MICROPROPAGATION OF GERBERA (*Gerbera jamesonii* Bolus) AND ASSESSMENT OF GENETIC STABILITY OF PLANTLETS USING ISSR ASSAY

By

AWCHAR DATTA MANIKRAO
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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
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DECLARATION

I hereby declare that the thesis entitled **"Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

Vellanikkara :

Date : 16/08/2014

Awchar Datta Manikrao

(2012-11-106)

CERTIFICATE

Certified that the thesis entitled **"Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay"** is a record of research work done independently by **Mr. Awchar Datta Manikrao** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship, fellowship to him.

15/8/2014

Vellanikkara
Date: 16/08/2014

Dr. M. R. Shylaja
(Chairman, Advisory Committee)
Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

CERTIFICATE

We, the undersigned members of the advisory committee of **Mr. Awchar Datta Manikrao** a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology,** agree that the thesis entitled **"Micropropagation of Gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay"** may be submitted by **Mr. Datta Manikrao Awchar** in partial fulfillment of the requirement for the degree.

5/9/2014

Dr. M. R. Shylaja
(Chairman, Advisory Committee)
Professor
Centre for Plant Biotechnology and Molecular Biology
College of Horticulture,
Vellanikkara, Thrissur.

5/9/2014

Dr. P. A. Valsala
(Member, Advisory committee)
Professor and Head
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

Dr. P. A. Nazeem
(Member, Advisory committee)
Professor and coordinator (DIC)
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur.

Dr. Deepu Mathew
(Member, Advisory committee)
Assistant Professor
Centre for Plant Biotechnology
and Molecular Biology
College of Horticulture
Vellanikkara, Thrissur

Dr. K. Ajith Kumar
(Member, Advisory committee)
Associate Professor
Pomology and Floriculture
College of Horticulture
Vellanikkara, Thrissur

5/9/2014

External examiner
Dr. N. K. Vijaykumar
Professor and Associate Dean (Rtd.)
College of Forestry
Kerala Agricultural University, Thrissur

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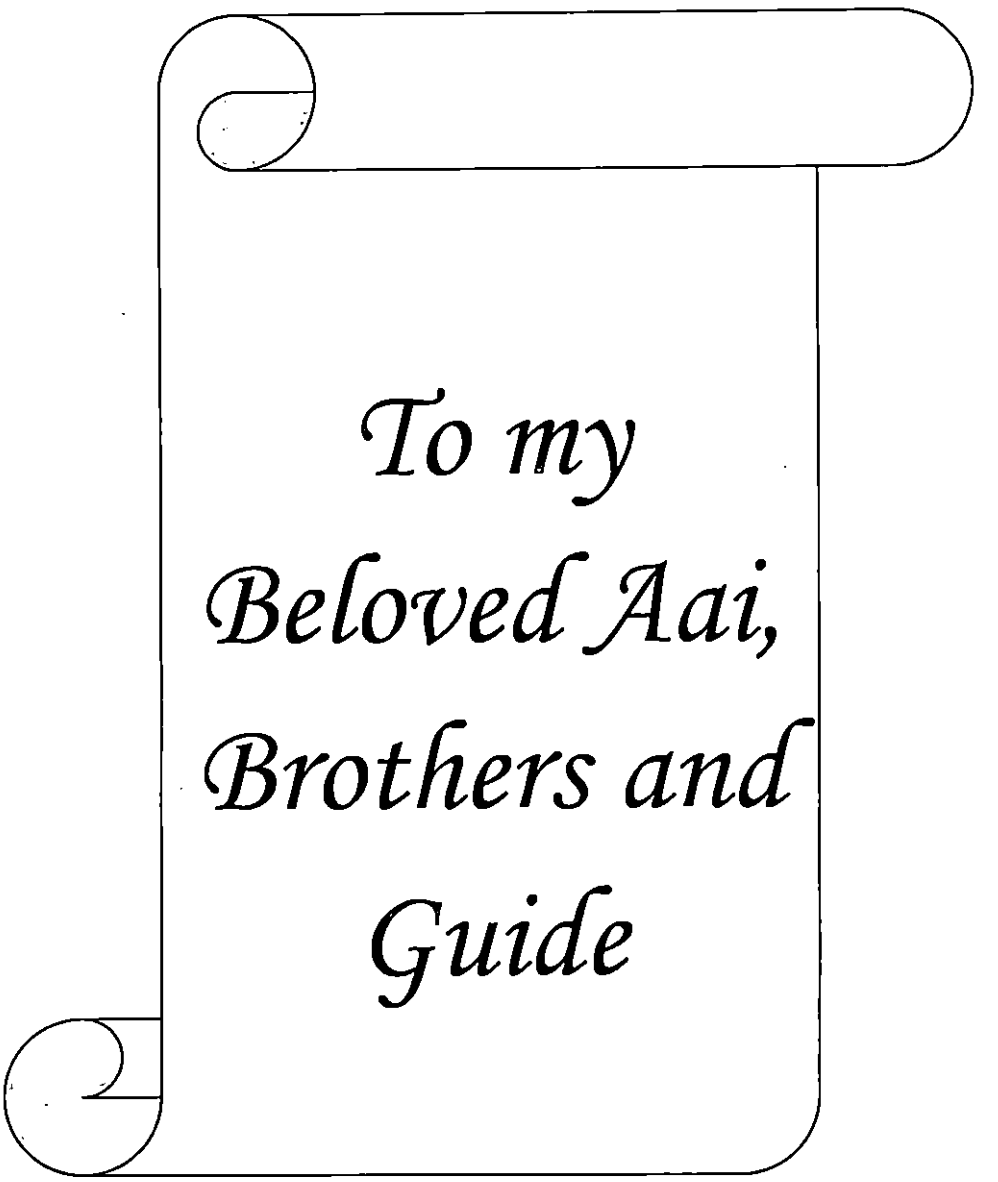
To my
Beloved Aai,
Brothers and
Guide

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ABBREVIATIONS

%	Percentage
µg	Microgram
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
ABA	Abscisic Acid
bp	Base pair
BAP	Benzyl amino purine
cc	*cubic centimetre*
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EMS	Ethyl methane sulphonate
EST	Expressed sequence tags
g	Gram
Gy	Gray
ha	Hectare
ISSR	Inter Simple Sequence Repeat
IAA	Indole acitic acid
IBA	Indole butyric acid
Kb	Kilo basepairs
Kin	Kinetin
2, 4-D	2, 4-Dichlorophenoxyacetic acid
L	Litre
M	Molar
mg	Milligram

ml	Millilitre
mM	Milli mole
ng	Nanogram
NAA	1-Naphthaleneacetic acid
°C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
P^H	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
TAE	Tris Acetate EDTA
TC	Tissue culture
TE	Tris EDTA
U	Unit
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus) commonly known as Transvaal Daisy, Barberton Daisy or African daisy, is an important commercial flower grown throughout the world in a wide range of climatic conditions. Gerbera ranks fifth in the global cut flower trade. It is a popular cut flower in Holland, Germany and USA. Gerbera is ideal for beds, borders, pots and rock gardens. The flowers are of various colours, suit very well in different floral arrangements and decorations. The cut blooms also have a long vase life of about 7 to 8 days.

The genus Gerbera was named in the honour of a German naturalist, Traugott Gerber. It is considered to be a native to South African and Asiatic regions. The plant is reported to be widely distributed in South Africa, Europe, Asia and Indonesia. In India, it is distributed from Kashmir to Kanyakumari, growing best at altitudes ranging from 1300 to 3200 meters above mean sea level. It is grown as a commercial crop in sub-tropical and temperate regions of India and in Southern Peninsula. It is commercially grown in and around Pune, Bangalore and Ooty as commercial cut flowers.

The genus Gerbera consists of about 40 species comprising half-hardy and perennial flowering plants. Among the different species, *Gerbera jamesonii* is the only species under cultivation. Some other important species are *G. aspleifolia, G. aurantiaca, G. kunzeana* and *G. viridifolia*. Gerbera belongs to the family Asteraceae with diploid chromosome number 2n=50. Plants are perennial, stem less and tender. Leaves are radical, petiolate, lanceolate, deeply lobed, sometimes leathery, narrower at the base and wider at the top and are arranged in a rosette at the base. Flower heads are solitary, many flowered, with conspicuous ray florets in one or two rows, florets of inner disc when present are very short, sub-tubular and two lipped. Based on flower heads, they may be grouped into single, semi-double and double cultivars. The flowers occur in a wide range of colours including yellow, orange, cream-white, pink, brick red, scarlet, salmon, maroon, terracotta and various other intermediate shades. The flower stalks are long, thin

and leafless. Best quality Gerbera flowers can be obtained under greenhouse conditions. A well drained, rich, light, neutral or slightly alkaline soil is most suitable for gerbera production. Day temperature of 22 - 25^{0}C and night temperature of 12 - 16^{0}C are ideal for its cultivation.

Gerbera is generally propagated by division of suckers or clumps. Propagation through seeds is not preferred as the plants exhibit heterozygosity and non-uniformity. Also, the improved semi-double and double cultivars do not set seeds. Propagation by division of suckers or clumps gives true to type plants, but the multiplication rate is very low. Many new varieties are being introduced every year. To popularize these varieties and also to meet the demand for quality planting material of elite varieties; there is a need to develop technology for rapid multiplication. This could be accomplished through micropropagation technique.

Micropropagation is practised in gerbera to mass multiply elite varieties. Though several explants like leaves (Jerzy and Lubomski, 1991; Parthasarathy and Nagaraju, 1999), petioles (Orlikowska *et al.,* 1999), capitulum (Pierik *et al.,* 1975), shoot tips (Murashige *et al.,* 1974; Aswath *et al.,* 2003) and flower buds (Son *et al.,* 2011) were tried for *in vitro* propagation, successful protocols were reported only from shoot tip and flower buds.

The successful micropropagation protocols from explants like shoot tip and flower buds are at the expense of flowers and further vegetative propagules. Till date, a successful micropropagation protocol based on leaf explant is not available, in most of the studies with leaf explant, only rhizogenesis has been reported. The inconsistent behaviour with respect to organ differentiation and very intricate *in vitro* rooting of shoots necessitated the use of alternative tissue and methods for mass multiplication of gerbera (Kanwar and Kumar, 2008; Gantait *et al.*, 2011). Also, the hardening and acclimatization techniques were not perfected in most of the protocols reported (Gantait *et al.*, 2011).

In any micropropagation protocol, assessment of genetic stability of the plantlets helps to know the true-to-type nature of the plantlets or variability

induced in tissue culture cycle. The occurrence of cryptic genetic changes arising via somaclonal variation in the regenerants can seriously limit the broader utility of the micropropagation system (Salvi *et al.*, 2001). A major problem associated with micropropagation is occurrence of somaclonal variation among the sub-clones parental lines, arising as a direct consequence of *in vitro* culture of plant cells, tissues and organs. The frequency of these variations varies with the source of explant and their pattern of regeneration (somatic embryogenesis/ organogenesis/ axillary bud multiplication), media composition and culture conditions (Damasco *et al.*, 1996; Salvi *et al.*, 2001). It is therefore, imperative to establish genetic uniformity of micropropagated plants to conform the quality of the plantlets for its commercial utility.

Polymerase chain reaction (PCR) based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) are immensely useful in establishing the genetic stability of *in vitro* regenerated plantlets in many crop species (Lakshmanan *et al.*, 2007; Joshi and Dhawan, 2007). Evaluation of genetic fidelity of *in vitro* propagated gerbera using RAPD and ISSR markers was attempted by Bhatia *et al.* (2010). Capitulum regenerated plants were subjected to molecular characterization and they observed monomorphic banding pattern in micropropagated plants which were similar to those of mother plant. Similar studies were conducted by Mata *et al.* (2009) in gerbera accessions. Gong and Deng (2010) described the development and characterisation of 99 EST-SSR markers in gerbera which could be utilized in breeding and genetic studies.

In this context, "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" was taken up at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University. There are various routes of micropropagation *viz.*, enhanced release of auxiliary buds, direct and indirect organogenesis and direct and indirect embryogenesis to regenerate whole plants. Two different explants like flower bud and leaf are used in the present investigation. Based on the type of

regeneration pathway achieved from flower bud/ leaf explants, the micropropagation protocol can be used either for commercial mass micropropagation or creating a variable population which will serve as the base material for further breeding programmes as there is high demand for new variants in gerbera.

The present study aims to micropropagate gerbera using flower buds and leaf explants and to test the genetic stability of the micropropagated plants using ISSR assay.

Review of literature

2. REVIEW OF LITERATURE

The research programme entitled "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2012- June 2014. Relevant literature on various aspects of the research is reviewed in this chapter.

2.1 General description

Gerbera (*Gerbera jamesonii* Bolus) is a herbaceous perennial flower crop with long leafless stalks and daisy-like flowers. A native of South Africa, it is a popular cut flower grown throughout the world in a wide range of climatic conditions. It is popularly known as 'Barbeton daisy' or 'Transvaal daisy'. It is ideal for flowerbeds, borders, pots and rock gardens. The gerbera plant also is used in the preparation of traditional Chinese medicine: tu-er-feng, derived from whole plants of gerbera, is used for curing cold with cough and for rheumatism (Ye *et al.*, 1990).

Gerbera jamesonii belongs to the family Asteraceae. It grows well in the open in tropical and subtropical regions, but in a temperate climate should be protected from frost and cultivated in glasshouses. The genus Gerbera consists of 30 species, which are of Asiatic and African origin. Among the different species, *G. jamesonii* is the only species under cultivation. The development of *G. jamesonii* as a floricultural crop is traced from its cultivation as a novelty in South Africa to its establishment as a commercial crop in the 1930s.

2.2 Botanical description

Gerbera is a stemless perennial herb; leaves radical, petiolated, lanceolate, lobed, narrower at the base and wider at tip and are arranged in a rosette at the base; flower heads solitary, showy, yellow, orange or scarlet many flowered. The

flower of gerbera is a large capitulum with striking, two-lipped ray florets in yellow, orange, violet, white, pink and red colours. The capitulum, which has the appearance of a single flower, is actually composed of hundreds of individual flowers. The morphology of the flowers varies depending on their position in the capitulum (Bose *et al.*, 2003).

2.3 Genetics

Gerbera is a diploid species with the somatic chromosome number 2n=50. The modern gerbera arose from *G. jamesonii* hybridized with *G. viridifolia* and possibly other species (Leffring, 1973). There is a wide range of variation available in this crop. Changes in chromosome number have influenced on the development of phenotype. Increased plant as well as flower size was reported in tetraploids (Pryor, 1972).

Productivity is mainly determined by the additive effect of genes. Genes with additive effect have the principle role in flower character inheritance. While studying the inheritance character, Kannan and Ramdas (1990) found significant difference in both general and specific combining ability. High heritability for inflorescence diameter and number of ray florets and relatively low for disc diameter and stem length was reported by Schiva, 1973. Self fertilization resulted in production of less number of viable seeds and high proportion of plants with poor characteristics. Heterosis and heritability for ligule length were high and well correlated with the certain other flower characters (Schiva, 1979).

2.4 Breeding

Numerous cultivars of gerbera have been evolved through hybridization. For breeding, flowers of *G. jamesonii* could be grouped into florets narrower than 5 to 5.5mm and those wider than 5.5mm. Tjia (1984) attempted to evolve varieties for flower form and colour, stem length and stiffness, keeping quality, period of flowering and resistance to biotic and abiotic factors. Leffring (1968) crossed several modern cultivars with wild species to develop a dark centred large flowered hybrid.

Sparnaaij *et al.* (1975) described a procedure for testing the resistance of gerbera seedling to *Phytophthora cryptogea*, causing wilt. Results of diallel cross indicated that in most cases genetic variation was likely to result from additive gene action.

Gagnon and Dansereau (1990) attempted to evolve varieties for low temperature. Genotypes adapted to cool temperature were crossed with 20 genotypes having good floral character in pairs to give 10 full sub families. Progeny was raised and assessed the floral attributes. Mean number of flowers produced by first eight families ranged from 8.09 to 12.90 / plant compared to 16.54 and 19.95 for two cool adapted parents.

2.5 Mutation

Ethyl methyl sulphonate (EMS) was effective than sodium azide in inducing single gene mutations in seeds of gerbera. Increasing the EMS concentration from one per cent to two per cent and that of sodium azide from 0.1mM to 1mM decreased germination, growth, survival, frequency of flowering plant, seed setting and number of seeds in M_1 generation and mutation occurring the M_2 generation. Schiva *et al* (1984) obtained a large non-lethal M_2 population using low concentrations of EMS for shorter duration.

Jain (1993) discussed possible approaches for creating genetic variability for the selection of useful plants, with reference to gerbera using mutagenesis, somaclonal variation and genetic transformation. Somaclonal variation may arise due to genetic or epigenetic changes, the latter being unstable and not transmitted to the progeny have to be identified before plants are multiplied for large-scale production using biochemical and molecular markers. It is suggested that genetic engineering has yet to provide a cost effective and reliable products and moreover unstable gene expression, especially for flower colour is a major problem in transgenic plants under field condition.

Shoots from *in vitro* plantlets of the pink gerbera cv. Rebecca were irradiated with a single dose of 20 Gy gamma rays (9.8 Gy/h) and then

micropropagated for two cycles; after rooting the plantlets were cultivated in the greenhouse and analysed for morphological mutations. Irradiation treatment induced a 25 per cent reduction of propagation rates in each cycle. Out of 424 variant flowers, 147 (35%) were chimeric. Of the uniformly variant flowers, 26 per cent showed only single morphological variations, 13 per cent only colour variations and the remaining flowers showed a combination. Laneri *et al.* (1990) concluded that in Rebecca more than two micropropagation cycles were necessary to increase the percentage of solid mutants.

2.6 Propagation

2.6.1 Seed propagation

Gerbera belongs to the family Asteraceae and can be propagated by both sexual and asexual methods. Propagation through seed are not adopted as the cultivated gerberas are extremely heterozygous and take more time to flowering. The advantages of seed propagation over vegetative propagation are simplicity and absence of pest and disease transmission. It can be produced both as indoor and outdoor plants. Artificial pollination is necessary during warm and sunny days between 10 and 12 hours and each flower should be pollinated 2-3 times (Stupak, 1985). Nicolini (1964) reported that seeds retain their full viability for only 15 days after harvesting.

According to Franceschetti (1973), the seeds stored at -5 to 5^0C retained high germinability even after two and a half years of storage compared with those stored at room temperature which lost their viability in approximately nine month period.

2.6.2 Vegetative propagation

2.6.2.1 Division

Most of the commercially grown cultivars are propagated through vegetative means to maintain uniformity and genetic purity (Peper *et al.*, 1971).

Among the vegetative means, multiplication through division of clumps is the most common method. It involves dividing large clumps into smaller units from March to June, when plants may be to set out in the field. Koek (1957) found that division of mother plants at the end of February resulted in six plants and they started flowering by May. Trimming of leaves and roots before planting improved establishment and care should be taken cover central part of sucker with soil.

2.6.2.2 Cutting

Gerbera can be multiplied by cutting. The buds in the leaf axial were detached from the mother plants after withholding water for three weeks, pruning of planted in peat substrate for rooting at 25-30^{0}C with 80 per cent RH. Under intermittent mist young stem cutting produced roots and shoots much easily.

2.6.2.3 Micropropagation

The goal of micropropagation is to obtain a large numbers of genetically identical, developmentally normal with high potential to survive in the *ex vitro* condition. Although true-to-type plantlets are the desired goal of micropropagation, but many times somaclonal variants are obtained due to involvement of tissue culture components. Micropropagation aims at producing exact replica of the original plant selected for its desirable characters in many plant species (Bhojwani, 1980; Wang and Hu, 1982). The micropropagation protocol can be used either for commercial mass micropropagation or crating variable population which will serve as the base material for further breeding programmes as this is high demand for new variants in gerbera.

For commercialization, quality planting material of gerbera is required on large scale, which requires the development of an easier, quicker and economically viable method of propagation. A tissue culture procedure has been proven to be commercially practical in gerbera propagation. Tissue culture method enables a million fold multiplication of a desired plant (Murashige *et al.*, 1974; Aswath *et al.*, 2002, 2003).

2.7. Studies on micropropagation

2.7.1. Micropropagation using different explants

The type of explant, its size, position, age, physiological state and the manner in which it is cultured can determine the morphogenesis in gerbera. Shoot tip, flower bud, flower, leaf, peduncle, petiole are the commonly used explants for *in vitro* gerbera multiplication (Cardoso and Silva, 2013).

Pierik and Segers (1973) reported that when inflorescence was cut into six instead of four explants, the total number of shoots per flower was the same, but it decreased when the inflorescence was cut into eight, ten or twelve explants. A slightly higher number of shoots per explant was obtained from the inflorescence collected in the month of June and July compared to those in other months. They concluded that there was a very small probability that these shoots were of adventitious origin. They insisted that shoot primordia in a quiescent state existed in the axils of the involucral bracts.

Pierik *et al.* (1975) propagated gerbera using fully developed flower. They found no differences in shoot formation between wide open and older flowers. However, they found closed flowers and very young flowers were inferior and had more infection than other flowers. Optimum rooting was obtained by IAA or IBA at a concentration of 10 mg/l, IAA induced a smaller number of long roots, whereas IBA induced larger number of short roots. Gregorini *et al.* (1976) opined that i*n vitro* culture of vegetative apices was a valid method for clonal multiplication of gerbera.

The culture of portions of young capitulum at the bud stage of inflorescence was proved more productive than using fully developed inflorescence (Laliberte *et al.*, 1985). They used immature inflorescence of 0.5 to 0.7 cm in diameter and after sterilization, involucral bracts were removed. The hypothesis of Pierik and Segers (1973) and Pierik *et al.* (1975), that the dormancy of pre-existing buds in the axils of the involucral bracts could be broken in the culture conditions, but this was excluded by Laliberte *et al.* (1985), since

involucral bracts had been removed from the explants. According to them the buds resulted from the reorientation of meristematic tissues in the explant.

Sitbon (1981), Meynet and Sibi (1984), Ahim and Vieth (1986) and Tosca *et al.* (1990) showed the feasibility of producing haploid plants through *in vitro* ovule culture.

Arello *et al.* (1991) used capitulum explants to produce a large translucent callus. Kafarski and Hauzinska (1974) also used young capitulum as explants for the multiplication of *Gerbera jamesonii.*

Gerbera plants were regenerated and propagated *in vitro* from floral peduncle explants by Le-CL *et al.* (1999). *De novo* regeneration of adventitious buds was possible by cutting peduncle tissues on a basal salt MS medium, which led to the production of axillary buds at the rate of 10 newly formed shoots per initial explant and per subculture.

Zhao *et al.* (2002) reported that the contamination rate could be lowered greatly if the receptacle was used as explants for gerbera. Maximum per cent contamination was observed in shoot tip and flower (fully developed) explants.

Modh *et al.* (2002) successfully used young capitulum having a diameter of 0.5 to 1.0 cm as an explant for gerbera cultivar Atella for *in vitro* culturing. The regenerated plantlets were transferred to poly-bags containing soil: sand: FYM (1:1:1) mixture and kept under high humidity for 3 days.

The shoot tips of 0.5 - 1.0 cm length and leaf discs and petiole of 1 - 2 cm length were used as explants for *in vitro* culturing by Mohanty *et al.* (2005). The shoot tip was widely used as explant for gerbera tissue culture studies by many workers (Murashige *et al.,* 1974; Gregorini *et al.,* 1976; Raalte-D-Van, 1978; Petru and Matous, 1984; Huang and Chu, 1985; Parthasarathy and Nagaraju, 1995).

Shoot tip was used as the primary explant for *in vitro* establishment in which MS medium supplemented with a low level of NAA (0.5 mg/l) and BAP

(1.5 mg/l) promoted earliest axillary bud initiation within 5 days in 91.6 per cent of the cultures (Gantait *et al.*, 2010). In the first phase of acclimatization, a balanced mixture of sand, soil and cow urine with tea leaves extract (1:1:1:1 v/v) provided optimum anchorage to the plants and helped in primary regeneration of roots within ten days.

Shabanpour *et al.* (2011) evaluated the organogenic response of *G. jamesonii*, orange and pink cultivars under *in vitro* culture. Different levels of Gamborg's medium (BA) (2.0, 4.0 and 6.0 mg/l) and thidiazuron (TDZ) (0.2, 0.4 and 0.6 mg/l) in combination with IAA (0 and 0.1 mg/l) in MS medium were evaluated for shoot induction. They also observed that orange and pink gerbera cultivars showed differences in shoot regeneration potential (88 and 44%, respectively) and number of shoots/explant (3.1 and 1.7 respectively) on MS medium with 4.0 mg/l BA and 0.1 mg/l IAA using capitulum explants

Bhargava *et al* (2013) were carried out study on micropropagation of *Gerbera jamesonii* Bolus cv. Harley. Capitulum explants were tried to develop protocol for organogenesis and plant establishment. They obtained callus on modified MS medium supplemented with BA (4.0 mg/l) and IAA (0.5 mg/l). The optimum number of quality shoots were obtained on MS medium containing 4 mg/l BA and 2 mg/l kinetin. For *in vitro* rooting, half strength medium supplemented with 2 mg/l IAA was found good. Maximum survival of plantlets (90%) after four weeks was observed in coco peat containing pots which were covered with polythene.

2.7.1.1 Micropropagation using flower bud explants

Schum and Busold (1985) observed *in vitro* shoot production to be higher and quicker from one to two cm long flower bud explants. They further noticed that shoot development in the axils of involucral bracts was most common in buds, while shoots from undifferentiated callus occurred more frequently in fully developed inflorescence. They used immature inflorescence of 0.5 to 0.7 cm in diameter and after sterilization involucral bracts were removed.

Shailaja (2002) studied the suitable explants for multiplication of gerbera at Department of Horticulture, UAS, Dharwad and found bud and flower as suitable explants for the establishment of *in vitro* gerbera culture. She also observed 100 per cent rooting, improved shoot and root growth on MS medium supplemented with 1 mg/l NAA. Maximum survival percentage (60%) with better plant vigour on perlite as hardening medium was recorded.

Flower bud age and position of the explants influences *in vitro* organogenesis in gerbera. Young flower buds (7 days) stimulated callus production while 10 to 14 days old flower buds stimulated direct shoot organogenesis on the same medium; the highest survival (98%), direct shoot induction and number of shoots/explant were observed from 10-days old flower buds in tTCL culture (Nhut *et al.*, 2007). They also observed that the multiplication rate was best in ½ MS medium containing 0.5 mg/l IBA, 0.5 mg/l BA and 3.0 mg/l Kin.

Son *et al.* (2011) used the flower buds of three varieties of gerbera as explants for culture establishment. MS medium supplemented with 3.0 mg/l BAP + 0.1 mg/l IAA was found best for culture establishment. More number of shoots (11.29) was produced in MS medium supplemented with 3.0 mg/l BAP + 0.1 mg/l NAA. They found better shoot multiplication rate in MS medium with 3.0 mg/l BA and 0.1 mg/l NAA, ranging between 5.8 and 15.5 in four gerbera cultivars. Effect of hardening media *viz.*, vermiculite alone, vermiculite + cocopeat (1:1 v/v) and sand + cocopeat (1:1 v/v) on survival percentage and growth of gerbera plantlets were observed. The best plant vigour and maximum survival of plants (67%) was observed in vermiculite medium followed by vermiculite + cocopeat (1:1 v/v) mixture (50%).

Akter *et al.* (2012) used three types of tender leaves as explants such as leaf tip, leaf with mid-rib and leaf blade segments, flower buds and flower stalks obtained from three selected varieties of gerbera (*Gerbera jamesonii* Bolus) for callus induction and *in vitro* regeneration of plantlets. Among the explants flower

bud and flower stalk were suitable and superior for callus induction and subsequent regeneration of *in vitro* shoots when cultured on MS medium supplemented with 5.0 mg/l BAP and 1.0 mg/l NAA. However, highest numbers of multiple shoots were obtained when the flower bud derived callus was subcultured on MS supplemented with 2 mg/l BAP.

Kadu (2013) developed protocol for rapid clonal micropropagation of gerbera by auxiliary bud. The basal medium containing MS + 2.0 mg/l BAP + 0.5 mg/l NAA, gave early bud initiation (19.40 days) with higher initiation per cent (91.66%) along with longer shoot length (2.5 cm). *In vitro* rooted plantlets was successfully transferred to sand + soil +FYM + leaf mould, plant showed highest survival rate (82.43%), longer plants (4.6 cm) and more number of leaves/plant with more number of roots/plant (3.467).

Shylaja *et al.* (2014) developed a highly efficient micropropagation protocol for multiplication of gerbera with unopened flower bud as explants. For culture establishment and propagule multiplication, MS medium supplemented with 3.0 mg/l BAP and NAA 0.1 mg/l was found good. Shoot were elongated in MS medium supplemented with 0.1 mg/l NAA. For root morphogenesis, MS medium supplemented with IBA 1 mg/l was found effective with more number of roots and longer roots.

Rahman *et al.* (2014) reported an *in vitro* propagation of a red gerbera (*Gerbera jamesonii*) variety by culturing flower bud, leaf segments and flower stalk segments in MS medium supplemented with different concentration (1-6 mg/l) of BA in combination with single concentration (1 mg/l) of NAA. Lower concentration of BA (1 and 2 mg/l) with NAA induced the explants to form callus. When the explants were cultured in higher concentration (5 mg/l) of BA, produced shoots and medium with 5.0 mg/l BA with 1.0 mg/l NAA was found best for shoot proliferation of the three explants, optimum response was obtained from flower buds. Further multiplication of shoots occurred upon transfer of shoot clumps to BA containing MS medium. Maximum frequency (81%) of rooting

with highest number (4) of roots per shoot was achieved in MS medium fortified with 0.3 mg/l IBA.

