INDUCTION OF VARIABILITY IN ANTHURIUM (*Anthurium andreanum* Lind.) THROUGH *IN VITRO* MUTAGENESIS

By

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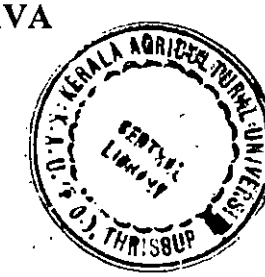
(2012-11-101)



THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis."** 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.

yashwant

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CERTIFICATE

Certified that the thesis entitled **"Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis."** is a record of research work done independently by **Mr. Yashawant Kumar Srivastava** 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.

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Yashwant

Yashawant Kumar Srivastava


Dedicated to my
loving family

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ABBREVIATIONS

%	Percentage
µg	Microgram
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
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
2ip	isopentenyl adenine
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
TILLING	Targeting Induced Local Lesions in Genomes
U	Unit
UV	Ultra violet
V	Volts
β	Beta
µl	Microlitre

Introduction

1. INTRODUCTION

Anthurium (*Anthurium andreanum* Lind.) belonging to family Araceae, is valued for their exotic flowers and foliage. The attractive characteristics of vibrant inflorescence with straight spathe, candle-like spadix, exotic foliage, and particularly the long lasting 'flower' have contributed to its great commercial importance; and therefore occupy a major share in flower industry, especially in terms of cut-flower and potted ornamentals.

Anthuriums rank ninth in the global flower trade and command a respectable price both for its cut flower and whole plant. Presently, Netherlands is the world's leading producer and exporter followed by Mauritius where it is also the national flower (Islam *et al.*, 2013). It is designated as second largest crop in the world among tropical flowers (http://www.tradenetsl.lk/Anthu/anthuriums.html.) and is cultivated throughout the tropics and temperate areas.

The main constraints faced by the anthurium sector presently are high cost of production, the high air-freight charges, lack of proper marketing system, lack of adequate technical support, no cold chain distribution facility etc. Also, the traditional locally grown commercial varieties have low market value. Anthurium is conventionally propagated through traditional techniques which are tedious and not practical when carried out on a large scale.

Plants derived from seeds show marked variation in colour, quality, yield and time of first flowering. Seed viability and germination percentage are also low. Seeds are viable only for two to three days and germination is as low as 20 to 30 per cent. Hence, there is a need to standardize a quicker method of propagation which may be achieved through *in vitro* techniques (Jahan *et al.*, 2009). Tissue culture greatly increases the normal multiplication rate of plants and can provide a source of clean planting material.

The method for *in vitro* production of plantlets of *Anthurium andreanum* was first developed by Pierik *et al.* (1974) and large no. of reports on *in vitro* culture exist since then. The production of *in vitro* plants directly from proliferating axillary buds (Kunisaki, 1980), adventitious buds (Cen *et al.*, 1993), leaf or petiole organogenic callus culture (Finnie and Van Staden, 1986 and Kuehnle and Sugii, 1991) and from somatic embryos derived from in vitro grown leaf blade explants (Kuehnle *et al.*, 1992) has been reported.

There exists great variation in the requirements of different genotypes with respect to spathe colour, spadix orientation, etc. Mutagenic agents have been used to induce useful phenotypic variations in plants for more than 70 years (Foster and Twell, 1996). A large number of mutant lines have been isolated from many plants and these have been used for plant research and crop breeding purposes (Evans, 1962). For anthurium, new techniques are needed for further improving crop cultivars apart from the traditional plant breeding. Mutation breeding is therefore being proposed as a means to create additional variation. The application of ionizing radiation, chemical mutagens as well as somaclonal variation from tissue culture is quite common in the creation of genetic variation.

Physical mutagens like ionizing radiations (X-rays, gamma rays and neutrons) and UV light, and also a series of chemical agents like EMS (Ethyl Methane Sulphonate, Colchicine and Sodium Azide (SA) are being employed for generating mutations in plants.

Ionizing radiation generates chromosomal breaks which, is not repaired, result in a variety of chromosomal aberrations, which include translocations, inversions, deletions and deficiencies. Mutations in the narrow sense affect parts or sections of a gene, either single base pairs or group of them. Exchange of base pairs or alterations of their sequence may change the primary gene product and by way of a more or less complicated chain reaction of events ultimately leading to a modified phenotypic expression of one or several traits (Yamaguchi, 2005).

The recovery of mutants induced by high levels of mutagens is limited by somatic effects, such as reduced viability, growth abnormalities and reduced fertility. Therefore, every mutagen has a most effective dose, which produces the maximum level of mutagenesis with minimal somatic effects.

Identification of desirable variants among the mutants is also very important. Traditionally, such screening has been based on morphological characters, but development of new techniques based on DNA information has made it quicker and precise. The PCR based technique, RAPD (Random Amplified Polymorphic DNA) (Williams *et al.*, 1990). Amplification fragment length polymorphism (AFLP) and SSCP (Single Strand Conformational Polymorphism), SCAR (Sequence Characterized Amplified Regions) and ISSR (Inter-Simple Sequence Repeats) have been used for the genetic characterization of mutant plants (Atak *et al.*, 2004; Pestanana *et al.*, 2011b).

In vitro mutagenesis has been exploited in different crop plants for improving traits; altered flower colour, shape and growth-habit (dwarf or trailing) are few among the successful examples of *in vitro* mutagenesis. According to the FAO/IAEA database, 465 mutants released in vegetatively propagated plants, most were in the floricultural plants. These included chrysanthemum (187), *Alstroemeria* (35), dahlia (34), bougainvillea (9), rose (27), *Achimenes* (8), begonia (25), carnation (18), *Streptocarpus* (30), and azalea (15) (Maluszynski *et al.*, 1992).

Considering its potential, the present investigation on "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" was taken up at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), with an intention to induce variability in anthurium and to characterise phenotypic variations at molecular level.

Review of literature

2. REVIEW OF LITERATURE

The research programme entitled "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2012-2014 and the relevant literature on various aspects of the research is reviewed in this chapter.

2.1 General description

Anthurium is a perennial herbaceous plant usually cultivated for its attractive, long lasting flowers which is not really a flower but an inflorescence rising from the base of a bract. The part which is considered the flower is composed of a colourful, modified leaf (spathe) and hundreds of small, botanical flowers on the pencil like protrusion (spadix) rising from the base of the spathe. Anthuriums grow under conditions of low light and high humidity.

The name anthurium is derived from Greek *anthos,* flower and *oura,* tail, referring to the spadix. Thus, anthurium is also known as 'tail flower' (Tajuddin and Prakash, 1996). The anthurium is native to Columbia, and belongs to the family Araceae which includes more than 100 genera and about 500-600 known species, mainly from the tropics (Bailey, 1963). *Anthurium andreanum* is one of the ten most cultivated ornamental plants for cut flower in the world (Jahan *et al.*, 2009).

Anthurium ranks ninth in the global flower trade and commands a respectable price both for its cut flower and whole plant. Netherlands is the world's leading producer and exporter followed by Mauritius and is the national flower of Mauritius (Islam *et al.*, 2013). It is contributed as second largest crop in the world among tropical flowers (http://www.tradenetsl.lk/Anthu/anthuriums.html.) and is cultivated throughout the tropics and in temperate areas.

In India, the anthurium cut flower industry is still in its infancy. At present anthuriums are mostly grown in some small gardens and nurseries. The important states cultivating anthuriums are Assam, Kerala, Tamilnadu (Salem) and Karnataka (Coorg), where the favourable climate exists (Agasimani *et al.*, 2011).

2.2 Botanical description

A common feature of the Araceae is the typical, cup-shaped inflorescence. It consists of numerous flowers closely arranged in a spadix together with an outer colourful heart shaped sheath called spathe. Flowers are inconspicuous, hermaphroditic with two carpelled ovary and four anthers. The sepals and petals are rudimentary; stigma appears as a round protuberance on the spadix when it is mature. Pollen matures at seven to ten days after stigma becomes receptive thus to prevent self pollination. The large variations in inflorescences are due to the difference in shape, colour and the size of the spathe and spadix (Croat, 1988).

2.3 Genetics

The basic chromosome number of anthurium is reported as n= 15, 16 and 22. Cytological analysis of anthurium revealed that *Anthurium* species have 2n = 30 chromosomes. Some are polyploid with 2n = 60, while a few species have 2n = 20 to 124 chromosomes (Petersen, 1989).

2.4 Propagation

Anthurium is propagated by seed, traditional vegetative means (offshoots and nodal cuttings) and through tissue culture. Most of the commercially grown anthuriums are hybrids with high genetic variability, thus cultivators multiply them through vegetative means (Kuehnle and Sugii, 1991). The seed propagation is not proper because of cross-pollination, poor germination rate, variation in colour, quality and yield of flowers, and low viability (2-3 days). Since the progenies are heterozygous, propagation by seed leads to genetic segregation (Martin *et al.*, 2003, Jahan *et al.*, 2009). In addition, seeds cannot be conserved,

and must be collected immediately after fruit maturation (Viegas *et al.*, 2007). Vegetative propagation of newly developed plants is thus laborious and time consuming (Kuehnle and Sugii, 1991; Reddy *et al.*, 2011).

2.5 *In vitro* propagation

Many researchers have applied *in vitro* culture methods for multiplication of anthurium using various media and explants. Micropropagation is being suggested as an alternative to increase the production of anthurium these days (Jahan *et al.*, 2009). Under the ideal environment, anthurium produces long lasting flowers all year round (Mairo *et al.*, 2010).

The *in vitro* culture/tissue culture greatly increases the normal multiplication rate of plants and can provide a source of clean material which has become increasingly important due to outbreak of bacterial and other diseases such as anthracnose, blight, leaf spot, root knot and bacterial wilt.

The method for *in vitro* production of plantlets of *Anthurium andreanum* was first developed by Pierik *et al.* (1974). The production of *in vitro* plants have been reported directly from proliferating axillary buds (Kunisaki, 1980), adventitious buds (Cen *et al.*, 1993), leaf or petiole organogenic callus culture (Pierik *et al.*, 1974; Pierik, 1975, 1976; Finnie and Van Staden, 1986) and from somatic embryos derived from *in vitro* grown leaf blade explants (Kuehnle *et al.*, 1992). Geier (1982) working on *Anthurium scherzeranium* was also able to develop plantlets from spadix explant and later from leaves.

Micropropagation of anthurium has been achieved with various tissues including lamina, petiole, seed, shoot tips, lateral bud, spadix and spathe (Martin *et al.*, 2003, Atak and Celik, 2009). Plant regeneration of *Anthurium andreanum* has been achieved through adventitious shoot formation from callus (Vargas *et al.*, 2004) and root explants (Chen *et al.*, 1997).

Raad *et al.* (2012) described a detailed protocol for *in vitro* regeneration of *Anthurium andreanum* cv. Antadra and Casino from micro-cuttings (lamina and petiole) and callus tissue through organogenesis in order to reduce the time required for the various production stages.

Geier (1986) showed that plant genotype and plant age influence plant regeneration of *Anthurium andreanum.* Multiplying anthuriums by callus induction implies a long explants-plant cycle (12 months). Various physical and biological factors play important roles during *in vitro* propagation of *Anthurium* (Silva *et al.*, 2005). Nowadays, most pot type anthuriums available in the market are produced by tissue culture methods (Mairo *et al.*, 2010).

