.beta.)- $$

Table 4.10: List of some qualitative characters of spots visualized on TLC plate after chromatographic development with different reagents. Within parenthesis is the band intensity

Reg. / Sol.	Ammonia (NH_3)	Iodine (I_2)	Ferric chloride ($FeCl_3$)	NP/PEG
Fr-A	Violet (+)	Brown (+)	Yellow (++)	–
Fr-B	Violet (+++)	Brown (++)	Yellow (++)	–
Fr-C	Violet(+)	–	Yellow (+)	–
Fr-D	Violet (+++)	Brown (++)	Yellow (++)	Orange (+)
Fr-E	Violet (+++)	Brown (++)	Yellow (+++)	Orange (+++)
Fr-F	Violet(+)	Brown (++)	Yellow (++)	Orange (+++)
Group	Polyphenolics	Unsaturated aromatics	Phenolics	Flavonoid glycosides

NB: Reg.– Reagent, Sol.- Solvent fraction, NP/PEG- Natural Product/ Polyethylene glycol

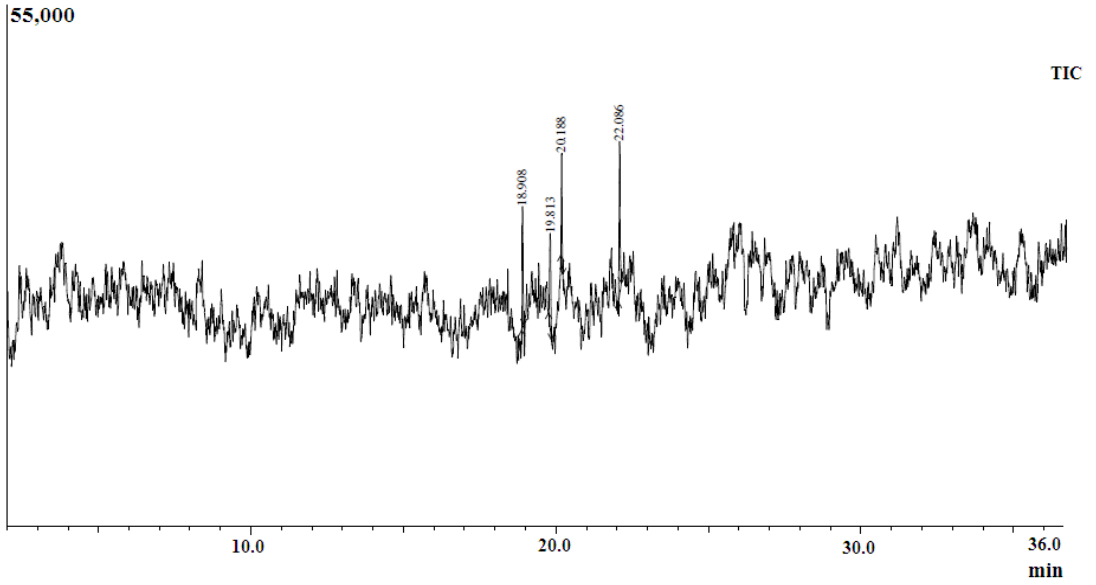


Figure 4.30: GC-MS Chromatogram of methanolic extract of *Canna edulis*

Cholesterol chloride $$ 3.beta.-Chlorocholest-5-ene (34.40%). The spectrum profile of GC-MS confirmed the presence of 3 compounds with retention time 18.908, 20.188 and 22.086 respectively. Besides, one more uncharacterized compound with retention time 19.813 was also identified. The individual fragmentation of the compounds is illustrated in figure 4.30.

In the present study, GC-MS analysis of the methanolic leaf extract of *C. edulis* identified some therapeutically significant components. Specifically the steroidal compound like 22,23-Dibromostigmasterol acetate $$ 22,23-Dibromostigmast-5-en-3-yl acetate ($C_{31}H_{50}Br_2O_2$) is known to possess antibacterial, antifungal properties (Bharat *et al*., 2013). Further, the methyl amine derivative compound i.e.

Table 4.11: Phytocomponents identified in the methanolic leaf extract of *Canna edulis* by GC-MS

Peak	R. Time	Compound	Mol. Form.	Mol. Wt.	Peak%
1	18.908	22,23-Dibromostigmasterol acetate $$ 22,23-Dibromostigmast-5-en-3-yl acetate	$C_{31}H_{50}Br_2O_2$	612	21.97
2	19.813	Unidentified	—	—	22.74
3	20.188	N-Heptyl-N'-{9-[2-(heptyl-methyl-carbamoyl)-acetylamino]-nonyl}-N-methyl-malonamide	$C_{31}H_{60}N_4O_4$	552	20.89
4	22.086	Cholest-5-ene, 3.beta.-chloro- $$ Cholesteryl chloride $$ Cholest-5-ene, 3-chloro-, (3.beta.)- $$ Cholesterol chloride $$ 3.beta.-Chlorocholest-5-ene	$C_{27}H_{45}C_l$	404	34.40

R. Time= Retention time; Mol. Form.= Molecular formula; Mol. Wt.= Molecular Weight

N-Heptyl-N'-{9-[2-(heptyl-methyl-carbamoyl)-acetylamino]-nonyl}-N-methyl-malonamide is proved to be a potent anticoccidal agent by reducing the parasitic activity in avian intestinal lining mainly in poultry industry (Scribner *et al.*, 2008). Anticoccidal activity of these compounds may be because of the inhibition of parasite specific cGMP dependent protein kinase activity (Gurnett *et al.*, 2002 and Donald *et al.*, 2002). As there is a growing awareness in correlating the phytochemical compounds and their biological activities, it is the first report of the presence of biologically significant molecules in *C. edulis* leaf, resolved by GC-MS analysis. Thus, the edible Canna variety may be considered as an important herbal medicine. However, it is also postulated that Dibromostigmasterol acetate has come cytotoxic property. Further studies is required to prove this.

4.6 *In vitro* culture studies

In vitro culture studies of Canna was done to standardize the protocol for regeneration of the whole plantlet in aseptic condition by using different explants and various hormone combinations in different culture media.

4.6.1 Establishment of aseptic culture

Young leaf, rhizome and seeds of healthy fruits were used as explants for *in vitro* culture studies of two different species of Canna (please refer Material and Methods section of *in vitro* culture studies). To manage the fungal and bacterial contamination problem efficiently and to establish aseptic culture, we tried the field collected explants with several disinfectants like extran, ethanol and mercuric chloride ($HgCl_2$) in varying concentration. However, none of them could eliminate the contamination completely alone. We found that a combination of surface sterilants like sterilization with 70% ethanol for 45 sec followed by 0.1% $HgCl_2$ for 1.5 min showed the best result. I didn't have much contamination problem during subculturing and maintaining of explants.