2.7.1.2 Micropropagation using leaf explants

Hedtrich (1979) observed the regeneration of adventitious shoots from leaf blades during *in vitro* propagation of variety Vulkun in modified MS medium supplemented with 1.0 mg/l BA and 0.1 mg/l GA3. Shoot formation from leaf calli was noticed during *in vitro* propagation of gerbera cultivar Vulkan.

Adventitious shoot formation from leaf explants occurred at the base of leaf petiole (Jezry and Lubomskii, 1991). Leaf, petiole and apex as explants of gerbera were used to establish tissue and cell cultures by Ruffoni and Massabo (1991). Leaf explants produced numerous roots and apex explants produced more shoots and calli.

Ruffoni and Massabo (1991) indicated that *in vitro* plant, leaf petiole and shoot apices were able to regenerate some buds, whereas leaf laminas were only able to regenerate callus and roots.

Jerzy and Lubomski (1991) examined the effects of preparation of leaf explants, composition of culture medium, the kind of *ex vitro* stock plant and low temperature pre-treatment on the formation of adventitious shoots in cv. Sardis. Adventitious shoot formation occurred at the base of leaf petiole with 0.5 mg/l IAA. They obtained the highest number of shoots/explant with 3 mg/l BA.

Reynoird *et al.* (1993) gave modified MS medium supplemented with 1.0 mg/l BA and 0.25 mg/l NAA for plant regeneration from *in vitro* leaf culture of several gerbera species. The morphogenetic potential varied with the developmental stage of the leaves and up to 90% of excised developing leaves formed 3-5 shoots per explant.

Efficient bud regeneration was obtained from leaf explants of a clone of *Gerbera hybrida* cultured on modified MS medium. Morphogenic potential varied with the development stage of the leaves. Up to 90 per cent of excised developing

leaves formed three to five shoots per plant. Bud regeneration was not obtained on same medium with fully expanded leaves (Reynoird *et al.*, 1993). They noticed the effect of developmental stage of leaf on morphogenic potential in clones 10 and 11 of *Gerbera hybrida*. Further, they observed that younger leaves located in the centre of the rosette were quite difficult to excise, but displayed the greatest ability to regenerate. A similar observation was made by Orlikowska *et al.* (1995) where, the production of adventitious shoots was best from the young leaf explants compared to the explants from petiole bases of old leaves.

Palai *et al.* (1998) developed an efficient protocol for mass cloning of gerbera cv. Fredaisy *in vitro* by manipulating growth regulators and culture conditions. Leaves were used as the explant source for callus culture. The cultures inoculated in the light exhibited a higher rate of shoot bud differentiation than those inoculated in the dark.

Parthasarathy and Nagaraju (1999) reported callus induction and subsequent plant regeneration from leaf explant of *G. jamesonii* in MS medium supplemented with NAA, BA, kinetin and IBA in different concentrations. Callus growth was greatest with 1 mg/l of NAA, BA and IBA. Adventitious shoots were observed in MS medium supplemented with 1.0 mg/l NAA and 0.75 mg/l IBA. More multiple shoots formed in varieties SWM and Dilmaya when shoot explants were inoculated in MS medium with 0.5-1.0 mg/l BA. Acclimatized plants transferred to polybags containing equal amount of sand + soil + FYM were established better with 95-100 per cent survival.

In vitro propagation of gerbera was studied by culturing leaf explants on a medium containing 0-4 mg/l BA and 0-1 mg/l IAA. Highest multiplication rates (9.5-11.2) were obtained on 1 mg/l BA, irrespective of the IAA concentration used. Highest root multiplication rates (9.5-11.2) were obtained on modified MS medium supplemented with 1 mg/l BAP, independently of the IAA concentration used in a study on *in vitro* propagation of gerbera cultivar Applebloesem using leaf explants (Barbosa *et al.*, 1993).

Orlikowska *et al.* (1999) produced callus from petiole explant of the youngest 3-4 leaves detached from axillary shoots produced *in vitro* with 2.3μM TDZ and 0.53μM NAA in variety Bal, Mariola and Rebecca. Direct shoot regeneration occurred during the first four weeks and the effectiveness of shoot regeneration depended on the cultivar, the sequence of passage on regeneration medium, growth regulators and duration of the induction period.

Aswath and Choudhary (2002a) obtained high leaf-derived-callus induction (65–90%), shoot regeneration frequency from callus (60–83%) and number of shoots per callus cluster (6–14) in gerbera cv. AV101 and AV108 from leaf explants. A high frequency of shoot organogenesis and plant establishment protocol was developed for *ex vitro* leaf derived callus with 0.4 mg/l BA and 4.0 mg/l NAA. The number and length of roots were best in half MS medium supplemented with 0.5 mg l-1 2, 4-D and 0.5 mg/l NAA and the root formation rate was 100 per cent.

Shiqing *et al* (2002) observed shoot regeneration from leaf and petiole segments of gerbera. They obtained regenerated shoots in media containing MS + 3.0 mg/l BA + 0.1 mg/l NAA and MS + 3 mg/l BA + 0.2 mg/l NAA for multiplication of plantlets. *In vitro* plantlets used for explants were at the stage with 3-4 leaves and suitable size of cut leaf segments was about 4×4 mm.

Xu *et al.* (2002) and Kumar *et al.* (2004) observed adventitious shoots from petiole and leaf pieces of *G. Jamesonii* in a medium supplemented with different concentrations of auxins and cytokinins.

Rapid *in vitro* multiplication of shoots was obtained using capitular section and leaf explants in *Gerbera jamesonii* (Tyagi and Kothari, 2004). They used three types of basal media including MS and two of its modified forms MSI and MSA. They obtained shoot induction in all three regimes but MSI medium supplement with kinetin (4.0 mg/l) and IAA (0.5 mg/l) was ideal for shoot initiation. They also observed that 2.0 mg/l kinetin combined with 0.5 mg/l

phenylacetic acid (PAA) promoted best multiplication rate and better recovery of shoots than 0.5 mg/l IAA.

Aswath *et al.* (2003) observed that ½ MS resulted in faster shoot multiplication while full-strength MS culture medium resulted in a higher number of shoots, longer shoots and greater shoot weight. They also observed that all three varieties of gerbera (GJ-1, GJ-2 and GJ-3) showed highest shoot number/explant in full-strength MS medium supplemented with 1.0 mg/l BA or 5.0 mg/l Kn, both combined with 0.1–0.2 mg/l IAA

Xi and Shi (2003) micropropagated four gerbera cultivars using 0.5–1.0 cm long young leaves with 1.0 mg/l BA and 0.1 mg/l NAA.

Prasanth and Sekar (2004) used leaf bits excised from cv. Mammut and cultured them on medium containing 0.1 mg/l BA and 0-2 or 3 mg/l NAA. The combination of BA and NAA was more effective in enhancing callus formation than either NAA or BA, and this effect increased with increasing concentrations of both growth regulators.

Kumar and Kanwar (2006) observed callus induction on leaf and petal explants on MS medium supplemented with 1, 1.5 and 2 mg/l 2, 4-D. The calli derived from leaf explants differentiated into roots with NAA. BA and kinetin failed to induce callus on leaf and petal explants in cut flower gerbera.

The maximum callus induction and growth in petal explants was observed in MS medium supplemented with 1, 1.5 and 2 mg/l 2, 4-D and in leaf with 1.5 and 2 mg/l 2, 4-D, however the callus failed to differentiate into shoots. The cytokinins (BA and Kin) failed to induce callus on leaf explant. This variation in callus induction/differentiation in different explants resulted due to the maturity of petiole and leaf explants in response to different growth regulators (Kumar and Kanwar, 2006). Similarly, Bonga (1987) reported that the type of explant used for induction of callus mainly depends upon the juvenility of the explants.

Kumar and Kanwar (2007) regenerated plants of *Gerbera jamesonii* from cell suspension cultures of calli derived from leaf tissue explants. Callus induction was best when the medium contained either 1.5 or 2.0 mg/l 2, 4-D. The percentage (68.7%) of calli that produced shoots and the number of shoots (10) per callus were highest in MS medium supplemented with 1.0 mg/l BA.

Callus induction from young leaf was found best on MS basal media containing 5.0 mg/l BAP + 0.25 mg/l IAA. Later they found best shoot regeneration medium containing MS basal salt supplemented with 5.0 mg/l BAP and 0.1 mg/l IAA (Kalra *et al.*, 2008).

Callus was formed from *in vitro* juvenile leaf explants of gerbera on 58 different MS-based media containing different concentrations and combinations of plant growth regulators *viz.*, BAP, Kin, Zeatin, NAA, IAA and ABA. Two different shoot formation media were evaluated, both contain 2 mg/l NAA but different concentration of BAP (2 and 4 mg/l). The highest shoot regeneration frequency was 36.6 % which was obtained in induction medium containing 2.0 mg/l BAP and 0.25 mg/l ABA and shoot formation in medium containing 2 mg/l of each of BAP and NAA (Hussein *et al.*, 2008).

Paduchuri *et al.* (2010) developed protocol for callus induction and root regeneration for *Gerbera jamesonii* using leaves as explants source. The optimal callus was developed on MS basal medium supplemented with BAP 2mg/l + Kinetin 1 mg/l + NAA 2mg/l + 2,4- D 2.5 mg/l. The formation of callus was positively correlated with the concentration of growth regulators in the nutrient media. The best roots were derived from callus in MS medium containing IBA 5 mg/l.

Shabbir *et al.* (2012) observed three types of callus depending on the plant growth regulator used in the medium: friable in medium with IBA (1-3 mg/l), friable and nodular in medium with NAA or 2,4-D (1-3 mg/l) and compact and nodular in medium with BA (1-4 mg/l).

Patidar *et al.* (2013) tried somatic tissues and seeds for raising reproducible cultures of *Gerbera jamesonii*. Mature embryos and leaf sections were used as explants and cultured in MS medium supplemented with various auxins and cytokinins in varying concentrations. Callusing was observed in both explants, however shoot regenerations was obtained only from mature embryo explants. For cultured leaf disc, MS basal medium amended with 2-4 mg/l auxins (2, 4-D and NAA) promoted higher degree of callus induction, however, regeneration was not attained. Higher in vitro rooting response was exhibited by rooting medium MS + 0.1 mg/l IBA.

Ying *et al.* (2014) studied effects of different genotypes, leaf ages, parts and hormones on callus induction and bud differentiation using leaf explants They found that induction rate was above 98 per cent when tender leaves were used the explants for callus induction and culture medium was MS + BA 1.0-5.0 mg/l + NAA 0.2 mg/l. The callus from petiole explants produced adventitious bud when subcultured in MS + TDZ 2.0 mg/l + NAA 0.2 mg/l, which had a low differentiation rate of 4.2 per cent.

Recently, Prasad (2014) reported callus regeneration from leaf and flower stalk explants cultured on the MS medium supplemented with 1.5 mg/l 2,4-D. The calli from leaf explants differentiated into shoots after 6-8 weeks of incubation in MS medium containing 1mg/l BAP, about 76.2 per cent of calli produced shoots.

2.7.1.3 Media manipulation and morphogenesis

Sahavacharin (1985) could multiply gerbera most rapidly in MS medium with 0.75 mg/l IAA and 12 mg/l Kin.

Huang and Chu (1985) found the best sucrose concentration to be 2 per cent for multiple-shoot induction (3 shoots/explant) in MS medium with half the concentration of macro-nutrients, 5.0 mg/l BA and 0.1 mg/l IAA, solidified with 10 g/l Bacto-agar.

Huang and Chu (1985) obtained 90 per cent rooting within a period of two weeks from explants of shoots of cultivars Arendsoog and Super Giant Yellow by treating with 0.1 per cent IBA solution for 30 seconds and planting into a sand bed. Meyer and VanStaden (1988) reported that shoots rooted *in vitro* on MS medium supplemented with 5 to 10 µM IBA or 5 to 10 µM NAA.

Zakharova (1987) reported that cultivars Auriga-Karmezin and Sonia produced best roots in MS medium supplemented with IAA at 1 mg/l. Mariska *et al.* (1989) obtained best rooting of shoots with IAA at 0.1 mg/l, while the use of NAA gave short swollen roots.

Szule and Rogozinska (1994) reported that more number of roots was obtained in cultivar Monaco with IBA at a concentration of 0.25 mg/l. They also reported that activated charcoal inhibited root formation and reduced number of roots.

Liqing *et al.* (1996) concluded that the rooting media supplemented with ABT (amino benzo triozole) at a concentration of 0.5 mg/l resulted in 100 per cent rooting and 98.9 per cent survival of transplanted plantlets.

Deepaja (1999) reported that the rooting response was better with IBA compared to NAA. MS basal media was found to be the best for *in vitro* rooting in GJ-1 and GJ-2. The cultivar GJ-3 rooted well at 0.5 mg/l IBA. The medium containing NAA induced swelling of roots in GJ-2 and callus production in GJ-3.

Posada *et al.* (1999) showed best multiple shoot induction in gerbera with 1 or 2 mg/l BA in MS-H medium.

Hitmi *et al.* (1999) observed that addition of NAA in propagation of *in vitro* was completely inhibitory to root initiation.

Budi and Suwandi (2000) obtained most multiple shoots in gerbera containing full-strength MS medium with 500 mg/l casein hydrolysate, 1.0 mg/l BA and 0.2 mg/l NAA.

Bouman *et al.* (2001) showed that MS was superior to DKW medium in shoot multiplication. Cytokinis such as Kinetin and TDZ can be used in the multiplication stage of gerbera micropropagation.

Sousa *et al.* (2006) showed that media containing 75 and 100% of MS salts was best for multiplication and obtained a high multiple shoot induction in gerbera using a combination of 0.5 mg/l IBA and 0.5 mg/l BA.

Chakabrarty and Datta (2008) also observed superiority BA for *in vitro* gerbera shoot multiplication than other Cytokinis, such as Kinetin and TDZ and showed that the best multiplication rate (17:1) took place in 2 mg/l BA.

A combination of 2.0 mg/l BA and 0.3 mg/l NAA resulted in more shoots/ explants when MS medium was used in the multiplication stage (Feng *et al.*, 2009).

Naz *et al.* (2012) were obtained best shoot multiplication using 10 mg/l BA (9 shoots/explant) without reporting any hyperhydricity. They also reported best rooting percentage (80%) using 10 mg/l NAA.

2.7.1.4 Hardening and acclimatization

Conti *et al.* (1991) reported that greenhouse acclimatization of plantlets was achieved in 30 days in a peat: perlite (1:1 ratio, v/v) substrate. Qianzhong *et al.* (1998) obtained better root growth and plant survival (93.8%) in rice chaff as hardening medium.

Plantlets were maintained under high relative humidity (90%) for three weeks. Acclimatized plants were kept under a natural photoperiod condition at a temperature of 25 ± 2 ^{0}C. Survival rate of plantlets was almost 100 per cent when plantlets after root development were transferred to plastic pots filled with coco peat, red soil, and sand in a 3:1:1 ratio (Aswath and Choudhary, 2002b).

Nga *et al.* (2005) reported that 100 per cent of micro-shoots of gerbera rooted in MS medium supplemented with NAA (0.1-1.0 mg/l) after 2 weeks of

inoculation, while 0.1 mg/l NAA gave higher number of roots and good plant vigour. Effect of hardening media *viz.,* soil, sand, rice husk, humus, soil + humus (1:1 v/v), sand + rice husk (1:1 v/v) and humus + rice husk (1:1 v/v) on survival percentage and root growth of gerbera plantlets were also studied. The best plant vigour and maximum survival of plants (93.25%) was observed in humus + rice husk (1:1 v/v) mixture followed by sand medium.

Chakabrarty and Datta (2008) regenerated plants with well established roots were transferred to potting mixture containing soil and sand (1:1 v/v) and kept in humidity chamber with 80-90 per cent relative humidity for 0, 5, 10, 15, 20 and 25 days before they were transferred to field. Survival percentage was higher when rooted plantlets were kept under humidity chamber for 15 days.

Hasbullah *et al* (2008) successfully established rooted plantlets of gerbera and all plantlets were transformed on to soil and maintained in the green house. The survival rate recorded plantlets was 75.03 per cent recorded.

Kadu (2013) was successfully transferred *in vitro* rooted plantlets to sand + soil +FYM + leaf mould, plant showed highest survival rate (82.43%), longer plants (4.6 cm), more number of leaves/plant with more number of roots/plant (3.467).

For hardening, rooted plants were transferred to different hardening media namely coco peat, coco peat: perlite (1:1v/v) and coco peat: perlite: vermicompost (1:1:1 v/v) in plastic pots with polythene cover. Out of the three potting mixture, maximum survival of plantlets (90%) after four weeks was observed in cocopeat containing pots which were covered with polythene (Bhargava *et al.*, 2013).

In vitro rooted plantlets of gerbera were hardened in sand, cocpeata and soil mixture with highest (90%) survival percentage (Shylaja *et al.*, 2014).

2.8. Genetic stability studies

The developed countries account for more than 90 per cent of the total world trade in floriculture products. About 156 ornamental genera are currently

being propagated using tissue culture in different commercial laboratories worldwide (Rout *et al.*, 2005).

Netherlands accounts for 33 per cent of the total global market for floriculture trade. Traditionally, new flower colours in ornamental plants are obtained through screening the natural occurring variation. However, traditional breeding methods have failed to produce cultivars with yellow or true red flower colours, resistance to gray mould disease or cold resistance, e.g. in saintpaulia (Kushikawa *et al.*, 2001).

Gerbera (*Gerbera jamesonii* Bolus) commonly known as Transvaal Daisy, ranks fifth in the international cut flower trade. Its commercial propagation through division of clumps and other conventional methods of propagation is slow and inadequate for the production of large number of uniform propagules. Micropropagation from different explants such as shoot tips, capitulum and leaf segments is a viable approach for large-scale multiplication of gerbera (Jerzy and Lubomski, 1991; Reynoird *et al.*, 1993; Aswath and Choudhary, 2002; Shylaja *et al.*, 2014).

A major problem associated with micropropagation is the occurrence of somaclonal variation among the sub-clones of parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissue and organs. The frequency of these variations varies with the source of explants and their pattern of regeneration (somatic embryogenesis/ organogenesis/ axillary bud multiplication), media composition and cultural conditions (Damasco *et al.*, 1996; Salvi *et al.*, 2001). The cryptic genetic defects arising via somaclonal variation in the regenerants seriously limits the utility of micropropagation system.

In any micropropagation protocol, assessment of genetic stability of the plantlets helps to know the true-to-type nature of the plantlets or variability induced in tissue culture cycle. Molecular markers like RAPD, ISSR and SSR are used widely for genetic stability analysis.

2.8.1 Methods of assessment of genetic stability

2.8.1.1 Morphological markers

The earliest studies of genetic characterization and divergence are based on morphological markers such as qualitative and quantitative traits (Arriel *et al.*, 2007). These markers are inexpensive and simple to score and are based on distinct phenotypes such as plant colour, plant height, seed characteristics, etc. The apparent disadvantage of such markers is prominent in studies of genetic diversity where the expression of the phenotype is highly influenced by environmental conditions.

Lack of adequate genome coverage because of limitation of the number of markers and problems of dominance can also be mentioned as weaknesses of morphological markers (Brown, 1978). Furthermore, the expression of these characters which are influenced by the environment may require that plants be grown to a suitable stage before certain characters can be scored.

Orlikowska *et al.* (1999) also observed morphological and physiological variations in micropropagated gerbera variety Mariola plantlets derived from indirect organogenesis of petioles, resulting in dwarf plants that did not flower.

Shiqing *et al.* (2002) reported that regenerable calli and plantlets were obtained from leaf and petiole segments of gerbera *in vitro* plantlets and those regenerated plantlets were morphologically normal to the mother plants.

Morphological characteristics were similar in both seed-propagated and micropropagated *Solanum melongena* L. plants (cv. Arka Shirish) (Mallaya and Ravishankar, 2013).

2.8.1.2 Molecular markers

Two types of molecular markers were generally used for detection such as hybridisation based Restriction Fragment Length Polymorphisms (RFLP) (Botstein *et al.*, 1980) and Polymerase Chain Reaction (PCR) based molecular

markers such as Random Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990), Simple Sequence Repeats (SSR) (Hearne *et al.*, 1992), Sequence-Tagged Sites (STS) (Fukuoka *et al.*, 1994), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al.*, 1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al.*, 1995).

2.8.1.2.1 Polymerase Chain Reaction (PCR) based detection

Polymerase Chain Reaction (PCR) was invented by Mullis and co-workers in 1983, and it is based on enzymatic *in vitro* amplification of DNA (Weising *et al.*, 2005). In PCR, DNA sequence is amplified primers and thermostable DNA polymerase. The PCR reaction involves three steps (denaturation, primer annealing and elongation steps).

2.8.1.2.1.1 Random amplified polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) markers is generated based on the probability that a DNA sequence, homologous to that of a short, oligonucleotide primer (tenmers for RAPDs) will occur at different sizes on opposite strands of a DNA template that is amplifiable by PCR (Williams *et al.*1990; Waugh and Powell 1992).

RAPD analysis is suitable for genotyping, phylogenetic analysis genetic diversity analysis and molecular selection (Williams *et al.*, 1990; Heun *et al.* 1994; Atak *et al.*, 2004).

Varshney *et al.* (2001) studied on RAPD markers for screening the clonal fidelity of *in vitro*-raised bulblets of *Lilium sp.* (Asiatic hybrids) produced through adventitious mode of propagation. Out of the 20 primers used to screen the samples, only 14 primers gave clear reproducible bands. Analysis of individual primers revealed the RAPD patterns produced were all shared by both the *in vitro*-raised bulblets and the mother bulb. There was no variation observed within the tissue culture-raised progenies.

Mandal *et al.* (2010) proved RAPD markers to be an effective and rapid technique for assessing the molecular stability of *in vitro* raised plants of gerbera at genomic level.

The plants regenerated from petiole explants via direct shoot formation were assessed using RAPD and SSR primers, which produced clear, reproducible and scorable bands. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. A similarity between the mother and the *in vitro* raised plants was 100 per cent, which confirmed the true-to-type nature of the *in vitro*-raised plants (Minerva *et al.*, 2012).

Six of 20 random amplified polymorphic DNA (RAPD) markers (Rusinowski and Domeradzka, 2012) were selected and useful to detect genetic variation among eight gerbera cultivars, resulting in polymorphic bands varying from 28.9 to 40% of differentiating bands, depending of the marker selected.

Genetic diversity of 12 accessions of gerbera was assessed through RAPD markers (Prajapati *et al.*, 2014).

2.8.1.2.1.2 Inter Simple Sequence Repeats (ISSR)

ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10-60 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored for the presence or absence of fragments of particular size.

The main advantage of ISSRs is that no sequence data for primer construction are needed. Because the analytical procedures include PCR, only low quantities of template DNA are required (5-50 ng per reaction). ISSRs are largely distributed throughout the genome.

It is a multi locus molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, and

dominant, provides Mendelian segregation and has been successfully applied in genetic and evolutionary studies of many species. It can also be applied in studies involving genetic identity, parentage, clone and strain identification and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Zietkiewicz *et al.*, 1994; Godwin *et al.*, 1997) and diversity analysis.

Polymerase chain reaction (PCR) based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) are immensely useful in establishing the genetic stability of *in vitro* regenerated plantlets in many crop species (Lakshmanan *et al.*, 2007; Joshi and Dhawan, 2007).

Joshi and Dhawan (2007) employed Inter simple sequence repeat (ISSR) marker assay to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication upto forty-two passages. A homogenous amplification profile was observed for all the micropropagated plants and results confirmed the clonal fidelity of the tissue culture-raised *S. chirayita* plantlets.

Alizadeh and Singh (2009) reported that micropropagated plantlets derived from three different grape rootstock genotypes namely, Dogridge (*Vitis champini*), SO4 (*V. berlandieri*×*V. rupestris*) and ARI-H-144 (*V. vinifera*×*V. labrusca*) were subjected to RAPD and ISSR analyses in order to evaluate their genetic stability and/or detect likely existing variations among *in vitro* derived plantlets. Although higher numbers of bands were obtained by ISSR rather than RAPD, but none of the primers showed polymorphism among micropropagated plantlets and their respective mother plants.

Gong and Deng (2010) described the development and characterisation of 99 EST-SSR markers in gerbera which could be utilized in breeding and genetic studies. Through *in silico* analysis of 8,670 unigene sequences assembled from 16,998 gerbera expressed sequence tags (ESTs) in the GenBank, they identified

893 SSR loci, corresponding to 10.3 per cent of the gerbera unigene sequences or one SSR in every 5.16 kb of the ESTs in the gerbera transcriptome.

Gantait *et al.* (2010) reported the clonal fidelity study of micropropagated and sustained cultured clones of *Allium ampeloprasum* L. using ISSR marker system and ensured the continuous supply of quality propagules retaining genetic uniformity.

Bhatia *et al.* (2010) reported true-to-type clonal fidelity is one of the most important pre-requisites in micropropagation of crop species. Genetic fidelity of *in vitro* raised plants of gerbera derived from three different explants, *viz.*, capitulum, leaf and shoot tips was assessed by 32 ISSR markers, for their genetic stability. Out of 32 ISSR markers, 15 markers produced clear, distinct and scorable bands. Fifteen ISSR markers generated monomorphic banding pattern all the clones. The clones derived from capitulum and shoot tip explants did not show any genetic variation, whereas, one of the leaf-derived clones exhibited some degree of variation.

The genetic fidelity of *in vitro*-raised gerbera derived from capitulum explants was assessed by random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers. Out of 35 RAPD and 32 ISSR primers screened, only 12 RAPD and 10 ISSR primers produced clear, reproducible and scorable bands. The number of bands generated per primer was greater in ISSR than RAPD. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Bhatia *et al.*, 2011).

Nadha *et al.* (2011) confirmed the true-to-type nature of the *in vitro* raised clones of *G. angustifolia* Kunth using DNA based RAPD and ISSR markers as they did not detect any variability in the tissue culture raised plantlets. Employin the axillary bud proliferation method for the commercial multiplication of *Guadua* without any risk of genetic instability.

Parida *et al.* (2011) assessed genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. using DNA based molecular markers. They used RAPD and ISSR

markers for assessment of genetic stability. All banding profiles from micropropagated plants were monomorphic and similar to the mother plant.

Borse *et al.* (2011) investigate clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) regenerants from six different *in vitro* subculture generations and in the explant suckers by using ISSR and REMAP molecular markers. Both types of markers revealed high degree of monomorphism. Very low variation was observed up to the eighth subculture generation with polymorphic bands being low in both ISSR (0.96%) and REMAP (0.95%) markers.

Khateeb *et al.* (2013) were assessed genetic stability of micropropagated plants using Inter-Simple Sequence Repeat (ISSR). The amplification products were monomorphic in all *in vitro* grown plants. No polymorphism was detected indicating the genetic integrity of *in vitro* propagated plants.

2.8.1.2.1.3 Simple sequence repeats (SSR) or Microsatellites markers

Simple Sequence Repeats (SSR) is tandem repeats of DNA sequence of only a few base pairs (1-6bp) in length. The term microsatellite was introduced to characterize the simple sequence stretches amplified by PCR (Hearne *et al.*, 1992). The most abundant being the dinucleotide repeat (McCouch *et al.*, 2002).

These are also known as Short Tandem Repeats (STR) or Simple Sequence Repeats (SSR) and differ from minisatellite in which repeated sequences are having repeat units ranging from 11-60bp in length (Edwards *et al.*, 1996). Microsatellite sequences are abundant, dispersed throughout the genome and are highly polymorphic in plant genomes, even among closely related cultivars, due to mutations causing variation in the number of repeating units in genomes (Condit and Hubbell, 1991).

Application of SSR markers could facilitate protection of gerbera varieties from illegal propagation and analysis of genetic diversity in cultivated and/or wild gerberas. These markers could be also very useful for assessment of clonal fidelity (Bhatia *et al.*, 2011) and future gerbera DUS tests (Tommasini *et al.*, 2003).

The success rate for EST-SSR primers (percentage of SSR primers producing discrete amplification products) in various reports was between 60 and 90% (Varshney *et al.* 2005).

Gong and Deng (2010) detected 67.7 per cent of working SSRs polymorphisms from seven cultivars/breeding lines, indicating a relatively high level of polymorphism that was revealed by gerbera EST-SSRs. Similar levels of polymorphism was detected in kiwifruit (Fraser *et al.* 2004) and tall fescue (Saha *et al.* 2004) using EST SSRs.

Gong and Deng (2012) selected 53 robust and informative EST-SSR markers for use in different breeding programme, genetic studies and detection of genetic fidelity in gerbera.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2012-June 2014, with the objective to develop an efficient micropropagation protocol for mass multiplication of gerbera (*Gerbera jamesonii* Bolus) and to test genetic stability of micropropagated plants. The study mainly focused on the following aspects:

1) Micropropagation studies using flower bud and leaf explants, hardening and acclimatization. 2) Genetic stability of micropropogated plants using ISSR assay. The materials used and methodologies adopted in the study are presented in this chapter.