In vitro regeneration in different varieties of anthurium has been reported earlier from Kerala Agricultural University. Sreelatha, 1992 has reported modified MS medium with reduced salt concentration as ideal for callus growth and regeneration. She has reported callus induction in medium supplemented with 2,4-D (0.5 mg/l) and BA (1.0 mg/l). Shoot regeneration and growth of the shoots were the best in MS medium with BA (0.5 mg/l) and IAA (2.0 mg/l). Anu, 1997 attempted micropropagation in anthurium and the callus production was achieved in explant from spadix in ½ MS medium supplemented with 2,4-D (2.0 mg/l) and kinetin (0.3 mg/l). Addition of casein hydrosylate in the medium is reported to improve callusing in leaf explants. Mini, 1998 has reported maximum shoot regeneration in Nitch medium supplemented with BAP 0.5 mg/l in the case of leaf callus of anthurium, Leena, 2003 reported that ½ MS medium supplemented with BA (6.0 mg/l) and NAA (3.0 mg/l) was effective for callus initiation. While BA 0.5 mg/l and IAA 1 mg/l proved good for regeneration and root induction.

2.5.1 Callus induction

Pierik *et al.* (1974) highlighted that good result could be obtained if MS/2 supplemented with 0.1 or 0.08 mg/l 2, 4-D was used. Pierik (1976) also attempted to optimize the shoot induction medium by adding 1.0 mg/l BAP.

Kuehnle and Sugii (1991) used 0.2 to 1.0 mg/l of cytokinin in a full strength MS medium for callus induction in anthurium. The importance of applying cytoknin for callus induction in anthurium was demonstrated by the better result obtained by Pierik *et al.* (1979) who added 10 mg/l of 2ip to the medium.

Prakash *et al.* (2001) obtained callus from *Anthurium andreanum* petioles after culture on MS medium containing 0.5 or 1.0 mg/l 2,4-D. Jahan *et al.* (2009) showed high frequency of callus on medium containing BAP and 2,4-D. Totally, most studies related to callus formation in anthurium have been performed on leaf explants using 1 mg/l BA or BAP and 0.1 mg/l 2,4-D (Zhao *et al.*, 2004; Puchooa, 2005).

Bejoy *et al.* (2008) showed that relatively older explants of leaf in *Anthurium andreanum* Hort. cv. Agnihothri, exhibited better responses to callus induction. However, studies of Reddy *et al.* (2011) on micropropagation of *Anthurium digitatum* revealed that the young leaf showed excellent callusing capacity.

2.5.2 Organogenesis

Montes *et al.* (1999), Trujillo *et al.* (2000), Bejoy *et al.* (2008) and Jahan *et al.* (2009) obtained the highest proliferation of anthurium on medium enriched with BAP. The combination of BA and NAA improved the shoot proliferation in *Anthurium andreanum*. The combination of 1 mg/l BA + 0.01 mg/l NAA with 22.83 shoots per explant was found to be the most suitable growth regulator regime. Some other researchers obtained the highest proliferation of anthurium in the presence of BA alone or in combination with other cytokinins or auxins (Yang *et al.*, 2000; Lee-Espinosa *et al.*, 2003; Martin *et al.*, 2003; Lara *et al.*, 2004; Duong *et al.*, 2007).

In some reports, 2,4-D in low concentrations has been used in combination with cytokinins to induce multiple shoots in anthurium (Orlikowska *et al.*, 1995; Atak and Celik, 2009).

Several researchers were reported the induction of callus on leaf explants of anthurium especially lamina (Nhut *et al.*, 2006; Bejoy *et al.*, 2008; Jahan *et al.*, 2009). Te-chato *et al.* (2006) used leaf, node and internodes of three genotypes of *Anthurium* and found internodes gave the highest callus formation.

Lamina explants exhibited more potential for callus formation when they contained midrib (visual observation), which was agreement with results reported by Kumar *et al.* (1992) and Bejoy *et al.* (2008).

2.5.3 *In vitro* rooting

As in any other crop auxin type and concentration significantly influenced rooting percentage and root length in anthurium. Rooting is usually induced by auxins and IBA is reported to be more effective for rooting compared with other auxins (Malhotra *et al.*, 1998; Puchooa and Sookun, 2003; Jahan *et al.*, 2009).

Puchooa (1996) reviewed root formation of three varieties of *Anthurium*, in regenerated shoots. They rooted readily on medium containing IBA (1.0 mg/l). Atta (1998) found that *in vitro* multiplied shoots when subsequently placed on different rooting medium containing 0.25 mg/l IBA which produced 3.6 roots per plantlet with 94 per cent success.

Martin *et al.* (2003) cultured shoots of *Anthurium andreanum* in medium supplemented with 0.54 µ M NAA and 0.93 µ M KIN for *in vitro* rooting. *In vitro* rooting percentages were 100 per cent. Also, Bejoy *et al.* (2008) achieved 98 per cent root from shoots in medium supplemented with 0.5 mg/l NAA. Generally, most studies related to rooting of anthurium shoots regenerated from callus have been performed using IBA and NAA (Zhang *et al.*, 2001; Joseph *et al.*, 2003).

Bautista *et al.* (2005) studied the effects of various concentrations of IAA (2.89 and 5.71 µM) and sucrose (30 and 40 g/l) on the micropropagation of *A. andraeanum* cultivars Sonate and Lambada. The length of buds, number of roots and length of roots were evaluated. At the rooting phase, 40 g sucrose/litre was optimum in the enhancement of plant height, number of roots, and length of roots in both cultivars

Islam *et al.* (2010) found MS media containing 1 mg/l IBA + 1 mg/l BAP showed the best performance in rooting of shoots (83.85%), highest number of roots (4.29/plantlet), root elongation (5.50 cm) were recorded at 60 DAI.

MuLan *et al.* (2012) found best medium for rooting as Nitsch basal medium with 0.5 mg/l NAA and 0.3 mg/l IBA. Raad *et al.* (2012) reported that shoots were rooted on the rooting induction media containing MS basal medium supplemented with IBA (0.5, 1.0 and 2.0 mg/l), NAA (0.05, 0.1 and 0.25 mg/l) and KIN (0.0 and 0.2 mg/l).

2.6 *In vitro* mutagenesis

The genetic improvement of ornamental plants for improving or developing new varieties requires genetic variation. However, the desirable genetic variation is most often lacking and that hampers the breeding of ornamental plants. This is due to existing germplasm fails to provide the desired recombinants and it is necessary to resort to other resources of variation. Since spontaneous mutations occur with extremely low frequency, mutation induction techniques provide tools for the rapid creation and increase in variability in crop species.

The genetic variability can be induced by mutagenic agents, such as **physical**: gamma rays, beta rays, neutrons, electron beams, ion beams, sodium azide and **chemical:** ethyl methane sulfonate and colchicines (Ahloowalia, 2001; Jain, 2007) and from which desired mutants could be selected (Datta, 1997; Jain and Spencer, 2006).

The mutagen treatment breaks the nuclear DNA and during the process of DNA repair mechanism, new mutations are induced randomly and are heritable. The changes can occur also in cytoplasmic organelles. The chromosomal or genomic mutations enable plant breeders to select useful mutants such as flower colour, flower shape, disease resistance, early flowering types etc. (Jain and Maluszynski, 2004).

A specific advantage of mutation induction is the possibility of obtaining unselected genetic variation, improvement of vegetatively propagated plants when one or few characters of an outstanding cultivar are to be modified.

2.6.1 Methods of *in vitro* mutagenesis

Mutations can be induced by treating plant material with chemical or physical mutagens or biological mutagenesis. Ionizing radiation and chemical mutagens damage the DNA in every cell. These damages include single and double stranded breaks, DNA protein cross links, Sl endonuclease sensitive sites indicative of clustered base damage and single-base damage such as 8-hydroxypurine, formamidopyrimidine and thymine or cytosine glycol. They interrupt the continuity of genetic information by inhibiting or preventing transcription, DNA replication and cell division (Yamaguchi, 2005).

The most common chemical mutagen used is ethyl methane sulphonate (EMS), which induce chemical modification of nucleotides, which result in mispairing and base changes. Strong biased alkylation of guanine (G) residue results, forming O6-ethyl guanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A/T. Ninety-nine per cent of mutations from alkylation of guanine induced by EMS are reported as G/C-to-A/T transitions (Greene *et al.*, 2003).

Among radiation sources, gamma radiation is most successful in inducing mutations in both seed and vegetatively propagated crops. There are two ways to irradiate with gamma radiation: a) acute radiation – high radiation dose is given

for a short period of time, seconds, minutes and hours. Gamma radiator is the main source for acute radiation treatment given to seeds, cell cultures, callus, and shoot buds. It is the most commonly used system for irradiation in seed and vegetatively propagated crops. b) Chronic radiation – low radiation dose treatment is given for a long period, e.g. days, weeks, months and years. There are different ways to give this treatment to *in vitro* shoot cultures or plantlets, seedlings, trees, potted plants. Gamma greenhouse is quite effective for irradiating seedlings, *in vitro* cultures and potted plants (Jain, 2007).

2.6.2 Mutation induction in cut and pot flowers

Commercially, cut flowers are important and have a worldwide market. The most important cut flowers are rose, chrysanthemum, gerbera, gladiolus, orchids, anthurium and carnation. In Japan, total production of cut flowers has risen to more than 40 per cent, which is lower than 70 per cent in Holland. The consumption of cut flowers and pot plants in Japan is mainly 40 per cent as gift-use, 25 per cent for commercial facilities, such as hotels, 25 per cent in home use and 10 per cent for educational purpose. As a result, there is a surge of demand for high grade flowers (Jain, 2007).

In vitro mass propagation of cut flowers has been successful and commercial companies largely multiply elite genetic material by micropropagation. In order to meet the demand of consumers, floriculture industry is always looking for new genetic material of ornamental plants to breed new cultivars, especially for flower colour, flower shape, long shelf life, etc., which require genetic variation for the selection of plant material with desirable commercial traits.

The rate of spontaneous mutation is very low which makes breeding new cultivars harder, and therefore, mutations are induced by physical and chemical mutagens to create more genetic variability in combination with *in vitro* culture techniques (Jain and Spencer, 2006).

It is logical to apply *in vitro* culture techniques, since most of the major ornamental plants can be micropropagated (Rout and Jain, 2004, 2005; Jain and Spencer, 2006). There has been a considerable work done on induced mutations in potted and cut flowers by using chemical and physical mutagens.

2.6.2.1 *In vitro* mutagenesis in cut flowers through gamma radiation

Commercially cut flowers have high value due to high economic returns, which are readily micropropagated (Rout and Jain, 2005) for large scale production. The major cut flowers are roses, orchids, chrysanthemum, gerbera, gladiolus and carnation, their production are outsourced in the developing countries due to low labour cost. The international trade in cut flowers is growing about 11 per cent annually and world market of cut flower products is worth about USD 8 billion.

Benetka (1985) irradiated single bud cuttings with 0, 20, 30, 40 and 60 Gy γ-rays and subsequently observed four bud propagated generations and the optimal doses were 40 and 50 Gy and chimerism decreased with successive generations. However, chimerism remains the main constraint in mutation breeding of vegetatively propagated crops.

Gerbera is one of the most important cut flowers in European countries. In view of its commercial importance, it is essential to improve continuously certain characters such as flower colour (with a wide range of variation), morphology, longevity and size of flowers, and disease resistance against *Phytophthora cryptogea* (a serious pathogen in greenhouse cultivation) (Jain *et al.*, 1998a). Additionally, gerbera is a highly heterozygous plant and mutagenesis should provide a good array of useful mutants for commercial growers.

Jain *et al.* (1998a) reported that gamma irradiation (10 and 20 Gy doses) of two gerbera cultivars produced mutants showing flower colour, flower morphology and plant morphology traits with an average of 8.6 per cent. The use of haploid tissues, unfertilized ovaries, haploid shoots and anthers would be

another approach to induced mutations in order to prevent chimerism and produce dihaploid mutants.