4.6.2 In vitro *regeneration*

4.6.2.1 Effect of medium and plant growth regulators on callusing, shooting and germination

The basal medium was used along with 3% sucrose, 0.7% agar, and 0.17% NH_4NO_3 and different plant growth regulators like BAP, NAA and 2,4-D. Hormone less control media was also

Table 4.12**:** Effect of basal medium and plant growth regulators on callus initiation, shooting and germination

Media	PGR	Explant		
		Leaf	Rhizome	Seed
MS	Control	No response	No response	Germination
	BAP	No response	Shooting	Germination
	BAP + NAA	No response	No response	Germination
	2,4-D	No response	No response	No response
BM	Control	No response	No response	Germination
	BAP	No response	Shooting	Germination
	BAP + NAA	Callusing*	No response	Germination
	2,4-D	No response	No response	No response
B5	Control	No response	No response	Germination
	BAP	No response	No response	Germination
	BAP + NAA	No response	No response	Germination
	2,4-D	No response	No response	No response

* used for somatic embryogenesis

used. The pH was adjusted to 5.6 to 5.8 before autoclaving. The cultures were maintained at 25°C±2°C under a photoperiod of 16 hours provided with cool white fluorescent light at a photon flux density of 2000-3000 lux. Initiation of callus depends on the type of media used for culture. Callus like structure was observed only when BM was used as basal medium along with BAP (2 mg/l) and NAA (0.5 mg/l) at different concentrations (figure 4.31). MS and B_5 media however, failed to induce callus. MS medium on the other hand helped in regenerating *in vitro* leaves which were further used for protoplast isolation. Apart from MS medium, shooting was also facilitated by Banana micropropagation medium containing BAP. Response of explants on different media and Plant growth regulators is represented in table 4.12. Control media (without hormone) of any kind failed to initiate shooting or callusing. Seed germination occurred in all the three media under study (MS, BM and B_5) with or without any hormone (table 4.12). From the table-4.12 it is evident that 2,4-D is not a preferred hormone by Canna for shooting or callusing.

4.6.2.2 Response of explants to culture media

Another important aspect of the response of explants to the type of culture media and phytohormone was studied from table 4.12. Of all the three explants, only leaf tissue was capable of producing callus in BM supplemented with BAP (2 mg/l) and NAA (0.5 mg/l). Though seed

germination occurred in all the three media, leaf and rhizome tissue were incapable of producing callus and shoot in B_5 medium. Shooting was initiated from rhizome, when these tissues were inoculated in MS and BM medium in presence of BAP. But the rhizome tissue did not have any potency to produce callus in any of the culture media supplemented with any combinations of phytohormone.

From the above experiment, BM was proved to be the best among the three media. For leaf tissue, combination both cytokinin (BAP) and auxin (NAA) proved to be the best among all the other combinations. So, leaf tissue in BM supplemented with BAP and NAA was considered for further *in vitro* culture study of Canna.

4.6.3 Somatic embryogenesis and regeneration

4.6.3.1 Somatic embryo formation

BM supplemented with 2 mg/l BAP and 0.5 mg/l NAA proved to be the best medium for callus induction from *Canna indica* leaf (table 4.12). Callus initiation from the explant was observed after 15 days of culture on the induction medium (Refer Material and Methods section). Fully developed callus was noticed after 10-15 days of callus initiation (figure 4.31).

Table 4.13: Duration of shoot and root initiation in different concentration of BAP

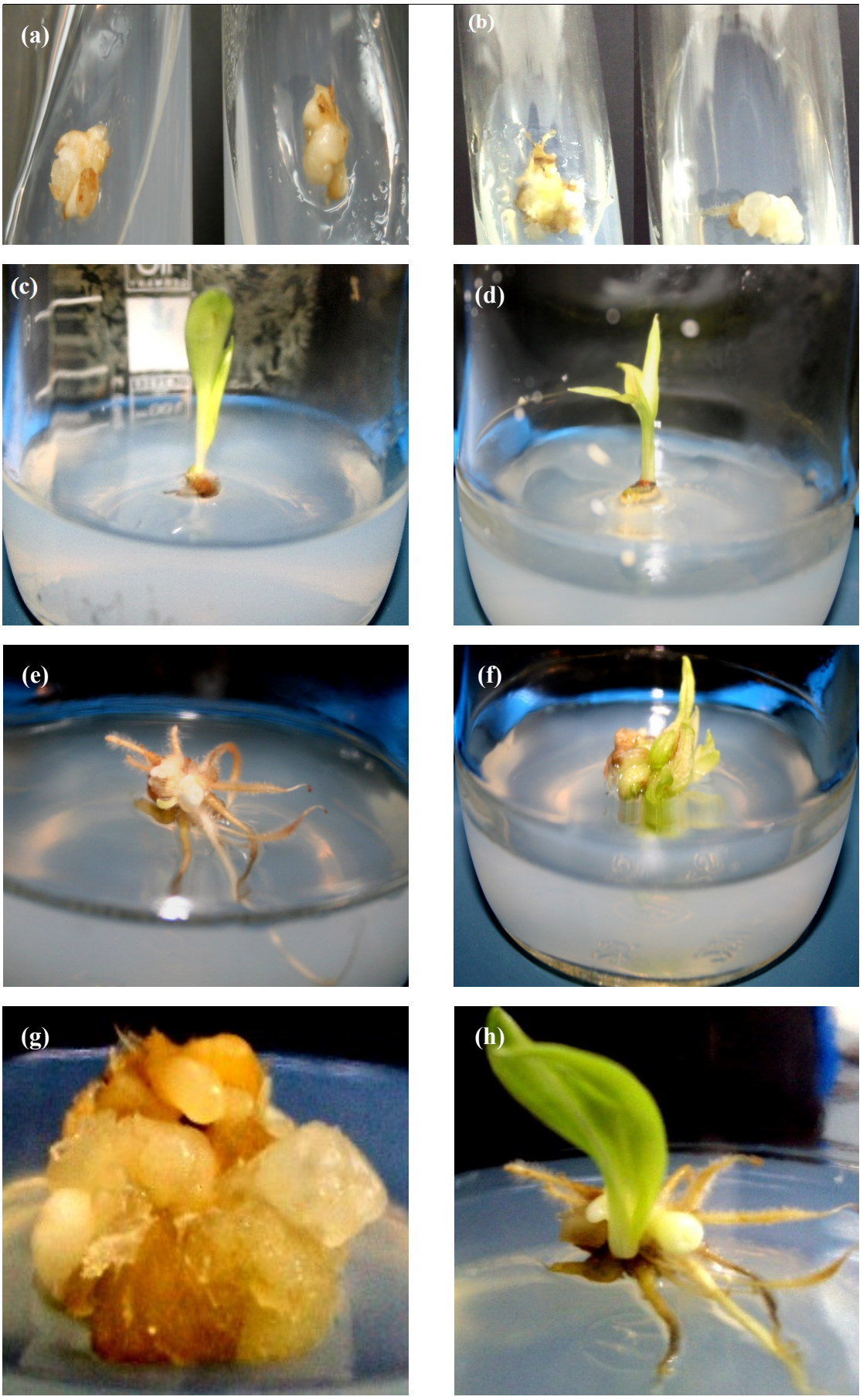
BAP mg/l	Shoot Initiation (Days)	Root Initiation (Days)
1	43.84	31.75
2	32.88	26.64
3	36.11	28.69
4	38.76	30.27

Callus tissue were cultured on the callus induction medium for about 2 weeks and then sub cultured on BM medium supplemented by BAP (2mg/l). After a period of about 35-40 days, embryo like structure was viewed from the callus tissue (figure 4.31). On an average, 4-5 somatic embryos were produced per flask. It was observed that subculture of callus in presence of both BAP and NAA obstructs the formation of somatic embryo. The number of embryos increased (9±3) up to 3rd subculture, while 4th subculture reduced the number of embryos (6±2).