3.1 Materials

3.1.1 Laboratory chemicals, glassware and plasticwares

Chemicals used for the present study were of good quality (AR grade) from various agencies like Merck India Ltd., HIMEDIA and SISCO Research Laboratories. The *Taq* DNA polymerase, dNTPs, *Taq* buffer and molecular marker (λDNA/*Hind*III+*Eco*RI double digest) were supplied by Bangalore Genei Ltd. All the plasticwares used were obtained from Axygen and Tarson India Ltd. The ISSR primers were obtained from Sigma Aldrich Chemicals Pvt. Ltd.

3.1.2 Equipment and machinery

The present research work was carried out using plant tissue culture and molecular biology facilities available at CPBMB, College of Horticulture. All the aseptic manipulations for micropropagation studies were carried out in laminar air flow (LABLIE INDUSTRIES). Media sterilization was done in autoclave (Nat steal equipment Pvt. Ltd.). Centrifugation was done in High speed refrigerated centrifuge (KUBOTA 6500)/ Dynamica Velocity 14R Refrigerated Centrifuge.

NanoDrop^{R} ND-1000 spectrophotometer was used for the estimation of quality and quantity of DNA. DNA amplification reaction was carried out in Agilent Technologies thermocycler (SureCycler 8800). Horizontal gel electrophoresis system (BIO-RAD, USA) was used for agarose gel electrophoresis. Gel Doc - BIO-RAD was used for imaging and documenting the agarose gel profile. The details are given in Annexure I.

3.2. Studies on micropropagation

3.2.1 Source of explants

One released variety of gerbera from IIHR Banglore, Arka Krishika and three varieties procured from A. V. Thomas and Company LTD. (AVT), Kochi *viz.,* Dubai, Shania and Hotspring were used for the study. Details of flower character are provided in Table 1. Source plants were maintained in net house of CPBMB, Collage of Horticulture. The varieties with different flower colours (white, yellow, red and violet) were included (Plate 1).

Table 1: Flower characters of selected gerbera genotypes

Character	Arka Krishika	Dubai	Shania	Hotspring
Type of flower	Double	Semi-double	Single	Single
Flower colour	Yellow	White	Red	Violet
Length of stalk (cm)	34	41	43	40
Girth of stalk (cm)	1.5	1.8	1.8	1.9
Diameter of flower (cm)	7	8	7	6.8
Disc florets colour	Yellow	Light green	Dark violet	Light red

3.2.2 Culture media

3.2.2.1 Preparation of MS medium

Standard procedures were followed for the preparation of MS (Murashige and Skoog, 1962) plant tissue culture media. Five stock (I, II, III, IV and V)

a) Variety Arka Krishika

b) Variety Dubai

c) Variety Shania

d) Variety Hotspring

Plate 1. Different gerbera varieties used for the study

solutions of major and minor nutrients were prepared and stored in pre cleaned glass bottles in refrigerated conditions. Stock III was stored in amber coloured bottle. Stock solutions of different growth regulators were stored under refrigerator conditions.

A clean beaker, rinsed with distilled water was used to prepare the medium. All stock solutions were pipetted in proportionate volume in the beaker. For preparing MS medium of full strength, 20ml from 50 X stocks and 10ml from 100 X stocks and required quantities of sucrose, inositol and hormones were added and dissolved in it. The desired volume was made up by adding distilled water. The pH of the medium was adjusted to 5.5 using 0.1 N NAOH.

The volume was finally made up and required quantity of agar was added to the medium. Agar in the medium was completely melted in a microwave oven. Then 15 - 20 ml of hot medium was poured into 25 x 150 mm pre-sterilized glass culture tubes and plugged with non-absorbent cotton and autoclaved. Chemical compositions of MS medium are given in Annexure II.

3.2.2.2 Autoclaving

The test tubes with nutrient media were autoclaved at a pressure of 1.06 kg cm^{-2} (121^0C) for 20 minutes. The tubes were then removed from the autoclave and allowed to cool. The inoculation was done 4 - 5 days after media sterilisation ensuring that the tubes were free of microbial contaminations.

3.2.3 Experimental conditions

3.2.3.1 Transfer area and aseptic manipulation

All the aseptic manipulations such as surface sterilisation of the explants and inoculation and subsequent sub-culturing were carried out in the laminar air flow cabinet. The work table of laminar air flow cabinet was sterilized by swabbing with 70 percent alcohol. The UV light was switched on for half an hour to achieve aseptic environment inside the cabinet and air was allowed to blow off for 20 minutes before working in the lamina air flow cabinet.

3.2.3.2 Culture conditions

The cultures were incubated at 26±2^{0}C in an air conditioned culture room with 16 h. photoperiod (1600 lux) from florescent tubes. Humidity in the culture room varied from 60 to 80 per cent according to the prevailing climate.

3.2.4 Methods

3.2.4.1 Preparation of explants

Explants were collected from source plants maintained in the net house of Centre for plant Biotechnology and Molecular Biology (CPBMB) and *in vitro* culture supplied from CPBMB.

3.2.4.1.1 Flower bud explants

Plantlets from flower bud explants were regenerated as per the procedure developed at CPBMB by Shylaja *et al.* (2014).

Immature unopened flower buds were collected from source plants maintained in the net house. After sterilization, the buds were cut vertically into four to eight pieces and each piece was inoculated separately on culture establishment medium.

3.2.4.1.2 Leaf explants

Leaf explants were collected from source plants maintained in net house and also from *in vitro* multiple shoots cultures supplied from CPBMB. Leaves were made into 4 - 5 mm pieces along with the portion of midrib. They were inoculated in such a way that the basal portion of the leaf was in contact with the medium.

3.2.4.1.3 Surface sterilization of explants

The explants from *in vivo* source were washed thoroughly in running tap water. Then they were immersed in a solution of Bavistin (0.1%) and two drops of Pril for 10 minutes and washed thoroughly with distilled water. They were later

sterilised with 0.1 per cent $HgCl_2$ for seven minutes and washed with sterile double glass distilled water for three to four times in laminar air flow cabinet to remove any traces of $HgCl_2$. After surface sterilization, the explants were allowed to dry by transferring them onto sterile tissue paper on a sterile petridish.

3.2.4.1.4 Culture establishment

The sterilized explants were inoculated to culture establishment medium. The cut ends of explants were kept in such a way that they had maximum contact with the medium.

3.2.4.1.5 Propagule multiplication

Micro shoots formed from flower bud/ leaf calli were taken out after three weeks of inoculation. The shoots were separated and inoculated to multiplication media for propagule multiplication.

3.2.4.1.6 Elongation

The healthy shoots were transferred to elongation medium for elongation of shoots.

3.2.4.1.7 Rooting

The *in vitro* micro shoots of three to four cm in length were taken out from multiple shoot cultures and kept for induction of rooting.

3.2.4.1.8 Hardening and acclimatization

The *in vitro* rooted plantlets were taken out of the culture vessels using forceps after soaking the culture in water for five minutes. The solidified medium from plantlets was washed out in running tap water. The plantlets were treated with 0.1 percent Bavistin for five minutes and were then planted in small earthen pots filled with different potting media and hardened in hardening unit.

3.2.4.1.8.1 Hardening media

Ten different potting media were studied for hardening micropropagated plantlets of gerbera. Plants derived through indirect organogenesis from leaf explants of variety Dubai were used for the study. The treatments were as follows:

T_1 - Cocopeat : Soil : Sand (1:1:1)

T_2 - Cocopeat : Soil : Sand (2:1:1)

T_3 - Cocopeat : Soil : Sand (3:1:1)

T_4 - Peat : Perlite : Vermiculite (3:1:1)

T_5 - Peat : Perlite : Vermiculite (2:1:1)

T_6 - Peat : Perlite : Vermiculite (1:1:1)

T_7 - Sand : Cocopeat (1:1)

T_8 - Vermiculite : Cocopeat (1:1)

T_9 - Vermiculite

T_{10} - Sand

Plants were observed for two months and survival rate in each potting medium was recorded.

Coco peat

Coco peat is coir fibre pith with coconut husk as its base. It is a soil conditioner and growing medium. Its uniqueness is that it can hold eight times of water of its own weight. It releases nutrients in solution over long intervals. It is best for commercial and home gardening application. It provides breathing space, *i.e.* letting in and letting out of air for roots which help better growth. Added to above mentioned characters, it encourages favourable micro organisms around the root zone and degrades slowly. It will re-wet easily without the use of chemical wetting as it is hydrophilic in nature. It can easily be mixed with other growing media.

Vermiculite

Vermiculite is common ingredient in growing medium. The origin of vermiculite is from mineral called mica. Mica is heated to 1800^0F, which causes it to expand resulting in good water holding ability and aeration. It is a light weight material and can easily be compressed. Vermiculite is available in different grades, ranging from a fine grade for use with seeds to a coarse grade measuring more than 0.5 cm in diameter. Vermiculite has a good cation exchange capacity with neutral to slightly alkaline pH (6.3 to 7.8) and provides small amounts of calcium, magnesium, and potassium.

Perlite

Perlite is an amorphous volcanic glass that has relatively higher water content, typically formed by the hydration of obsidian. It occurs naturally and has the unusual property of greatly expanding when heated. Perlite softens when heated to temperature of 850-900°C. Water trapped in the structure of the material vaporises and escapes and causes the expansion of material to 7–16 times of its original volume. The expanded material is brilliant white, due to the reflectivity of the trapped bubbles. In horticulture, perlite can be used as a soil amendment or alone as a medium for hydroponics or for rooting cuttings. When used as an amendment, it has high permeability/ low water retention and helps to prevent soil compaction.

3.2.5 Assessment of growth of plantlets

Growth parameters *viz.* plants height, number of leaves and leaf length and width were recorded in plants survived in each potting medium after four weeks of transfer to hardening medium.

3.2.5.1 Growth parameters

1. Plant height

Recorded from base of the plant to the tip of the last leaf

2. Number of leaves

All green leaves except the young unfolded one were counted

3. Leaf length and width

Length and width of the leaves were recorded from top three fully opened leaves

3.3 Genetic stability studies using ISSR molecular marker

For analysis of genetic stability of micropropagated plants, ISSR molecular assay was carried out. Source plants, regenerated plants from flower buds (direct organogenesis) and leaf calli (indirect organogenesis) were subjected to genetic stability studies using ISSR assay with seven ISSR primers reported for gerbera by Bhatia *et al.* 2010. The main advantage of ISSR (Inter Simple Sequence Repeat) markers is very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample (5-50 ng per reaction) and they do not need any prior sequence information to design the primer. They do not use radioactive probes as in restriction fragment length polymorphism (RFLP) (Lakshmanan *et al.* 2007). ISSRs are largely distributed throughout the genome. Thus, they are suitable for the assessment of the genetic fidelity of *in vitro* regenerated plants.

3.3.1 Genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important pre requisites for doing ISSR analysis. The CTAB procedure reported by Rogers and Bendich (1994) was used for the extraction of good quality genomic DNA. The young tender leaves were used for genomic DNA isolation.

Reagents (Details of composition of reagents are provided in the Annexure III)

1. 2 X CTAB extraction buffer
   - 2 per cent CTAB (w/v)

- 100mM Tris (pH 8.0)
- 20mM EDTA (pH 8.0)
- 1.4M NaCl
- 1 per cent PVP

2. CTAB (10 per cent)
   - 10 per cent CTAB (w/v)
   - 0.7M NaCl.
3. TE buffer
   -10mM Tris (pH 8.0)
   -1mM EDTA (reagent 1 and 3 autoclaved and stored at room temperature)
4. β- mercaptoethanol
5. Chloroform : isoamyl alcohol (24:1)
6. Isopropanol (chilled)
7. Ethanol (70 per cent)
8. Distilled water

3.3.1.1 Procedure for DNA isolation

Procedure

Young and tender leaf tissue (0.25g) was weighed and ground in a pre-chilled mortar and pestle in the presence of liquid nitrogen and a pinch of PVP. Extraction buffer (2X) 4ml and 50µl of β-mercaptoethanol were added to it. The homogenized sample was transferred to an autoclaved 2ml centrifuge tube and added 3ml of pre-warmed extraction buffer (total 7ml). After mixing thoroughly, the mixture was incubated at 65°C for 20 to 30 minutes with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added and the mixture was mixed by inversion to emulsify. Then it was spun at 10,000rpm for 15 minutes at 4°C. After the centrifugation, the top aqueous layer was transferred to a clean centrifuge tube and 1/10th volume of ten per cent CTAB was added. Equal volume of chloroform: isoamyl alcohol (24:1) was added

and mixed by inversion and centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a clean centrifuge tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till the DNA was precipitated. The mixture was again centrifuged at 10,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off. The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol, spun for 5 minutes at 10,000 rpm and decanted the ethanol. The DNA pellet was air dried for 30 minutes and the pellet was dissolved in 50µl of TE buffer or sterilized water and stored at - 20°C.

3.3.1.2 Purification of DNA

The DNA isolated would contain RNA as contaminant and was hence purified by treatment with RNase and further precipitated (Sambrook *et al.*, 1989).

Reagents

1. Chilled isopropanol

2. 70 % ethanol

3. TE buffer

4. Chloroform: Isoamyl alcohol (24:1, v/v)

5. RNase (1 %)

The RNase-A from Sigma, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase-A in autoclaved distilled water. The solution was dispensed into aliquots and stored at -20°C.

Procedure

RNase solution (2 µl) was added to 100µl DNA sample and incubated at 37°C in dry bath (GeNei, Thermocon) for one hour. The volume was made up to 250µl with distilled water and equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. This was then centrifuged at 10,000 rpm for 15

minutes at 4°C. The aqueous phase was collected into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added. Again it was centrifuged at 10,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a fresh micro centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by gentle inversion till the DNA was precipitated. The mixture was incubated at -20°C for half an hour for complete precipitation and centrifuged at 10,000 rpm for 15 minutes at 4°C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellet was air dried and dissolved in 50µl of autoclaved distilled water.

3.3.2 Assessing the quality of DNA by Electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Materials for agarose gel electrophoresis

1. Agarose
   - 0.8 per cent (for genomic DNA)
   - 1.5 per cent for ISSR
2. 50X TAE buffer (pH 8.0)
   - Tris buffer
   - Acetic acid
   - 0.5mM EDTA

3.6X Loading /Tracking dye
   - 0.03% bromophenol blue
   - 0.03% xylene cyanol
   - 60% glycerol

4. Ethidium bromide (0.5 µg/ml)

5. Electrophoresis unit, power pack, gel casting tray, comb

6. UV transilluminator

7. Gel documentation and analysis system

Chemical composition of buffers and dye are given in Annexure IV. The procedure followed for agarose gel electrophoresis was as follows:

1 X TAE buffer was prepared from the 50 X stock solution. Agarose (0.8%) was weighed and dissolved in TAE buffer (1X) by boiling, then ethidium bromide was added at a concentration of 0.5µg/ml and mixed well. The open end of the gel casting tray was sealed with cello tape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (4 µl) along with tracking dye (1 µl) was loaded into the wells using a micropipette carefully. λDNA/*Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (80V) and current (50 A). The power was turned off when the tracking dye reached 2/3rd length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV transilluminator for presence of DNA. The DNA fluoresces under UV light due to ethidium bromide dye. The image was documented in gel documentation system. The gel profile was examined for intactness and clarity of DNA band.

3.3.2.1 Assessing the quality and quantity of DNA by NanoDrop method

The quality and quantity of genomic DNA was estimated using NanoDrop^{R} ND-1000 spectrophotometer. Before taking sample readings, the instrument was set zero by taking 1µl autoclaved distilled water as blank. One micro litre of nucleic acid sample was measured at a wavelength of 260 nm and 280 nm and OD_{260}/ OD_{280} ratios were recorded to assess the purity of DNA.

A ratio of 1.8 to 2.0 for OD_{260}/OD_{280} indicated good quality of DNA. The quantity of DNA in the pure sample was calculated using the formula OD_{260}= 1 is equivalent to 50 µg double stranded DNA/ µl sample.

1OD at 260 nm = 50 µg DNA/ml

Therefore $OD_{260} \times 50$ gives the quantity of DNA in µg/ml.

3.3.4 Molecular Marker assay

Inter Simple Sequence Repeats (ISSR) was used for assessing genetic stability of micropropagated plants.

3.3.4.1 Inter Simple Sequence Repeats (ISSR) analysis

ISSR assay was performed to detect the polymorphism in amplification patterns in the region between two SSR's. This was carried out by amplifying the DNA by using specific primers relating to the SSR region flanking the ISSR.

Good quality genomic DNA (30 to 50ng/µl) isolated from gerbera leaf samples of plantlets regenerated from buds and leaf explants (direct and indirect organogenesis) were subjected to ISSR assay. ISSR primers supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' with good resolving power were used for amplification of DNA.

3.3.4.2 ISSR primers selected for the study

Based on earlier reports on ISSR assay in gerbera by Bhatia *et al* (2010), seven ISSR primers were selected for the study. Details of selected primers are presented in Table 2. The selected primers were checked for amplification using bulked DNA from different groups of plants in the present study.

Table 2. Details of primers selected for ISSR assay

Sl. No.	Primer	Nucleotide Sequence (5'-3')
1.	ISSR-08	5'- ATTATTATTATTATTGTA -3'
2.	ISSR-09	5'- ACACACACACACACACC -3'
3.	ISSR-15	5'- AGAGAGAGAGAGAGAG -3'
4.	ISSR-18	5'- GAGAGAGAGAGAGAGA -3'
5.	ISSR-21	5'- TCTCTCTCTCTCTCTC -3'
6.	ISSR-25	5'- TGTGTGTGTGTGA -3'
7.	ISSR-31	5'- AGAGAGAGAGAGAGAGC -3'

The amplification was carried out in an Agilent thermocycler. Amplification was performed in a 20 µl reaction mixture as shown below:

Composition of the reaction mixture for PCR

Materials	Quantity (µl)
a) Genomic DNA (30 ng/ µl)	- 2.0
b) 10X *Taq* assay buffer B	- 2.0
c) $MgCl_2$	- 2.0
c) dNTP mix (10mM each)	- 1.5
d) *Taq* DNA polymerase (3U)	- 0.4
e) Decamer primer (10 pM)	- 1.5
f) Autoclaved distilled water	- 10.6
Total volume	- 20.0

The thermo cycler was programmed as follows:

94°C for 4 minutes - Initial denaturation

94°C for 1 minute - Denaturation

40-60°C for 1 minute - Primer annealing } 35 cycles

72°C for 2 minutes - Primer extension

72°C for 8 minutes - Final extension

4°C for infinity to hold the sample

The amplified products were run on 1.5 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA/*Eco*RI+*Hind*III double digest). The profile was visualized under UV transilluminator and documented using gel documentation. The documented ISSR profiles were carefully examined for polymorphism.

3.3.4.3 ISSR assay

ISSR assay was performed with DNA samples isolated from four different source plants and micropropagated plants derived from flower bud and leaf calli using seven primers.

3.3.4.4 ISSR data analysis

Amplification profile of source plants, micropropagated flower bud derived plants and leaf calli derived plants were compared with selected primers. Bands of DNA fragments were scored manually as (1) for presence and (0) for absence. The DNA amplification pattern with selected primers in different groups of plants were analysed and exhibiting variability at DNA level was calculated.

Results

4. RESULTS

The results of the investigations on "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2012 to June 2014 are presented in this chapter under different sub headings.

4.1 Studies on micropropagation

One released variety of gerbera from IIHR Banglore, Arka Krishika and three other varieties procured from A. V. Thomas and Company LTD. (AVT), Kochi *viz.*, Dubai, Shania and Hotspring were used for the study. The flower colour of variety Arka Krishika is yellow while Dubai white, Shania red and Hotspring is violet.

Different explants were used for micropropagation studies *viz.*, leaf and flower bud explants. Leaf explants were collected from *in vitro* multiple shoot culture and *in vivo* grown mother plants maintained at CPBMB, College of Horticulture. Bud explants were collected from mother plants of different varieties maintained in the net house of CPBMB. The micropropagation protocol developed at CPBMB by Shylaja *et al.* (2014) was used to regenerate plantlets from flower bud explants of various genotypes. The protocol for indirect organogenesis from leaf explants was standardized in the present study.

4.1.1 Micropropagation using flower bud explants

The micropropagation protocol developed at CPBMB by Shylaja *et al.* (2014) was used for production of plantlets from flower bud explants. The IIHR Variety Arka Krishika along with three varieties procured from AVT, Kochi was used for micropropagation studies using flower bud explants.

4.1.1.1 Response of different gerbera varieties for shoot regeneration from flower bud explants

The unopened 15 days old flower buds collected from source plants maintained at CPBMB net house were made into vertical segments and they were inoculated to MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA (Plate 2). Shoot regeneration was observed in the culture establishment medium itself (Plate 3a, 3b and 3c). Shoot morphogenesis and response of varieties are presented in Table 3.

Shoot regeneration was recorded three weeks after inoculation. The variety Dubai recorded the highest shoot regeneration percentage (62.5%) followed by Arka Krishika (55%) and Shania (50%). Shoot regeneration was not observed in the variety Hotspring. The number of shoots regenerated was also high in the variety Dubai (32) followed by Arka Krishika (26) and Shania (16). As the flowering was just started for the variety Hotspring, only one flower could be inoculated.

Table 3. Response of different gerbera varieties for shoot regeneration from flower bud explants

Variety	No. of flower bud inoculated	No. of flower bud segment inoculated	No. of flower buds shoots regenerated	Percentage of regeneration	No. of shoots regenerated
Arka Krishika	5	20	11	55.0	26
Dubai	6	24	15	62.5	32
Shania	4	16	08	50.0	19
Hotspring	1	04	-	-	-

Plate 2. Unopened flower bud (15 days old)

a) Variety Dubai

b) Variety Arka Krishika

c) Variety Shania

Plate 3. Shoot regeneration from flower bud explants in diferent gerbea varieties

4.1.1.2 Response of different gerbera varieties for shoot proliferation from flower bud explants

For shoot proliferation, the regenerated shoots of varieties *viz.*, Dubai, Arka Krishika and Shania were transferred to medium of same composition (Plate 4a, 4b and 4c). Number of shoots regenerated was recorded for a period of three subculture cycles and the data is presented in Table 4.

Subculturing was done at one month interval. Progressive total of number of shoots regenerated from a single culture showed that the variety Dubai recorded highest proliferation rate (53.33) followed by Arka Krishika (23.00). Shoot multiplication was found very low in red flowered variety Shania (11.00).

Table 4. Response of different gerbera varieties for shoot proliferation from flower explants

Sl. No.	Variety	No. of shoots in single base culture	1st sub-culture	2nd sub-culture	3rd sub-culture	Mean No. of shoots multipled in each subculture
1.	Dubai	2	14	48	162	53.33
2.	Arka Krishika	3	8	26	72	23.00
3.	Shania	2	7	24	-	11.00

4.1.2 Micropropagation using leaf explants

4.1.2.1 *In vitro* leaf

Multiple shoot culture of variety Dubai obtained from CPBMB was used as source for collecting *in vitro* leaf explants. All the stages of micropropagation were standardised using the variety Dubai and the protocol was further tested in other varieties.

a) Variety Dubai

b) Variety Arka Krishika

c) Variety Shania

Plate 4. Multple shoot cultures of variety Dubai, Arka Krishika and Shania from flower bud explants

4.1.2.1.1 Callusing and callus growth from *in vitro* leaf explants

4.1.2.1.1.1 Effect of various levels of auxin on callusing and callus growth

The *in vitro* leaf segments when cultured on MS medium supplemented with different auxins (IAA, NAA and 2, 4-D) at various levels produced calli three weeks after inoculation. Callusing started from cut ends of leaf segments. Callus growth was scored visually and a maximum score of four was given when calli spared the whole surface of the medium in the tube, three for growth in ¾th of the surface, two for half surface and one for ¼th of the surface. Calli were produced in all media investigated but maximum callus growth (2.32) was observed in MS medium supplemented with 1 mg/l NAA and minimum (0.11) was in MS medium supplemented with 3 mg/l 2, 4-D (Table 5).

Callusing percentage and calls growth were found low in higher concentrations of 2, 4-D (2-3 mg/l). MS medium supplemented with 1 mg/l NAA was found best with respect to callusing and callus growth followed by MS medium with 1 mg/l IAA. In medium supplemented with all levels of NAA, rhizogenesis was observed.

Table 5. Effect of various levels of auxin on callusing and callus growth in variety Dubai

Sl. No.	Media combination	Percentage callusing (P)	Callus growth (G)	Callus index (P x G)
1.	MS+1mg/l IAA	100	2.22	222
2.	MS+2mg/l IAA	100	2.11	211
3.	MS+3mg/l IAA	100	1.61	161
4.	MS+1mg/l NAA	100	2.32	232
5.	MS+2mg/l NAA	100	1.72	172
6.	MS+3mg/l NAA	94.44	1.55	146.38
7.	MS+1mg/l 2, 4-D	100	1.50	150
8.	MS+2mg/l 2, 4-D	33.33	0.33	10.99
9.	MS+3mg/l 2, 4-D	11.11	0.11	1.22

4.1.2.1.1.2 Effect of various levels of auxin and cytokinin on callusing and callus growth

Various combinations of auxins and cytokinins were tried to achieve callusing and callus growth from *in vitro* leaf explants of variety Dubai. IAA-BAP, IAA-kinetin, NAA-BAP and NAA-kinetin combinations were supplemented to basal MS medium.

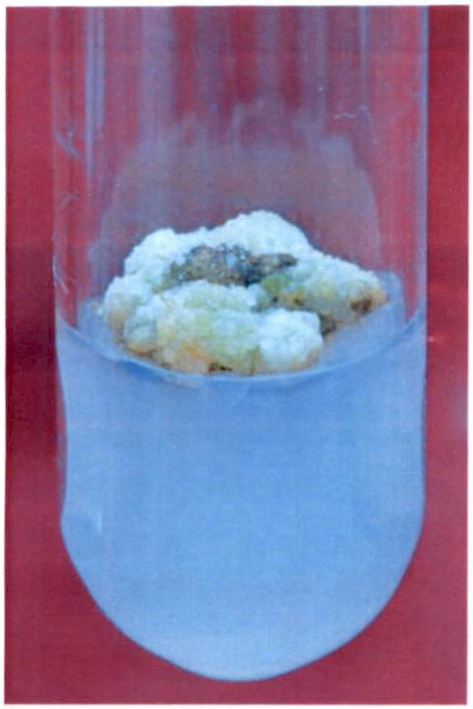
MS medium supplemented with 3.0 mg/l NAA and 0.3 mg/l BAP recorded highest callus growth (2.94) and callusing percentage (100%), while lowest callus growth score (0.42) and callusing percentage (83.33) were observed in 3.0 mg/l IAA and 0.3 mg/l Kinetin. MS medium supplemented with various concentrations of auxins *viz.*, IAA and NAA (3-5 mg/l) along with various concentration of cytokinin BAP (0.3-0.5 mg/l) also found to give good callusing percentage (100%) (Table 6).

MS medium supplemented with NAA and BAP was found to give highest callusing and callus growth. For further studies, calli induced in MS medium supplemented with 3.0 mg/l NAA and 0.3 mg/l BAP was used.

4.1.2.1.1.3 Response of different gerbera varieties for callusing and callus growth

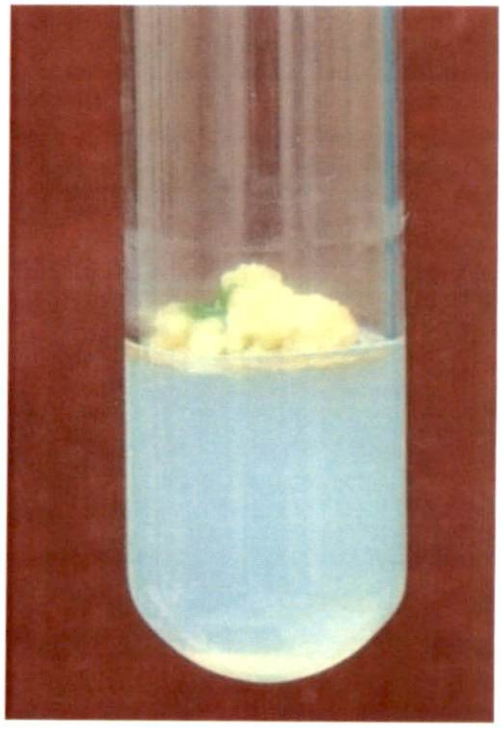
In vitro leaves from other selected types *viz.*, Shania and Hotspring were also studied for callusing and callus growth. Leaf explants were inoculated to MS media containing 3.0 mg/l BAP with 0.1mg/l NAA and 3.0 mg/l NAA with 0.3 mg/l BAP for callus induction. Observations were recorded after three weeks of inoculation (Table 7). The *in vitro* leaf derived calli of different varieties are shown in Plate 5.