Laneri *et al.* (1990) obtained several flower mutants in gerbera by gamma irradiation and suggested that shoots should undergo 3-4 cycles of micropropagation after mutagenic treatment in order to minimise chimerism. During 1993, a Japanese group headed by S. Nagatomi, (1995), developed six flower colour mutants of chrysanthemum by chronic irradiation of plants.

Single cell mutagenesis induced by irradiation of petiole explants from adventitious shoots of cut flower chrysanthemums (*Chrysanthemum morifolium*) with 8-15 Gy γ radiation from a ^{60}Co source. Variation was approx. five per cent with regard to flower type, flower colour and flowering date. Different cultivars exhibited different degrees of variability, with the most extreme cases being flower colour in 'white' cultivars and flower type with 'quilled-flower' cultivars. Eleven new cultivars were derived from these mutants (Wei *et al.*, 1996).

Rooted cuttings of *Chrysanthemum morifolium* cv. Maghi, a small flowered, late blooming cultivar, were treated with different doses of gamma rays such as 1.5, 2.0 and 2.5 krad (^{60}Co radiation source). Somatic mutations in flower colour (light mauve, white, light yellow and dark yellow) and chlorophyll variegation in leaves were detected as chimeras in treated populations. Plants with chlorophyll variegation in leaves and two new flower colours (light mauve and white) were isolated in pure form with 64 per cent and 100 per cent efficiency of mutant recovery (Mandal *et al.*, 2000).

Arunyanart and Soontronyatara (2002) induced mutations in tissue cultured lotus (*Nelumbo nucifera*) by treating plantlets with either acute γ-rays at doses of 0, 2, 3, 4, 5 and 6 krad or X-rays at doses of 0, 1, 2, 3, 4 or 5 krad. The 2-krad dose of either γ- or X-ray treatments resulted in a 50 per cent survival rate. The use of γ- and X-rays to induce mutation in lotus resulted in 21 altered characteristics. Mutants from 1- and 2-krad of either γ- or X-rays had long

secondary roots and numerous adventitious roots. These mutants also exhibited good shoot growth and healthy rhizome development.

Misra *et al.* (2003) developed two chrysanthemum mutants by γ-irradiation (0.5 Gy). Both mutants were yellow but one had flat spoon shaped ray florets similar to the original cultivar, while the other had tubular florets.

Lamseejan *et al.* (2003) have used chrysanthemum var. 'Taihei' for mutation induction with chronic and acute gamma irradiation treatment and obtained mutants with different traits such as flower colour, form and size. The mutation frequency for flower colour was higher than for other traits.

Gamma rays have been widely used for mutation induction in cut flowers and recently, heavy-ion beam treatment has generated interesting results in chrysanthemum (Nagatomi *et al.*, 2003). Several mutants have been isolated in chrysanthemum for flower colour and flower shape.

Pathania and Mishra, (2003) have reported irradiation of *in vitro* multiplied shoot clumps of gladiolus 'Eurovision' and 'Wine & Roses' with different doses of ^{60}Co gamma rays. Survival of irradiated shoots decreased with increased dose of ^{60}Co gamma irradiation. A dose of 50 Gy had the lowest survival rate but was sub-lethal. *In vitro* selection by challenging with fusaric acid (1-1.5 mM) and culture filtrate (20 %) of *F. oxysporum* f. sp. *gladioli* resulted *Fusarium* yellows insensitive mutants at the end of the 3rd selection cycle.

Another important popular cut flower is rose, which is widely grown worldwide and is one of most important commercial ornamental flowers. Rose propagation is done by grafting or budding and the breeding programs mainly focus on the improvement of various characteristics to enhance the ornamental value, including flower colour, size, keeping quality of the bloom and response to various environmental stresses (Rout and Jain, 2004).

Datta *et al.* (2005) established the adventitious bud technique to avoid unstable chimeras by forcing regeneration of buds from single cells in chrysanthemum. They obtained five solid flower colour/floret shape mutants with slight changes in ray floret morphology. *In vitro* mutagenesis through direct regeneration helped in the development of solid mutants without diplontic selection in a relatively short period of time.

More than 30 rose mutant varieties have been released and commercialised (Datta and Chakrabarty, 2005), mainly for changed flower colour, higher oil content and better quality oil. Recently, under the INS/5/031 TC project, Indonesians released three rose mutant varieties- Rosmarun, Yulikara and Rosanda with specific traits of flower colour and flower shape induced by gamma radiation treatment (Jain and Spencer, 2006).

Hossain *et al.* (2006) developed a stable NaCl-tolerant mutant (R1) of *Chrysanthemum morifolium* Ramat by *in vitro* mutagenesis with gamma radiation (5 Gy). Salt tolerance was evaluated by the capacity of the plant to maintain both flower quality and yield under NaCl stress.

A study on the effects of gamma irradiation on mutagenesis of *Curcuma alismatifolia* was conducted by Abdullah *et al.* (2009) to determine the optimal dose for radio sensitivity test (LD_{50}) of the plants and determine the effects of induced mutation on the species. Ten levels (dose rate) of gamma irradiation were used. Results showed that the highest survival rate was 67 per cent obtained from the non-irradiated rhizomes (0 Gy), 50 per cent when were treated with 10 Gy and 63 per cent survival rate when treated at 20 Gy. Mean survival rate fell sharply from 63 per cent at 20 Gy and 7 per cent at 30 Gy. This decreasing trend was followed by 2 per cent survival at 40 Gy. Results indicated that the radio sensitivity test (LD_{50}) for *C. alismatifolia* was approximately at 25 Gy.

Zalewska *et al.* (2011) have reported *in vitro* mutagenesis in three cultivars of *Chrysanthemum* Albugo, Alchimist, Satinbleu. Gamma radiation of 15 Gy obtained from ^{60}Co source. 'Albugo' and 'Satinbleu' micro cuttings cultured on

MS medium were exposed to irradiation. From the mutants obtained in vM2 generation five genotypes were selected: Albugo Sunny, Alchimist Tubular, Alchimist Golden Beet, Satinbleu Minty and Satinbleu Honey.

Singh *et al.* (2011) have induced variations for floral traits in *Gerbera jamesonii*. Seeds obtained from controlled crossing among white gerbera genotypes were irradiated with different doses of gamma rays (1 to 5 kR). Induced floral fasciations were observed in M1 seed raised plants of gerbera. Ring-fasciation and linear-fasciation were observed in mutant types which lead to deformed and asymmetric flower heads which are mostly male and female sterile.

Barakat and Sammak, (2011) have induced mutation in *Gypsophila paniculata* through *in vitro* mutagenesis by treating the shoot tips and lateral buds with four doses of gamma irradiation (0.25, 0.5, 0.75 and 1 Gy) The lateral bud explants gave significantly higher number of shoots (19.28) compared to shoot tip explants (14.68). In conclusion, gamma rays irradiation can induct mutations which can be carefully acclimatized and commercially propagated under suitable condition.

Kahrizi *et al.* (2012) determined the effect of gamma rays on nuclear DNA content as an index of radiation damage in different genotypes of Rosa hybrida was investigated and the morphological characteristics of the resulting mutants were compared with their own progenitors. The *in vitro* nodal sections of five rose cultivars ("Apollo", "Maroussia", "Dolce vita", "Black baccara" and "Beauty by ogre") were irradiated by 0, 30, 60 and 90 Gy gamma rays. Nuclear DNA content of the putative mutants and their original plants was estimated by flow cytometry. The results revealed that nuclear DNA content was influenced by different doses of gamma rays.

Gamma radiation treatment in anthurium has been reported by Puchooa, 2005. Different explants (leaves, seeds, *in vitro* plantlets) were irradiated, best response was observed with the 5 Grays (Gy) treatment in terms of callus formation and regeneration while the 15 Gy dose was lethal to the anthurium

tissues. The phenotypic results indicated a boosting effect of the 5 Gy dose on the leaf tissues. The variability in the responses observed seemed to indicate some mutation, both positive and negative, at the cellular level of the tissues.

2.6.2.2 *In vitro* mutagenesis in pot flowers through gamma radiation

Another group of ornamental plants, pot flowers, are commercially produced in developed and developing countries for higher economic returns. They are improved for quality attributes such as leaf types, flower colour, longevity and form, plant shape and architecture (Jain and Spencer, 2006).

The flower colour spectrum was compared in carnation mutants induced by gamma rays and carbon ion beam. Okamura *et al.* (2001) found that the flower colour spectrum induced by carbon ion beam was much broader as compared to that induced by gamma irradiation in carnation. Similar results were obtained in chrysanthemum (Nagatomi *et al.*, 1995).

Gamma radiation treatment of rhizomes of curcuma varieties 'Chiang Mai Pink', 'Doi Tung Purple Bright' and 'Bua Khao' with chronic and acute gamma rays resulted in several mutants with variation in bract colour. Similarly, Sharma *et al.* (2002) isolated bract colour mutant 'Palekar' Bougainvillea plants by gamma radiation, which is the first establish mutant for this trait.

The major pot flowers are anthurium, begonia, cyclamen, *Dianthus* spp., *Ficus* spp., pelargonium, and poinsettia. Just like in cut flowers, good quality planting material is the basic need of growers for increasing production and profit. Many commercial companies worldwide use *in vitro* culture systems for rapid plant multiplication of elite germplasm including mutant lines. The great potential of micropropagation for large scale plant multiplication might be exploited even more provided the cost of plant production is reduced (Jain *et al.*, 2006).

A range of flower colour and shape mutants of *Canna hybrida* and *Canna generalis* have been isolated and 22 mutant varieties have been released in Thailand (visit http://gisc.rdi.ku.ac.th).

Although desirable traits were introduced by conventional breeding, rose improvement was hampered due to a limited gene pool. Mutation breeding has been most effective in developing a large number of new rose varieties, which are being grown commercially. The mutations were mostly in flower colour and shape as a result of both chemical and physical mutagens.

Up to now, induced mutations have helped to induce flower colour variants of commercialized cultivars, similar to those obtained by spontaneous mutation (Datta *et al.*, 2005).

In orchids, two mutant varieties were released at MINT, Malaysia (Jain, 2005). More than 13 carnation mutant varieties have been released with changed flower colour by chemical mutagen treatment and gamma irradiation (Datta and Chakrabarty, 2005).

The combination of biotechnology, induced mutations and molecular tools can develop and multiply elite mutants in a short period of time in most of the ornamental plants (Datta and Chakrabarty, 2005; Jain and Spencer, 2006).

2.6.3 Chemical mutagenesis

Chemical mutagens have also been used for mutation induction in ornamental plants. Singh *et al.* (2000) used auxiliary bud explants of carnation for mutagenic treatment with ethyl methane sulphonate (EMS) under in *vitro* conditions. EMS at the dose rates of 0.025 and 0.050 per cent in culture medium and 0.25 per cent used for explant agitation, showed stimulatory effect on sprouting, number of shoots, number of flowers and flower initiation, while higher doses of 0.075 and 0.100 per cent caused damage to all vegetative and floral

characters. Two colour mutants, viz. red with white stripes and pink with white stripes were isolated from the mutagen treated population.

A 0.50 per cent EMS solution in 3 per cent DMSO was applied for 90 min to shoot internodes 0.5 cm long of *in vitro* propagated weigela plants. After three rinse in sterile water the explant were laid on MS salt culture medium supplemented with kin 2 mg/l and IBA 0.3mg/l for two months to promote adventitious shoot formation and among 388 mutants six mutants behaved like solid mutants (Duron, 1992).