4.6.3.2 Plantlet regeneration

4.6.3.2.1 Shoot and root initiation

The embryos so formed were further sub cultured in every 15 days to observe the initiation and development of shoot and root growth. BAP (2mg/l) alone was supplemented in BM for shoot initiation. Interestingly, roots appeared in the same medium without any rooting hormone and within a very short duration profuse rooting was



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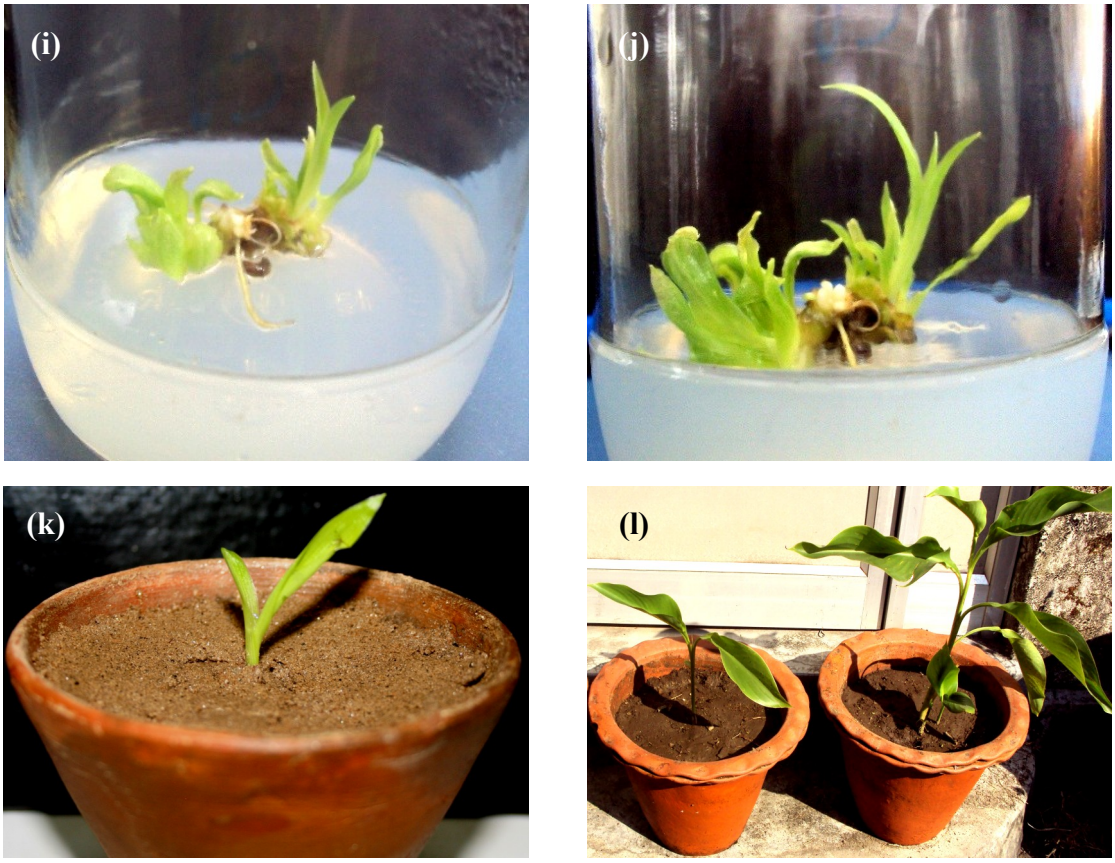


Figure 4.31: Stages of Callus initiation and regeneration, somatic embryogenesis and plantlet formation. (a) Callus initiation (b) Matured callus (c) Formation of *in vitro* shoot (d) Multiple shooting (e) Profuse *in vitro* rooting (f) Plantlet regeneration from callus (g) Somatic embryo formation (h) *In vitro* shooting and rooting from embryo (i) Fifteen days old plantlet (j) Plantlet after 1 month (k) Acclimatization of plant in clay pot containing mixture of soil and sand (l) Transfer and hardening of *in vitro* grown plant

observed. This indicated that Canna didn't require any specific hormone for initiation of *in vitro* rooting. Kambaska and Santilata, (2009) and Tyagi *et al.*, (2007) also reported similar phenomemon in ginger and turmeric respectively. Average number of days for the initiation of shoot and root was found to be 38±5 days and 29±3 days respectively. Duration of both shoot and root initiation varied in different concentration of BAP (Table 4.13). Rooting started earlier than shooting. It was further observed that early rooting had no affect on the formation of *in vitro* shoot (Mondal *et al.*, 1990).

Table 4.14: Subculturing and hormone effect on shoot growth

Media	BAP	Shoot length (cm)					Shoot No				
		15 D	30 D	45 D	60 D	75 D	15 D	30 D	45 D	60 D	75 D
BM*	1 mg/l	0.66	1.8	3.5	5.1	5.9	3.29	8.96	13.44	18.88	19.56
	2 mg/l	1.04	3.22	4.96	6.5	7.35	4.38	11.98	16.56	22.24	24.32
	3mg/l	0.51	2.4	3.2	4.32	4.88	3.43	9.01	12.88	17.02	19.42
	4mg/l	0.42	1.7	2.61	3.8	4.64	2.27	7.99	10.42	13.62	15.31

*BM=banana medium; D=No. of days

4.6.3.2.2 Subculturing and hormone effect on shoot growth

Number and length of shoots were dependant on the concentration of the phytohormone used in this study (Table 4.14). Highest number of shoot was observed in 2mg/l of BAP. Table 4.14 shows that, the number of shoot first increases as concentration of BAP increases from 1mg/l to 2mg/l and then gradually decreases. Lowest number of shoots was seen in BAP 4mg/l. It was found that, with increase in the concentration of BAP, number of shoot increased up to a certain optimum limit and then decreased. Higher concentration of BAP may have some inhibitory effect hampering the formation of shoot (Hussey, 1977). Shoot length showed a similar pattern when studied for concentrations of phytohormones. Thus, BAP (2mg/l) was considered to be the optimal concentration of growth regulator for shooting, beyond which the regeneration of plantlet decreased.

Response of primary, secondary and successive cultures were shown in table 4.14. The number of shoot showed an increasing trend up to 4^{th} subculture. But in 5^{th} and subsequent subculture (data not given after 5^{th} subculture) the number decreased remarkably. Growth rate of shoot was also observed a similar pattern with increase in the number of subculture. The above result indicated that *in vitro* growth of Canna gradually increases up to a certain limit in response to the fresh medium. The reduction in growth after the specific period gave an idea about adaptation of the explant to the culture media and the diminished effect of sterilants. Decline in the regeneration may be an effect of gradual aging and decrease in totipotency of the plant explant (Murashige and Nakano, 1965).