In the medium MS + 3.0 mg/l BAP + 0.1 mg/l NAA variety Hotspring recorded highest callusing percentage (88.88%) followed by variety Shania (85.71). Maximum callus growth (0.62) was found in variety Hotspring followed by Variety Shania (0.31).



a) Variety Dubai

b) Variety Shania



c) Variety Hotspring

Plate 5. *In vitro* leaf derived calli of different gerbera varieties

Table 6. Effect of various levels of auxin and cytokinin on callusing and callus growth (var. Dubai)

Sl. No.	Media combination	Percentage Callusing (P)	Callus growth score (G)	Callus Index (P x G)
1.	MS + 3.0mg/l IAA + 0.3mg/l BAP	100	0.46	46.00
2.	MS + 4.0mg/l IAA + 0.4mg/l BAP	100	0.50	50.00
3.	MS + 5.0mg/l IAA + 0.5mg/l BAP	100	0.57	57.00
4.	MS + 3.0mg/l IAA + 0.3mg/l Kin	83.33	0.42	34.99
5.	MS + 4.0mg/l IAA + 0.4mg/l Kin	94.44	0.48	45.33
6.	MS + 5.0mg/l IAA + 0.5mg/l Kin	94.44	0.44	41.55
7.	MS + 3.0mg/l NAA + 0.3mg/l BAP	100	2.94	294
8.	MS + 4.0mg/l NAA + 0.4mg/l BAP	100	2.44	244
9.	MS + 5.0mg/l NAA + 0.5mg/l BAP	100	2.16	216
10.	MS + 3.0mg/l NAA + 0.3mg/l Kin	94.44	2.11	199.26
11.	MS + 4.0mg/l NAA + 0.4mg/l Kin	100	2.44	244
12.	MS + 5.0mg/l NAA + 0.5mg/l Kin	100	2.33	233

Table 7. Response of different gerbera varieties for callusing and callus growth

Media combination	Variety	Percentage callusing (P)	Callus growth score (G)	Callus index (P x G)
MS + 3.0 mg/l BAP + 0.1 mg/l NAA	Shania	85.71	0.31	26.57
	Hotspring	88.88	0.62	55.10
MS + 3.0 mg/l NAA + 0.3 mg/l BAP	Shania	100	0.64	64.00
	Hotspring	88.88	0.77	68.43

In the medium MS + 3.0 mg/l NAA + 0.3 mg/l BAP, variety Shania recorded 100 per cent callusing followed by Hotspring (88.88). In case of callus growth, variety Hotspring recorded maximum callus index (68.43) followed by variety Shania (64.0).

4.1.2.1.2 Shoot regeneration

4.1.2.1.2.1 Effect of various levels of auxins and cytokinins on shoot regeneration

The calli obtained from *in vitro* leaf segments of variety Dubai were used for shoot morphogenesis. MS medium supplemented with various levels of BA and NAA were used for shoot induction (Plate 6). Only 5.5 per cent calli showed regeneration in MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA. producing on an average 25 shoots/ culture, three weeks after inoculation (Table 8).

Table 8. Effect of various levels of cytokinin and auxin on initial shoot regeneration in gerbera variety Dubai

Sl. No.	Media combination	No. of calli inoculated	Percentage of shoot regeneration	No. of shoots regenerated /culture
1.	MS + 3.0 mg/l BAP + 0.1 mg/l NAA	18	-	-
2.	MS + 3.0 mg/l BAP + 0.2 mg/l NAA	18	5.55	25
3.	MS + 3.0 mg/l BAP + 0.3 mg/l NAA	18	-	-
4.	MS + 4.0 mg/l BAP + 0.4 mg/l NAA	64	-	-
5.	MS + 5.0 mg/l BAP + 0.5 mg/l NAA	64	-	-

Plate 6. Shoot regeneration from *in vitro* leaf derived calli in gerbera variety Dubai

4.1.2.1.2.2 Response of different gerbera varieties for shoot regeneration

The calli induced for the varieties *viz.*, Shania and Hotspring were inoculated to MS medium containing various combinations of auxins and cytokinins for shoot morphogenesis. MS medium supplemented with kinetin (1.0, 2.0, 3.0 and 4.0 mg/l) and IAA (0.1 mg/l each), BAP (1, 2, 3 and 4 mg/l) and NAA (2 mg/l each), BAP (2 and 4 mg/l) and kinetin (4 and 4 mg/l) and BAP (3.0, 3.0 mg/l) and NAA (0.1 and 0.2 mg/l) were tried for shoot morphogenesis.

All the calli inoculated of the two genotypes did not respond with respect to shoot morphogenesis in the different media tried.

4.1.2.1.3 Shoot proliferation

4.1.2.1.3.1 Effect of BAP and NAA on shoot proliferation in gerbera variety Dubai

The regenerated shoots of variety Dubai were transferred to MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA and MS medium supplemented with 3.0 mg/l BAP and 0.2 mg/l NAA for propagule multiplication (Plate 7). The number of shoots proliferated was recorded three weeks after inoculation and medium with low level of NAA (0.1 mg/l) produced more number of shoots (24.64). The data is shown in Table 9.

Table 9. Effect of BAP and NAA on shoot proliferation in gerbera (var. Dubai)

Sl. No.	Media combination	Mean No. of shoots at the time of subculturing	Mean No. of shoots after 3 weeks
1.	MS + 3.0 mg/l BA + 0.1 mg/l NAA	6.21	24.64
2.	MS + 3.0 mg/l BA + 0.2 mg/l NAA	7.36	19.62

Plate 7. Multiple shoot culture from *in vitro* leaf derived calli of gerbera variety Dubai

4.1.2.1.3.2 Shoot proliferation in subsequent subculture cycles in gerbera variety Dubai

The multiple shoots were subcultured to MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA for four subculture cycles and the data in Table 10 shows the shoot proliferation from single culture. From a single culture with an induced shoot of 25 numbers, in initial culture, a total of 2862 shoots could be regenerated in four subculture cycles.

Table 10. Shoots regeneration in different subculture cycles in gerbera (var. Dubai)

Culture details →	Initial culture	Subculture cycles			
		I	II	III	IV
No. of cultures	1	2	19	82	129
No. of shoots proliferated in subculture cycle	25	56	486	1113	2862

4.1.2.1.4 Elongation of proliferated shoots

The proliferated shoots were kept for elongation in the elongation medium (0.1 mg/l NAA) developed for micropropagation of gerbera at CPBMB. On an average shoot elongation of 3.0 cm was observed in the elongation medium. Shoot elongation in elongation medium is shown in Plate 8.

4.1.2.1.5 Induction of rooting

The *in vitro* micro shoots of 3.0 - 4.0 cm length were used for induction of rooting. Based on the trials at CPBMB, MS medium supplemented with 1 mg/l IAA was used for induction of rooting (Plate 9). Total number of shoots kept for rooting and number of shoots rooted, rooting percentage, mean number of roots/plantlets, and mean root length were recorded after three weeks of inoculation (Table 11).

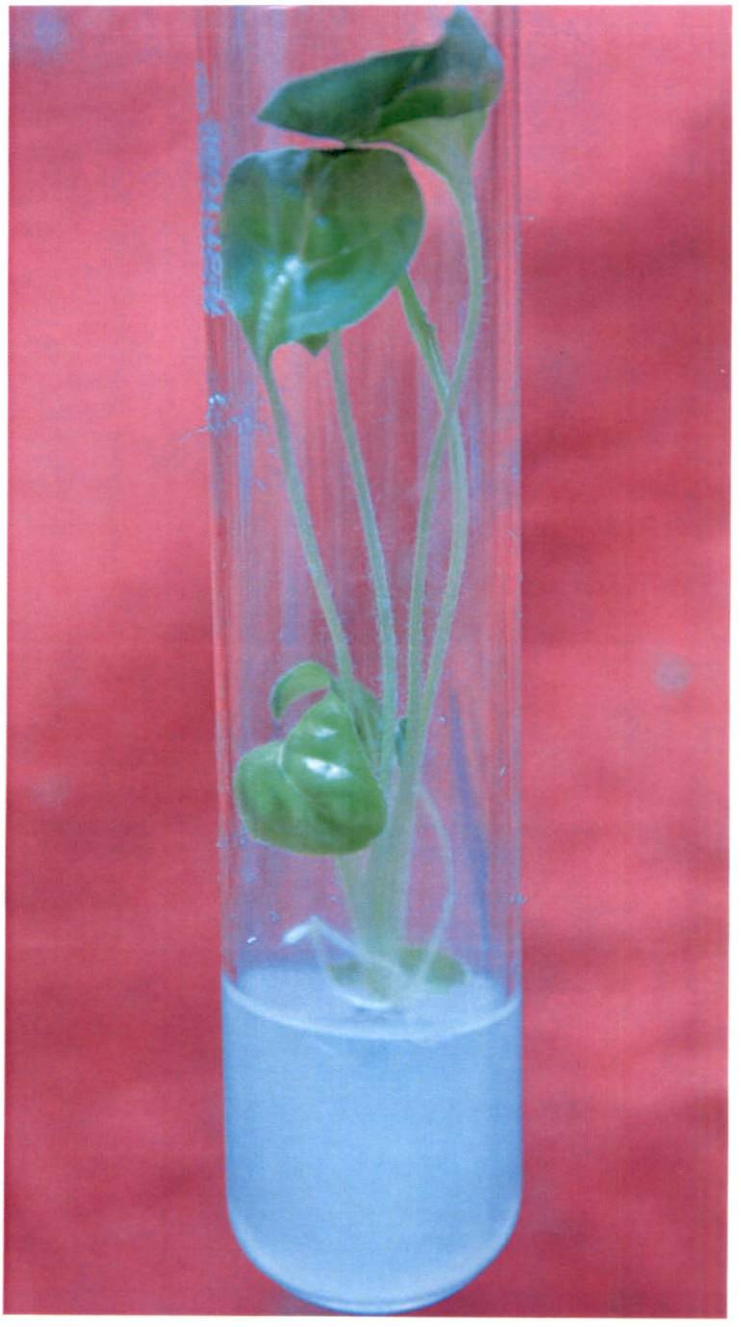


Plate 8. Shoot elongation in elongation medium in gerbera variety Dubai

Plate 9. Rooted culture of gerbera variety Dubai

In the medium, 89.22 per cent rooting was observed. Root initials were observed ten days after inoculation. Plantlets recorded good root characters with 4.7 roots/ plant with mean root length of 2.61 cm.

Table 11. Rooting and root characters in *in vitro* plantlets of gerbera (var. Dubai)

Media composition	No. of shoots inoculated for root induction	No. of shoots rooted	Rooting (%)	Mean No. of roots/plantlets after 3 weeks	Mean root length (cm)
MS + 1mg/l IAA	464	414	89.22	4.7	2.61

4.1.2.1.6 Hardening and acclimatization

Ten different potting media with different proportion of sand, soil, peat, perlite and vermiculite were used for hardening micropropagated plantlets (Plate 10). The survival and growth of plantlets in different potting media are presented in Table 12.

The survival rate differed among the treatments. The maximum survival rate (76%) was noticed in T_4 medium (peat: perlite: vermiculite with proportion 3:1:1, v/v respectively) after four weeks of transfer to hardening medium. Minimum survival (40%) was recorded in T_3 (cocopeat: soil: sand in 3:1:1, v/v proportion respectively).

There is significance in plant height in the different media. Plant height recorded was highest (10.03) in T_1 (Cocopeat: soil: sand) followed by T_9 (Vermiculate) with 9.55 cm. All potting media except sand recorded plant height from 7.27-8.64 cm. The height recorded was lowest (6.86 cm) in T_{10} in which sand alone was used as the medium.

Number of leaves recorded was highest in T_1 (9.0) followed by T_9 (8.0). The leaf size was also found highest in T_1 and T_9. Irrespective of the medium,

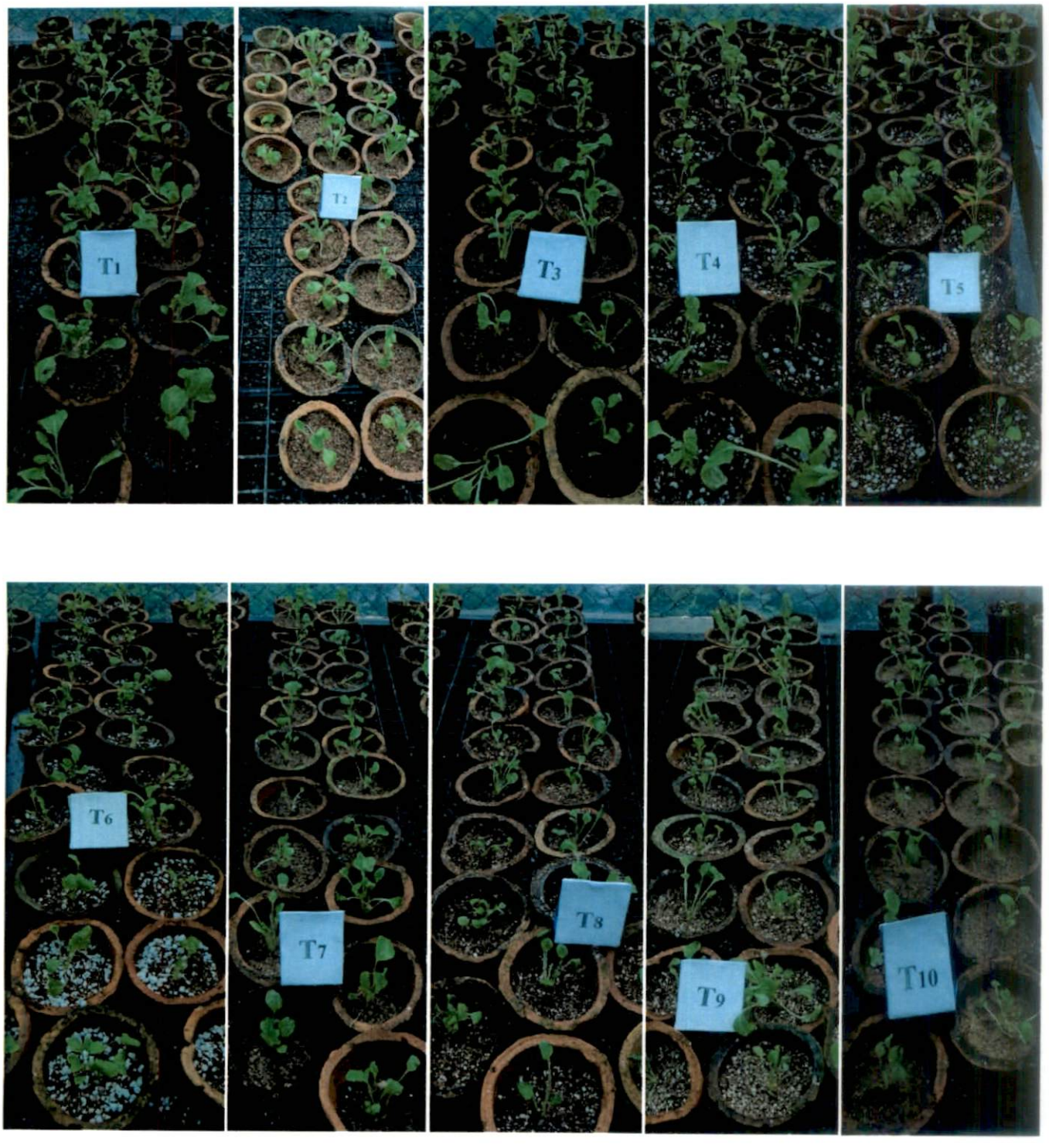


Plate 10. Hardening and acclimatization of gerbera plantlets variety Dubai

Table 12. Survival and growth of micropropagated plants in different hardening medium (var. Dubai)

Treatment	Potting media	Ratio	No. of plants planted out	Survival (%) 4WAP	Mean plant height (cm)	Mean No. of leaves/plant	Mean length of leaf (cm)	Mean width of leaf (cm)
T_1	Cocopeat:soil:sand	1:1:1	25	68	10.03	9.0	4.28	3.47
T_2	Cocopeat:soil:sand	2:1:1	25	44	7.70	5.45	3.37	2.73
T_3	Cocopeat:soil:sand	3:1:1	25	40	8.64	6.60	4.15	3.21
T_4	Peat:perlite:vermiculite	3:1:1	25	76	8.51	7.89	3.80	3.07
T_5	Peat:perlite:vermiculite	2:1:1	25	52	7.43	6.30	3.59	2.88
T_6	Peat:perlite:vermiculite	1:1:1	25	56	8.17	6.78	3.61	2.91
T_7	Sand:cocopeat	1:1	25	68	7.27	7.52	3.33	2.82
T_8	Vermiculite:cocopeat	1:1	25	52	7.39	6.46	3.26	2.58
T_9	Vermiculite	-	25	60	9.55	8.0	4.15	2.99
T_{10}	Sand	-	25	44	6.86	5.54	3.30	2.49
CD (0.05)					**2.014**	**NS**	**NS**	**NS**

plantlets exhibited a mean plant height of 8.15 cm with 6.94 number of leaves with leaf length of 3.68 cm and leaf width of 2.62 cm, four WAP. The survived plants (200 Nos.) were transferred to potting mixture and maintained in net house of CPBMB (Plate 11).

4.1.2.2 Micropropagation using *in vivo* leaf explants

4.1.2.2.1 Response of variety Arka Krishika for callusing and callus growth

Leaf explants were collected from mother plants maintained in the net house of CPBMB. Newly emerged young leaf was cut into 4 - 5 mm pieces along with midrib and inoculated to three different media selected based on report which is shown in Table 13. After three weeks of inoculation, MS medium supplemented with 3 mg/l NAA and 0.3 mg/l BAP recorded highest callusing percentage (75%) with highest callus index (283.5) (Plate 12a).

Table 13. Response of variety Arka Krishika for callusing and callus growth

Sl. No.	Media combination	No. of leaf segment inoculated	No. leaf segment callused	Callusing percentage (P)	Callus growth score (G)	Callus index (PxG)
1.	MS + 10 mg/l BA + 2 mg/l IAA	18	11	61.11	0.75	45.83
2.	MS + 3.0 mg/l NAA + 0.6 mg/l BA	18	13	72.22	3.30	238.32
3.	MS + 3.0 mg/l NAA + 0.3mg/l BA	36	27	75	3.78	283.5

4.1.2.2.2 Response of different gerbera varieties for callusing and callus growth

In vivo leaf explants collected from other three varieties *viz.* Dubai, Shania and Hotspring were inoculated to MS medium supplemented with 3.0 mg/l NAA

Plate 11. Micropropagated plants from *in vitro* leaf derived calli of gerbera variety Dubai established in net house

and 0.3 mg/l BAP (Plate 12b, 12c and 12d). After 3 weeks of inoculation, callus growth score and callusing percentage were recorded and data are presented in Table 14.

The data showed that the highest callusing percentage (74%) and callus growth score (3.62) was observed in variety Dubai, while lowest callusing (51.85%) and callus growth score (2.16) was observed in variety Shania in MS medium supplemented with 3.0 mg/l NAA and 0.3 mg/l BAP.

4.1.2.2.3 Response of different gerbera varieties for shoot regeneration

The calli obtained from *in vivo* leaf explants were transferred to MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA. Shoot morphogenesis was nil in all varieties except Arka Krishika in which 2.72 per cent of cultures showed shoot regeneration (Table 15). The shoot regeneration from *in vivo* leaf derived callus is shown in Plate 13.

4.2 Genetic stability studies using ISSR molecular marker

4.2.1 Genomic DNA isolation

4.2.1.1 Source of DNA

For isolation of genomic DNA, leaf samples were collected from source plants maintained at CPBMB net house and plantlets regenerated from flower buds and leaf explants. As only up to shoot multiplication was achieved from flower bud explants in the present study. Ten flower bud regenerants each of variety Dubai and Shania supplied from CPBMB were used for marker analysis. Ten plants of leaf calli derived from variety Dubai regenerated as per the standardised protocol of the present investigation were also used for marker analysis.

a) Variety Arka Krishika

b) Variety Dubai

c) Variety Shania

d) Variety Hotspring

Plate 12. *In vivo* leaf derived calli from different gerbera varieties

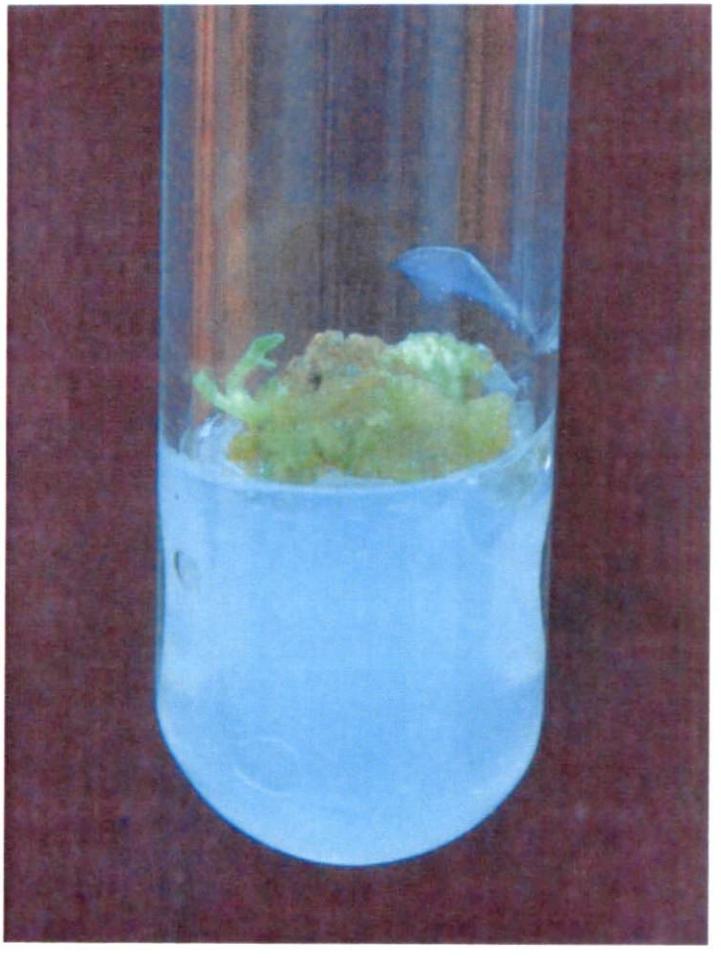


Plate 13. Shoot regeneration from *in vivo* derived calli in gerbera variety Arka Krishika

Table 14. Response of different gerbera varieties for callusing and callus growth in MS medium supplemented with 3.0 mg/l NAA and 0.3 mg/l BAP

Sl. No.	Variety	No. of leaf segments inoculated	No. of leaf segment callused	Callusing percentage (P)	Callus growth score (G)	Callus index (P x G)
1.	Dubai	27	20	74	3.62	267.8
2.	Shania	27	14	51.85	2.16	111.9
3.	Hotspring	18	13	72.22	2.45	176.9

Table 15. Response of different gerbera varieties for shoot regeneration from *in vivo* leaf derived calli in MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA

Sl. No.	Variety	No. of calli segments inoculated	Percentage of regeneration	No. of shoots regenerated/ culture
1.	Arka Krishika	36	2.77	2
2.	Dubai	18	-	-
3.	Shania	18	-	-
4.	Hotspring	18	-	-

4.2.1.2 Isolation and purification of DNA

Genomic DNA isolated through CTAB method reported by Roger and Bendich (1994) was not pure and had slight RNA contamination. RNase treatment after DNA isolation resulted in good quality DNA (Plate 14).

4.2.1.3 Quantification of DNA

The quality and quantity of isolated DNA was analysed using both electrophoresis and NanoDrop spectrophotometer. Intact clear bands indicated that DNA extracted was non-degraded and was of good quality. Spectrophotometric analysis gave ratio of UV absorbance ($A_{260}/_{280}$) between 1.8 and 2.0. The DNA after appropriate dilution was used as template for ISSR analyses.

4.2.2 ISSR analysis

Seven ISSR primers reported by Bhatia *et al* (2010) were selected. The details of selected ISSR primers are given in Table 2 under section 3.3.4.2.

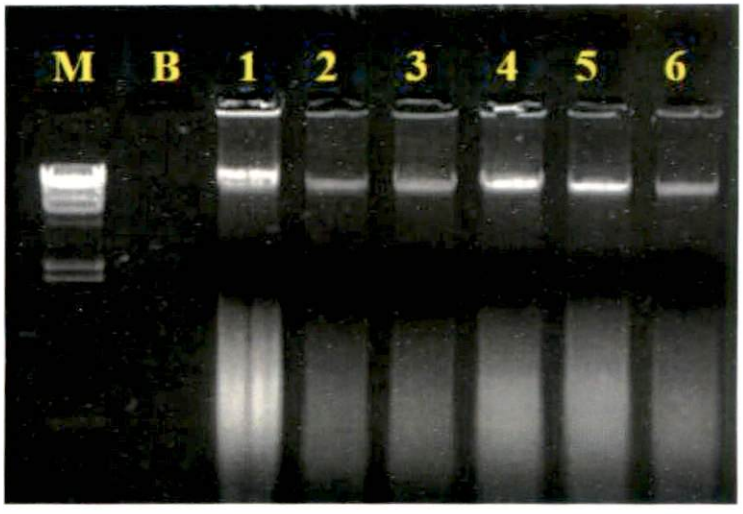
Genomic DNA isolated from various sources *viz.*, mother plants, reregenerants from direct and indirect organogenesis were subjected to ISSR analysis.

4.2.2.1 Regenerants from flower bud and leaf explants of variety Dubai

The amplification pattern obtained for plants derived from flower bud and leaf explants of variety Dubai with seven selected ISSR primers is provided in Plate (15-18). Primer specific amplification details are as shown below:

ISSR 08:

A total of eleven clear amplicons were obtained with the primer ISSR 08. The pattern of amplification is shown in Plate 15a. The molecular weight of amplicons ranged between 300 to 2900 bp. There was no polymorphism, DNA

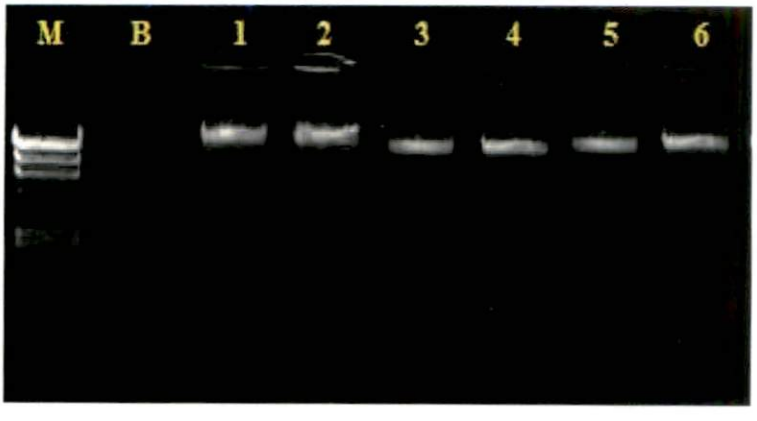


M: Marker Lambda DNA (*Eco*RI/ *Hind* III digest 1000bp), B: Blank

Mother plant: (1 – Arka krishika, 2 – Dubai, 3 – Shania, 4 – Hot spring)

Regenerants: 5 – flower bud, 6 – leaf callus of variety Dubai

a. Isolated DNA from different gerbera varieties and regenerated plants



M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000bp), B: Blank

Mother plant: (1 – Arka krishika, 2 – Dubai, 3 – Shania, 4 – Hot spring)

Regenerants: 5 – flower bud, 6 – leaf callus of variety Dubai

b. Isolated DNA from different gerbera varieties and regenerated plants after RNase treatment

Plate 14. Isolated DNA from different gerbera varieties and regenerated plants

from three groups of plants produced monomorphic bands when resolved with the primer ISSR 08.

ISSR 09

Amplification with the primer ISSR 09 generated seven clear amplicons. The pattern of amplification is shown in Plate 15b. The amplicons obtained with the primer were monomorphic. The molecular weight of amplicons ranged from 375 to 1450 bp.

ISSR 15

A total of fifteen clear amplicons were obtained when DNA was amplified with primer ISSR 15. The amplicons produced were monomorphic. The pattern of amplification is shown in Plate 16a. The molecular weight of amplicons ranged between 200 and 2225 bp.

ISSR 18

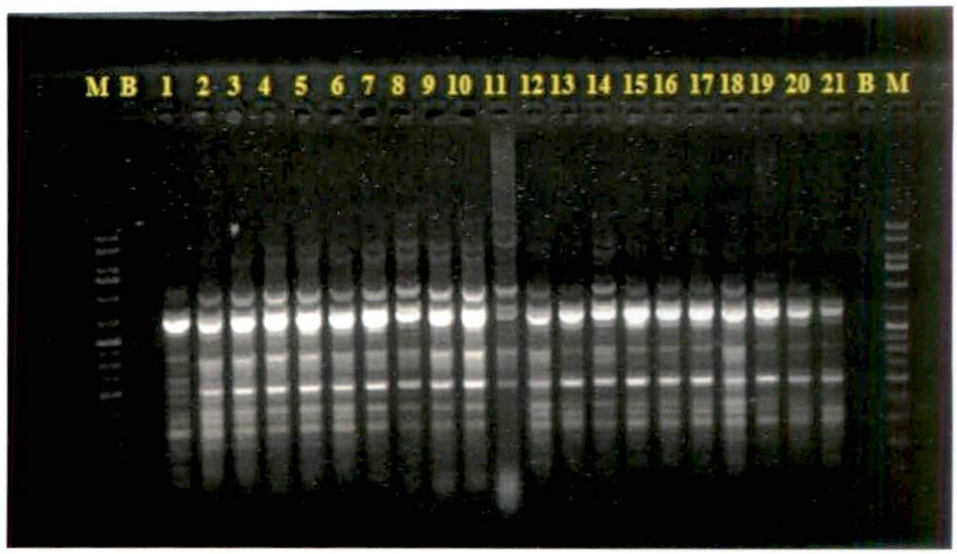
Amplification with primer ISSR 18 generated six clear amplicons, of which two were polymorphic and remaining were monomorphic. The pattern of amplification is shown in Plate 16b. The molecular weight of amplicons ranged from 300 to 1185 bp. Two amplicons of size 600 and 700 bp were absent in two of the bud regeneants examined.