Latado *et al.* (2004) induced mutations in immature floral pedicels of chrysanthemum by ethyl methane sulphonate (EMS) (0.77%) treatment for 105 min and developed adventitious buds through in vitro. Forty eight mutants were identified from 910 plants which deviated in petal colour. Most of their phenotypes were uniform.

Tejaswini *et al.* (2006) treated nodes of carnation (*Dianthus caryophyllus*) from *in vitro* shootlets with EMS at the concentrations of 0.1, 0.5 and 1 per cent. At each concentration, explants were incubated for 15, 30 and 60 minutes. Survival percentage and percentage of explants showing growth was reduced with the increased concentration of EMS in media.

In vitro mutagenesis study was conducted by Senapati and Rout (2008) in some commercially important rose cultivars. *In vitro* grown meristems of *Rosa hybrida* cvs. First Red, Cri and Pusa Gaurav were used with various concentrations of ethyl methane sulfonate (EMS) to develop mutants. Depending on the concentration used, fifty per cent survivality was obtained in micro shoots pre-treated with 1 per cent EMS in 6 hours. The treated microshoots were used further for shoot multiplication. About 70 to 75 per cent of rooted plantlets survived in the greenhouse.

Gang *et al.* (2009) treated corm bud of *Gladiolus hybridus* Hort with EMS of different consistency. The result showed that EMS has a very effective

mutagenic agent for the corm bud of *G. hybridus* Hort. with the increase of consistency, the mutagenic range increased first, then decreased, among which 0.6 per cent EMS treatment had the biggest influence.

Shufang *et al.* (2010) treated corm bud of *G. hybridus* Hort with different concentrations of EMS. Results showed that EMS has a very effective mutagenic agent for the corm bud of *G. hybridus* Hort. with the increase of EMS concentration, the mutagenic rate was increased, among which 1.0 per cent EMS treatment had the biggest influence. However, with the same EMS concentration, there is no close relationship between the amount of mutagenic agent and the divergence of plants genomes, which offered a molecular basis for selecting plants with good mutation.

Mekala *et al.* (2010) have induced mutation in *Jasminum sambac* cv. Gundumalli through gamma rays alone and in combination with Ethyl Methane Sulphonate (EMS) at different doses including control. Higher the dose of mutagens, there was reduction in the expressivity of the traits. The mean stalk length decreased with the increase in the irradiated population and it was minimum with 2.5 kR+30 mM EMS (0.89 ± 0.021 cm). The mutagenic treatment 2.0 kR followed by 1.5 kR+30 mM EMS manifested higher variability in terms of PCV and GCV recording the maximum flower range of (3-35) and (5-27).

Fang (2011) have exposed *in vitro* leaf sections of Saintpaulia cv. Crystobal to various EMS treatments at (0.2 to 0.6 % for 30 to 240 min), after which adventitious shoots were recovered from the treated explants. Shoots producing at least six leaves were induced to root and the resulting plantlets were transplanted to soil. A total of 1838 plantlets were grown to flowering stage and 10 mutants were identified. Four of the mutants were variegated leaf chimeras and the remaining six presented variations at the level of flower colour.

Roychowdhury and Tah, 2011 treated *Dianthus* seeds with three different concentrations (0.1, 0.4 and 0.7%) of colchicine (Col), ethyl methane sulphonate

(EMS) and sodium azide (SA), to assess seed germination behaviour, lethality, pollen sterility and mutagenic effectiveness. Higher lethality over control (32.89) was shown by 0.7 per cent EMS. Pollen sterility also increased with increasing mutagenic doses. The maximum pollen sterility (61.1%) was observed under 0.7 per cent colchicine. The highest mutagenic frequency (13.953) was observed at 0.4 per cent Col and lowest one (4.464) at 0.1 per cent Col. The mutagenic effectiveness was maximum (86.42 per cent) at 0.1 per cent EMS and minimum (13.824 per cent) in 0.7 per cent Col. The highest mutagenic efficiency (6.977) was recorded in 0.4 per cent Col and lowest (0.995) in 0.7 per cent SA on the basis of survivability. The effectiveness of the three chemicals on *Dianthus* is ranked as EMS>Col>SA.

The phenotypic response of *Delphinium malabaricum* to chemical mutagens (EMS and SA) and physical mutagen (gamma rays) were studied by (Kolar *et al.*, 2011). Eleven different types of chlorophyll mutants namely albina, albina-green, xantha, aurea, chlorina, viridis, yellow viridis, tigrina, striata, maculata and variegated type were identified in the treated populations. The highest frequency of chlorophyll mutations (9.74%) was reported in the 0.25 per cent EMS.

Ghani *et al.* (2013) induced mutation in gerbera (*Gerbera jamesonii*) cv. 'Harley' through physical and chemical mutagenesis. *In vitro* raised shoot cultures of gerbera, established from petiole explants, were exposed to different doses of γ-rays (1.5 to 30.0 Gy) using a ^{60}Co source. To induce mutations through chemical mutagenesis, different concentrations of ethyl methane sulphonate [EMS; 0.1 to 1.0% (v/v)] were administered for 10 min or for 20 min. Morphological variants showing changes in leaf shape, leaf size, scape length, flower diameter, and flower colour were obtained.

Bhajantri and Patil (2013b) reported variability induced by ethyl methane sulphonate (EMS) for fifteen quantitative traits in M_1V_1 generation of gladiolus varieties Ethyl Cav Cole and white prosperity during Kharif 2011-12. Corm

weight and corm diameter recorded high phenotypic and genotypic coefficient of variation at 0.50 per cent EMS treated population in both genotypes. The highest variability for days to spike initiation was at 0.50 per cent EMS. High heritability estimates was found for most of the characteristics *viz.*, number of leaves per plant, leaf width, days to full spike emergence days to first floret to show colour, spike length, florets per spike and floret diameter in all treated mutant populations.

Corms of two gladiolus (*Gladiolus hybrida* L.) cultivars (Cavcole and White Prosperity) were exposed to gamma rays (10, 20 and 30 Gy) and Ethyl methane sulphonate (EMS; 0.25, 0.50 and 0.75%). Most of the morphological characters were improved at higher doses of both the mutagens, but at higher concentrations they were injuries to most of the seedling characters. Treatments of 10 Gy and 0.25 per cent EMS were found to be suitable for induction of mutants (Bhajantri and Patil, 2013a).

In vitro mutagenesis with Ethyl methane sulphonate (EMS) has been done in anthurium by Te-chato and Susanon (2005). Meristematic nodular calli of anthurium cv. Valentino were treated with ethyl methane sulfonate (EMS) at various concentrations for 90 min in order to find the optimum concentration (LD50). Plantlets regenerated from treated calli were grown in the field until flowering. The results revealed that EMS at concentration 0.5 and 0.75 per cent gave survival percentages of the calli of 60 and 34 per cent, respectively (LD50 = 0.62%). Treatment with EMS at 0.75 per cent gave smaller size of spathe compare with the control having 14.2 per cent yellow spadix. EMS at 1 per cent gave 60 per cent yellow spadix, whereas the control treatment gave pink spadix. Moreover, spadices from those treated with EMS were shorter with more erect angle (45-90°) than the control treatment (25°). Development of female flower was 0-50 per cent, which untreated plants produced 100 per cent female flower.

2.6.3.1 Induction of mutation in other crops

Dwimahyani and Ishak (2004) have induced mutations in *Jatropha curcas* for improvement of agronomic characters with an irradiation dose of 10 Gy and identified mutant plants with early maturity and better branch growth.

In several investigations report, low doses of gamma radiation were found to be stimulative dose for flowering, fruits and seed yield in several crops such as *Pinus kesiya* and *P. wallichiana* (Thapa, 2004), *Anacardium occidentale* (Klarizze, 2005), *Vitis vinifera* (Charbaji and Nabulsi, 1999), *Eruca veisicaria* (Moussa, 2006), *Pisum sativum* (Zaka *et al.*, 2004) and *Triticum durum* (Melki, 2009).

It was noted by Hameed *et al.* (2008) that final germination per cent was decreased significantly after higher irradiation dose ranging from 350-500 Gy. A maximum decreased in seed germination percentage was observed after 500 Gy doses. Gamma radiation had inhibitory effect on physiological and quantitative traits (Khan and Goyal, 2009).

Dhakshanamoorthy *et al.* (2010) have induced mutation in *Jatropha curcas* by exposing the healthy and dry seeds to gamma rays viz., 5, 10, 15, 20 and 25 Kr doses and ethyl methane sulphonate (EMS) viz., 1, 2, 3 and 4 per cent. The results conclude that treatments of gamma rays were found to be greater compared to those of EMS treatments. Based on the variation in flowering and yield traits of gamma rays and EMS treated plants.

The seeds of ashwagandha (*Withania somnifera* L.) were irradiated with different dose of gamma rays *viz.*, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50KR. The seedling survival also increased at lower doses, below 50 per cent observed in 25KR of gamma rays treatment (Bharathi *et al.*, 2013).

Dhakshanamoorthy *et al.* (2013) have induced mutation in *Jatropha curcas* L. through different concentrations (0, 1, 2, 3, and 4%) of EMS. The

improved agronomic traits such as germination, flowering, maturity, seed traits, and oil content were recorded in 1 per cent EMS treated plants.

Cowpea (*Vigna unguiculata* L. Walp) was subjected to different treatment levels of gamma rays and ethyl methane sulphonate (EMS). The treated and untreated plants were self-fertilized for five generations to observe different morphological characters in M6 generation. Highest mutation frequency for flower colour and seed characteristics was induced by 25mM EMS (Girija *et al.*, 2013a).

The seed germination, seedling survival, days to first flower, plant height, number of branches per plant, number of leaves per plant, number of berries per plant, seed yield per plant(gm) and root yield per plant (fresh weight (gm)) were gradually decreased with increase in various dosage of gamma rays. Similar results in morphological parameters were analyzed in cowpea (Gnanamuthy *et al.*, 2013) and also in sesame (Anbarasan *et al.*, 2013).

2.6.4 *In vitro* mutagenesis for biotic and biotic stress tolerance

Application of mutagens is reported for inducing cold and salt tolerance of rice (Sallem *et al.*, 2005; Rakotoarisoa *et al.*, 2008), cold tolerance of canola (Madinchey and Kottliama, 2007), drought-resistance in wheat (Khan *et al.*, 2001), tolerance to heat, salinity and disease in potato (Sharabash, 1998; Das *et al.*, 2000), and tolerance to cold and salinity in cauliflower (Fuller *et al.*, 2006).

Akhar *et al.* (2011) treated chickpea genotypes with different doses of gamma radiation (60, 100, 140 and 180 Gy). The highest survival percentage, 80.1 and 64.6, were observed in the two genotypes of MCC741 in 180 Gy dose and MCC495 in 140 Gy dose, respectively. The increased survival of these two genotypes up to more than 50 per cent could show the increased tolerance to freezing.

2.7 Methods of detecting mutants

2.7.1 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.

2.7.1.1 Morphological markers for mutant detection

Variation of M2 lines of chickpea with morphological characteristics showed a reasonable difference in M2 generation (Schum, 2003). Evaluation of the M2 mutant flax plants (produced by crossing of a NIAB-75 cultivar with rayed pollen of REBA-288 cultivar) using morphological characteristics was carried out and observed noticeable variation (Aslam *et al.*, 2009). Sarwar and Ahmad (2003) by evaluating grow indexes in M2 mutant green pea plants could choose desirable lines for next generation selections.