4.6.4 Hardening of in vitro *regenerated plants*

In plant tissue culture, the ultimate success of *in vitro* propagated plantlets lies in their adaptation to the external environmental conditions. The *in vitro* propagated plantlets with well developed root and shoot system were successfully transferred to clay pots containing autoclaved mixture of soil and sand. They were maintained for about 2 months under plastic covers in order to avoid their desiccation. Then these plantlets were transplanted to pots containing a mixture of farm yard manure and soil in a ratio of 1:1 (figure 4.31) in a greenhouse condition which showed a survival percentage of about

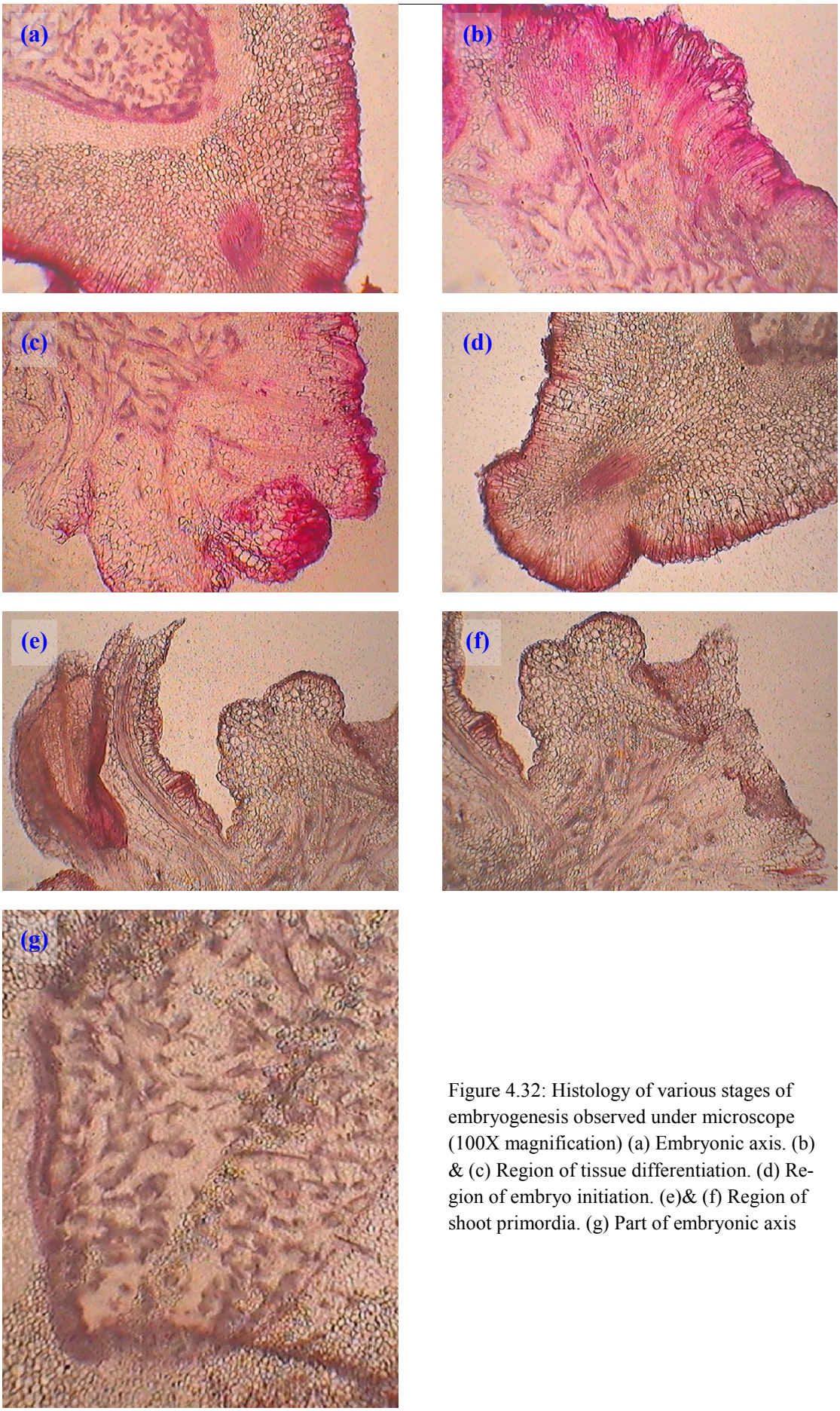


Figure 4.32: Histology of various stages of embryogenesis observed under microscope (100X magnification) (a) Embryonic axis. (b) & (c) Region of tissue differentiation. (d) Region of embryo initiation. (e)& (f) Region of shoot primordia. (g) Part of embryonic axis

80%. After a period of 1 month, the acclimatized plants were transferred to the field.

4.6.5 Histological studies of cultured embryo

Transverse and longitudinal sections of somatic embryo generated from the leaf tissue revealed the following structure under 100X magnification in a microscope (figure 4.32).

1. Anthocyanin pigmentation was visualized on the edges of the section in the form of red spots as stained by safranin.

2. Primordium of embryo development was observed from the surface of the cotyledon slices, in the form of compact mass of cells arranged sequentially on the edge of section.

3. Differentiation and separation of proembryonic globules.

4. Proembryonic globules were viewed with well differentiated epidermis.

5. Details of epidermal cell divisions like periclinal, anticlinal and mitotically active cells were observed.

Embryonic axis with shoot meristem, cortical tissues, central cylinder and procambial strands were clearly visible.

Table 4.15: PCR amplicons obtained from RAPD primers in *in vitro* raised *C. indica*

Primer	Bands	Band size (bp)
OPA01	7	330-1120
0PA03	6	400-1260
OPA07	6	500-1550
0PA11	8	420-1380
OPA17	7	760-1440
OPB01	5	410-1350
OPF09	6	610-1500
OPG19	7	500-1280
OPH04	4	300-1050
OPN04	4	450-1300
Total	**60**	

Table 4.16: PCR amplicons obtained from ISSR primers in *in vitro* raised *C. indica*

Primer ID	Total bands	Band size (bp)
UBC810	3	550-800
UBC815	6	375-1100
UBC822	8	390-1280
UBC824	6	450-1110
UBC825	8	540-920
UBC856	5	410-980
UBC873	9	390-1240
Total	**45**	

4.6.6 Somaclonal variation among in vitro *raised plantlets*

In the present study an attempt was made to screen the *in vitro* raised *Canna indica* plantlet for somaclonal variations (if any) by using molecular markers like RAPD and ISSR. Ten RAPD and 7 ISSR primers (which already successfully amplified the genomic DNA of all the 20 accessions of Canna) were used to detect somaclonal variations in *Canna indica* (Table 4.15 and 4.16). A total of 60 scorable bands with an average of 6±2

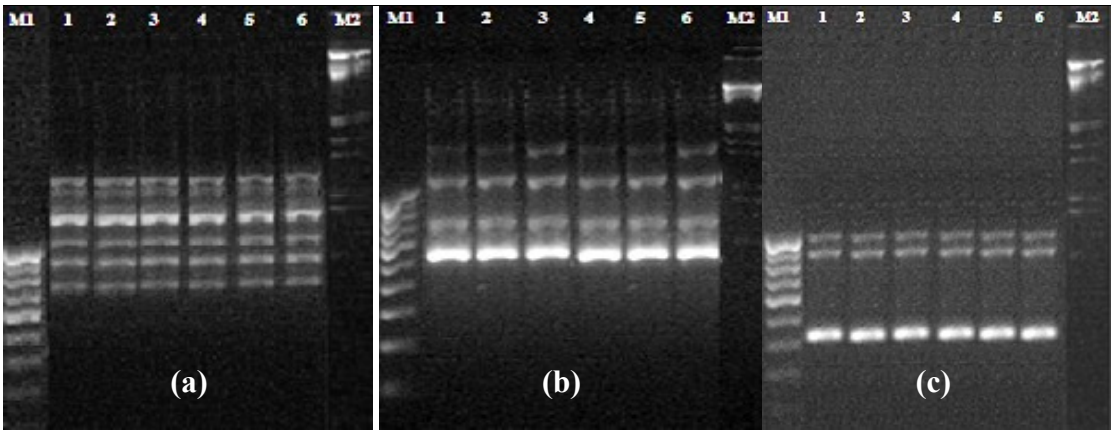


Figure 4.33: DNA fingerprinting pattern of *in vitro* callus regenerated plantlets of *C. indica*. RAPD primers (a) OPA 17 (b) OPB01(c) OPH04. Lane 2-6: micropropagated plantlets compared with mother plant (lane1); Lane M1: 100 bp molecular marker; Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