ISSR 21

Four clear amplicons were observed in agarose gel for DNA amplified with the primer ISSR 21 (Plate 17a). One amplicon of size 2500 bp was polymorphic in calli regenerated plants; the rest were monomorphic. The molecular weight of the product ranged from 1600 to 2500 bp.

ISSR 25

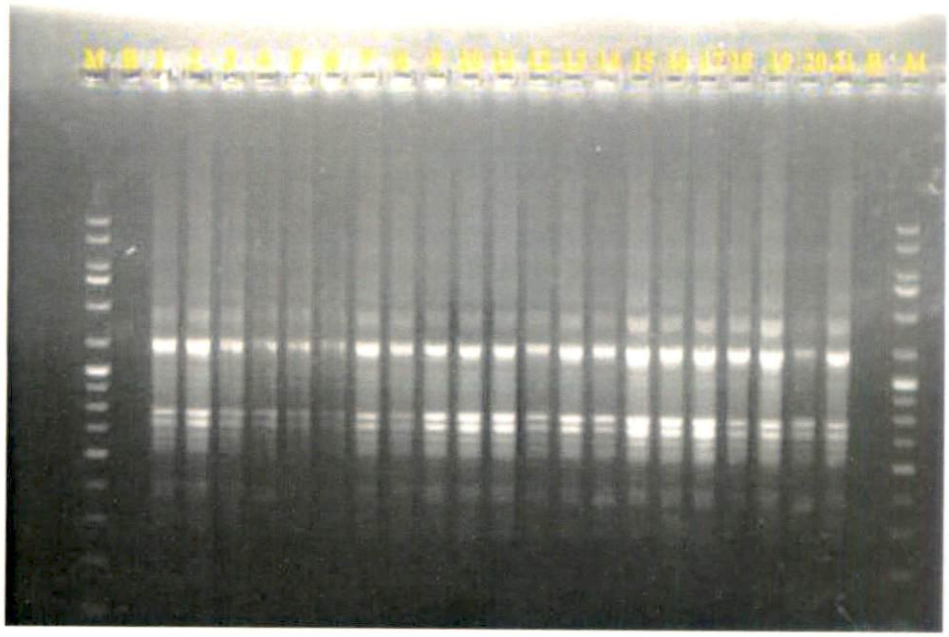
Amplification with primer ISSR 25 generated seven clear amplicons. The pattern of amplification is shown in Plate 17b. Only one amplicon was



M- Marker(1.3 kb ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

a - Amplification with ISSR 08

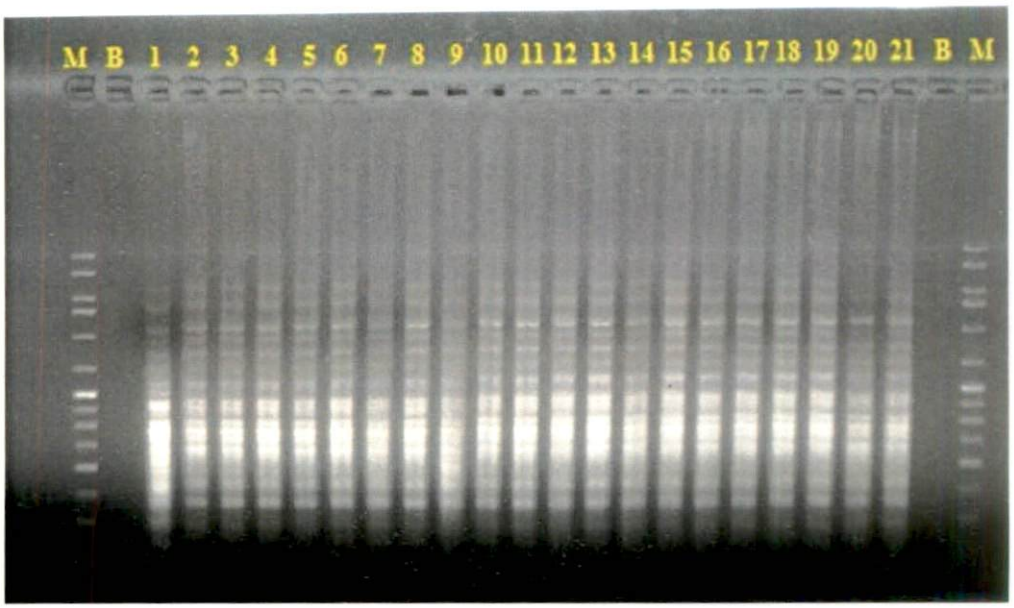


M- Marker (1.3 kb ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

b – Amplification with ISSR 09

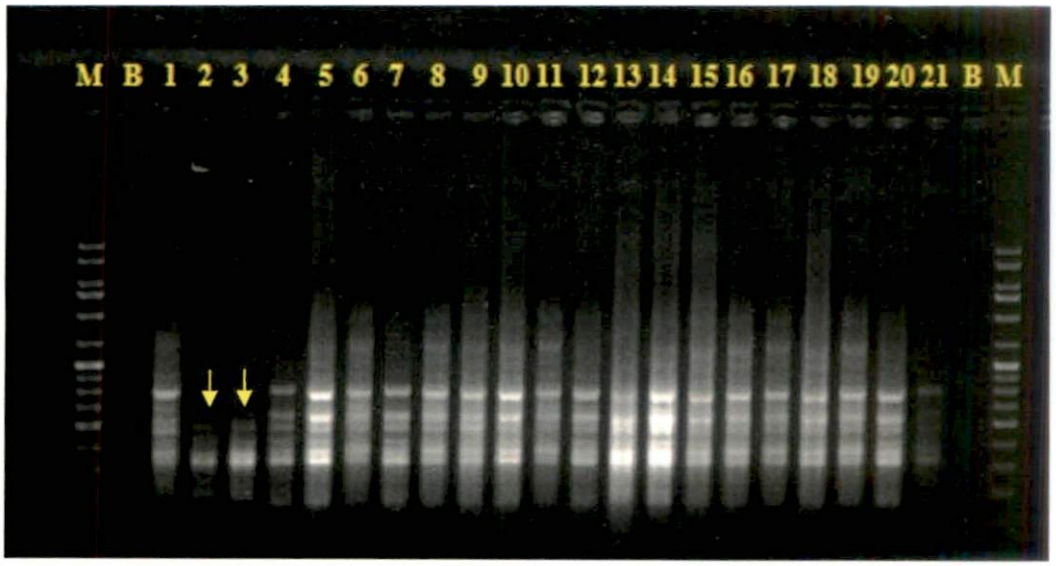
Plate 15: ISSR amplification pattern in source plants and regenerants from flower bud and leaf calli in variety Dubai with primer ISSR 08 and ISSR 09



M- Marker(1.3kb ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

a – Amplification with ISSR 15

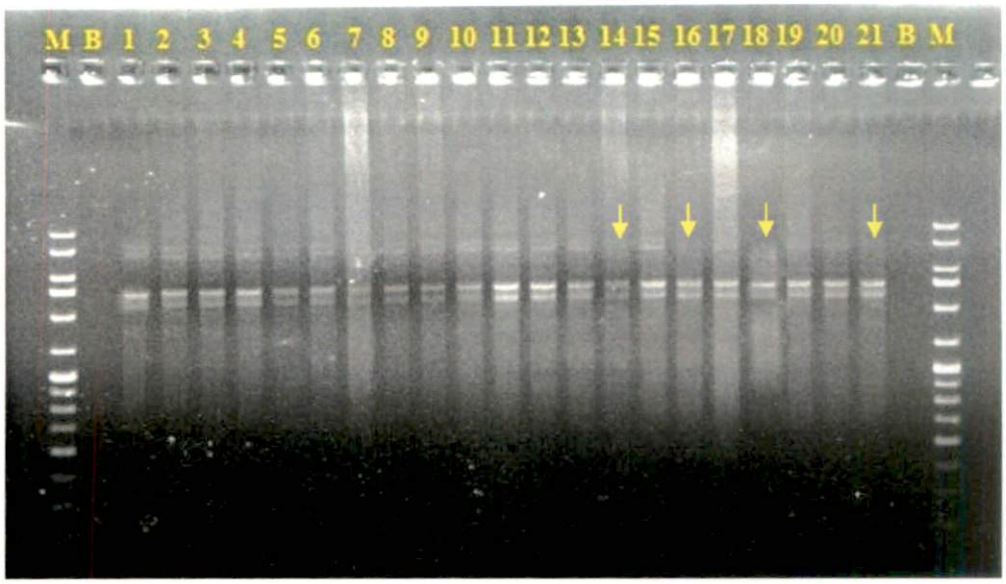


M- Marker(1.3 kb ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

b – Amplification with ISSR 18

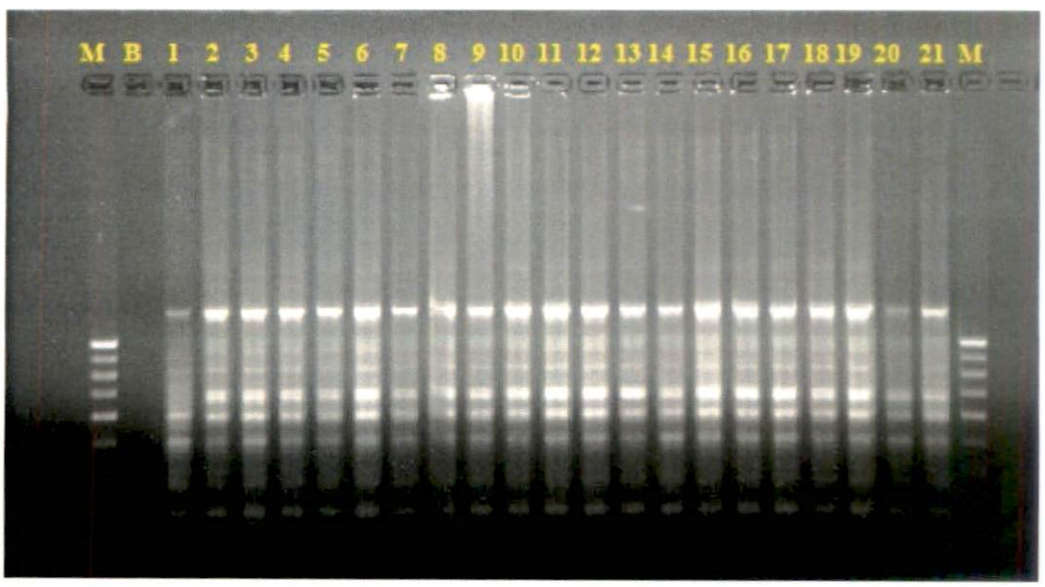
Plate 16: ISSR amplification pattern in source plants and regenerants from flower bud and leaf calli in variety Dubai with primer ISSR 15 and ISSR 18



M- Marker (1.3 kb ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

a – Amplification with ISSR 21

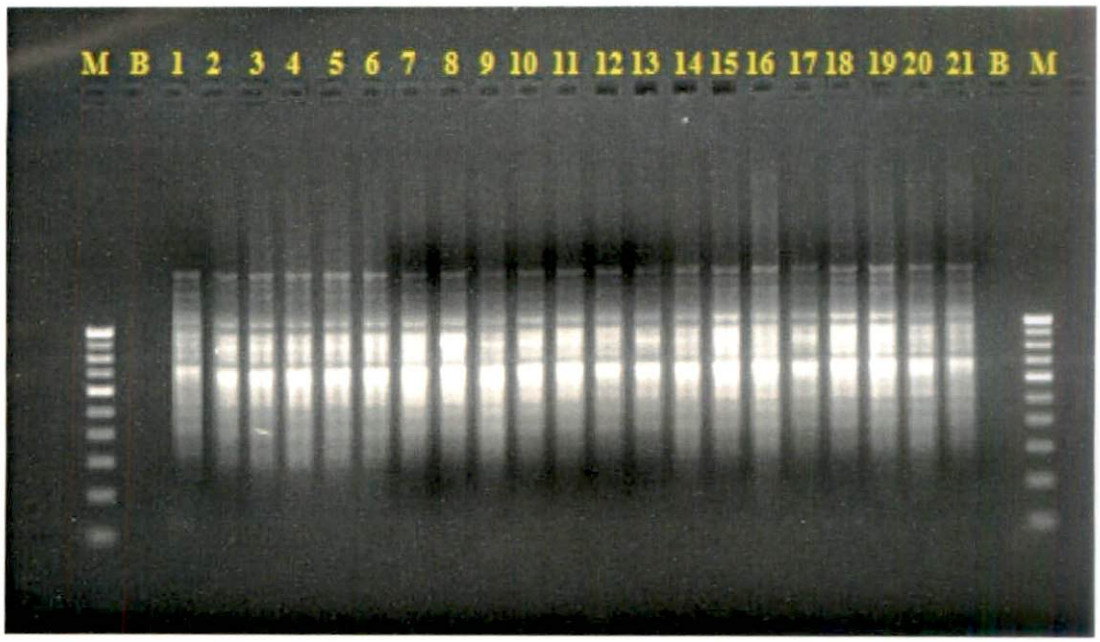


M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

b – Amplification with ISSR 25

Plate 17: ISSR amplification pattern in source plants and regenerants from flower bud and leaf calli in variety Dubai with primer ISSR 21 and ISSR 25



M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerants, 12-21-Leaf calli regenerants

Amplification with ISSR 31

Plate 18: ISSR amplification pattern in source plants and regenerants from flower bud and leaf calli in variety Dubai with primer ISSR 31

polymorphic; the rest were monomorphic. The molecular weight of the product ranged from 200 to 1200 kb. An amplicon of size 2500 bp was absent in plant numbers 14, 16, 18 and 21.

ISSR 31

A total of eight clear amplicons were obtained with the primer ISSR 31. The amplicons obtained were monomorphic. The pattern of amplification is shown in Plate 18. The molecular weight of amplicons ranged from 415 to 2150 bp.

4.2.2.2 Regenerants from flower buds of variety Shania

The mother plant and bud regenerants of gerbera were compared for their DNA amplification pattern with different primers.The amplification pattern obtained for plants derived from flower bud of variety Shania is provided in Plates (19-22). Details are as given below:

ISSR 08

Amplification with the primer ISSR 08 generated four clear amplicons which were monomorphic. The pattern of amplification is shown in Plate 19a. The molecular weight of amplicons ranged from 500 to 900 bp.

ISSR 09

A total of ten clear amplicons were obtained with the primer ISSR 09. The ampicons were monomorphic. The pattern of amplification is shown in Plate 19b. The molecular weight of amplicons ranged from 350 to 1450 bp.

ISSR 15

A total of eight clear amplicons were obtained after DNA amplification with the primer ISSR 15. Two amplicons were found polymorphic; the rest were monomorphic. The pattern of amplification is shown in Plate 20a. The molecular

weight of amplicons ranged between 275 and 1500 bp. Two amplicons of size 280 bp and 680 bp were absent in Plant No. 9 and Plant No. 11.

ISSR 18

Amplification with the primer ISSR 18 generated five clear amplicons which were monomorphic. The pattern of amplification is shown in Plate 20b. The molecular weight of amplicons ranged from 750 to 1900 bp.

ISSR 21

Four amplicons were observed on the agarose gel for the DNA amplified with the primer ISSR 21 (Plate 21a). All amplicons were monomorphic. The molecular weight of the product ranged between 325 to 1185 bp.

ISSR 25

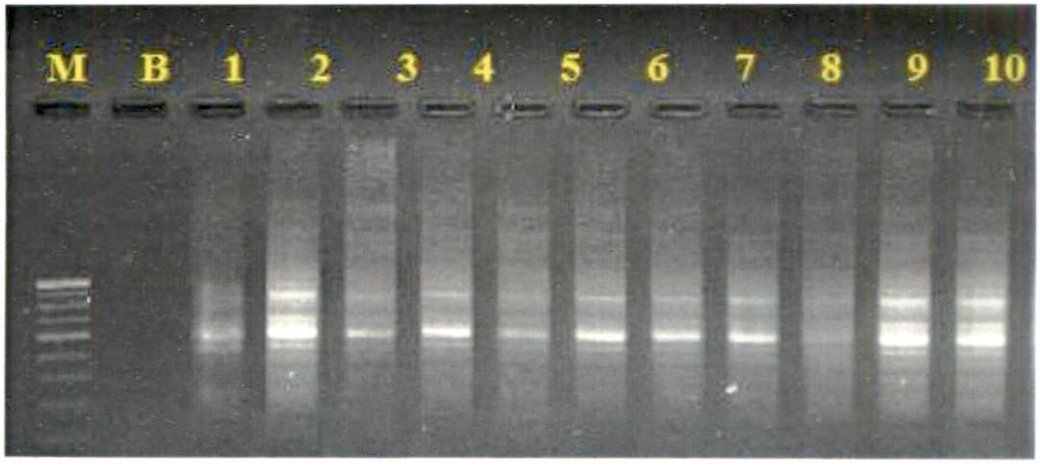
A total of nine clear amplicons were obtained with the primer ISSR 25. Three amplicons were polymorphic; the rest were monomorphic. The pattern of amplification is shown in Plate 21b. The molecular weight of amplicons ranged from 270 to 1815 kb. Two amplicons of size 1500, 1650 bp and 1815 were absent in Plant No. 9.

ISSR 31

Amplification with the primer ISSR 31 generated twelve clear amplicons which were monomorphic. The pattern of amplification is shown in Plate 22. The molecular weight of amplicons ranged from 100 bp and 1815 bp.

4.2.3 DNA Amplification pattern in micropropagated plants of variety Dubai with ISSR primers

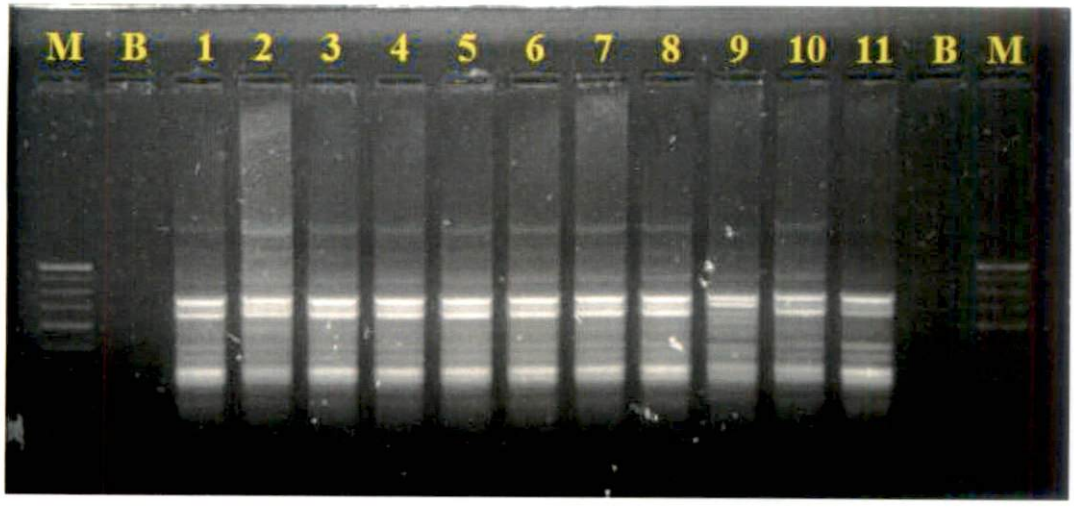
Details of DNA amplification pattern in micropropagated plants of variety Dubai are presented in Table 16. In variety Dubai; three groups of plants *viz.* mother plant regenerants from flower bud and leaf calli were subjected to ISSR assay. Out of the seven gerbera specific primers tested, two primers ISSR 18 and



M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

a - Amplification with ISSR 08

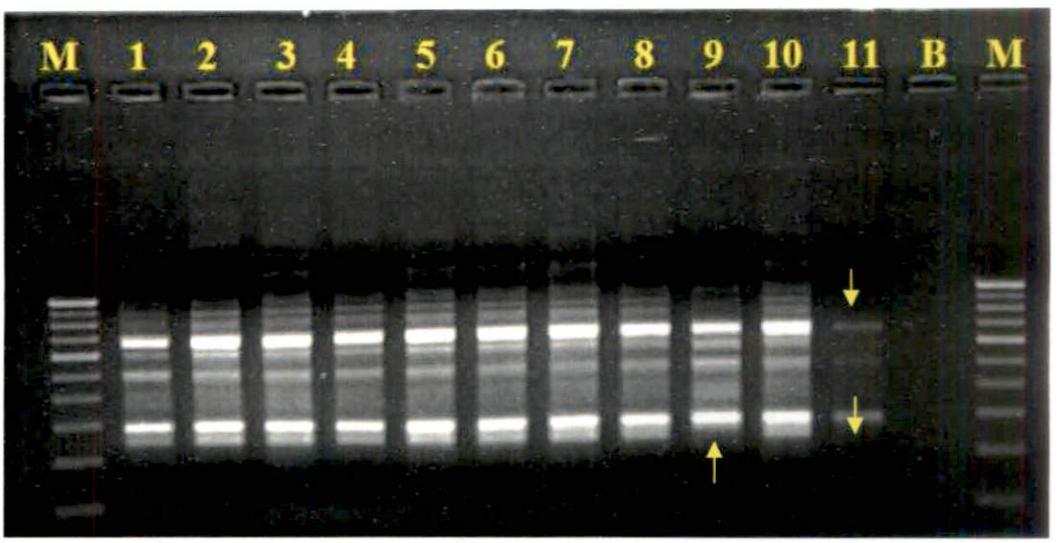


M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

b - Amplification with ISSR 09

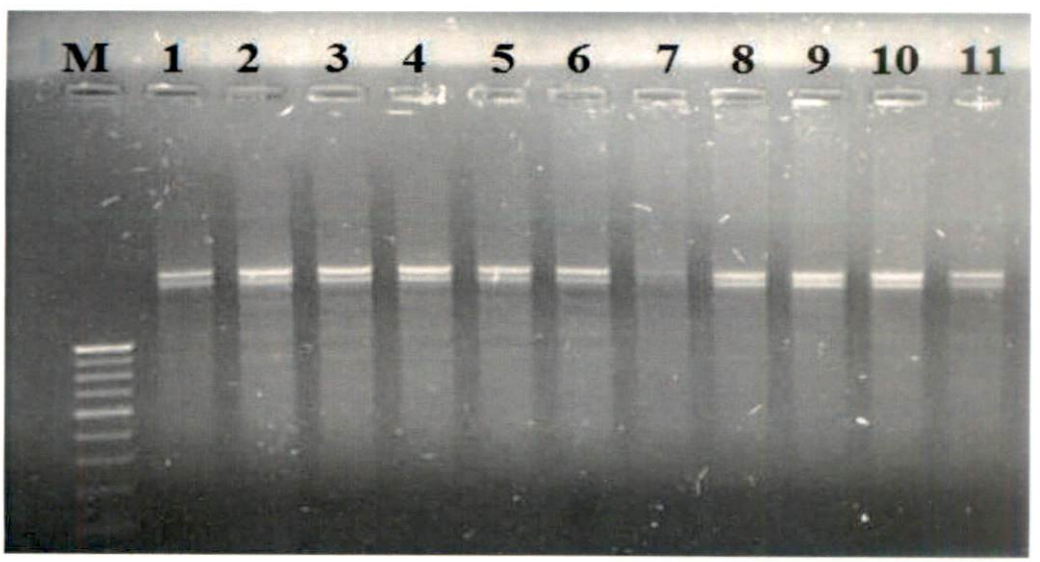
Plate 19. ISSR amplification pattern in mother plant and regenerants from flower bud in variety Shania with primer ISSR 08 and ISSR 09



M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

a - Amplification with ISSR 15

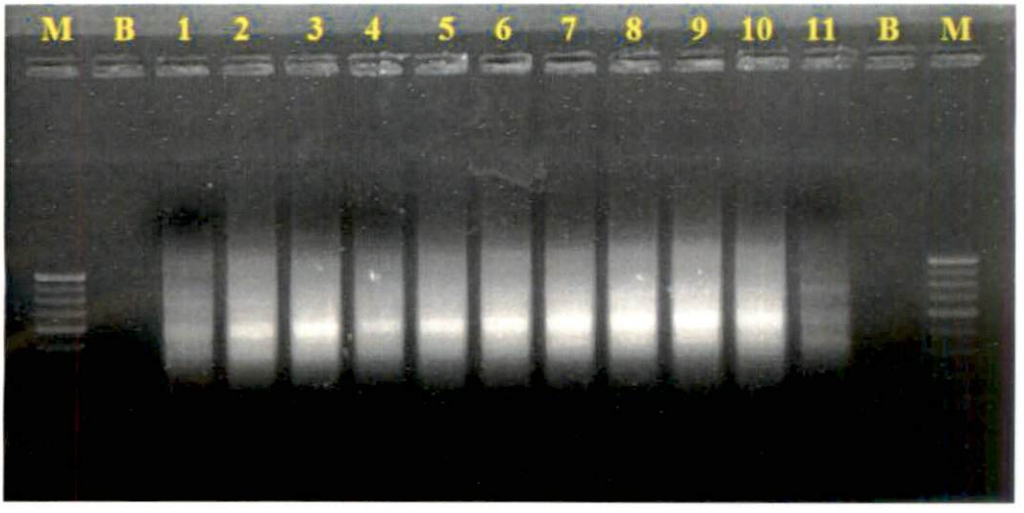


M- Marker/100bp ladder, B- Blank

1- Source plant, 2-11 – Flower bud regenerated

b - Amplification with ISSR 18

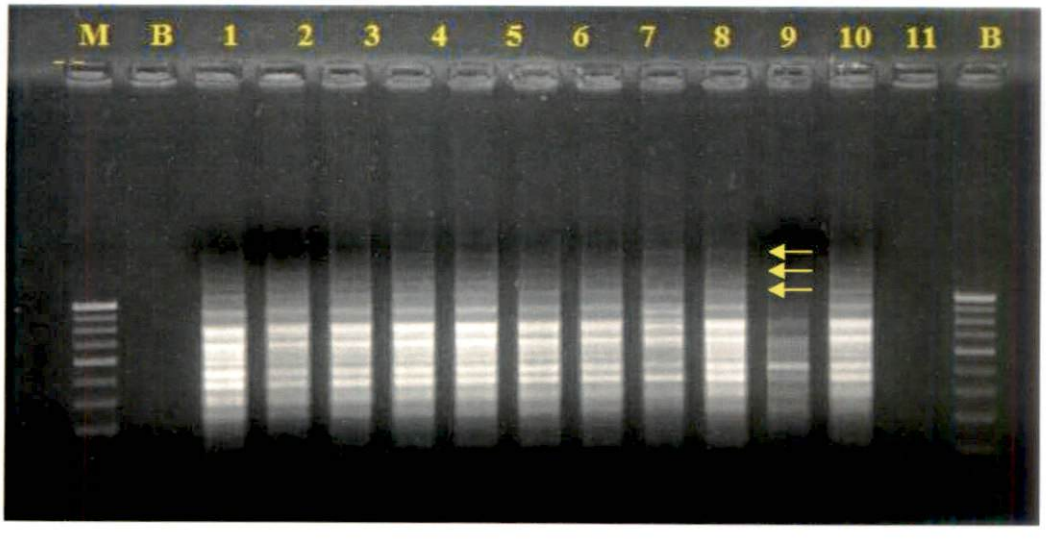
Plate 20: ISSR amplification pattern in mother plant and regenerants from flower bud in variety Shania with primer ISSR 15 and ISSR 18



M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

a - Amplification with ISSR 21

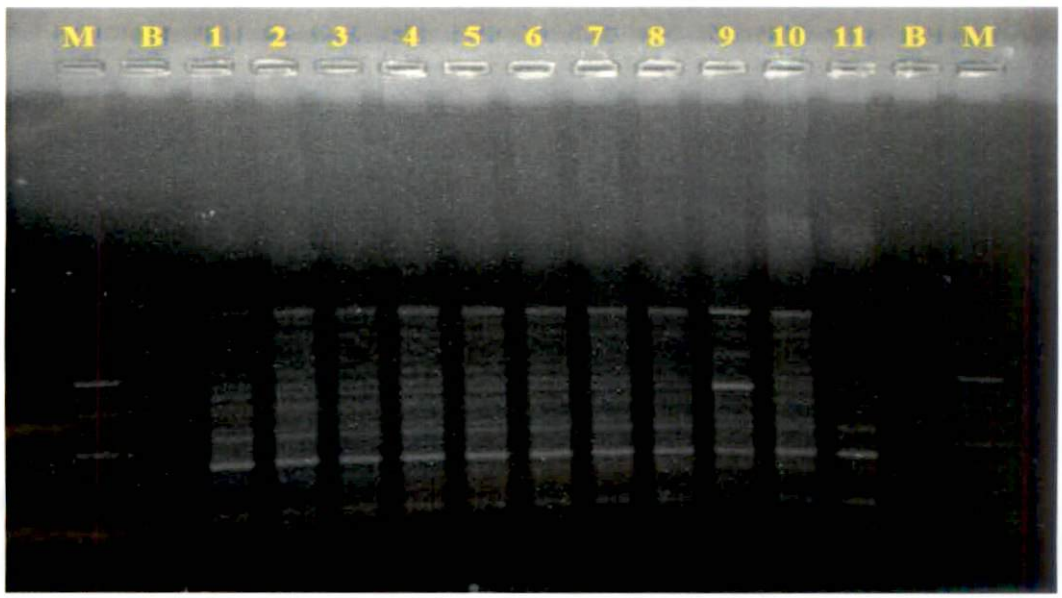


M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

b - Amplification with ISSR 25

Plate 21: ISSR amplification pattern in mother plant and regenerants from flower bud in variety Shania with primer ISSR 21 and ISSR 25



M- Marker (100bp ladder), B- Blank

1- Mother plant, 2-11 – Flower bud regenerated

Amplification with ISSR 31

Plate 22: ISSR amplification pattern in mother plant and regenerants from flower bud in variety Shania with primer ISSR 31

ISSR 21 exhibited polymorphism. The average polymorphism observed in flower bud regenerants was 3.44 per cent and in leaf calli regenerants was 1.72 per cent.