Sabaghi *et al.* (2013) have evaluated the morphological characteristics of induced mutant lines of stock var. Centum white. Horticultural traits including plant height, inflorescence length, leaf length and width, inter node space, number of flower in florescence, number of petals, the number of true leaves below the flower, flower diameter and number of days to flowering, were assessed. Results

showed that mutation breeding effectively increased the morphological variation in the second generation of stock plants.

2.7.2 Biochemical markers

Biochemical markers are markers derived from study of the chemical products of gene expressions. These are also termed as isozyme/allozyme markers or protein markers. According to Gottlieb (1981) the oldest biochemical technique used to study variations is isozyme analysis. It has the power to reveal polymorphism of alleles at particular locus on the basis of protein mobility (Brown *et al.*, 1978).

Biochemical genetic markers offer specific advantage in assessment of genetic diversity and trait-specific crop improvement. Such markers can facilitate appropriate choice of parents for crosses to mapping/tagging of gene blocks associated to economically important traits and in turn permits marker assisted selection (MAS) in backcross, pedigree and population improvement programs (Mohan *et al.*, 1997).

As biochemical markers, isozymes have advantages compared to morphological markers. The alleles (allozymes) at most isozymes loci are co-dominant with a simple mendelian inheritance, so that the frequency of individual alleles is directly counted. This co-dominant causes no deleterious changes in plant phenotype through recessiveness or pleotrophy and allows heterozygous to be distinguished from homozygous. Isozymes rarely exhibit epistatic interaction so that a genetic stock containing an infinite number of markers could be constructed (Tanksley and Rick, 1980).

The use of SDS-PAGE and isozymes were the cheapest and simplest methods that offer sufficient information and serve as a starting point for DNA-based studies (Veasey *et al.*, 2008; Kavita *et al.*, 2011).

These biochemical markers were also been used in anthurium by Kobayashi *et al.* (1987), they were used different isozyme gel electrophoresis systems to identify seven *A. andreanum* cultivars. The identification of seven cultivars was undertaken using seven enzyme systems. Bands were observed in four of these systems, i.e. glutamate-oxaloacetate transaminase, malate dehydrogenase (MDH), peroxidase (Px), and phosphoglucose isomerase (PGI). All seven cultivars were characterized by the combined data for Px, MDH and PGI.

2.7.2.1 Biochemical markers for mutant detection

Azzam and El-Sawy (2005) studied the protein banding patterns of seed storage proteins in peanut and indicated that differences caused by gamma ray irradiation were due to real genetic structure changes. They added that, the densitometric analysis of the SDS protein banding patterns was found to be useful in identifying the induction of variations in the irradiated populations. The similarity index indicated that Giza 6 and R 92 are more sensitive genotypes to irradiation with gamma rays than Giza 4. It is important to determine the suitable dose, which induces the needed variation to start a breeding program that depends on inducing mutations.

Khaleifa *et al.* (2006) have screened ten mutants of peanut and their parental variety (Giza-5) against damping off and root rot diseases to determinate specific biochemical changes in resistant and susceptible mutants. Studying the SDS protein banding patterns of the ten peanut mutants leaves (as a result of gamma irradiation) and their parental variety grown under normal (non stressed) and disease stress conditions was found to be useful in selection for resistance against damping off and root rot diseases.

Sen and Alikamanoglu (2014), characterized drought tolerant sugar beet mutants induced with gamma radiation using biochemical analysis and isozyme variations. As for isozyme variations, two new POX isozyme bands (POX5 and

POX1) were detected in all mutants but not the control and Fe-SOD was observed in one out of ten mutants (M8), while the intensity of Cu/Zn-SOD was found to be variable in all experimental samples. Furthermore, CAT and APX isozymes were detected at different intensities on native gels.

2.7.3 Molecular markers

Two types of molecular markers are 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).

2.7.3.1 Hybridisation based molecular marker

2.7.3.1.1 Restriction Fragment Length Polymorphism (RFLP)

RFLPs were developed by Botstein *et al.* (1980). RFLP uses restriction enzymes that cut the DNA molecule at specific sites, called restriction sites, resulting in different fragments of variable lengths. After separation by electrophoresis, fragments are transferred to nitrocellulose or nylon filters through southern blotting followed by hybridization with radioactively labelled DNA probes and visualisation using photographic film (Varshney *et al.*, 2004).

They are co-dominant markers that are more or less specific depending on the probe used (cDNA, genomic DNA, etc.). These markers are difficult and quite expensive to use. They require the extraction of a large quantity of good quality DNA and operations are lengthy. It takes around two weeks after DNA extraction to read the bands. Cytoplasmic genomes are usually much less polymorphic than the nuclear genome.

2.7.3.2 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 with the adding of primers and thermo stable DNA polymerase. The PCR reaction involves three steps (denaturation, primer annealing and elongation steps).

2.7.3.2.1 Random amplified polymorphic DNA (RAPD)

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).

RAPD among other molecular marker methods has considerable advantages because it is fast, not expensive, applicability to any organism without prior information on the nucleotide sequence and in the potential detection of DNA damage and mutation (Ahloowalia and Maluszynski, 2001; Atienzar *et al.*, 2002).

The previous reports on molecular characterisation of anthurium by Ranamukhaarachchi *et al.* (2001) examined the genetic relationships of nine morphologically similar pot plant cultivars of *A. andraeanum* and concluded that there was a close genetic relationship among the cultivars.

Buldewo and Jaufeerally (2002), Puchooa and Sookun (2003), Nowbuth *et al.* (2005) showed less than 20 per cent variation among the anthurium accessions tested by the RAPD analysis.

Khan *et al.* (2010) used RAPD markers to examine genetic relationship among twelve accessions of anthurium varieties along with a hybrid as an out group check. A high degree of polymorphism was observed.

Buldewo *et al.* (2012) assessed the genetic diversity of 12 *A. andraeanum* cultivars in Mauritius using random amplified polymorphic DNA. RAPD were found to be useful tools in differentiating locally grown *A. andraeanum.*

2.7.3.2.1.1 RAPD for mutation detection

RAPD can quickly detect a large number of genetic polymorphisms and has been employed to detect mutations and assess somaclonal variants (Hashmi *et al.*, 1997; Atienzar *et al.*, 2002; Gesteira *et al.*, 2002; Atak *et al.*, 2004).

Atanassov *et al.* (1996) used gamma rays and SA (sodium azide) to create somaclonal mutation in barley. They suggested that RAPD assay is a sensitive and representative approach to distinguish the variability created by tissue culture and mutagenesis.

Sandhu *et al.* (2002) irradiated seeds of 21 rice lines with 200, 250 and 300 Krad/h with ^{60}Co source. The differences between resistant and susceptible lines for glyfosate were analysed with RAPD method.

The differences among the atrazine mutants were examined by using RAPD and the differences between herbicide resistant mutants were shown (Atak *et al.*, 2004).

Lema-Rumińska *et al.* (2004) have found genetic variations of *Dendranthema grandiflora* Tzvelev irradiated with X or 15 Gy gamma radiation

dose. They obtained polymorphic bands from the mutant plants with eight RAPD primers.

Kumar *et al.* (2006) detected genetic variability among chrysanthemum radio mutants (flower shape, floret shape and flower colour) with RAPD. The cluster analysis of chrysanthemum radio mutants separated into different groups but the genetic distance, which was observed between them, was low except two mutants.

Selvi *et al.* (2007) used RAPD analysis for identification of DNA polymorphism of gamma ray treated *Emblica officinalis* scions (V1M1 V2M).

Khan *et al.* (2007) have studied genetic variability in sugarcane induced somatic mutations using gamma ray and RAPD data showed that the similarity between mutants and parents decreased while the irradiation doses increased.

Senapati and Rout (2008) used RAPD marker for early detection of EMS derived mutants in *Rosa hybrida* cvs. First Red, Cri and Pusa Gaurav.

Gang *et al.* (2009) have analysed the EMS treated mutants of *Gladiolus hybridus* Hort through RAPD and the result shows that the RAPD analysis could be used for mutant identification.

Barakat *et al.* (2010) have induced mutation in *Chrysanthemum morifolium* cv. 'Delistar white' through *in vitro* mutagenesis by treating the ray florets with two doses of gamma irradiation. Mutation was detected through RAPD analysis. The genetic similarity among the fourteen genotypes ranged from 0.43 to 0.95. The chrysanthemum cultivar and its 13 somaclones were classified into five clusters.

Atak *et al.* (2011) used RAPD analysis to show the differences between *Rhododendron* mutants and control plants. The mutant plantlets were selected due to their better *in vitro* shoot and root regeneration relative to control in both varieties. Genetic variability induced with gamma ray and RAPD methods were

used to detect mutations at the *Rhododendron* shoot culture. Results show that *Rhododendron* mutants were district from controls.

Barkat and Sammak, (2011) have induced mutation in *Gypsophila paniculata* through *in vitro* mutagenesis by treating the shoot tips and lateral buds with four doses of gamma irradiation (0.25, 0.5, 0.75 and 1 Gy) and applied RAPD analysis for the detection of genetic polymorphism among *Gypsophila* mutants and their parent. Result shows that RAPD technique could be successfully applied to the newly *Gypsophila* variants and can differentiate mutants from their parents.

Qassar *et al.* (2011) attempted *in vitro* mutagenesis in tomato *(Lycopersicon esculentum* Mill.) through gamma radiation and chemical (sodium azide) and RAPD technique was performed to detect genetic mutation in extracted DNA samples. The total number of amplification products was 27 bands, of which 22 bands were monomorphic bands which indicates the presence of genetic mutations.

Salt-tolerant mutants of potato (*Solanum tuberosum* L. 'Marfona') were obtained via gamma irradiation. Molecular level differences between the control and mutant plants were elucidated using RAPD-PCR method and the polymorphism rate according to the selected primers was calculated as 89.66 per cent. The greatest difference encountered between the control and mutants was 47 per cent, which was detected in mutant plants produced by 20 or 30 Gy (Yaycili and Alikamanoglu, 2012).

Girija *et al.* (2013b) analysed the cowpea seeds to identify DNA polymorphism among the mutants treated with gamma rays and EMS through a RAPD marker analysis. The mutants showing the differences in morphological traits showed DNA polymorphism in PCR profile amplified by RAPD marker.

Yunus *et al.* (2013) have attempted *in vitro* mutagenesis in *Etlingera elatior* (Jack) through gamma radiation and the early detection of mutants was

performed by using RAPD analysis. The result shows that early screening of mutants can speed up the breeding program.

Randomly Amplified Polymorphic DNA (RAPD) marker analysis was carried out to assess the DNA polymorphism induced by different concentrations (control, 1, 2, 3, and 4%) of EMS in *Jatropha curcas* L. (Dhakshanamoorthy *et al.*, 2013).

Random amplified polymorphic DNA (RAPD) has been used in anthurium by Puchooa, (2005) and he did not observed any differences of RAPD profiles between mother anthurium plant and mutant plants irradiated with 5 Gy gamma radiation dose which showed phenotypic differences.

2.7.3.2.2 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 as the presence or absence of fragments of particular size (Zietkiewicz *et al.*, 1994).

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, 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 (Godwin *et al.*, 1997; Zietkiewicz *et al.*, 1994) and diversity analysis.

In anthurium the Inter Simple Sequence Repeats (ISSR) and Random Amplified Polymorphic DNA (RAPD) markers were found to be useful in current breeding programs of *A. andraeanum,* for estimating the genetic similarity among genotypes and for the identification of cultivars (Jau-Yueh *et al.*, 2001).

Gantait *et al.* (2008) used ISSR marker to assess the genetic identity of both *ex vitro* hardened clones and *in vitro* sustained clones with their mother plant, displayed no polymorphism.