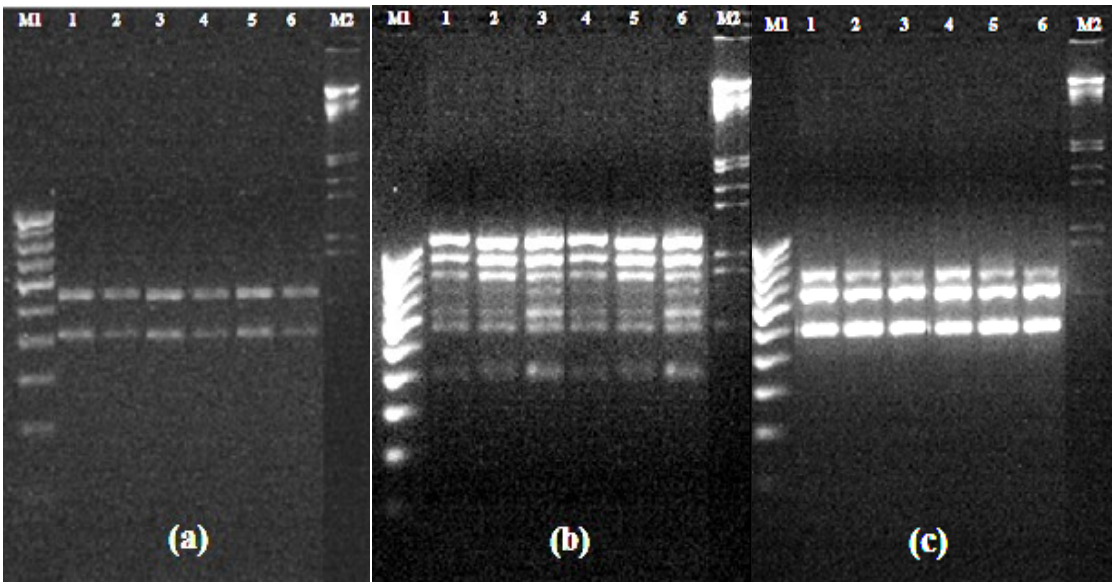


Figure 4.34: DNA fingerprinting pattern of *in vitro* callus regenerated plantlets of *C. indica.* ISSR primers (a) UBC810 (b) UBC815(c) UBU825. Lane 2-6: micropropagated plantlets compared with mother plant (lane1); Lane M1: 100 bp molecular marker; Lane M2: λ DNA/*Eco*RI/*Hind*III double digest DNA ladder

bands per primer were obtained from the RAPD analysis, where as in case of ISSR markers, total 45 bands were produced from 6 primers showing an average of 7±2 bands per primer. All most all the bands generated through both RAPD and ISSR analysis were common in parental genotypes and the regenerated plants of different sub cultures. Amplified products exhibited monomorphic pattern across all the sub cultures of the *in vitro* plants for both the markers. A representative of RAPD and ISSR profile is represented in figure 4.33 and 4.34. Though the plantlets were produced indirectly from the leaf tissue through callus formation, RAPD and ISSR analysis showed no

evidence of polymorphism between parent plant and the regenerated plants. Clonal fidelity was discussed in some Zingiberales like banana and turmeric (Tyagi *et al.*, 2007; Rout *et al.*, 2009 and Purohit *et al.*, 2012).

4.6.7 Isolation and fusion of protoplasts

4.6.7.1 Protoplast isolation

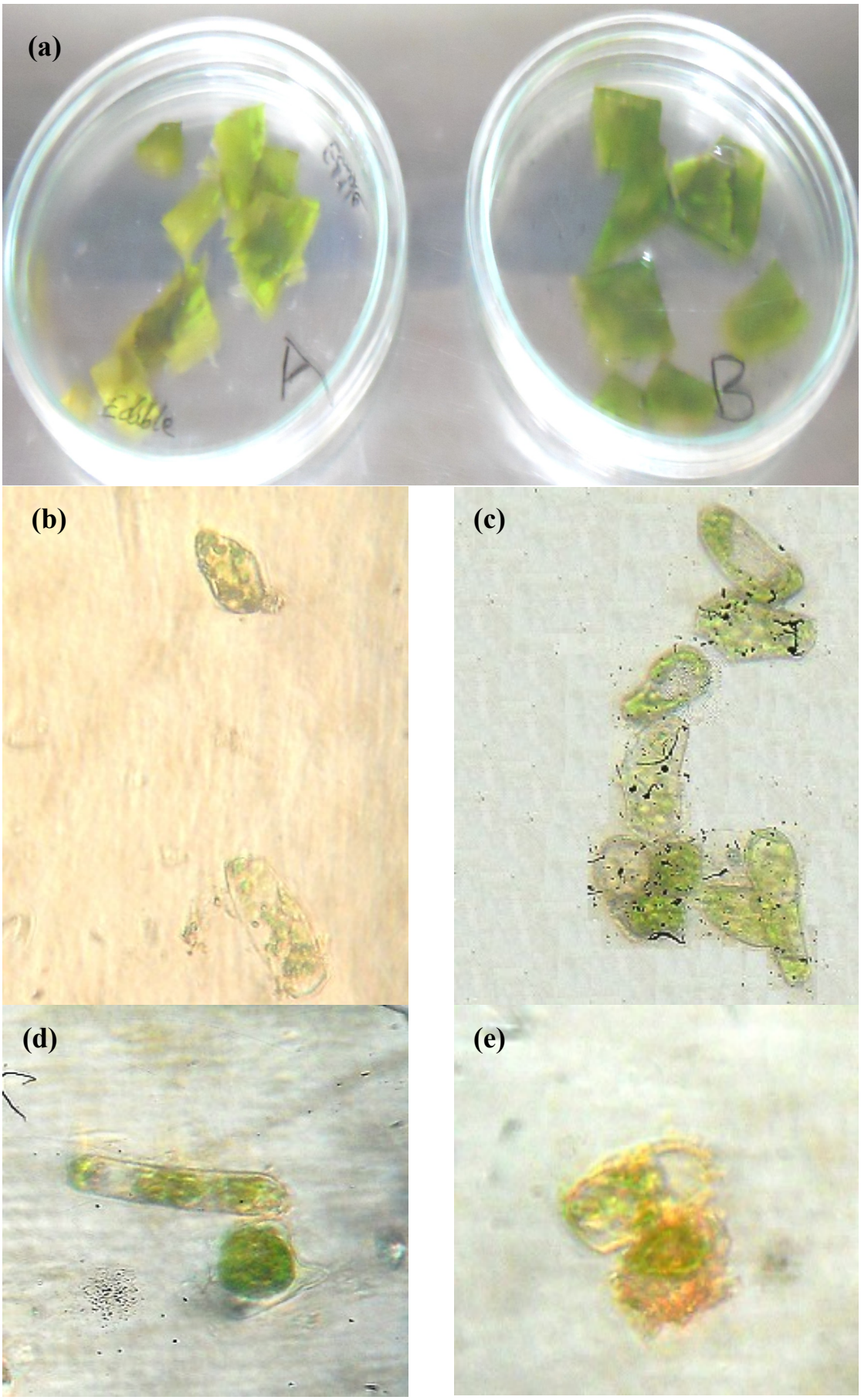
In vitro germinated leaves of *Canna indica* and *in vitro* regenerated shoots of *Canna edulis* were used as the source for isolation of protoplasts. It was observed that in both the plants, good quality of protoplasts was produced from *in vitro* leaf tissue (figure 4.35). Of all the combinations of enzymes, 1% cellulase + 0.5% pectinase generates good quality protoplasts in both the plants. As per the literature, the viable protoplasts were viewed to be unstained with phenosafranin (De, 1997). The viability range of freshly isolated leaf protoplasts was 60-75%. Mesophyll protoplasts of *Canna edulis* were found to be more uniform in size [20-30 micro meter (μm)] than that of *Canna indica*.