4.2.4 Amplification pattern in micropropagated plants of variety Shania with ISSR primers

In variety Shania, two groups of plants *viz.* mother plants and flower bud regenerants were compared using the seven selected primers. Of the seven primers tested, ISSR 15 and ISSR 25 exhibited polymorphism. The average polymorphism was observed was 9.61 per cent (Table 17).

4.2.5 Variation at DNA level observed in gerbera micropropagated plants

Number of plants exhibiting variation at DNA level is presented in Table 18. The flower bud regenerants of two varieties (Dubai and Shania) showed 20 per cent variability while the callus regenerants exhibited more vaiability (40 %).

Table 16. DNA Amplification pattern in micropropagated plants of variety Dubai with ISSR primers

Sl. No.	Primer name	Flower bud regenerants				Leaf calli regenerants			
		Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)	Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)
1	ISSR 08	11	0	11	-	11	0	11	-
2	ISSR 09	7	0	7	-	7	0	7	-
3	ISSR 15	15	0	15	-	15	0	15	-
4	ISSR 18	6	2	4	33.33	6	0	6	-
5	ISSR 21	4	0	4	-	4	1	3	25.00
6	ISSR 25	7	0	7	-	7	0	7	-
7	ISSR 31	8	0	8	-	8	0	8	-
Total		58	2	56	3.44	58	1	57	1.72
Average		8.28	0.28	8		8.28	0.14	8.14	

Table 17. Amplification pattern in micropropagated plants of variety Shania with ISSR primers

Sl. No.	Primer	Total No. of amplicons	No. of polymorphic amplicons	No. of monomorphic amplicons	Polymorphism (%)
1	ISSR 08	4	0	4	-
2	ISSR 09	10	0	10	-
3	ISSR 15	8	2	6	25.00
4	ISSR 18	5	0	5	-
5	ISSR 21	4	0	4	-
6	ISSR 25	9	3	6	33.33
7	ISSR 31	12	0	12	-
Total		52	5	47	9.61
Average		7.42	0.71	6.71	-

Table 18. Variation at DNA level observed in gerbera micropropagated plants

Sl. No.	Particulars	No. of plant analysed	No. of plants showing polymorphism	Primer showing polymorphism	Plant Number	Plants exhibiting variability (%)
1	Flower bud regenerants var. Dubai	10	2	ISSR - 18	2 and 3	20
2	Leaf callus regenerants var. Dubai	10	4	ISSR - 21	14, 16, 18 and 21	40
3	Flower bud regenerants var. Shania	10	2	ISSR -15	9 and 11	20
				ISSR - 25	9	

Discussion

5. DISCUSSION

The present studies on "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" were carried out in order to develop an efficient micropropagation protocol for mass multiplication of gerbera and to test genetic stability of the micropropagated plants using ISSR assay. The results of the experiments are discussed in this chapter.

5.1 Studies on micropropagation

Four different varieties of gerbera *viz.,* Arka Krishika, Dubai, Shania and Hotspring with different flower colours *viz.,* yellow, white, red and violet, respectively, were used for the study. Flower bud and leaf explants from different varieties were used for micropropagation. Leaf explants were collected from *in vitro* multiple shoot culture and *in vivo* leaf explants and flower bud explants were collected from mother plants maintained in the net house of CPBMB. The micropropagation protocol developed at CPBMB by Shylaja *et al.* (2014) was used for investigating plantlets from flower buds. Indirect organogenesis from leaf explants was standardised in the present investigation.

5.1.1 Micropropagation using flower bud explants

5.1.1.1 Response of different gerbera varieties for shoot regeneration from flower bud explants

Cytokinins play a crucial role in the morphogenesis of gerbera. Various combinations of auxins and cytokinins have been tried to achieve multiple shoot induction in gerbera (Murashige *et al.,* 1974; Barbosa *et al.,* 1993). In the present study, MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA was used for culture establishment and shoot proliferation. The number of adventitious shoots produced *in vitro* also depends on the plant genotype. In the present study, Dubai variety gave higher number of shoots (32) from flower bud explants

followed by Arka Krishika (26). The growth response is also said to be cultivar specific (Schiva *et al.*, 1982; Pierik *et al.*, 1982 and Harel *et al.*, 1993). Explants of cv. Marleen formed 14.8 and 13.2 shoots on the initiation medium containing BA and kinetin respectively; those of cv. Victoria formed 11.8 and 2.2 shoots, respectively (Hempel *et al.*, 1985). Deepaja (1999) also reported that, every genotype has a specific range of optimum growth regulator concentration. The same trend also observed in the present investigation.

Flower bud age and position of the explants influences *in . vitro* organogenesis in gerbera. Young flower buds (7 days) stimulated callus production while 10 to 14 days old flower buds stimulated direct shoot organogenesis (Nhut *et al.*, 2007). In present study also, similar results were found.

Son *et al.* (2011) also used flower buds of three varieties of gerbera as explants for culture establishment. More number of shoots (11.29) were recorded in MS medium supplemented with 3 mg/l BAP and 0.1 mg/l NAA. In the present study also, best shoot regeneration was obtained in the same medium.

According to Akter *et al.* (2012),. flower bud and flower stalk explants were suitable and superior for callus induction and subsequent regeneration of *in vitro* shoots, when cultured in MS supplemented with 5.0 mg/l BAP and 1.0 mg/l NAA. However, highest numbers of multiple shoots were obtained when the flower bud derived callus was subcultured on MS supplemented with 2.0 mg/l BAP. Direct shoot regeneration were observed in MS medium containing 3.0 mg/l BAP and 0.1 mg/l NAA in the present study also.

Kadu (2013) also found highest shoot regeneration percentage (91.66%) in MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA from flower bud explants. Similar results were also found in the present study with low concentration BAP and NAA. Similar results also found by Rahman *et al.* (2014).

Shylaja *et al.* (2014) developed a highly efficient micropropagation protocol for multiplication of gerbera with unopened flower bud as explants. For culture

establishment, MS medium supplemented with 3.0 mg/l BAP and NAA 0.1 mg/l was found highest shoot regeneration percentage (95.83%) and high number of shoot regeneration (42.33) in variety Dubai. Genotype differences were observed in all stages of *in vitro* propagation. Same protocol was followed in the present studies and with respect to variety Dubai, shoot regeneration percentage (62.5%) and number shoot (32) was found to be maximum.

5.1.1.2 Response of different gerbera varieties for shoot proliferation from flower bud explants

For shoot proliferation, MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA was employed. Variety Dubai recorded the highest proliferation rate (53.33) followed by Arka Krishika (23.00). Shoot multiplication was found very low in red flowered variety Shania (11.00). The proliferation rate differed from genotype to genotype.

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and the growth substances produced endogenously by the cultured cells. Various combinations of cytokinins and auxins have been tried to achieve multiple shoot induction in gerbera (Murashige *et al.*, 1974; Schiva *et al.*, 1982; Barbosa *et al.*, 1993). Apart from the direct effect on cellular mechanisms, many synthetic growth regulators may in fact modify the level of endogenous substances (George and Sherrington, 1984).

In present studies, MS medium supplemented with 3.0 mg/l BAP and 0.1 mg/l NAA was used. However, optimum concentration of growth regulators varies with different varieties. Among the different varieties, the Dubai variety was the best since it gave the highest proliferation rate (53.33). Harel *et al.* (1993) also obtained the highest multiplication rate (8 shoots) in cultivars Ansofie, Terracerise and Lablinel, moderate rate (6 - 7 shoots) in varieties Maria, Shangha and Fresultane, while the lowest rate (4-5 shoots) in varieties Raisa, Fredigor, Terramaxima and Fredibel. This difference indicated that the rate of

multiplication through micpropagation also depends on the cultivars. Also every genotype has a specific range of optimum growth regulator concentration (Deepaja, 1999).

In a previous study, Budi and Suwandi (2000) obtained most multiple shoots in full-strength MS medium with 1 mg/l BA and 0.2 mg/l NAA. Son *et al.* (2011) obtained a better shoot multiplication rate in MS medium supplemented with 3.0 mg/l BA and 0.1 mg/l NAA, ranging between 5.8 and 15.5 in four gerbera varieties. In present studies, also better shoot multiplication was observed in BAP-NAA combination.

As reported by Shylaja *et al.* (2014), Dubai variety exhibits higher shoot proliferation (42.33) as compared to Shania variety (36.86). In present, investigation same result were found and further, variety Dubai recorded high shoot proliferation (53.33) as compared to Hotspring variety (11).

5.1.2 Micropropagation using leaf explants

5.1.2.1 Callusing and callus growth from leaf explants

5.1.2.1.1 Effect of various levels of auxin on callusing and callus growth

In the present experiment, *in vitro* leaf segments when cultured on MS medium supplemented with different auxins (IAA, NAA and 2, 4-D) at various levels, have produced calli three weeks after inoculation. Callusing started from cut ends of leaf segments. Calli were produced in all media investigated but maximum callus growth (2.32) was observed in MS medium supplemented with 1 mg/l NAA and minimum (0.11) was in MS medium supplemented with 3 mg/l 2, 4-D.

Parthasarathy and Nagaraju (1999) also reported callus induction from leaf explants of *G. jamesonii* in MS medium supplemented with NAA, BA, kinetin and IBA in different concentrations. Callus growth was greatest with 1 mg/l of NAA. Similar were results also found in present study, when MS medium supplemented with 1 mg/l of NAA recorded highest callus growth (2.32). But

rhizogenesis was reported in medium containing NAA. Similar rhizogenesis from leaf calli were reported by Ruffoni and Massabo (1991), Kumar and Kanwar (2007), Bhatia *et al.* (2008) and Paduchuri *et al.* (2010).

Reports are available on indirect organogenesis in gerbera, from different of explants (Kanwar and Kumar, 2008). Aswath and Choudhary (2002a) obtained high leaf-derived-callus induction (65–90%) in gerbera varieties AV101 and AV108, from leaf explants.

Kumar and Kanwar (2007) also reported that the percentage of explants producing callus was highest with 1.5 mg/l 2, 4-D. Callus growth was best with 2.0 mg/l 2, 4-D. But in the present study, MS medium supplemented with (2 and 3 mg/l) 2, 4-D to produced lowest callus growth and callusing percentage but MS medium supplemented with 1 mg/l 2, 4-D produced 100 per cent callusing.

5.1.2.1.2 Effect of various levels of auxin and cytokinin on callusing and callus growth

The classical findings of Skoog and Miller (1957) reported that organogenesis in tissue culture was governed by the balance of auxin and cytokinin in the medium. May of the, this may not be demonstrated universally, due to the explant sensitivity or the original content of the endogenous growth regulators in explants. The development processes of gerbera callus required both cytokinins and auxins for callusing and callus growth.

In the present study, various combinations of auxins and cytokinins were tried to achieve callusing and callus growth for *in vitro* leaf explants of variety Dubai. IAA-BAP, IAA-kinetin, NAA-BAP and NAA-kinetin combinations were supplemented to basal MS media. MS medium supplemented with 3.0 mg/l NAA and 0.3mg/l BAP recorded highest callus growth (2.94) and callusing percentage (100%), while lowest callus growth score (0.42) and callusing percentage (83.33) were observed in 3.0 mg/l IAA and 0.3 mg/l kinetin.

Reynoird *et al.* (1993) gave modified MS medium supplemented with 1.0 mg/l BA and 0.25 mg/l NAA for plant regeneration from *in vitro* leaf culture of several gerbera species. Kalra *et al.* (2008) reported that, callus induction from young leaf was found best on MS basal medium containing 5.0 mg/l BAP + 0.25 mg/l IAA. In the present study, BAP and IAA combination has recorded 100 per cent callusing but callus growth was poor.

In another study by Hussein *et al.* (2008), callus was formed from *in vitro* juvenile leaf explants of the gerbera on 58 different MS-based media containing different concentration and combinations of plant growth regulators *viz.*, BAP, Kin, Zeatin, NAA, IAA and ABA. It was observed callus formation percentage varied from 0 to 95 among the media. It was recorded that only five media showed high percentage of callus formation ranging between 90 - 95 per cent.

The result of the present study were in close agreement with those of Bhatia *et al.* (2008) showing that induction of callus formation by cytokinin was promoted by the addition of an auxin, especially by NAA in combination with BAP. Aswath and Choudhary, (2002) also found that the combination of NAA and BAP was more effective for callus induction than either IAA or BAP alone. But highest callus induction and growth of callus were observed in MS medium supplemented with BAP 2 mg/l + kinetin 1 mg/l + NAA 2 mg/l + 2,4- D 2.5 mg/l by Paduchuri *et al.* (2010).

5.1.2.2 Effect of various levels of auxin and cytokinin on shoot regeneration

In the present study, calli obtained from *in vitro* leaf segments of variety Dubai were used for shoot morphogenesis. MS medium supplemented with various levels of BA and NAA were used for shoot induction. Only 5.5 per cent calli showed regeneration, with an average of 25 shoots/ culture, three weeks after inoculation. Twelve other media combinations were also tried for shoot morphogenesis and varieties failed in shoot morphogenesis.

Topoonyanont and Dillen (1988) observed that shoot induction from capitulum tissue was observed only in cultivars with orange, yellow and red flowers, but no regeneration was possible from cultivars with pink flowers, suggesting a relation between flower colour and organogenic capacity in gerbera. In the present investigation, only Dubai variety observed shoot regeneration but variety Shania and Hotspring failed to shoot morphogenesis.

In some of the previous reports, response to regeneration was significantly influenced by the concentrations and combinations of cytokinins and auxins. Pierik *et al.* (1973) reported that addition of strong auxin (NAA) with BAP promoted better shoot formation as compared to weak auxin (IAA). In many of the previous reports also, potential to induce callus and regenerate shoots is also dependent on the genotype (Reynoird *et al.*, 1993; Jerzy and Lubomski, 1991; Orlikowska *et al.*, 1999). Similar trend was observed in the present study.

Jerzy and Lubomski (1991) showed a useful protocol for direct and indirect regeneration of shoots of 28 cultivars of gerbera from petiole explants on MS medium supplemented with 3.0 mg/l BA and 0.5 mg/l IAA. Orlikowska *et al.* (1999) observed differences in the regeneration potential of petiole explants of cv. Mariola (94.1%), Boy (55.3%) and Rebecca (47.5%). Reynoird *et al.* (1993) observed high differences in young leaves (≤2 mm) that regenerated shoots between two clones, clone 10 (83.8–97.8%) and clone 11 (36.6–49.4%), both from the same cross between *G. jamesonii and G. viridifolia*, relative to two wild species *G. viridifolia* (50–65.6%) and *G. piloselloides* (7.1–39.7%).

Most researchers used combination of cytokinins (2 to 15 mg/l BA or Kin) and auxin (0.1 to 0.5 mg/l IAA) for shoot regeneration in capitulum explants (Modh *et al.*, 2002; Zheng *et al.*, 2002; Tyagi and Kothari, 2004). (Nhut *et al.*, 2007). Misra *et al.* (2010) observed differences in shoot regeneration capacity when using MS medium with 0.5 mg/l BA, 0.5 mg/l TDZ and 1.0 mg/l IAA, was able to rank seven cultivars based on their regeneration ability.

Hasbullah *et al.* (2008) also reported that addition of auxins along with cytokinins was essential for shoot induction in gerbera. When BAP was supplemented with IAA, callus was induced but no shoot proliferated. On the other hand when BAP was supplemented with NAA, in some combinations, callus induction as well as shoot proliferation occurred.

In vitro shoot regeneration of gerbera is dependent on cultivar and hormonal combination. In gerbera micropropagation, cytokinin plays a significant role in the morphogenesis of capitulum explants (Shabanpour *et al.*, 2011). In this experiment, BA and NAA produced shoots form calli in the variety Dubai. The number of adventitious shoots regenerated depended on the plant genotype.

5.1.2.3 Effect of BAP and NAA on shoot proliferation in gerbera variety

In the present experiment, the regenerated shoots of variety Dubai were transferred to MS medium supplemented with 3 mg/l BAP and 0.1mg/l NAA and MS medium supplemented with 3 mg/l BAP and 0.2 mg/l NAA for propagule multiplications. When the number of shoots proliferated was recorded three weeks after inoculation, medium with low level of NAA (0.1 mg/l) produced more number of shoots (24.64).

Sahavacharin (1985) could multiply gerbera most rapidly on MS medium with 0.75 mg/l IAA and 12 mg/l kinetin. Topoonyanont and Dillen (1988) could induce multiple shoots of orange, red and yellow cultivars, but not of a pink cultivar, even when a high concentration of kinetin (2.5 mg/l) was used.

In a previous study conducted by Harel *et al.* (1993), differential responses in the multiplication rate among 10 cultivars was observed and they classified them as high multiple shoot-induction (8) cultivars (Ansofie, Terracerise and Lablinel), moderate (6–7) (Maria, Shangha and Fresultane) and low multiple shoot induction cultivars (4–5) (Raisa, Fredigor, Terramaxima and Fredibel).

Reynoird *et al.* (1993) used 0.25 mg/l BA combined with 0.25 mg/l kinetin and 0.45 mg/l IAA for the multiplication stage of *G. hybrida (G. jamesonii* × *G.*

viridifolia). Posada *et al.* (1999) showed best multiple shoot induction with 1 and 2 mg/l BA in MS-H medium. Parthasarathy and Nagaraju (1999) observed that in varieties SWM and Dilmaya more multiple shoots formed when shoot explants were propagated *in vitro* in MS medium with 0.5-1.0 mg/l BA.

Budi and Suwandi (2000) obtained most multiple shoots in full strength MS medium with 500 mg/l casein hydrolysate, 1 mg/l BA and 0.2 mg/l NAA. Bouman *et al.* (2001) reported that MS medium was superior to DKW medium in shoot multiplication. Cytokinins such as kinetin and TDZ were used in the multiplication stage of gerbera micropropagation. Shailaja (2002) and Jerzy and Lubomski (1991) obtained the highest number of shoots/explant with 3 mg/l BA.

Aswath *et al.* (2003) observed that half MS medium resulted in faster shoot multiplication while full-strength MS culture medium resulted in a higher number of shoots, longer shoots and greater shoot weight. But some authors also observed that highest shoot number/explant in full strength MS medium supplemented with 1 mg/l BA or 5 mg/l kinetin, both combined with 0.1–0.2 mgl/ IAA in all three varieties (GJ-1, GJ-2 and GJ-3). Tyagi and Kothari (2004) observed that 2.0 mg/l kinetin combined with 0.5 mg/l phenylacetic acid (PAA) promoted best multiplication rate and better recovery of shoots than 0.5 mg/l IAA.

However, in previous reports by Nhut *et al.* (2007) and Gantait *et al.* (2010), a higher number of multiple shoot induction were observed. In varieties Sciella, 14 shoots/explant and by progenies from cross II, 22.2 shoots/explants were obtained. Chakabrarty and Datta (2008) also observed that BA was superior for shoot multiplication than other cytokinines, such as kinetin and TDZ and showed that the best multiplication rate (17:1) took place in 2.0 mg/l BA.

A combination of 2.0 mg/l BA and 0.3 mg/l NAA resulted in the 12 shoots/ explant when MS medium was used in the multiplication stage (Feng *et al.*, 2009). Son *et al.* (2011) also obtained a better shoot multiplication rate in MS with 3.0 mg/l BA and 0.1 mg/l NAA, ranging between 5.8 and 15.5 in four gerbera cultivars. In the present study, same medium was used for shoot

proliferation which observed high proliferation rate. From a single culture with induced 25 shoots in initial culture, a total of 2862 shoots was regenerated in four subcultures.

Naz *et al.* (2012) obtained best shoot multiplication using 10 mg/l BA (9 shoots/explant), without reporting any hyperhydricity.

5.1.2.4 Induction of *in vitro* rooting

In the present experiment, MS medium supplemented with 1 mg/l IAA was used for the induction of rooting. In this medium, 89.22 per cent rooting was observed. Root initials were observed ten days after inoculation. Plantlets recorded good root characters with 4.7 roots/ plant with mean root length of 2.61 cm.

Sahavacharin (1985) reported that MS media best with 0.5 mg/l IAA has induced adventitious roots in gerbera. But Pierik and Sprenkels (1984) and Murashige *et al.* (1974) reported 100 per cent rooting in shoots cultured in MS medium containing 1 or 3 mg/l NAA, better than IAA for Fleur and Florence and the choice of genotype affected the rooting response to different types of auxins. But in present study, MS medium supplemented with 1 mg/l IAA was found good response.

Some authors did not observe an effect of mineral concentration of MS medium on the rooting percentage of the gerbera variety Appelbloesem (Barbosa *et al.*, 1992, 1993), suggesting the genotype dependence of the rooting response and nutrient requirements for this growth stage. Posada *et al.* (1999) observed improved rooting percentage in gerbera shoots when MS salt concentration was reduced. But results were obtained with half MS medium without IAA.

Shailaja (2002) also observed 100 per cent rooting on MS with 2 mg/l NAA. The media used in stages prior to rooting, such as shoot multiplication media, tended to affect the subsequent rooting stages. But according to Sousa *et al.* (2006), use of cytokinine in the rooting media, such as 0.5 mg/l BA, can

reduce the percentage of rooting in gerbera. But in the present investigation, IAA found best for root induction (89.93%).

In gerbera variety Jaguar Cream, an increasing concentration of NAA (0-4 mg/l) in half MS also had shown increase in the number (1.6–5.0) and length (3.4–5.0 cm) of roots (Rezende *et al.*, 2008). Feng *et al.* (2009) observed that half MS medium was best for rooting gerbera stem segments, when 0.2 mg/l NAA was added. Son *et al.* (2011) also obtained the best rooting percentage (86, 96 and 100%) and number of roots/shoot (3.8, 9.4 and 7.2) in MS medium with 2.0 mg/l NAA in three gerbera cultivars (Arianna, Bonnie and Tobia).

Shabanpour *et al.* (2011) showed that 100 per cent of two cultivars (Pink and Orange) could be successfully rooted in MS media with IAA or NAA, but most roots (4.6–5.1 roots/shoot) could be obtained with 3 mg/l IAA. Shabbir *et al.* (2012), testing different concentrations of NAA and IAA for rooting of Sunglow shoots, obtained best rooting percentage (97.7%), number of roots/shoot (7.6) and root length (7.33 cm) in MS medium with 1.5 mg/l IAA. NAA (0.5–2.0 mg/l) resulted in lower rooting percentage (36.7–60.7%) than IAA, independent of the concentration. Similar results were recorded in present study, and low level of IAA (1 mg/l) was found better.

Naz *et al.* (2012) showed that best rooting percentage (80%) was obtained using 10 mg/l NAA. IBA resulted in 100 per cent adventitious root induction in cv. AL101 (Cardoso and Silva, 2012) in which good quality plantlets and roots were obtained with a low concentration of IBA (0.05 mg/l). Similarly, maximum frequency (81%) of rooting with highest number (4) of roots per shoot was achieved in MS medium fortified with 0.3 mg/l IBA (Shylaja *et al.*, 2014). But in the present study, IAA medium recorded the highest best root/ plantlets ratio (4.76) with good (2.71 cm) root length.

5.1.2.5 Hardening and acclimatization

In the present studies, ten different potting media with different proportion

of sand, soil, peat, perlite and vermiculate were used for hardening micropropagated plantlets. The survival rates obtained have differed among the treatments. The maximum survival rate (76%) was noticed in T_4 medium (peat: perlite: vermiculite with proportion 3:1:1 v/v), after four weeks of transfer to hardening medium. Minimum survival (40%) was recorded in T_3 (cocopeat: soil: sand in 3:1:1 v/v).

Conti *et al.* (1991) reported that greenhouse acclimatization of plantlets was achieved in 30 days in a peat: perlite (1:1 v/v) substrate. In other reports, rooted plantlets were first transferred to Jiffy-7 peat pellets in a glass covered acclimatization chamber and later to a mixture of perlite: sphagnum moss (1:1 v/v), in greenhouse. The losses at this stage were less than 5 per cent (Laliberte *et al.*, 1985). Qianzhong *et al.* (1998) obtained better root growth and plant survival (93.8%) in rice chaff as hardening medium. Parthasarathy and Nagaraju (1999) obtained 95-100 per cent success at the acclimatization stage, using polyethylene bags and soil: sand: farmyard manure (1:1:1 v/v) as the organic-mineral substrate in gerbera cv. SWM and Dilmaya.

Rooted plantlets of micropropagated gerbera were successfully acclimatized (95% survival rate) using plastic pots with coco peat, red soil and sand (3:1:1v/v) (Aswath and Choudhary, 2002b). Nga *et al.* (2005) investigated the effect of hardening media, *viz.*, soil, sand, rice husk, humus, soil + humus (1:1 v/v), sand + rice husk (1:1 v/v) and humus + rice husk (1:1 v/v) on survival percentage and root growth of gerbera plantlets. The best plant vigour and maximum survival of plants (93.25%) was observed in humus + rice husk (1:1, v/v) mixture followed by sand medium.

Taha *et al.* (2010) used garden soil, vermiculite, black soil and autoclaved garden soil to acclimatize gerbera plantlets that had previous rooted *in vitro*. They observed that garden soil (black soil: red soil 2:1 v/v), showed best results with 86 ± 0.9% plantlet survival, followed by vermiculite with 73 ± 1.3 per cent survival. In present study, sand potting medium observed minimum survival percentage (44%).

Son *et al.* (2011) reported in their study that, sand and cocopeat mixture 1:1 (v/v) showed the worst result, with a 27 per cent survival of plantlets, whereas the maximum survival rate was noticed in vermiculite medium (67%), after four weeks. But in the present study, vermiculite recorded minimum survival percentage as compared to other potting media.

Kadu (2013) successfully transferred the *in vitro* rooted plantlets to sand + soil +FYM + leaf mould, and the highest survival rate (82.43%), longer plants (4.6 cm), more number of leaves/plant with more number of roots/plant (3.467) were obtained.

For hardening, rooted plants were transferred to different hardening media namely coco peat: perlite (1:1, v/v) and coco peat: perlite: vermicompost (1:1:1, v/v) in plastic pots with polythene cover. Out of the three potting mixture, maximum survival of plantlets (90 %) after four weeks was observed in coco peat containing pots which were covered with polythene (Bhargava *et al.*, 2013). Similar result found in present investigation, peat: perlite: vermiculite (2:1:1 v/v) found highest survival percentage (76%).

Shylaja *et al.* (2014) reported highest (90%) survival percentage in sand, cocpeata and soil mixture (1:1:1 v/v), where as in the present investigation; same potting medium recorded 68 per cent survival.

The various stages of indirect organogenesis from leaf explants are depicted in Fig. 1.

5.2. Genetic stability studies using ISSR molecular marker

5.2.1 DNA isolation

DNA was isolated from young tender leaves of gerbera plants derived from mother plants, flower bud regenerants and leaf calli derived plantlets. The protocols reported by Rogers and Bendich, (1994) with 4X CTAB extraction buffer yielded

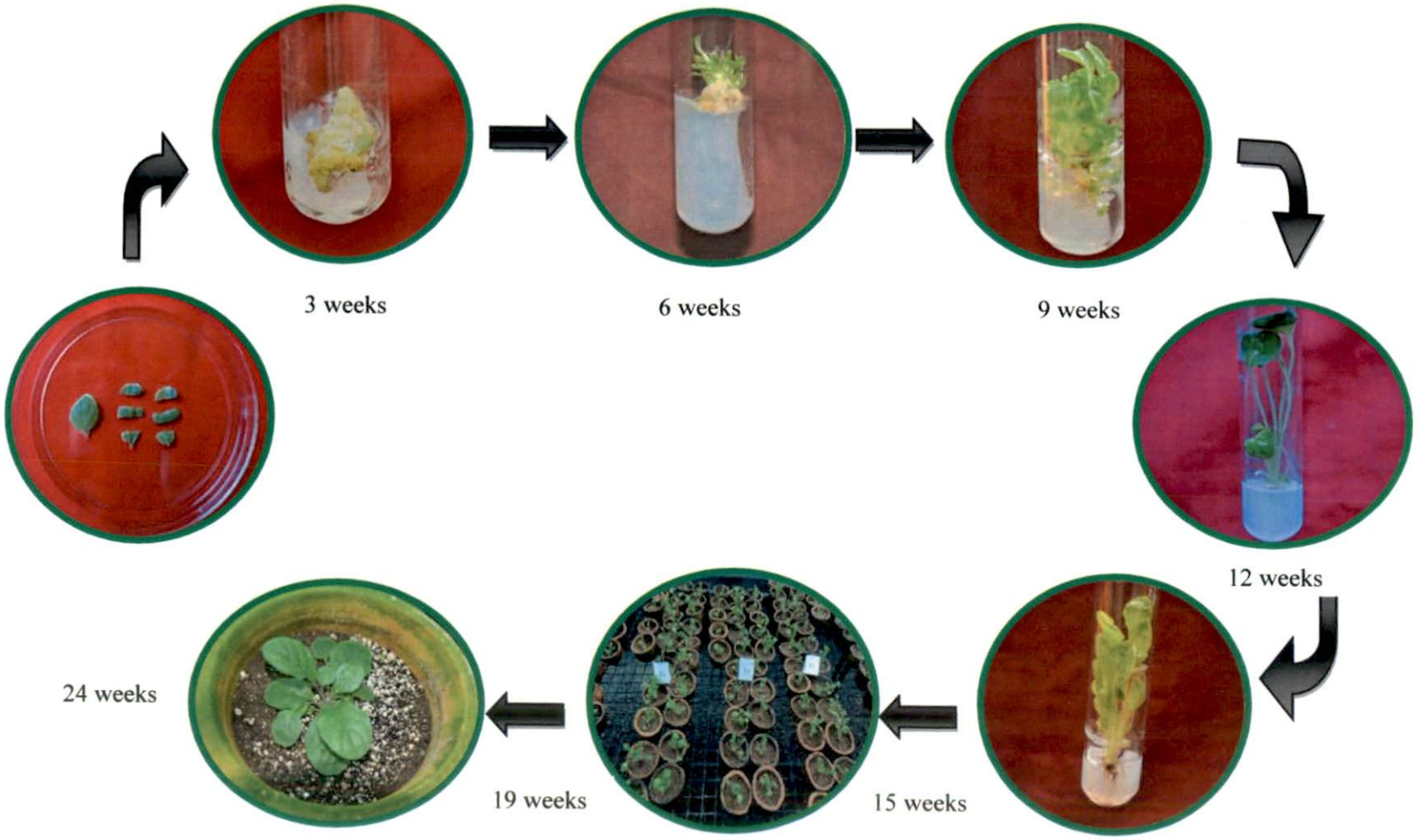


Fig. 1. Stages of indirect organogenesis from *in vitro* leaf explants of variety Dubai in gerbera

good quality DNA. The electrophoressed DNA showed distinct bands without shearing.