Gantait and Sinniah (2011) used inter simple sequence repeat (ISSR) markers to assess the variation between *ex vitro* grown micropropagated anthurium and seed germinated plants. Assessment through ISSR showed no polymorphism in banding pattern.

Buldewo *et al.* (2012) assessed the genetic diversity of 12 *Anthurium andraeanum* cultivars in Mauritius using RAPD and ISSR markers and identify cultivar specific markers for the genetic profiling of *A. andraeanum* cultivars. Polymorphism among the 12 cultivars was assessed using RAPD and ISSR primers. Both ISSR and RAPD were found to be useful tools in differentiating the *A. andraeanum* varieties.

2.7.3.2.2.1 ISSR for mutant detection

Shufang *et al.* (2010) treated the corm bud of *Gladiolus hybridus* Hort. The mutants from M_1 generation were analyzed through ISSR. Results showed that EMS was a very effective mutagenic agent for the corm bud of *G. hybridus* Hort. There was no close relationship between the amount of mutagenic agent and the divergence of plants genomes, which offered a molecular basis for selecting plants with good mutation.

ISSR markers were used to detect the differences between mutants and their parents in different species. This technique was successfully used to show genetic differences between control and putative banana mutants which were obtained from Pacovan, Preciosa, Basrai and GCTCV-215 banana cultivars via *in vitro* mutagenesis (Khatri *et al.*, 2011; Pestanana *et al.*, 2011a; Pestanana *et al.*, 2011b).

Mudibu *et al.* (2011) have used ISSR markers in soybean to show polymorphism ratios and genetic differences between gamma radiation induced mutants and it was reported that polymorphism ratios and genetic distances between the samples varied from 70 to 90 per cent and from 0.00 to 0.46, respectively.

Sen and Alikamanoglu, (2012) selected drought tolerant sugar beet (*Beta vulgaris* L.) mutants induced with gamma radiation using ISSR markers and SDS-PAGE. Leaf soluble proteins obtained from the control and drought-tolerant mutants were analyzed by SDS-PAGE. The ISSR markers and SDS-PAGE analysis were able to show the genetic distance between mutant and control plants in the earlier stages of development.

Xi *et al.* (2012) treated the *Lilium longiflorum* Thunb. cv. White fox with different doses of gamma radiations and the genetic variations among the morphological mutants were evaluated by DNA fingerprinting using ISSR molecular marker. The genetic variation frequency reached 36.06 per cent using seven ISSR primers. Out of the 50 mutant, nine were observed to have significantly different morphological characters than those of the controls.

Taheri *et al.* (2013a) have used ISSR assays to identify DNA polymorphism among the mutant varieties of *Curcuma alismatifolia* (Chiang Mai Red, Sweet Pink and Kimono Pink) and one hybrid (Doi Tung 554). The study revealed that DNA polymorphism detected by ISSR analysis offered a useful molecular marker for the identification of mutants in gamma radiation treated plants.

2.7.3.2.3 Amplified Fragment Length Polymorphism (AFLP)

AFLPs are DNA fragments (80-500 bp) obtained from digestion with restriction enzymes, followed by ligation of oligonucleotide adapters to the digestion products and selective amplification by the PCR. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. AFLPs therefore involve both RFLP and PCR. The technique involves three steps:

(i) Restriction of the DNA and ligation of oligonucleotide adapters
(ii) Selective amplification of sets of restriction fragments and
(iii) Gel analysis of the amplified fragments.

PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence. The method allows the specific co-amplification of high numbers of restriction fragments. The number of fragments that can be analyzed simultaneously is dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Vos *et al.*, 1995).

The strengths of AFLPs lie in their high genomic abundance, considerable reproducibility, the generation of many informative bands per reaction, their wide range of applications, and the fact that no sequence data for primer construction are required. AFLPs may not be totally randomly distributed around the genome as clustering in certain genomic regions, such as centromers, has been reported for some crops (Alonso-Blanco *et al.*, 1998; Saal and Wricke, 2002).

2.7.3.2.3.1 AFLP for mutant detection

AFLP has also been used to probe the relationship between various chrysanthemum species (Zhou and Dai, 2002), and Sung *et al.* (2010) reported the genetic relationship between the original chrysanthemum 'Argus' and four mutant lines using an AFLP analysis.

The analysis of Amplified Fragment Length Polymorphism (AFLP) was used to estimate genetic diversity in common bean (*Phaseolus vulgaris* L.) variety Hwachia and in 34 NaN3-induced mutants and 11 commercial varieties introduced from China. The calculated Jaccard similarity coefficients based on AFLP data ranged from 0.47 to 0.84. The molecular profiles obtained from eight AFLP primer pairs indicated a high genetic diversity among Hwachia, NaN3-induced mutants and introduced varieties (Chen *et al.*, 2011).

Kang *et al.* (2013) have used AFLP marker to compare the polymorphisms, genetic diversity and genetic distances between the original variety and gamma irradiated *in vitro* plants of *Phaseolus vulgaris* L. The resulting genetic variations were more diverse in the *in vitro* populations than in the plants derived from conventional cuttings. They identified 83 per cent (866 bands) of the polymorphisms using 12 primer combinations.

2.7.3.2.4 Simple sequence repeats (SSR) or Microsatellites markers

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

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).

SSR markers have been widely applied to construct linkage maps of the rice (McCouch *et al.*, 2002) and tomato (Frary *et al.*, 2005) genomes. Such markers can also be used to assist selection in breeding programme and backcrossing for a specific trait or quantitative traits, in genotyping species (Zorrilla-Fontanesi *et al.*, 2011) and in cultivars identification (Tantasawat *et al.*, 2010; Zhang *et al.*, 2010) and serve as effective tools in plant germplasm diversity analysis (Mujaju *et al.*, 2010).

Simple sequence repeat (SSR) markers are relatively abundant in plant genomes (Wang *et al.*, 1994), these markers show hypervariability, are multiallelic in nature, representing co-dominant genetic traits and are suitable for high throughput analysis (Rajwant *et al.*, 2011).

Wang and Chuang (2013) conducted the study to develop and characterize novel microsatellite markers using the Araceae sequences in GenBank of the NCBI. 100 pairs of SSR primers were screened against a set of 28 diverse genotypes with a mutant line. From the selected six polymorphic SSR loci, 52 alleles were amplified and 27 distinct genotypes were found, except for 'Tropical' and its mutant with a mean number of eight alleles per locus. The polymorphism information content (PIC) ranged from 0.86 to 0.93.

2.7.3.2.4.1 SSR in mutant detection

Taheri *et al.* (2013b) studied the effect of eight different doses of acute gamma irradiation in *Curcuma alismatifolia* and 20 Gy dose was effective to influence morphological and molecular characteristics of studied *C. alismatifolia* varieties. The DNA polymorphism was detected Simple Sequence Repeats (SSR) markers. The genetic similarity coefficient among the 44 individuals amplified

using eight SSR primers varied from 0.0 to 1.0. The use of microsatellite markers as a co-dominant marker will facilitate the exploration of genetic variability among treated and non-treated plants will help to distinguish the plants showing differences in morphological characters.

2.8 TILLING

TILLING (Targeting induced local lesions in genomes) a newly developed general reverse genetic strategy helps to locate an allelic series of induced point mutations in genes of interest. It allows the rapid and inexpensive detection of induced point mutations in populations of physically/chemically mutagenized individuals (Colbert, 2001). In addition to allowing efficient detection of mutations by TILLING approach, EcoTILLING technology is also ideal for examining natural variation. Endonuclease CEL I cut effectively the multiple mismatches in a DNA duplex. Therefore, heteroduplex DNA of unknown sequence with to that of a known sequence reveals the positions of polymorphic sites. Both nucleotide changes and small insertions/deletions are identified.

It can be performed more inexpensive than full sequencing the methods currently used for the most single nucleotide polymorphism (SNP) discovery. SNP variation can provide clues to the adaptive strategies and population history that undoubtedly played roles in specie's evolution. It is also used for screening and detection of plants with desired traits by knockdown and knockout mutations in specific genes. This makes TILLING and EcoTILLING an attractive strategy for a wide range of applications from the basic functional genomics study to practical crop breeding (McCallum, 2000).

It was first explored in the late 1990's by the efforts of Claire McCallum and his collaborators (Fred Hutchinson Cancer Research Centre and Howard Hughes Medical Institute) who were experimenting on *Arabidopsis* (Borevitz *et al.*, 2003).

This technique was developed by pooling chemically mutagenized plants together, creating heteroduplexes among the pooled DNA, intensify the region of concern and using dHPLC (denaturing high performance liquid chromatography) to identify the mutants by chromatographic variations (McCallum *et al.*, 2000). A less expensive and faster modification of the TILLING protocol was published later, which uses a mismatch specific celery nuclease (CEL I) and LI-COR gel analyzer system (Alonso and Ecker, 2006; Oleykowski *et al.*, 1998). In 2001, the standard procedure was developed with practical software that makes the TILLING technique a more routine method to detect mutations to get satisfactory results (Colbert, 2001).

This technique was first utilized in *Arabidopsis* TILLING Project (ATP) during 2001. The ATP project has detected, sequenced, and delivered over 1000 mutations in more than 100 genes (Till *et al.*, 2003).

TILLING is one of the methods found to be best for mutation detection and it is being successfully applied in several crops such as *Lotus japonicas* (Perry *et al.*, 2003), maize (*Zea mays* L.) (Till *et al.*, 2004), wheat (*Triticum aestivum* L.) (Slade *et al.*, 2005) and rice (McCallum, 2000). In maize 17 independent induced mutations from 11 genes were obtained from a population of 750 pollen-mutagenized plants (Till *et al.*, 2004).

Through induced mutation, a large number of plant varieties have been developed with improved traits such as high yield, early maturity, as well as high protein content, biotic and abiotic resistance (Ahmad *et al.*, 2012). Malaysian Nuclear Agency (Nuclear Malaysia) has produced more than 20 new varieties of ornamental and industrial crops.

According to IAEA Mutant Varieties Database (http: //www-mvd.iaea. org), 2,570 mutant varieties have been officially released worldwide. Of these, 625 varieties are ornamental and decorative plants and the improved characters include compact growth, attractive variegated leaves, novel flower colour and shapes.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2012-2014, with the objective to induce variation in anthurium var. Tropical through *in vitro* mutagenesis and to characterise the variability through morphological and molecular assay. The materials used and methodologies adopted in the study are provided in this chapter.

3.1 Materials

3.1.1 Plant material

In vitro culture of *Anthurium andreanum* var. Tropical obtained from the Centre for Plant Biotechnology and Molecular Biology (CPBMB), CoH were multiplied in MS medium and utilized for the study.

3.2 Laboratory chemicals, Glass ware and Plastic ware

The chemicals used in the present study were of good quality (AR grade) from various agencies including 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 plastic ware used was obtained from Axygen and Tarson India Ltd. The RAPD and ISSR primers were obtained from Sigma Aldrich Chemical Pvt. Ltd.

3.2.1 Equipment and Machinery

The present research work was carried out using plant tissue culture and molecular biology facilities and equipments available at CPBMB, College of Horticulture. All the aseptic manipulations for *in vitro* mutagenesis studies were carried out in laminar air flow (LABILE INDUSTRIES-HLF). 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. The amplification reaction was carried out in Veriti Thermal Cycler (Applied Biosystem, USA) and the 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. The details are given in Annexure I. Gamma chamber were utilized for irradiation available at Radio Tracer Laboratory, College of Horticulture and laminar air flow for maintaining aseptic condition. Ethyl methane sulphonate (EMS) were obtained from HIMEDIA.

3.3 Preparation of MS medium

Standards procedures were followed for the preparation of plant tissue culture media. Five stock (I, II, III, IV and V) 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.