The isolation of protoplasts in Canna depends significantly on both donor material and genotype used. It is essential to standardize the enzymatic mixture for the donor material to isolate high quality of protoplasts. This indicated that the digestion of cell wall components depends on enzymatic mixture. It was found in the literature that, in banana and other monocotyledons such as rice, maize and wheat, lower yield and lower viability of protoplasts were observed when derived from mesophyll tissue (Assani *et al.*, 2002; Jain *et al.*, 1995; Prioli and So¨ndahl, 1989 and Vasil *et al.* 1990). The less viable protoplasts of mesophyll tissue could be connected to the sensitivity of leaves to enzymatic stress, resulting in breakage and damage during enzymatic digestion.

In the present study, a standard protocol for isolation of protoplasts has been developed for the ornamental monocot like Canna. Since protoplast regeneration is a prerequisite for somatic hybridization, efficient isolation of protoplasts would be important for producing new cultivars of Canna through protoplast fusion.

4.6.7.2 Culture of protoplasts

Callus like whitish mass of tissue was observed after 9-10 days the culture of Protoplasts. The frequency of formation of the above whitish tissue



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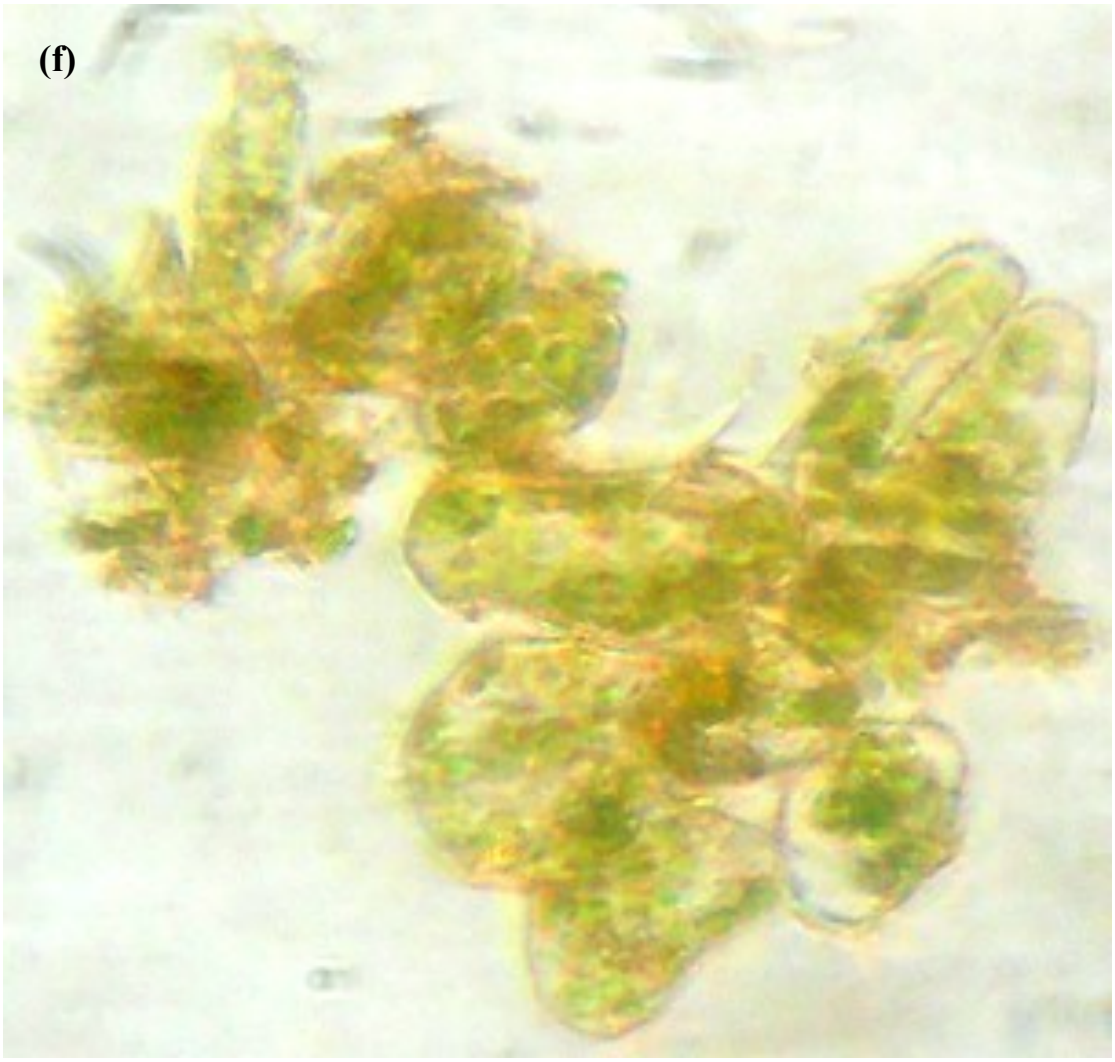

Figure 4.35: (a) Leaf pieces of *C. edulis* and *C. indica* for enzymatic digestion; (b) Freshly isolated mesophyll protoplasts (c) Chain of protoplasts (d) Protoplasts coming closer to each other after application of PEG (e) Fused protoplasts (f) Fused protoplasts chain

was very low i.e. 25-30%. The callus like tissue was able to divide and increase in their size, although they did not differentiate further and died thereafter. This showed that the calli produced from the protoplast did not regenerate; however, they were alive for about 15 days while on the feeder layer. The whitish tissue may represent a type of wound tissue, which stimulates callus formation but was incapable of further differentiation. Lower regenerating capacity of isolated plant protoplasts might be due to the suppression of totipotency. Reduced viability and cell division potential in isolated protoplasts may be because of the presence of reactive oxygen species and reduced cellular antioxidant mechanism. Similar problems were found in the regeneration of isolated plant protoplasts in tobacco, grapevine

and mustard etc. (Papadakis *et al.*, 2001 and Yasuda *et al.*, 2007).