The homogenisation, pulverisation and uniformity of grinding of plant tissue were essential during DNA extraction. Excess liquid nitrogen was used for the homogenisation of the leaf tissue. Liquid nitrogen helped in maintaining the frozen tissue, preventing nucleic acid degradation and effect of secondary metabolites and a better mechanical disruption of tissues (Hernandez and Oyarzum, 2006). The problem of polyphenols was overcome by the addition of β- mercaptoethanol and poly vinyl pyrrolidone (PVP) along with the extraction buffer. β- Mercaptoethanol disrupted the protein disulphide bond and was thereby capable of initiating protein degradation. Poly vinyl pyrrolidone (PVP) has removed polyphenols and inhibited co-precipitation of polysaccharides which resulted in good quality DNA. That were previously confirmed by Matasyoh *et al.* (2008).

The detergent present in the extraction buffer, CTAB helped in the release of nucleic acids into buffer disruption of the cell membrane. The released DNA was protected from the action of DNase enzyme by EDTA present in the extraction buffer. It is a chelating agent, which efficiently blocks Mg^{2+}, the major cofactor of DNase enzyme. The DNA isolated by CTAB method was made free of chlorophyll by using the chloroform: isoamyl alcohol which aided in the separation of organic mixture and aqueous phase of the DNA isolation. EDTA was also a major component of TE buffer in which the DNA dissolved and stored as was reported by Sambrook *et al.* (1989).

The yield of DNA and its purity varied with plants. The yield in the present study ranged from 430.2 ng/µl to 3236 ng/µl and the purity (A_{260}/ A_{280}) of DNA ranged from 1.82 to 2.15.

5.2.2 ISSR analysis

In the present study, PCR-based technique, ISSR was adopted to characterise the *in vitro* regenerants. Seven ISSR primers reported by Bhatia *et al.* (2010) were

selected for the study. Genomic DNA isolated from various sources *viz.*, mother plants, reregenerants from direct and indirect organogenesis were subjected to ISSR analysis.

ISSRs are DNA fragments of about 100-3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). It is a multi locus, dominant molecular based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, whether that segregated in Mendelian ratio and has been successfully applied in genetic and evolutionary studies of many species. ISSR primers are now proved to be much more efficient in assessing the genetic integrity among clonally propagated plants as reported by many workers in different species (Zietekiewicz *et al.*, 1994; Bhatia *et al.*, 2009; Mohanty *et al.*, 2010; Bhatia *et al.*, 2011).

In the present study three groups of plants of the variety Dubai *viz.*, mother plant, flower bud and leaf calli derived plants were subjected to ISSR assay. Out of seven primers tested, ISSR 18 and ISSR 21 exhibited polymorphism (25 - 33.33 %) while ISSR 08, ISSR 09, ISSR 15, ISSR 25 and ISSR 31 gave monomorphic amplicons. The seven ISSR primers produced 58 distinct and scorable bands in the size range of 200 bp to 2900 bp. The number of scorable bands for each primer varied from 4 (ISSR-21) to 15 (ISSR-15) with an average of 8.28 bands per primer. A total of 58 bands were generated from mother plants, flower bud regenerants and leaf calli regenerants out of which 55 were monomorphic. However, one band was polymorphic in flower bud regenerants and two were polymorphic in leaf calli regenerants.

In the variety Shania, mother plant and regenerants from flower bud were subjected to ISSR assay. Out of seven primers tested, ISSR 15 and ISSR 25 exhibited polymorphism (25 - 33.33 %) while ISSR 08, ISSR 09, ISSR 18, ISSR 21 and ISSR 31 gave monomorphic amplicons. The seven ISSR primers produced

52 distinct and scorable bands in the size range of 270 bp to 1900 bp in variety Shania. The number of scorable bands for each primer varied from 4 (ISSR-21) to 12 (ISSR-31) with an average of 7.42 bands per primer. A total of 52 bands were generated from mother plants and flower bud regenerants, out of which 50 were monomorphic. However, two bands were polymorphic in flower bud regenerants.

The polymorphism in flower bud and leaf calli regenerated plants when amplified with ISSR 15, ISSR 18, ISSR 21, ISSR 25 primers, could result from change in either the sequence of primer binding site (e.g. point mutations) or change which alter the size and prevent successful amplification of target DNA (e.g. insertion, deletion, inversions). The variations in flower bud and leaf calli regenerated plants might be due to gene amplification, chromosomal irregularities, point mutation and alteration in DNA methylation during *in vitro* culture (Saker *et al.*, 2000). Although the direct formation of plant structures without any intermediate callus phase, minimises the possibility of instability (Karp, 1994).

In the previous studies it was reported that the presence or absence of variations during *in vitro* propagation depends upon the source of explants and the method of regeneration (Goto *et al.* 1998). The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martin *et al.* 2004). Even at optimal levels, longterm multiplication and high chromosome number of the plant may often lead to somaclonal or epigenetic variations in micropropagated plants, thus, questioning the varies fidelity of their clonal nature. In present study, plantlets were obtained from flower buds regenerants and leaf calli regnerants, in both explants regenerants variation at DNA level was observed.

Gross and cryptic chromosomal changes, or extensive changes in chromosome number, occur early during induction in an *in vitro* culture (Larkin and Scowcroft, 1981). Moreover, the rapid multiplication of a tissue or long-term cultures may affect genetic stability and thus lead to somaclonal variation

(Reuveni and Israeli, 1990). Genetic fidelity also depends on explant source (Krikorian *et al.*, 1993). Highly differentiated tissues, such as roots, leaves, and stems, generally produce more variants, probably due to the callus-phase, than explants that have pre-existing meristems (Sharma *et al.*, 2007). *In vitro*, the conditions of culture can be mutagenic and regenerated plants derived from organ cultures, calli, protoplasts and somatic embryos sometimes can show phenotypic and genotypic variation (Orbovic *et al.*, 2008).

In some reports, plants derived from organised meristems are not always genetically true-to-the type in many crops (Devarumath *et al.* 2002). Plants regenerated from adventitious shoots from axillary buds or from other well developed meristematic tissue showed the lowest tendency for genetic variation (Joshi and Dhawan, 2007). Hence, it becomes imperative to regularly check the genetic purity of the micropropagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation

Joshi and Dhawan (2007) have employed ISSR marker assay to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication up to forty-two passages. Gantait *et al.* (2010) reported the clonal fidelity of micropropagated and sustained cultured clones of *Allium ampeloprasum* L. using ISSR primers.

Bhatia *et al.* (2010) reported genetic fidelity of *in vitro* raised plants of gerbera derived from three different explants *viz.*, capitulum, leaf and shoot tips using ISSR assay. The clones derived from capitulum and shoot tip explants did not show any genetic variation, whereas one of the leaf-derived clones exhibited some degree of variation. The genetic fidelity of *in vitro*-raised gerbera derived from capitulum explants was assessed by using RAPD and ISSR markers (Bhatia *et al.*, 2011). In another report by Borse *et al.* (2011) clonal fidelity of banana (*Musa acuminata* cv. Grand Naine) regenerants from six different *in vitro* subculture generations and in the explant suckers were evaluated by using ISSR and REMAP molecular markers. Very low variation was observed up to the

eighth subculture generations with polymorphic bands both ISSR (0.96%) and REMAP (0.95%) markers system. Similar results have also been found in the present studies, with the flower bud regenerants showing less (20%) variation, while leaf calli regenerants showing high variability (40%) in the two tested gerbera varieties Dubai and Shania.

Parida *et al.* (2011) assessed genetic fidelity of *in vitro* regenerated *Alpinia galanga* L. subjected to RAPD and ISSR markers to assess genetic stability. Nadha *et al.* (2011) were confirmed the true-to-type nature of the *in vitro* raised clones of *G. angustifolia* Kunth using RAPD and ISSR markers for variability in the tissue culture raised plantlets. Khateeb *et al.* (2013) assessed genetic stability of micropropagated plants of *Moringa peregrina* using ISSR. In all reported studies, they found the monomorphic amplification in different explants regenerants and ensured true-to-type nature of micropropagated plants.

In the present investigations micropropagation was attempted through two different pathways *viz.*, direct organogenesis from flower bud explants and indirect organogenesis from leaf explants.

The micropropagation protocol developed at CPBMB was used to regenerate plantlets from flower buds. As flower production was late and less in mother plants maintained at CPBMB net house, only less number of flower buds were available for experimentation and hence only up to shoot multiplication could be achieved in the present study. The protocol for regeneration from leaf explants was standardised in the present study.

The most important problem in gerbera micropropagation is the genotypic difference in response and till date a protocol which can be utilized for multiple genotypes has not been reported (Cardoso *et al.*, 2013). The regeneration from flower bud explants obtained in the present study is working in different genotypes even though there is difference in multiplication rates. However, for effective mass multiplication, genotype specific optimization in protocol holds good.

Another striking feature of the present study is the regeneration from leaf explants both from *in vitro* and *in vivo* sources which was not achieved in most of the studies done in gerbera and only rhizogenesis was reported in most of the cases.

The high chromosome number (2n = 50) of gerbera and the high proliferation rate observed in the present protocol might have contributed for the variation observed in ISSR assay. Even in direct regenerated plants from flower bud explants, ISSR assay showed 20 per cent variation. The variation observed in the DNA level sometimes may not bring about phenotypic variation which could be conformed only after phenotypic evaluation of the regenerants.

Majority of the gerbera specific primers exhibited monomorphic banding pattern both in plants regenerated from flower bud explants and leaf calli regenerants which showed their uniformity. Out of the seven ISSR primers tested in three groups of plants in variety Dubai (mother plant, flower bud regenerants and leaf calli regenerants), five primers exhibited monomorphic banding pattern. Similarly in the variety Shania also, out of seven primers tested, five exhibited monomorphic banding pattern when mother plant and bud culture regenerants were analysed for genetic stability.

However, thorough phenotypic evaluation of the micropropagated plants is to be undertaken to know the variation if any in flower morphology and other floral characters before recommending the protocol for commercial micropropagation. The DNA level variation was found high for callus mediated organogenesis. The protocol developed from leaf calli is to be further investigated for creating a base population for future breeding programmes.

Summary

6. SUMMARY

The study entitled "Micropropagation of gerbera (*Gerbera jamesonii* Bolus) and assessment of genetic stability of plantlets using ISSR assay" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period August 2012- June 2014, with the objective to develop an efficient micropropagation protocol for mass multiplication of gerbera (*Gerbera jamesonii* Bolus) and to test genetic stability of the micropropagated plants using ISSR assay. Two different explants *viz.*, flower bud and leaves were used for micropropagtion. Two different pathways *viz.*, direct and indirect organogenesis were attempted to produce plantlets.

The salient findings of the study are as follows:

1. The IIHR gerbera variety Arka Krishika and three other varieties procured from AVT, Kochi *viz.*, Dubai, Shania and Hotpsring were utilised for the investigations.
2. The micropropagation protocol developed at CPBMB was used for production of plantlets from flower bud explants.
3. Regeneration from flower bud explants was achieved through direct organogenesis and cultures regenerated were multiplied up to three subculture cycles.
4. The protocol for indirect organogenesis using leaf explants was standardised.
5. All the stages of indirect organogenesis was standardised using the gerbera variety Dubai.
6. For callusing and callus growth, MS medium supplemented with 3 mg/l NAA and 0.3 mg/l BAP was found good.
7. For induction of shoots, MS medium supplemented with 3 mg/l BAP + 0.2 mg/l NAA was found good.
8. For shoot proliferation, MS medium supplemented with 3 mg/l BAP + 0.1 mg/l NAA was found good.

9. For elongation of shoots, MS medium supplemented 0.1 mg/l NAA was found good.
10. For rooting, MS medium supplemented with 1 mg/l IAA was found good.
11. The protocol for indirect organogenesis standardised in variety Dubai was applied to other varieties like Arka Krishika, Shania and Hotspring.
12. Shoot morphogenesis was not obtained in the varieties Shania and Hotspring from *in vitro* leaf calli.
13. Shoot morphogenesis was obtained in variety Arka Krishika from *in vivo* leaf derived calli.
14. High genotypic difference was observed in shoot regeneration and proliferation in different gerbera varieties studied. The white flowered variety Dubai showed high rate of multiplication in both the routes of micropropagation *viz.*, indirect organogenesis using leaf explants and direct organogenesis using flower bud explants.
15. The potting media for hardening micropropagated plants were standardised and media with peat, perlite and vermiculite mixture (3:1:1 v/v) was found good which gave 76 per cent survival four months after planting.
16. For the growth of plantlets, the medium with cocopeat, sand and soil in proportion 1:1:1 v/v was found good.
17. Plantlets produced were successfully acclimatised and 200 plants were established in net house.
18. Genetic stability studies using ISSR assay were carried out in three groups of plants *viz.*, mother plants, flower bud regenerants and leaf calli regenerants.
19. The protocol suggested by Rogers and Bendich (1994) was used for extraction of genomic DNA from young tender leaves. Seven ISSR primers already reported for gerbera by Bhatia *et al.* (2010) were used for genetic stability analysis. Of the seven primers tested, five primers showed monomorphic banding pattern in different groups of regenerants of variety Dubai and variety Shania.

20. In variety Dubai, mother plant and regenerants from flower bud and leaf calli showed 25 to 33.33 per cent polymorphism when DNA was resolved with primer ISSR 18 and ISSR 21.
21. In variety Shania, mother plant and flower bud regenerants exhibited 25-33.33 per cent polymorphism with primers ISSR 15 and ISSR 25.
22. Genetic stability studies conducted using ISSR assay showed that mother plant and regenerants derived from flower buds were uniform to the extent of 80 per cent.
23. Variation at DNA level observed was more (40%) for plantlets regenerated through indirect pathway.

References

REFERENCES

Ahim, M. and Vieth, J. 1986. Production of haploids plants gerbera (*Gerbera jamesonii*) par culture *in vitro* ovules. *Canadian. J. Bot.* **54** : 2355-2357.

Akter, N., Hoque, M. I. and Sarker, R. H. 2012. *In vitro* propagation in three varieties of gerbera (*Gerbera jamesonii* Bolus) from flower bud and flower stalk explants. *Plant Tissue Cult. Biotech.* **22**(2): 143-152.

Alizadeh, M. and Singh S K. 2009. Molecular assessment of clonal fidelity in micropropagated grape (*Vitis* spp.) rootstock genotypes using RAPD and ISSR markers. *Iranian J. Biotech.* **7**: 37-44.

Arello, E. F., Pasqual, M., Pinto, J. E. B. P. and Barbosa, M. H. P. 1991. *In vitro* establishment of explants and seedling regeneration in gerbera (*Gerbera jamesonii* Bolus ex Hook) by tissue culture. *Pesquisa Agropecuaria Brasileira* **26**: 269-273.

Arriel, N. H. C. , Mauro, A. O. D., Arriel, E. F., Uneda-Treviso, S. H., Costa, M. M., Barbaro, I. M. and Muniz, F. R. S. 2007. Genetic divergence in sesame based on morphological and agronomic traits. *Crop Breed. Appl. Biotech.* **7**: 253-261.

Aswath, C. R. and Choudhary, M. L. 2002a. Rapid plant regeneration from *Gerbera jamesonii* Bolus callus cultures. *Acta. Botanica. Croatica.* **61**: 125-134.

Aswath, C. R. and Choudhary, M. L. 2002b. Mass propagation of gerbera (*Gerbera jamesonii*) through shoot tip culture. *Indian J. Hort.* **59**: 95-99.

Aswath, C. R., Deepa, S. N. and Choudhary, M. L. 2003. Commercial multiplication of gerbera (*Gerbera jamesonii* Bolus) through *in vitro* shoot tips culture. *J. Ornamental Hort.* **6**: 303-309.

Atak, C. and Celik, O. 2009. Micropropagation of *Anthurium andraeanum* from leaf explant. *Pak. J. Bot.* **41**(3): 1155-1161.

Barbosa, M. H. P., Pasqual, M., Pinto, J. E. B. P., Arello, E. F., Barros, I. De. and Barros I. 1993. Effects of benzyl amino purine (BAP) and 3-indole acetic acid (IAA) on *in vitro* propagation of Gerbera (*Gerbera jamesonii* Bolus ex Hook) cv. Appelbloesem. *Pesquisa Agropecuaria Brasileira* **28**: 15-19.

Bhargava, B., Dilta, B. S. and Gupta, Y. C. 2013. Studies on micropropagation of gerbera (*Gerbera jamesonii* Bolus). *Agriculture* 3: 8-11.

Bhatia, R., Singh, K. P., Jhag, T. and Sharma, T. R. 2010. Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers. *Sci. Hort.* **199**: 208-211.

Bhatia, R., Singh, K. P., Sharma, T. R. and Jhang, T. 2011. Evaluation of the genetic fidelity of *in vitro* propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers, *Plant Cell Tiss. Org. Cult.* **104**: 131-135.

Bhojwani, S. S. 1980. Micorpropagation method for a hybrid for a hybrid willow (*Salix matsudama* xalba NZ looz). *N2 Bot.* **18**: 2009-2011.

Bonga, J. M. 1987. Tree tissue culture applications. In: Advances in cell culture. K. Maramorosch (ed). *Academic Press, New York.* pp 209-239.

Borse, N., Chimote, V. P. and Jadhav, A. S. 2011. Stability of micropropagated *Musa acuminata* cv. Grand Naine over clonal generations: A molecular assessment. *Scientia Hort.* **129**: 390–395.

Bose, T. K., Yadav, L. P. and Pal, P. 2003. *Commercial flowers. Vol. 2.* Naya Udgog. Kolkata, India, 660p

Botstein, D., White, R. L. and David, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphism. *Am. J. Hum. Genet.* **32**: 314-331.

Bouman, H., Morris, B. and Tiekstra, A. 2001. Development of new tissue culture media, using the relation between mineral composition of plant and medium. *Acta. Hort.* **560**: 373–6.

Brown, A. H. D. 1978. Isozymes plant population genetic structure and genetic conservation. *Theor. Appl. Genet.* **52**:145-157.

Budi, R. S. and Suwandi, T. C. K. H. 2000. Micropropagation and acclimatization of gerbera (*Gerbera jamesonii* Bollus). *J. Penelit Pertan.* **19**: 40-47.

Cardoso, J. C. and Silva, J. A. T. 2013. Gerbera Micropropagation. *Biotech. Adv.* **31**: 1344-1357.

Chakabrarty, D. and Datta, S. K. 2008. Micropropagation of gerbera: lipid peroxidation and antioxidante enzyme activities during acclimatization process. *Acta. Physiol. Plant* **30**: 325–31.

Condit, R. and Hubbell, S. P. 1991. Abundance and DNA sequences of two base repeat regions in tropical tree genomes. *Genome* **34**: 66-71.

Conti, L., Frangi, P., Tosca, A. and Verga, P. 1991. Breeding clones of *Gerbera jamesonii* Hybrida. Suitable to micropropagation and pot cultivation. *Acta Hort.* **300**: 103-105.

Damasco, O. P., Graham, G. C., Henry, R. J., Adkins, S. W., Smith, M. K. and Godwin, I. D. 1996. Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* spp.) bananas. *Plant Cell Rep.* **16**: 118-123.

Deepaja, S. M., 1999, Micropropagation of Gerbera (*Gerbera jamesonii* Bolus), *M.Sc. Thesis*, University of Agricultural Sciences, Bangalore.

Feng, X., Lai, C. and Lai, Z. 2009. Optimization of micropropagation conditions in *Gerbera jamesonii*. *Subtropical Agric. Res.* 4: 5-9.

Franceschetti, U. 1973. Observation on the longevity of gerbera seed. *Sementi-Elette*. **19**(6): 7-10.

Fraser, L. G., Harvey, C. F., Crowhurst, R. N., De Silva, H. N. 2004. EST–derived microsatellites from *Actinidia* species and their potential for mapping. *Theor. Appl. Genet.* **10**(8): 1010-1016.

Fukuoka, S., Inoue, T., Miyao, A., Monna, L., Zhong, H. S., Sasaki, T. and Minobe, Y. 1994. Mapping of sequence-tagged sites in rice by single strand confirmation polymorphism. *DNA Res.* **1**: 271-277.

Gagnon, S. and Dansereau, B. 1990. Influence of light and photoperiod on growth and development of gerbera. *Acta. Hort.* **272:** 145-151.

Gantait, S., Mandal, N., Bhattacharyya, S. and Das, P. K. 2010. An elite protocol for accelerate quality cloning in *Gerbera jamesonii* Bouls cv. Sciella. *In vitro cell Dev. Biol.* **46**: 537-548.

Garthwaite, J.M., 1959. Observations on gerbera growing. *Exp. Hort.* **2:** 12-15.

Godwin, I. D., Aitken, E. A. B. and Smith, L. W. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. *Electrophoresis* **18**: 1524-1528.

Gong, L. and Deng, Z. 2010. EST-SSR marker for gerbera *(Gerbera Hybrida). Mol. Breed.* **26**: 125-132.

Gong, L. and Deng, Z. 2012.Selection and application of SSR markers for variety discrimination, genetic similarity and relation analysis in gerbera. *Sci. Hort* .**138**: 120-127.

Gregorini, G., Lorenzi, R. and Lansioni, G. 1976. The propagation of gerberas by *in vitro* culture of vegetative apices. *Rivista-della-Ortoflorofrutticoltura-Italiana* **60**: 282-288.

Harel, D., Kuzmicic, I., Jug-Dujakovic, M. and Jelaska, S. 1993. The effect of genotype on gerbera shoot multiplication *in vitro*. *Acta. Bot. Croatia* **52**: 25-32.

Hasbullah, N. A., Taha, R. M. and Awal, A. 2008. Growth optimization and organogenesis of *Gerbera jamesonii* Bolus ex. Hook f. *in vitro*. *Pak. J. Biol. Sci.* **11**:1449–54.

Hearne, C. M., Ghosh, S. and Todd, J. A. 1992. Microsatellites for linkage analysis of genetic traits. *Trends Genet.* 8: 288-294.

Hedtrich, C. M. 1979. Production of shoots from leaves and propagation of *Gerbera jamesonii*. *Gartenbauwissenschaft* **44**: 1-3.

Hempel, M., Petos, W. B. and Tymoszuk, J. 1985. The influence of cytokinins on multiplication and subsequent rooting of gerbera *in vitro*. *Acta Hortic.* **167**: 301- 305.

Heun, M., Murphy, J. P. and Phillips, T. D. 1994. A comparision of RAPD and isozyme analyses for determining the genetic relationships among *Avena sterilis* L. accessions. *Theor. Appl. Genet.* **87**: 689-696.

Hitmi, A., Barthomeuf, C. and Sallanon, H. 1999, Rapid mass propagation of *Chrysanthemum cinerariafolium* by callus culture. *Plant Cell Rep.* ***19:*** 156-160.

Huang, M. C. and Chu, C. Y., 1983, *In vitro* formation of gerbera (*Gerbera hybrida* Hort.) plantlets through excised scape culture. *J. Japanese Soc. Hort. Sci.* **52**: 94-100.

Huang, M. C. and Chu, C. Y. 1985. A scheme for commercial multiplication of gerbera through shoot tip culture. *J. Japanese Soc. Hort. Sci.* **54**: 94-100.

Hussein, G. M., Ismail, I. A. Hashem, M. E. Miniawy, M. E. and Abdallah, N. A. 2008. *In vitro* regeneration of gerbera. *Agric. For.. Res.* **58**: 97-102.

Jain, S.M. 1993. Studies on somaclonal variation in ornamental plants. *Acta Hort.* **336:** 365-372.

Jerzy, M. and Lubomski, M. 1991. Adventitious shoot formation on *ex vitro* derived leaf explants of *Gerbera jamesonii. Sci. Hort.* **47**: 115-124.

Jerzy, M. and Lubomskii, M. 1992 *In vitro* adventitious bud techniques for mutation of *Gerbera jamesonii. Acta. Hort* **314**: 269-274.

Joshi P, Dhawan, V. 2007. Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. *Biol. Plant.* **51**:22-26.

Kadu, A. R. 2013. *In vitro* micropropagation of gerbera using auxillary bud. *Asian J. Biol.Sci.* **8**: 15-18.

Kafarski, H. and Hauzinska, E. 1974. Nowa metada romnazamia wegetatywnego gerberyhodowla pakow kwiatostarowych *in vitro*. *Ogrodnictwo* **11**: 345-347.

Kalra, M., Sehrawat, S. K., Batra, P. Kumar, S. Dahiya, D. S. and Gupta, A. K. 2008. Micropropagation studies in gerbera (*Gerbera jamesonii* Bolus). *Haryana J. Hort. Sci.* **37**: 78-79.

Kannan, M. and Ramdas, S. 1990. Variability and heritability studies in gerbera (*Gerbera jamesonii*). *Prog. Hort.* **22** (14): 72-76.

Kanwar, J. K. and Kumar, S. 2008. *In vitro* propagation of gerbera - A review. *Hort. Sci.* **35**(1): 35-44.

Karp A. 1994. Origins, causes and uses of variation in plant tissue cultures. In: Vasil IK, Thorpe TA (eds) Plant cell and tissue culture. Dordrecht: Kluwer Academic Publishers. pp139-152.

Khateeb, W. A., Bahar, E., Lahham, J., Schroeder, D. and Hussein, E. 2013. Regeneration and assessment of genetic fidelity of the endangered tree

Moringa peregrina (Forsk) Fiori using Inter Simple Sequence Repeat (ISSR). *Physiol. Mol. Biol. Plants*. **19**(1): 157–164.

Koek, P. C. 1957. Gerbera. *Jversl. Proefst. Bloem. Aalsmeer*. pp 49-52

Krikorian, A. D., Irizarry, H., Cronauer-Mitra, S. S. and Rivera, E. 1993. Clonal fidelity and variation in plantain (*Musa* AAB) regenerated from vegetative stem and floral axis tips *in vitro*. *Annals of Botany* **71**:519-535.

Kumar, S. and Kanwar, J. K. 2006. Regeneration ability of petiole, leaf and petal explants in gerbera cut flower cultures *in vitro*. *Folia Hort.* **18**: 57-64.

Kumar, S. and Kanwar, J. K. 2007. Plant regeneration from cell suspensions in *Gerbera jamesonii* Bolus. *J. Fruit Ornamental Plant Res.* **15**: 157-166.

Kumar, S, Kanwar, J. K. and Sharma, D. R. 2004. *In vitro* regeneration of *Gerbera jamesonii* Bolus from leaf and petiole explants. *J. Plant Biochem. Biotech.* **13**: 73-75.

Kushikawa, S., Hoshino, Y. and Mii, M. 2001. *Agrobacterium*-mediated transformation of *Saintpaulia ionantha* Wendl. *Plant Sci.* **161**:953-960.

Lakshmanan, V., Venkataramareddy, S. R. and Neelwarne, B. 2007. Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. *Electron J. Biotech.* **10**: 1-8.

Laliberte, S., Chretien, L. and Vieth, J. 1985. *In vitro* plantlet production from young capitulum explants of *Gerbera jamesonii*. *Hort. Sci.* **20**: 137-139.

Laneri, U., Franconi, R. and Altavista, P. 1990. Somatic mutagenesis of *Gerbera jamesonii* hybrid: irradiation and *in vitro* cultures. *Acta Hort.* **280**: 395-402.

Larkin, P. and Scowcroft, W. 1981. Somaclonal variation a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* **60:** 197-214.

Le-CL, Julmi, C., Thomas, D. and Tschuy, F. 1999. *In vitro* regeneration and multiplication of *Gerbera jamesonii* Bolus. *Revue-suisse-de-viticulture-d'Arborticulture-etd'Horticulture* **31**: 207-211.