A clean beaker, rinsed with distilled water was used to prepare the medium. All stock solutions were pipette in proportionate volume in the beaker. For preparing media of full strength, 20ml pipette from 50 X stocks and 10ml from 100 X stocks and required quantities of sucrose, inositol and hormone were added and dissolve 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.

CleriGel was added as solidifying agent at 2g/l concentration, after adjusting pH. The medium was stirred and heated to melt the CleriGel, poured in to culture vessels (jam bottles) for fresh media and in conical flask to prepare antibiotic media. Vessels containing media were sterilised in an autoclave at 121^{0}C in 15 psi for 20 min. The medium was allowed to cool at room temperature and stored in media store room until used. Chemical compositions of MS medium are given in Annexure II.

3.3.3 Transfer area and aseptic manipulation

All the aseptic manipulation such as preparation, inoculation, subsequent sub culturing and preparation of antibiotic media were all carried out under laminar air flow.

3.3.4 Culture room

The cultures were incubated at 26 ± 2°C in an air conditioned 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 climate prevailing.

3.4 Methods

3.4.1 Gamma irradiation

The gamma irradiation was performed at room temperature using a gamma chamber equipped with ^{60}Co source (GC-900) at Radio Tracer Laboratory, Kerala Agricultural University. The five vessels containing 10 to 12 *in vitro* culture of anthurium were exposed with different dosage of gamma rays (0, 5, 10, 15 and 20 Gy). Immediately after irradiation the cultures were kept in dark room for 48 hrs and then all the cultures were inoculated into fresh MS medium. The survival of irradiated cultures were observed after two weeks and after 45 days interval cultures were sub cultured, healthy and rooted shoots were planted out and hardened successfully The plants were observed for the variation at both morphological and molecular level.

3.4.2 Chemical mutagenesis

EMS is a potential carcinogen so its preparation and handling were conducted very carefully inside a laminar air flow and different concentration (0, 0.1, 0.2 and 0.5%) of EMS was first prepared using distilled water. The EMS solutions were filter-sterilized (through a 0.2-µm membrane) before use. The *in vitro* cultures of anthurium were immersed in 0, 0.1, 0.2, and 0.5 per cent EMS solutions for 0 and 30 min with constant swirling throughout the treatment. After

the treatments, the cultures were rinsed three times with sterile distilled water and blotted dried on a sterile tissue paper. They were inoculated on MS medium for shoot regeneration. The frequency of culture survival was recorded at 2 week after and the frequency of culture producing shoots was observed at one month after EMS application.

3.5.1 *In vitro* multiplication

The *in vitro* cultures of anthurium var. Tropical provided from the tissue culture lab of CPBMB were multiplied in the medium already standardized at the centre. The medium composition consisted of full MS semisolid media supplemented with 3 per cent sucrose, 0.1g inositol and 2mg/l 2ip. Since rooting was observed for healthy shoots in the same media no separate media was tried.

3.5.2 Hardening

The plantlets were hardened in a mist chamber; moist cocopeat was used as substrate for planting. Two weeks after hardening the plantlets were transferred into small earthen pots filled with a mixture of charcoal, brick pieces, coconut husk chips and potting mixture (1:1:1:1). Two months after secondary hardening the plants were transfer to larger pot with same media composition for further evaluation.

3.6. Morphological characterisation

Morphological observations were recorded at two months after plantout in single pots and observations were recorded at every two months interval. Six months old hardened morphological variant plants were selected and subjected to molecular assay. The major morphological characters for which observations were taken include:

3.6.2 Vegetative characters

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 recorded

3. Leaf length and width

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

3.6.3 Morphological data analysis

Statistical analysis of entire morphological data recorded was done by Duncan test

3.6.4 Flower characterization

Flowering observations were recorded nine months after planting

3.7. Molecular characterisation

Molecular analysis of the plants derived from different subcultures of mutagen treated *in vitro* cultures was carried out with two molecular markers RAPD and ISSR.

3.7.1 Genomic DNA extraction

Isolation of good quality genomic DNA is one of the most important pre requisites for molecular marker analysis. The CTAB procedure reported by Rogers and Bendich (1994) used for the extraction of good quality genomic DNA from *in vitro* mutagenesis derived hardened plants. The young tender leaves were used for the 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 (pH8)
   - 20mM EDTA (pH8)

- 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 (pH8)
   -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

3.7.1.1 Procedure for DNA isolation

Young and tender leaf tissue (0.2g) was weighed and ground in liquid nitrogen using mortar and pestle along with 50 µl of β-mercaptoethanol and a pinch of Poly Vinyl Pyrrolidone (PVP). The sample was ground into fine powder using excess of liquid nitrogen and the powder was transferred to a sterile 2ml centrifuge tube containing 1ml of pre-warmed extraction buffer (total 2ml). The homogenate was incubated for 30 minutes at 65°C with occasional mixing by gentle inversion. Equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 minutes at 4°C. The contents got separated into three distinct phases. The aqueous phase containing DNA was pipetted out into a fresh 2ml centrifuge tube and this step was repeated tow times. Supernatant was collected, 0.6 volume of chilled isopropanol was added and incubated at -20°C for 30 minutes. The precipitated DNA was pelleted by centrifugation at 8,000 rpm for 15 minutes at 4°C. The pellet was collected and washed first with

70 per cent alcohol and then with 100 per cent alcohol. Pellet was air dried for few minutes at room temperature and dissolved in 50 µl distilled water.

3.6.2 Purification of DNA

The DNA which had RNA as contaminant was purified by RNase treatment and further precipitation. The RNase treatment was carried out during the DNA isolation steps, before pelleting the DNA.

3.7.3 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 (SRL, low EEO)
   - 0.8 per cent (for genomic DNA)
   - 1.5 per cent for RAPD
   - 2.0 per cent for ISSR
2. 50X TAE buffer (P^H 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 (BIO-RAD), gel casting tray, comb
6. UV transilluminator (Wealtec)
7. Gel documentation and analysis system (BIO-RAD)

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 (BIO-RAD, USA). The gel profile was examined for intactness, clarity of DNA band, presence of RNA and protein.

3.7.4 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 (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to 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.

3.8. Molecular Markers assay

Two different types of markers viz., Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) were used for the study in order to check the variations at molecular level.

3.8.1 RAPD (Random Amplified Polymorphic DNA) analysis

RAPD assay was performed to detect the mutant plants.

The good quality genomic DNA (40 to 50ng/µl) isolated from young tender leaves of variants and control plants were subjected to RAPD. Random decamer primers supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' with good resolving power was used for amplification of DNA. The decamer primers for RAPD assay were selected after an initial screening.

3.8.2 Screening of RAPD primers

For initial screening seven RAPD primers reported by Khan and Pankajasan, (2010) were selected. After initial screening five primers were selected. List and sequence information of screened RAPD primers is given in Table 1. These five selected primers were again screened with two different varieties (Tropical (Red) and Meringue White) of anthurium to confirm whether these primers can differentiate between two varieties if they can, there is more chance of differentiating between mutant and control plants and those primers which were able to differentiate were utilised for mutant detection.

Table 1. Details of RAPD primers screened with anthurium samples

Sl. No.	Primer	Nucleotide Sequence
1	OPB6	5'- GTGATCGCAG -3'
2	OPB 8	5'- CAGCACCCAC -3'
3	OPA 10	5'- CCACAGCAGT -3'
4	OPA 13	5'- GGACCCTTAC -3'
5	OPB 15	5'- TGCTCTGCCC -3'
6	OPB 18	5'- GTCCACACGG -3'
7	OPB 20	5'- GGAGGTGTT -3'

The amplification reaction was carried out in Veriti Thermal Cycler (Applied Biosystem, USA). A master mix without the template DNA was prepared using the reaction mixture for the required number of reaction. From that master mix, 18µl was pipetted out into each PCR tube and finally 2µl of template DNA was added. Then PCR amplification was performed in 20µl reaction mixture as detailed below:

Composition of the reaction mixture for PCR (20µl)

a) Genomic DNA (30-50ng/µl)	- 2.0 µl
b) 10X *Taq* assay buffer B	- 2.0 µl
c) $MgCl_2$	- 1.8 µl
c) dNTP mix (10mM each)	- 1.5 µl
d) *Taq* DNA polymerase (3U)	- 0.3 µl
e) Decamer primer (10 pM)	- 2.0 µl
f) Autoclaved distilled water	- 10.4 µl
Total volume	=20.0 µl

The thermo cycler was programmed as follows:

94°C for 2 minutes	-	Initial denaturation	
94°C for 1 minute	-	Denaturation	40 cycles
37°C for 1 minute	-	Primer annealing	
72°C for 2 minutes	-	Primer extension	
72°C for 10 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 system BIO-RAD. The documented RAPD profiles were carefully examined for polymorphism. The primers gave good amplification with good polymorphism were selected for further studies.

3.8.3 RAPD with selected primers

RAPD assay was performed with DNA samples isolated from few selected variants as well as from control plants using five selected primers with same PCR reaction mixture composition and programme.

3.9. Inter Simple Sequence Repeats (ISSR) analysis

ISSR was performed to characterise the selected *in vitro* mutants.

The good quality genomic DNA (30 to 50ng/µl) isolated from young tender leaves of variants and control plants were subjected to ISSR. ISSR primers supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' with good resolving power were used for amplification of DNA. The primers for ISSR assay were selected after an initial screening.

3.9.1 Screening of ISSR primers

For initial screening eight ISSR primers reported by Buldewo *et al.* (2012) were selected. After initial screening five primers were selected. List and sequence information of screened ISSR primers is given in Table 2. These five selected primers were again screened with two different varieties (Tropical (Red) and Meringue White) of anthurium to confirm whether these primers can differentiate between two varieties if they can, there is more chance of differentiating between mutant and control plants and those primers were able to differentiate they were utilised for mutant detection.

Table 2. Details of ISSR primers screened with anthurium samples

Sl. No.	Primer	Nucleotide Sequence (5'-3')
1	B2	5'- GGCGTCGGTTTCCATTAT -3'
2	B11	5'- TGTGCCGACGATGTTGATGCAAT -3'
3	AN3	5'- ACTTCATGCTATGTGGCGACT -3'
4	AM7	5'- TTGTTGCTGTGGGTATAGCATCA -3'
5	ISSR1	$(GTT)_5$
6	ISSR2	$(CTGA)_4$
7	ISSR3	$(CCA)_5$
8	ISSR4	$(GTC)_5$

Amplification was performed with the same PCR reaction mixture composition and programme. The amplification was carried out in an Applied Biosystem PCR. Amplification was performed in a 22 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR

a) Genomic DNA (30-50ng/ µl)	- 2.0 µl
b) 10X *Taq* assay buffer B	- 2.0 µl
c) $MgCl_2$	- 1.8 µl
c) dNTP mix (10mM each)	- 1.8 µl
d) *Taq* DNA polymerase (3U)	- 0.4 µl
e) Decamer primer (10 pM)	- 2.0 µl
f) Autoclaved distilled water	- 12.0 µl
Total volume	- 22.0 µl

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 } 40 cycles

72°C for 2 minutes - Primer extension

(Depend on primers)

72°C for 8 minutes - Final extension

4°C for infinity to hold the sample

The amplified products were run on 2.0 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 system BIO-RAD. The documented ISSR profiles were carefully examined for polymorphism. The primers gave good amplification with good polymorphism were selected for further studies. ISSR assay was performed with DNA samples isolated from variants as well as from control plants using five selected primers with the same PCR reaction mixture composition and programme.