4.6.7.3 Fusion and culture of protoplasts

Somatic hybridization could be an excellent tool for the breeding of ornamental and cultivated Canna. But till date there are very few reports of successful somatic hybridization in monocots. In the present experiment, protoplast fusion in Canna was standardized using Polyethylene glycol (PEG). Different steps of protoplast fusion are represented in figure 4.35. A number of fused protoplasts were clearly seen under microscope. The rate of cell divisions of fused protoplasts may be affected by the toxic level of PEG, which hampers the viability of protoplasts. Less viable protoplasts do not participate in the fusion process and ultimately lowers down the mitotic activities (Mercer & Schlegel, 1979).

The fused protoplasts were cultured in the same media as described for the culture of single isolated protoplasts. But unfortunately the fused protoplasts failed to regenerate even after 25-30 days of their culture. This might be due to the loss of viability of the fused protoplasts with due course of time due to different biotic and abiotic factors. The incapability of regeneration of fused protoplasts was described by many authors (Evans *et al.*, 1984) and Schieder & Kohn 1986). This may be because of the effect of PEG, which ceases the development of fusion product and finally limiting the formation of callus and somatic embryos (Assani *et al.*, 2005).

Conclusion

Canna is a common ornamental plant presents almost every part of the world. There are various species and cultivars of Canna also found in the eastern part of India. Canna is an annual shrub with colorful flowers. Though it is introduced in India but in course of time it has almost became naturalized. However, virtually no biological documentation has been done on these beautiful ornamental plants. Besides it has been found that some hilly tribes of eastern Indian also used Canna as an edible and medicinal plant. Therefore, in the spring of 2009, when I started my work to obtain my PhD degree, I thought of working on various aspects of Canna biology including molecular diversity, *in vitro* tissue culture, and production of non-conventional hybrid plants through protoplast fusion and determination of medicinal and edible properties.

Since Canna is an introduced plant, people used to think that molecular diversity in these ornamental plants will be low. However, I for the first time could be able show that a good deal of diversity does exist among Canna varieties and species. For instance, with 18 RAPD primers a total of 159 bands were found out of which 143 were polymorphic i.e. the amount of polymorphism was 89.93%. The ISSR marker also revealed polymorphism, though at a lesser extent (88.17%). I have also analyzed a portion of chloroplast genome through PCR-RFLP. However, polymorphism is very less over there. Therefore it may be concluded that in the process of naturalization the introduced varieties of Canna have evolved substantially.

Hill tribes of north-eastern Indian for example, Lepchas and Bhutias etc. regularly use Canna rhizomes for food and medicinal purposes. I found that some Canna varieties like, *Canna edulis* and its cultivars are rich in edible starch content. Besides various Canna varieties and species are routinely used as medicinal plants. It has been observed that most of the Canna rhizomes contain high dose of antioxidants. The experiments like DPPH scavenging activity, ferric reducing power assay (FRP), H_2O_2 scavenging activity confirm the fact. The amount of polyphenolics like total phenol and flavonoids also proved the presence of considerable amount of natural antioxidants. Separation of antioxidant molecules of the rhizome of *Canna edulis* through silica gel column chromatography helps in isolating various solvent fractions, which were further investigated to test their medicinal value. On the basis of various parameters like DPPH radical scavenging activity, total flavonols and total proanthocyanidin contents, nitric oxide scavenging activity, hydroxyl radical scavenging activity and anti lipid peroxidation

activity, *C. edulis* was established as a medicinal plant. It was further confirmed through thin layer chromatography.

GC-MS analysis identified the presence of some therapeutically significant molecules, which further validated *C. edulis* to be an important medicinal plant.

Though Canna grows well in most of the soil, the techniques of micropropagation of Canna must be standardized for the genetic manipulation of these ornamental plants. So far, there was no reliable protocol for *in vitro* growth of *Canna Indica* or *Canna edulis*. I have standardized the protocol. I found that banana micropropagation medium supplemented with 3% sucrose, 0.7% agar, and 0.17% NH_4NO_3 and different plant growth regulators like BAP and NAA was found to be effective in inducing callus. Callus culture medium along with BAP was ideal for somatic embryogenesis and plantlet regeneration in Canna propagation. Tissue culture generated plants were quite stable because I tried several RAPD and ISSR primers to detect polymorphism but did not get any variation. After standardizing the micropropagation, I tried to develop some hybrid varieties of Canna through protoplast fusion. So far my knowledge goes this is the first attempt to fuse Canna protoplast *in vitro*. Viable protoplasts were isolated as a result of enzymatic digestion of the *in vitro* leaves of *Canna indica* or *Canna edulis*. Fusion of mesophyll protoplasts was standardized using Polyethylene glycol (PEG).

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Index

Appendix-A

Thesis related publication till November, 2013

Mishra T, AP Das and A Sen (2012) Phytochemical screening and *in vitro* antioxidant profiling of solvent fractions of *Canna edulis* Ker Gawler. *Free radicals and antioxidants*, 2(1): 13-20.

Mishra T, AK Goyal, SK Middha and A Sen (2011) Antioxidative properties of *Canna edulis* Ker Gawl.. *Indian Journal of Natural Product Resources*, 2: 315-321.

Mishra T, AK Goyal, P Mondal and A Sen (2011) Free radical scavenging activity of ornamental and edible cultivars of Canna found in Eastern India. *NBU Journal of Plant Science*, 5: 41-45.

Appendix-B

Buffers and chemicals used for DNA fingerprinting studies & Protoplast fusion

CTAB- buffer

100mM Trizma Base (Sigma, Cat# T1503) (pH-8.0)

20mM EDTA (Merck India, Cat# 60841801001730) (pH-8.0)

1.4 M NaCl (Merck India, Cat#60640405001730)

2% (w/v) CTAB (Hexadecyl cetyl trimethyl ammonium bromide) (Sigma, Cat# H6269)

12.11g of molecular grade Trizma base was dissolved in 400 ml double distilled water, pH was adjusted to 8.0 and was divided into two parts of equal volume. To one part 7.44g EDTA was added and to the other part 81.8g NaCl and 20g CTAB. Both the parts were than mixed and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

Note: Add 1% PVP (Polyvinylpyrrolidone) (Sigma, Cat #P5288) and 0.3% β-mercaptoethanol (Sigma, Cat# M3148) just before use.

1X TE

Tris- Cl (pH 8.0) (i.e. 10Mm) =0.6055gm

EDTA (pH 8.0) (i.e. 1mM) =0.186 gm

Both the reagents were dissolved separately and finally mixed together and the final volume was made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

PEG solution

30% (w/v) PEG 8000 (SIGMA, Cat#P5413)

1.6 M NaCl (Merck India, Cat#60640405001730)

2.34gm of NaCl was dissolved in 15ml double distilled water and autoclaved. PEG (7.5gm) was first dissolved in 15ml of NaCl solution and then the final volume was made to 25ml with sterile water. PEG solution was stored at 4ºC for indefinite period.

5X TBE (Tris-borate-EDTA) buffer

Trizma base (Sigma, Cat# T1503) = 27 gm

Boric acid (Sigma, Cat# 15663)= 13.75 gm

0.5M EDTA (pH 8.0)=1.86 gm

All the reagents were dissolved separately and finally mixed together and the final volume was

made up to 1000ml with double distilled water prior to autoclaving. The buffer was autoclaved at 121ºC and 15 psi for 20 mins and stored at room temperature for further use.