Leffring, L. 1968. Vegetative propagation of Gerbera. *Bloemist.* **26:** 9-12.

Leffring, L. 1973. Flower production in Gerbera I correlation between shoot, leaf and flower formation in seedling. *Scienta. Hort.***1:** 221-229.

Leffring, L. 1975. Effects of daylength and temperature on shoot and flowerproduc- tion of gerbera. *Acta Hort.* **51**:263-265.

Liqing, Y., Zhang, J. J., Chen, Q. Q., Wan, C. Z. and Fan, K. H. 1996. Effect of ABT (amino benzo triozole) on rapid propagation of Barberton Daisy (*Gerbera jamesonii*). *Acta Hort.*. **12**: 55-58.

Loeser, H. 1986. New Gerbera cultivars at Heidelberg. *Deutscher Gartenbau* **40** (32): 1461-1464.

Mallaya, N. P. and Ravishankar, G. A. 2013. *In vitro* propagation and genetic fidelity study of plantregenerated from inverted hypocotyl explants of eggplant (*Solanum melongena L.*) cv. Arka Shirish. *Biotechnology* **3**: 45-52.

Mandal, A. B., Das, S. and Biswas, A. 2010. *In vitro* propagation of gerbera and assessment of clonal fidelity of the microclones. *Indian J. Hort.* **67**: 357-361.

Mariska, I., Gati, E. and Sukmadjaja, D. 1989. *In vitro* clonal propagation of gerbera (*Gerbera jamesonii* Bolus). *Bulletin Penelitaion Hort.* **17**: 34-43.

Mata, T. L. D., Segeren, M. I., Fonseca, A. S. and Colombo, C. A. 2009. Genetic divergence among gerbera accessions evaluated by RAPD. *Sci. Hort.* **121**: 92-96.

McCouch, S. R., Teytelman, L., Xu, Y., Lobos, K. B., Clare, K., Walton, M., Fu, B., Maghirang, R., Li, Z., Xing, Y., Zhang, Q., Kono, I., Yano, M., Fjellstrom, R., DeClerck, G., Schneider, D., Cartinhour, S., Ware, D. and Stein, L. 2002. Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). *DNA Res.* **9**: 199-207.

Meng, X. C. and Wang, X. J. 2004. Regulation of flower development and anthocyanin accumulation in *Gerbera hybrida*. *J. Hort. Sci. Biotech.* **79**: 131-137.

Meyer, H. J. and VanStaden, J. 1988. The *in vitro* culture of *Gerbera aurantica*. *Plant Cell Tiss. Org. Cult.* **14**: 25-30.

Meynet, J. and Sibi, M. 1984. Haploid plants from *in vitro* culture of unfertilized ovules in *Gerbera jamesonii*. *Zeitschrift-fur-Pflanzenzuchtung* 93: 78-85.

Minerva, G., Surinder, K. and Thakur, M. 2012. *In vitro* propagation and assessment of plant genetic fidelity by RAPD and SSR markers in gerbera (*Gerbera jamesonii*). *Res. J. Biotech.* 7(4): 53-57.

Misra, P., Purshottam, D. K., Siddiqui, S., Jain, M. B. and Toppo, D. B. 2010. A comparative study of in vitro regeneration of shoots in different cultivars of *Gerbera jamesonii* H. Bollus ex Hook f. *Prop. Orn. Plants* **10**: 156–62.

Modh, F. K., Dhaduk B. K. and Shah R. R. 2002. Factors affecting micropropagation of Gerbera from capitulum explants. *J. Ornamental Hort* **5**: 4-6.

Mohanty, B. K., Kumar, S., Srivastava, R. and Chand, S. 2005. *In vitro* studies on somatic embryogenesis and shoot proliferation in Gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) *cv.* Alsmeera. 8(3): 196-200.

Murashige, T. 1977. Plant propagation through tissue culture. *Annual Review of Plant Physiology* **25**: 135-136.

Murashige, T., Sepra, M. and Jones, J. B. 1974a. Clonal multiplication of gerbera through tissue culture. *Hort. Sci.* **9**: 175-180.

Murashige, T., Serpa, M. and Jones, J. B. 1974b. Clonal propagation of Gerbera through tissue culture. *Nauchnye-Trudy-Moskovskii-Leso Tekhnicheskii-Institut*, **188**: 72-75.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum* **15**: 473-497.

Nadha, H. K., Kumar, R., Sharma, R. K., Anand, M. and Sood, A. 2011. Evaluation of clonal fidelity of in vitro raised plants of *Guadua angustifolia* Kunth using DNA-based markers. *J.Medicinal Plants Res.* **5**(23): 5636-5641.

Naz, S, Naz, F., Tariq, A., Aslam, F., Ali, A. and Athar, M. 2012. Effect of different explants on *in vitro* propagation of gerbera (*Gerbera jamesonii*). *African J. Biotechnol.* **11**:9048-53.

Nga, H. T., Hoa, N. T. P., Giang, N. T., Thach, N. Q. and Ly Anh, N. T. 2005. Establishment of the protocol for *Gerbera jamesonii* propagation *in vitro* culture technique. *Vietnamese J. Agri.* **4**: 76-82.

Nhut, D. T., Thuy An T. T., Dieu Huong, N. T., Don N. T., Hai, N. T., Thien, N. Q. and Vu, N. H. 2007. Effect of genotype, explant size, position and culture medium on shoot generation of *Gerbera jamesonii* by receptacle transverse thin cell layer culture. *Scientia Horticulturae* **111**: 146-151.

Nicolini, G. 1964. Response of seed germination in gerbera. *Ital. Agric.* **101**: 833-841.

Orbovic, V., Calovic, M., Viloria, Z., Nielsen, B., Gmitter, F., Castle, W. and Grosser, J. 2008. Analysis of genetic variability in various tissue culture-derived lemon plant populations using RAPD and flow cytometry. *Euphytica* **161**: 329-335.

Orlikowska, T., Nowak, E., Marasek, A. and Kucharska, D. 1999. Effects of growth regulators and incubation period on *in vitro* regeneration of adventitious shoots from *gerbera* petioles. *Plant Cell Tiss. Org. Cult.***59**: 95- 102.

Orlikowska, T., Sabala, I., Nowak, E., Vainstein, A. and Weiss D. 1995. Adventitious shoot regeneration on explants of Anthurium, Codiaeum, Dieffenbachia, Gerbera, Rosa and Spathiphyllum for breeding purposes. *Acta Hortic.* **420**: 115-117.

Paduchuri, P. Y., Deogirkar, G. V., Kamdi, S. R., Kale, M. C. and Madhavi, D. R. 2010. *In vitro* callus induction and root regeneration studies in *Gerbera jamesonii*. *Int. J. Adv. Biotech. Res.*1: 87-90

Palai, S. K., Pattnaik, S., Patnaik, A. K. and Das, P. 1998. Efficient plant regeneration through callus culture in gerbera (*Gerbera jamesonii*). *Orissa J. Hort.* **26**: 62–67.

Parida, R., Mohanty, S. and Nayak, S. 2011. Evaluation of genetic fidelity of *in vitro* propagated greater galangal (*Alpinia galanga* L.) Using DNA based markers. *Int. j. Plant Animal Environmental Sci.* **1**: 123-133.

Parthasarathy, V. A. and Nagaraju, V. 1995. Morphogenetic response of Gerbera shoots to BAP (benzyl amino purine). *Ann. Plant Physio.* **9**: 10-12.

Parthasarathy, V. A. and Nagaraju, V. 1999. *In vitro* propagation in *Gerbera jamesonii* Bolus. *Indian J. Hort.* **56**: 82-85.

Parthasarathy, V. A., Parthasarathy, U. and Nagaraju, V. 1996. Morphogenetic response of Gerbera shoots to benzyl amino purine. *Ann. Plant Physio.* **10**: 34-39.

Patidar, H., M. Vidhyasankar, M., Tripathi K. and Patel, R. P.2013. Effect of auxins and cytokinins on shoot formation of *Gerbera jamesonii. Plant Cell Biotech. Mol. Biol.***14**(3): 128-138

Pawlowska, H. 1977. Trials on Gerbera propagation *in vitro. Hod-Rosl-Aklimat-Nasiem* **21**: 177-181.

Penningsfeld, F., Forchthammer, L., Fischer, P. and Kalthoff, F. 1976. Cultural comparison of Gerbera clones. *Deutscher Gartenbau* **30**(35): 11-23

Peper, H., Brandis, A. V. and Dopke, H. 1971. Clonal propagation of gerberas can be profitable. Result from Ahlem on the culture and clonal propagation of gerbera. *Taspo 105*: 7.

Petru, E. and Matous, J. 1984. *In vitro* cultures of Gerbera (*Gerbera jamesonii* Bolus). *Sbornik-UVTIZ-Zahradnictvi* **11**: 309-314.

Pierik, R. L. M., Jansen, J. L. M., Masdam, A. and Binnendijk, C. M., 1975. Optimization of gerbera plantlet production from excised capitulum explants. *Scientia Horticulture* **3:** 351-357.

Pierik, R. L. M. and Segers, T. A. 1973. *In vitro* culture of midrib explants of Gerbera: Adventitious shoot formation and callus induction. *Z. Pflanzenphysiology* **69**: 204- 221.

Pierik, R. L. M. and Sprenkels, P. A. 1984 Improvement of the rooting of Gerbera *in vitro* by NAA. *Vakblad-voor-de-Bloemisterij* **39**: 18-19.

Pierik, R. L. M., Steegmans, H. H. M., Verhaegh, J. A. M. and Wouters, A. N. 1979. New developments in the vegetative propagation of Gerberas in test-tubes. *Vakblad-voorde- Bloemisterij* **34**: 36-37.

Pierik, R. L. M., Steegmans, H. H. M., Verhaegh, J. A. M. and Wouters, A. N. 1982. Effect of cytokinin and cultivar on shoot formation of *Gerbera jamesonii in vitro*. *Netherlands J. Agri. Sci.* **30**: 341-346.

Posada, M., Ballesteros, N., Obando, W. and Angarita, A. 1999. Micropropagation of gerbera from floral buds. *Acta Horticult.* **482**: 329-331.

Prajapati, P., Singh, A., Patel, N. L., Singh, D. And Srivastav, V. 2014. Evaluation of genetic diversity in different genotypes of *Gerbera jamesonii* Bolus using random amplified polymorphic DNA (RAPD) markers. *Afr. J. Biotech.* **13**: 1117-1122.

Prasad, M. P. 2014. *In vitro* optimization of growth hormones in the micropropagation of *gerbera* species. *Int. J.Curr. Biotechnol.* **2**(2):1-5.

Prasanth, M. and Sekar, K. 2004. Studies on age of explant on callus induction in gerbera cv. Mammut. *Scientia Horticulturae* **9**: 207-211

Pryor, R. L. 1972. A tetraploid Gerbera. *Hort. Sci.* **7**: 197-198.

Qianzhong, L., Fadi, C., Weiming, F. and Rong, L. 1998 Effect of various media on plantlet training of *Gerbera jamesonii* in tissue culture. *Advances in Horticulture* **2**: 723-726.

Raalte-D-Van., 1978, Gerbera breeding and propagation. *Deuscher-Gartenbau* **32**: 1754- 1755.

Rahman, M., Ahmed, B., Islam, R., Mandal, A. and Hossain, M. 2014. A biotechnological approach for the production of red gerbera (*Gerbera*

Jamesonii Bolus). *Nova Journal of Medical and Biological Sciences.* 2(1): 1-6

Ranjan, S. and Gaurav S. 2005. Somatic embryogenesis in Gerbera (*Gerbera jamesonii* Bolus ex Hooker f.) as influenced by explants. *J. Ornamental Horticulture* **8**: 128-130.

Reuveni, O., Israeli, Y. Golubowicz, S. 1993. Factors influencing the occurrence of somaclonal variations in micropropagated bananas. *Acta Hort.* **336**: 357-364.

Reynoird, J. P., Chriqui, D., Noin, M., Brown and Marie, D. 1993. Plant regeneration from *in vitro* leaf culture of several Gerbera species. *Plant Cell Tiss.Org. Cult.* **33**: 203-210.

Rezende, R. K. S., Paiva, L. V., Paiva, R., Chalfun, A., Torga, P. P. and Castro, E. M. 2008. Organogênese emcapítulos florais e avaliação de características anatômicas da folha de *Gerbera jamesonii* Adlam. Ciênc Agrotéc **32**: 821-827.

Roberts, V. A. and Smith F. E. 1990. The preparation *in vitro* of chrysanthemum for transplantation to soil: 1. Protection of roots by cellulose plugs. *Plant Cell Tiss.Org. Cult.* **21**: 129-132.

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi; In: Gelvin, S.B. and Schilperoort, R.A. (eds.), *Plant Molecular Biology manual*, MA Kluwer Academic Publishers, Boston, pp. 1-8.

Rout, G. R., Mohapatra, A., Jain, S. M. 2006. Tissue culture of ornamental pot plant: a critical review on present scenario and future prospects. *Biotech. Adv.* 24: 531–60.

Ruffoni, B., Damano, C., Silvano, G. and Bregliano, R. 1987. The sterilization and micropropagation of *Gerbera jamesonii* Hybrida. *Annali-dell'Istituto-Sperimentale-perla- Floricoltura* **18**: 21-42.

Ruffoni, B. and Massabo, F. 1991 Tissue culture in *Gerbera jamesonii* Hybrida. *Acta Horticulturae*, **289**: 147-148.

Rusinowski, Z. and Domeradzka, O. 2012. Evaluation of the utility of the random amplified polymorphic DNA method and of the semi-specific PCR to assess the genetic diversification of the *Gerbera jamesonii* Bolus line. *Scient. World J.* 2012: Article ID 450920, 5 pages. doi:10.1100/2012/450920.

Saha, M. C., Mian, R. A., Eujayl, I., Zwonitzer, J. C., Wang, L. and May, G. D. 2004. Tall fescue EST–SSR markers with transferability across several grass species. *Theor. Appl. Genet.* **109**:783-791.

Sahavacharin, O. 1985. Clonal propagation of gerbera (*Gerbera jamesonii* Hort) through tissue culture. *Research Report. Bangkok: Kasetsart University* p. 95.

Saker, M. M., Bekheet, S. A., Taha, H. S., Fahmy, A. S., Moursy, H. A., 2000. Detection of somaclonal variations in tissue cultured-derived date palm plants using isoenzyme analysis and RAPD fingerprints. *Biol. Plant* **43** (3): 347–351.

Salvi, N. D., George, L. and Eapen, S. 2001. Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tiss Organ Cult.* **66**:113–119.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Mannual.* Academic Press, New York, USA, 1322p.

Schiva, T, 1973. Metric analysis of the characters of commercial importance of *Gerbera jamesonii* and consequences dell'inicrocio. *Ann. Fac. Agric. Sci. Uni. Stud. Turin* **8:** 355-366.

Schiva, T. 1975. Vegetative propagation in gerbera improvement. *Annali dell'Instituto Sperimentale per la Floricultura* **6**: 133-135.

Schiva, T, 1979. *EUCARPIA meet. Genet. Carnation and Gerbera.* April 1978, Italy. pp 211-221.

Schiva, T., Lercari, B. and Giusta, R. 1982. Micropropagation of Gerbera: variable response to *in vitro* culture. *Annali-dell'Istituto-Sperimentale-per-la-Floricoltura* **13**: 56-61.

Schiva, T. Ruffoni, B. Vaccarino, R. 1984a. Genetic variability induced by chemical compounds E.M.S. in a *Gerbera jamesonii* var. hybrida H. Bolus. *Annali dell Istituto Sperimentale per la Floricoltura* **15**(1): 29-71.

Schiva, T., Ruffoni, B. and Vaccarino, R. 1984b. Genetic variability induced by chemicals (E.M.S.) [ethyl methane sulphonate] on *Gerbera Jamesonii* hybrid. Annali dell Istituto Sperimentale per la Floricucoltura. **11**: 65-75.

Schleiden, M. J. 1838. Beitrage zur phytogenesis. *Muller-Arch-Anat-Wiss* **11**: 137-176.

Schum, A. and Bichler, I. 1986. Rooting and quality of Gerbera *in vitro* effects of various nutrient ingredients. *Dentscher Gartenbau,* **40**: 140-144.

Schum, A. and Busold, M., 1985. *In vitro* shoot production from inflorescences of Gerbera. *Gartnerborse Gartenwelt* **85:** 1744-1746.

Shabanpour, K., Sharifi, A., Bagheri, A. and Moshtaghi, N. 2011. Effect of genotypes and culture medium on shoot regeneration and proliferation of Gerbera jamesonii. *Afr. J. Biotech.* **10**(57): 12211-12217.

Shabbir, K., Ahmad, T., Hafiz, I. A., Hussain, A., Abbasi, N. A. and Ahmad, J. 2012. *In vitro* regeneration of *Gerbera jamesonii* cv. Sunglow. *Afr. J. Biotech.* **11**: 9975-9984.

Shailaja, V. P. 2002. Studies on *in vitro* propagation of *Gerbera jamesonii* Bolus. *M.Sc. Thesis*, University of Agricultural Sciences, Dharwad.

Sharma, S., Bryan, G., Winfield, M. and Millam, S. 2007. Stability of potato (*Solanum tuberosum* L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. *Planta* **226**:1449-1458.

Shiqing, X., Shihu, Y., Dan, N. And Jianmin, W. 2002. *In vitro* micropropagation of gerbera leaf. *Acta Horticulturae Sinica* **29** (5): 493-494.

Shylaja M. R., Sashna P., Chinjusha V., Nazeem P. A. 2014. An efficient micropropagation protocol for *Gerbera jamesonii* Bolus from flower bud. *Int. J. Plant Animal Environmental Sci.* **4**(3): 641-643.

Sitbon, M. 1981. Production of haploid *Gerbera jamesonii* plants by *in vitro* culture of unfertilized ovules. *Agronomie* **1**: 807-812.

Skoog, F. and Miller, C.O. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* **11**: 118-131.

Soczek, V. and Hempel, M. 1988. The influence of some organic medium compounds on multiplication of Gerbera *in vitro*. *Acta Horticulturae*, **226**: 643-646.

Son, N. V., Mokashi, A. N., Hegde, R. V., Patil, V. S. and Lingaraju, S. 2011. Response of gerbera (*Gerbera jamesonii* Bolus) varieties to micropropagation. *Karnataka J. Agric. Sci.* **24**(3): 354-357

Sousa, C. M., Santos, R. P. and Miranda, R. M. 2006. Optimization of salts concentration of medium MS in the micropropagation of gerbera, var. Ornela. *Agronomia* **40**:52-58.

Sparnaaij L. D., Frida Garretsen, Bekker W., 1975. Additive inheritance of resistance to *Phytophthora cryptogea* Pethybirdge and Laffery in *Gerbera jamesonü* Bolus. *Euphytica* **24**: 551-556.

Sreelatha, U., Nair, R. S. and Rajmohan, K. 1998. Factors affecting somatic organogenesis from leaf explants of Anthurium species. *J. Ornamental Hortic.* **1**: 48-54.

Stupak, N. N. 1985. Pollination studies in gerbera. *Rrferativnyi Zhurnal* **55**: 798-802.

Szule, P. and Rogozinska, J. 1994. Effect of auxins and activated charcoal on rooting of Gerbera *in vitro*. *Zeszyty-Probleowe-Postepow-Nauk-Roliniczych*, **414**: 371-377.

Tanaka, K., Kanno, Y., Kudo, S. and Suzuki, M. 2000. Somatic embryogenesis and plant regeneration in chrysanthemum (*Dendranthema grandiflorum* (Ramat.) Kitamura). *Plant Cell Reports* **19**: 946-953.

Tjia, B. 1984. Gerbera production and its problems. *Intl. Proc. Prop. Soc.* **34:** 365-375.

Tommasini, L., Batley, J., Arnold, G. M., Cooke, R.J., Donini, P., Lee, D., Law, J. R., Lowe, C., Moule, C., Trick, M., and Edwards, K. J. 2003. The development of multiplex simple sequence repeat (SSR) markers to complement distinctness, uniformity and stability testing of rape (*Brassica napus* L.) varieties. *Theor. Appl. Genet.* **106:** 1091-1101.

Topoonyanont, N., Ampawan, R. and Debergh , P. C. 1999. Bushiness in *Gerbera jamesonii:* Abnormal shoot development during micropropagation. *J. Hortic. Sci. Biotech.* **74**: 675-679.

Topoonyanont, N. and Dillen, W. 1988. Capitulum explants as a start for micropropagation of Gerbera: culture technique and applicability. *Mededelingen-van-de-Faculteit-Landbouwwetenschappen-Rijksuniversiteit-Gent* **53**: 169-173.

Tosca, A., Lombardi, M., Marinoni, L., Conti, L. and Frangi, P. 1990. Genotype response of *in vitro* gynogenesis technique in *Gerbera jamesonii. Acta Horticulturae* **280**: 337-339.

Tui, R., Prasenjit, S. and Roy, S. C. 2005. *In vitro* plant regeneration from young capitulum explants of *Gerbera jamesonii. Plant Cell Biotech. Mol. Biol.* **6**: 35-40.

Tyagi, P. and Kothari, S. L. 2004. Rapid in vitro regeneration of *Gerbera jamesonii* (H. Bolus ex Hook. f.) from different explants. *Indian J. Biotechnol.* 3: 584–588.

Varshney, A., Lakshmikumaran, M., Srivastava, P. S. and Dhawan, V. 2001. Establishment of genetic fidelity of *in vitro* raised *Lilium* bulblets through RAPD markers. *In vitro Cell, Dev.Biol. Plant* **37**: 227-231.

Varshney, R., K., Graner, A. and Sorrells, M. E. 2005. Genic microsatellite markers in plants: features and applications.*Trends Biotech.* **23**:48-55.

Villalobos, A. V. M. 1986. Obtaining virus free carnation plants by *in vitro* culture of meristerm and apices. *In Proceedings of The Tropical Region, Am. Society Hortic. Sci.* **23**: 280-283.

Vos, P., Hogers, R., Bleeker, M., Reljans, M., Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **21**: 4407-4414.

Wang, P. J. and Hu, C. Y. 1982. *In vitro* mass tuberization and virus free seed potato production in Taiwan. American *J. Potato Res.* **59**: 33-37

Waugh, R. and Powell, W. 1992. Using RAPD markers for crop improvement. *Trends Biotechnol.* **10**: 186-191.

Weising, K., Nybom, H., Wolff, and Kahl, G. 2005. DNA fingerprinting in plants: principles, methods and applications.CRC press, New York. pp 472.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**: 6531-6535.

Xi, M. and Shi, J. S. 2003. Tissue culture and rapid propagation of *Gerbera jamesonii. J. Wanjing Forest. Uni.* **27**: 33-36.

Xu, S., Yang, S., Ni, D. and Wan, J. 2002. *In vitro* micropropagation of gerbera Leaf. *Acta Horticulturae Sinica.* **29**: 493 494.

Ye, J. N., Wang, T. Z., Cai, S. Q., Mikage, K. K. M. and Namba, T. 1990. Pharmacological studies on folk medicine in Sichuan province, China. II: on Tu-er-fang derived from gerbera plants. *J. Pharmac. Soc. Japan.* **110** (6):374-382.

Ying, B. X., Dong, Z. X. and Xiu, W. J. 2014. Callus Induction and bud differentiation of *Gerbera jamesonii. J. Jilin Agric. Uni.* **36**(1): 66-70

Zakharova, I. K., 1987: Effect of nutrient media on the morphogenesis of gerbera *in vitro. Nauchnye Trudy, Moskovskii Lesotekhnicheskii Institut.* **188**: 72-75.

Zhao, J. C., Zhang, G. Y., Xi, M. L. and Yulan, X. 2002. Tissue culture and rapid propagation of *Gerbera jamesonii*. *J. Nanjing Forest. Uni.* **25**: 40-44.

Zietkiewicz, E., Rafalski, A. and Labuda, D. 1994. Genome fingerprinting by inter simple sequence repeat (ISSR)–anchored polymerase chain reaction amplification. *Genomics* **20**: 176-183.

Appendices

Gel documentation system	:	Biorad, USA

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge	:	Kubota, Japan
Horizontal electrophoresis System	:	Biorad, USA
Thermal cycler	:	Veriti Thermal Cycler (Applied Biosystem, USA)
Gel documentation system	:	Biorad, USA
Nanodrop® ND-1000 spectrophotometer USA	:	Nanodrop®Technologies Inc.

ANNEXURE II

Chemical composition of the Murashige and Skoog medium

Stock	Chemical	mg/litre	Stock concentration	Stock
I	$(NH_4)NO_3$	1,650	50 X	82.5g/l
	KNO_3	1,900		95.0g/l
	KH_2PO_4	170		8.5g/l
	$MgSO_4.7H_2O$	370		18.5g/l
II	$CaCl_2.2H_2O$	440	50 X	22.0g/l
	(Prepare the stock separately or it may precipitate)			
III	Na_2EDTA	37.3	100 X	3.7g/l
	$FeSO_4.7H_2O$	27.8		2.8g/l
(Remember to prepare this as described under stock solution preparation)				
IV	$MnSO_4.7H_2O$	22.3	100 X	2.23g/l
	$ZnSO_4.7H_2O$	8.6		860mg/l
	H_3BO_3	6.2		620mg/l
	Kl	0.83		83.0mg/l
	$Na_2MoO_4.2H_2O$	0.250		25.0mg/l

	$CuSO_4.5H_2O$			
	$CoCl_2.2H_2O$			
V	Vitamins			
	Glycine	2.0	100X	200mg/l
	Nicotinic acid	0.5		50 mg/l
	Pyridoxine acid – HCL	0.5		50 mg/l
	Thiamine – HCL	0.1		10 mg/l
	100 mg/l myo – inositol			
	30 g/l sucrose			
	2 g/ l CleriGel			
	pH 5 – 7 – 5.8			

ANNEXURE III

Reagents required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

CTAB	:	2g
(Cetyl trimethyl ammonium bromide)		
Tris HCl	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 %, 100 ml)

CTAB	:	10 g
NaCl	:	4.09 g

3. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0°C and was used for the study.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM)	:	0.1576 g
EDTA (1 mM)	:	0.0372 g

The solution was prepared, autoclaved and stored at room temperature.

ANNEXURE IV

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

MICROPROPAGATION OF GERBERA (*Gerbera jamesonii* Bolus) AND ASSESSMENT OF GENETIC STABILITY OF PLANTLETS USING ISSR ASSAY

By

AWCHAR DATTA MANIKRAO
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ABSTRACT OF THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
COLLEGE OF HORTICULTURE
VELLANIKKARA, THRISSUR – 680656
KERALA, INDIA

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ABSTRACT

Gerbera jamesonii Bolus commonly known as African daisy is an important cut flower ranking fifth in the global cut flower trade. Gerbera is also used as potted plant due to its attractive flowers of varying colours. It is generally propagated by division of suckers or clumps, but the multiplication rate is found very slow. New varieties are introduced every year in large numbers for commercial cultivation through high-tech system. Development of an efficient micropropagation protocol is of great significance for meeting the large scale demand of quality planting material and to popularise the new varieties. As there is high demand for new variants in gerbera, tissue culture induced variability also could be exploited in breeding programmes.

The investigations on micropropagation of gerbera (*Gerbera jamesonii* Bolus) were hence taken up at Centre for Plant Biotechnology and Molecular Biology, College of Horticulture from 2012 to 2014. The present study aims to micropropagate gerbera using flower buds and leaf explants and to test the genetic stability of the micropropagated plants using ISSR assay. The IIHR variety Arka Krishika and three other varieties procured from AVT, Kochi *viz.,* Dubai, Shania and Hotpsring were utilised for the study. Micropropagation protocols were attempted in different gerbera varieties using two different pathways *viz.,* direct and indirect organogenesis with explants like flower bud and leaves.

The micropropagation protocol developed at CPBMB by Shylaja *et al.* (2014) was used to regenerate plants from flower bud explants. Regeneration from flower bud explants was achieved through direct organogenesis in three gerbera varieties *viz.*, Arka Krishika, Dubai and Shania.

An efficient protocol was standardised from *in vitro* leaf explants through indirect organogenesis and from initial cultures to establishment of micropropagated plants it took only six months.

High genotypic difference was observed in propagule multiplication in different gerbera varieties studied. The white flowered variety Dubai showed high rate of multiplication in both the routes of micropropagation *viz.,* indirect organogenesis using leaf explants and direct organogenesis using flower bud explants.

The potting media for hardening micropropagated plants were standardised and plantlets produced were successfully acclimatized.

Genetic stability studies using ISSR assay were carried out in three groups of plants *viz.,* mother plants, flower bud regenerants and leaf calli regenerants. Of the seven gerbera specific ISSR primers tested, five primers showed monomorphic banding pattern. In variety Dubai, primers ISSR 18 and ISSR 21 exhibited polymorphism to the extent of 25 to 33.33 per cent. In variety Shania, the primers ISSR 15 and ISSR 25 exhibited polymorphisms to the extent of 25 to 33.33 per cent.

Genetic stability studies showed that mother plant and regenerants derived from flower buds were uniform to the extent of 80 per cent. Variation at DNA level observed was more (40%) for plantlets regenerated through indirect pathway.

The established micropropagated plants (200 Nos.) and plants showing variation at DNA level are to be evaluated further for flower production and floral characters before recommending the protocol for commercial micropropagtion. Genotype specific optimisation should be done in micropropagation protocol for effective large scale multiplication of different varieties.