6X gel loading buffer:

TYPE 3:

0.25% Bromophenol blue (Sigma, Cat# B0126)

0.25% Xylene cyanol FF (Sigma, Cat# X4126)

30% Glycerol (Merck India, Cat#61756005001730) in water

Store at 4ºC.

RNase A:

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

Note: Both 0.01M sodium acetate and 1M Tris-Cl were prepared and autoclaved at 121ºC and 15 psi for 20 mins prior to use.

Solution for Protoplast Isolation

Chemical/Reagent	Quantity
KH_2PO_4	27.2mg/l
KNO_3	101mg/l
$CaCl_2,2H_2O$	1480mg/l
$MgSO_4,7\ H_2O$	246mg/l
KI	0.16 mg/l
$CuSO_4,5\ H_2O$	0.025mg/l

The pH of the solution was adjusted to 5.8.

Solution I: To the above CPW solution 13% mannitol was mixed and autoclaved.

Solution II: To the above CPW solution 21% sucrose was mixed and autoclaved.

Appendix-C

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

Macroelements	**Amount required (mg/l)**
KH_2PO_4	170.00
KNO_3	1900.00
$MgSO_4$	180.54
NH_4NO_3	1650.00
Microelements	**Amount required (mg/l)**
$CoCl_2$, $6H_2O$	0.025
$CuSO_4$, $5H_2O$	0.025
$FeSO_4$, $7H_2O$	27.80
Na_2 . EDTA	37.30
H_3BO_3	6.20
KI	0.83
$MnSO_4$, H_2O	16.90
Na_2MoO_4, $2H_2O$	0.25
$ZnSO_4$, $7H_2O$	8.60
Vitamins	**Amount required (mg/l)**
Glycine	2.00
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thyamine HCl	0.10

Composition of Banana micropropagation medium (Hi media Cat# Himedia, Cat#PT076)

Macroelements	**Amount required (mg/l)**
KH_2PO_4	44.00
KNO_3	2020.00
$MgSO_4$	120.33
$CaCl_2$, $2H_2O$	220.50
Microelements	**Amount required (mg/l)**
$CoCl_2$, $6H_2O$	0.24
$CuSO_4$, $5H_2O$	0.245
$FeSO_4$, $7H_2O$	27.80
Na_2 - EDTA	37.26
H_3BO_3	1.24
KI	0.83
$MnSO_4$, H_2O	8.40
Na_2MoO_4, $2H_2O$	0.13
$ZnSO_4$, $7H_2O$	0.72
Vitamins	**Amount required (mg/l)**
Glycine	2.00
Myoinositol	100.00
Nicotinic acid	0.50
Pyridoxine HCl	0.50
Thyamine HCl	0.10

Composition of Gamborg (B5) medium (Hi media Cat# Himedia, Cat#TS1014)

Macroelements	**Amount required (mg/l)**
NaH_2PO_4	130.42
KNO_3	2500.00
$MgSO_4$	122.09
$(NH_4)_2SO_4$	134.00
$CaCl_2$, $2H_2O$	150.00
Microelements	**Amount required (mg/l)**
$CoCl_2$, $6H_2O$	0.025
$CuSO_4$, $5H_2O$	0.025
$FeSO_4$, $7H_2O$	27.80
Na_2 . EDTA	37.30
H_3BO_3	3.00
KI	0.75
$MnSO_4$, H_2O	10.00
Na_2MoO_4, $2H_2O$	0.25
$ZnSO_4$, $7H_2O$	2.00
Vitamins	**Amount required (mg/l)**
Myoinositol	100.00
Nicotinic acid	1.00
Pyridoxine HCl	1.00
Thyamine HCl	10.00
Sucrose	20000.00

To it, 3% sucrose (Hi media Cat# RM134) and 0.332 mg/l $CaCl_2$ (Merck India Cat# 61764405001730) were added in Murashige and Skoog medium. For Banana micropropagation medium and B5 medium, 0.17% NH_4NO_3 (Merck India Cat#17804) was added in place of Calcium chloride. pH was adjusted to 5.6±0.1 and the volume was made up to 1000ml with double distilled water. It was then autoclaved for 20 minutes at 121ºC and 15psi and cooled and plant growth regulators are added (if any) as per requirement.

Note: In case of solid media agar (Hi media Cat#RM026) is added at the rate of 0.7%.

Appendix-D

Chemicals and buffers used for antioxidant profiling

2-deoxy-D-ribose (Himedia, Cat# RM452)
Acetone (Merck India, Cat# 60001405001730)
Aluminium Chloride (Sd Fine, Cat# 37073)
Ammonia (Merck India, Cat# 17500)
Ascorbic acid (Himedia, Cat# CMS1014)
Benzene (Merck India, Cat# 60178325001730)
Butanol (Merck India, Cat# 17419)
Butylated hydroxytoluene (BHT) (SD fine chem Limited, Cat# 38067)
Cathechin (Sigma, Cat#C0567)
Chloroform (Merck India, Cat# 82226505001730)
Diethyl ether (SD fine chem Limited, #38132)
DPPH (Himedia, Cat# RM2798)
Ethyl acetate (SD fine chem Limited, 20108)
Ethylenediamine tetra acetic acid (EDTA) (Merck India, Cat# 60841801001730))
Ferric chloride (Himedia, Cat# RM1379)
Ferrous sulphate (Merck India, Cat# 62840005001046)
Folin Ciocalteu's reagents (SRL, Cat# 062015)
Gallic acid (Himedia, Cat# RM233)
Glacial acetic acid (SD fine chem Limited, Cat# 37013)
Hexane (SD fine chem Limited, Cat# 38485)
Hydrochloric acid (Merck India, Cat# 61762505001730)
Hydrogen peroxide (Merck India, Cat# 61765305001730)
Iodine solution (SD fine chem Limited, Cat# 25008AM)
Methanol (Merck India, Cat# 60600905001730)
Naphthyl ethylene diamine dihydrochloride (Himedia, Cat# RM1073)
Orthophosphoric acid (SD fine chem Limited, Cat# 20173)
Phosphate buffer (0.2M) pH 6.6
    a. Potassium dihydrogen phosphate (Merck India, Cat# 60487305001730)
    b. Dipotassium hydrogen phosphate (Merck India, Cat#61788005001730)
Phosphate buffer (pH 7.4)
    a. Potassium dihydrogen phosphate (Merck India, Cat#60487305001730)
    b. Potassium hydroxide (Merck India, Cat#60503305001730)
Potassium chloride (Merck India, Cat# 61779205001730)
Potassium ferrocyanide (Merck India, Cat# 61843605001730)
Quercetin (Himedia, Cat# RM6191)
Silica gel (SD fine chem Limited, 200-300 mesh size)
Sodium acetate (Sigma, Cat# S9513)
Sodium Carbonate (Merck India, Cat# 61778705001730)
Sodium hydroxide (Merck India, Cat# 6184305001730)
Sodium Nitrite (Himedia, Cat# RM417)
Sodium nitroprusside (Merck India, Cat# 61761501001730)
Sulphanilamide (Himedia, Cat# RM1558)
Thiobarbituric acid (TBA) (Himedia, Cat# RM1594)
Trichloro acetic acid (Qualigens, Cat# 28445)
Vanillin (Himedia, Cat# RM616)