Results

4. RESULTS

The results of research programme on "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2012-2014 are presented in this chapter under different sub headings.

4.1 *In vitro* multiplication

The *in vitro* cultures of var. Tropical supplied from Centre for Plant Biotechnology and Molecular Biology when inoculated in full MS medium supplemented with 2 mg/l 2ip gave good multiplication and plant development. The shoots when developed 2 to 3 healthy leaves, started producing normal roots and hence, no different rooting media was tried for rooting. The rooted plantlets thus developed when separated and planted out gave 85 per cent hardening success. The details are provided in Table 3 and Plate 1.

4.2 *In vitro* mutagenesis

4.2.1 Gamma irradiation

Irradiated cultures differed much in their response when sub cultured in the regeneration media. Response after two weeks is provided in Table 4 and Plate 2. The cultures treated with 5 Gy behaved almost similar to the control with slight increase in the number of shoot initials. The survival rate was low at higher doses, when evaluated two weeks after irradiation. Only 25 per cent cultures survived after two weeks when irradiated with 20 Gy and the number of shoot initials was also very low in the surviving cultures. The new shoot initials were also very poor at higher doses. The details of effect of gamma irradiation on *in vitro* cultures of anthurium are provided in Table 4 and Plate 2.

a- Culture after inoculation

b- Multiplying culture

c- Rooted culture

d- Plant out of TC derived plants

e- Primary hardened plants

f- secondary hardened plants

Plate 1: *In vitro* multiplication of anthurium var. Tropical

a- Control

b- 5 Gy

c- 10 Gy

d- 15 Gy

e- 20 Gy

Plate 2: Response of anthurium cultures two weeks after *in vitro* irradiation

Table 3. Response of anthurium cultures in regeneration medium and success at hardening stage

Details		Subculture 1	Subculture 2	Subculture 3
No. cultured		20	20	20
No. survived		20	20	20
No. multiplied		20	20	20
No. of shoots initials/culture	initial	6.32	7.21	9.57
	after 1 month	20.65	26.73	30.15
Multiplication rate		3.33	3.71	3.33
No. of healthy shoots produced/culture		8.74	11.05	16.61
No. of shoots rooted/ culture		-	4.83	7.16
Total no. planted		-	80	156
No. survived		-	62	138
Survival %		-	77.5	88.46

4.2.1.1 Shoot regeneration in gamma irradiated *in vitro* cultures

The irradiated *in vitro* anthurium cultures were subjected to different subculture cycles. The cultures treated at 0 and 5 Gy doses multiplied and produced good plantlets in further subcultures and the 5 Gy treated cultures produced more and vigorous plantlets than control. The dose of 10 Gy gamma irradiation was lethal to anthurium tissue and only 66 per cent cultures were survived and they started producing plantlets only after 2nd subculture while higher doses at 15 and 20 Gy the survival was low (25%) and most of the cultures lost their green appearance (turned blackish) and were not capable of shoot regeneration. In later subcultures the remaining part of the callus of 15 and 20 Gy were inoculated into fresh medium and after 3rd subculture the culture were revived and producing shoots were planted out. The details of shoot regeneration at different subcultures are provided in Table 5 and Plate 3, 4.

4.2.1.2 *In vitro* rooting

In vitro rooting in gamma irradiated cultures were observed only 4th week after treatment. In control shoots 16.66 per cent rooting was observed and in 5 Gy treatments the rooting was 21.05 per cent. In 10 Gy treatment the rooting was very less 11.11 per cent and because of higher doses of irradiation in 15 and 20 Gy initially rooting was not observed. The details of *in vitro* rooting are provided in Table 6.

4.2.1.4 Hardening

In vitro derived plantlets of anthurium from different gamma irradiation treatment were planted in primary hardening unit. Rooted shoots were hardened successfully, control plants with 88.46, 5 Gy with (75.92), 10 Gy with (50.77), 15 Gy with (34.56) and 20 Gy gave 20 per cent hardening success. Later the primary hardened plants were transferred to secondary hardening, gave more than 90 per cent hardening success. The details of hardening are provided in Table 7.

a- Control

b- 5 Gy

c- 10 Gy

d- 15 Gy

e- 20 Gy

Plate 3: Shoot regeneration in gamma irradiated anthurium cultures in 1st subculture

a- Control

b- 5 Gy

c- 10 Gy

d- 15 Gy

e- 20 Gy

Plate 4: Shoot regeneration in gamma irradiated anthurium cultures in 3rd subculture

Table 4. Effect of gamma irradiation on *in vitro* cultures of anthurium two weeks after irradiation

Treatment	No. of cultures irradiated	Average no. of shoot initials at the time of treatment	Survival after 2 weeks (%)	Mean no. of shoots/culture	
				Initials	Healthy
Control	12	7.25	100	13.02	5.61
5 Gy	12	9.52	100	15.13	4.32
10 Gy	12	8.76	66.66	6.43	1.24
15 Gy	12	8.50	50	3.61	-
20 Gy	12	9.36	25	2.27	-

Table 5. Shoot regeneration in gamma irradiated anthurium cultures at different subculture cycles

Treatment	Subculture 1			Subculture 2			Subculture 3		
	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials
Control	12	3.64	15.31	18	5.04	20.61	22	7.36	24.06
5 Gy	12	4.37	17.81	22	6.53	23.09	26	10.12	28.47
10 Gy	8	1.82	10.16	12	3.13	15.13	16	4.71	15.32
15 Gy	6	-	4.21	5	2.34	6.21	10	3.19	8.78
20 Gy	3	-	2.09	3	-	3.73	4	2.07	4.21

Table 6. *In vitro* rooting of healthy shoots in gamma irradiated cultures

Treatment	No. of shoots		No. of shoots rooted within 4 weeks	% rooting	No. of healthy roots/ shoots
	Initials	Healthy			
Control	13	5	3	16.66	3
5 Gy	15	4	4	21.05	3
10 Gy	6	1	1	11.11	2
15 Gy	3	-	-	-	-
20 Gy	2	-	-	-	-

Table 7. Hardening success of anthurium plantlets derived from gamma irradiated cultures

Treatment	No. planted	No. survival		% survival	
		Primary hardening	Secondary hardening	Primary hardening	Secondary hardening
Control	156	138	125	88.46	90.57
5 Gy	162	123	115	75.92	93.49
10 Gy	193	98	90	50.77	91.80
15 Gy	81	28	25	34.56	89.28
20 Gy	85	17	15	20	88.23

4.2.2 Chemical mutagenesis

Three different concentrations (0, 0.1, 0.2 and 0.5%) of ethyl methane sulphonate (EMS) were used for treatment of *in vitro* culture of anthurium. Early response of *in vitro* cultures to EMS could be detected within two weeks of treatment. Some cultures started to turn blackish. However, in control and 0.1 per cent, the cultures started to producing shoots. All the cultures of control and 0.1 per cent EMS survived but in higher doses treatment only 40 per cent cultures survived after two weeks. Cultures treated at higher doses (0.2 and 0.5%), producing shoots were very poor and rooting was very less in such cultures and they started producing few roots only after 3rd sub culture. The details of effect of EMS on *in vitro* cultures of anthurium are provided in Table 8.

4.2.2.1 Shoot regeneration in EMS treated *in vitro* cultures

Different concentrations of EMS (0, 0.1, 0.2 and 0.5%) treated *in vitro* cultures of anthurium were subjected to different subculture intervals. The control and 0.1 per cent treatment cultures were well multiplied in regeneration media and produced healthy plantlets and the rooted plantlets were planted out. Treatment at 0.2 and 0.5 per cent cultures produced enough shoot initials but the rooting was very less in initial sub culture (Table 7). Details of EMS treated cultures of anthurium at different sub cultures cycles are provided in Table 9 and Plate 5, 6.

4.2.2.2 *In vitro* rooting

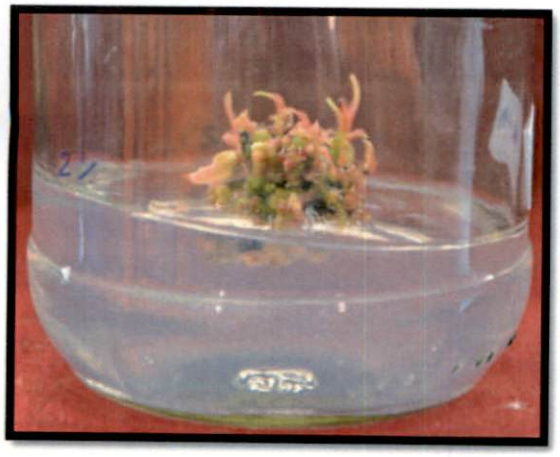
The EMS treated cultures at 0.1 per cent gave 15.38 per cent rooting and treatment at 0.2 and 0.5 per cent produced shoot initials with few roots. Rooted plantlets were planted out but none of the plants were survived at hardening stage. The details of *in vitro* rooting are provided in Table 10.

4.2.2.3 Hardening success of plantlets derived from EMS treated cultures

In vitro derived plantlets of anthurium from different EMS treatment were planted in primary hardening unit. Rooted shoots were hardened successfully. The details of hardening are provided in Table 11.

a- Control

b- 0.1 per cent EMS



c- 0.2 per cent EMS

d- 0.5 per cent EMS

Plate 5: *In vitro* cultures of anthurium var. Tropical after EMS treatment

a- Control

b- 0.1 per cent EMS

c- 0.2 per cent EMS

d- 0.5 per cent EMS

Plate 6: Shoot regeneration in EMS treated anthurium cultures in 3rd subculture

Table 8. Effect of ethyl methane sulphonate (EMS) treatment on *in vitro* cultures of anthurium two weeks after treatment

Treatment	No. of cultures treated	Average no. of shoot initials at the time of treatment	Survival after 2 weeks (%)	Mean no. of shoots/culture	
				Initials	Healthy
Control	10	7.15	100	14.3	3.13
0.1%	10	6.75	100	10.67	2.79
0.2%	10	8.32	60	6.12	-
0.5%	10	7.45	40	4.39	-

Table 9. Shoot regeneration in ethyl methane sulphonate (EMS) treated anthurium cultures at different subculture cycles

Treatment	Subculture 1			Subculture 2			Subculture 3		
	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials	No. of cultures	Mean no. of healthy shoots	Mean no. of shoot initials
Control	10	3.25	13.23	16	4.18	16.12	20	6.21	25.42
0.1%	10	2.86	10.86	12	2.75	14.50	18	4.49	20.84
0.2%	6	-	8.73	8	1.56	8.36	10	2.36	12.32
0.5%	4	-	6.08	5	-	5.13	6	1.25	8.19

Table 10. *In vitro* rooting of healthy shoots in EMS treated cultures

Treatment	No. of shoots		No. of shoots rooted within 4 weeks	% Rooting	No. of healthy roots/ shoots
	Initials	Healthy			
Control	14	4	3	16.66	3
0.1%	10	3	2	15.38	2
0.2%	6	-	-	-	-
0.5%	4	-	-	-	-

Table 11. Hardening success of Anthurium plantlets derived from ethyl methane sulphonate (EMS) treated cultures

Treatment	No. planted	No. survival		% survival	
		Primary hardening	Secondary hardening	Primary hardening	Secondary hardening
Control	54	48	45	88.88	93.75
0.1%	25	11	11	44.00	44.00
0.2%	13	0	0	0	0
0.5%	8	0	0	0	0

4.3 Morphological data analysis

The mutant plants derived from *in vitro* culture of anthurium obtained from different subculture intervals were hardened successfully. The morphological data were recorded at every two months for six months. The morphological data recorded were analysed and are provided in Table 12, 13 and 14 and Plate 7.

4.3.1 Two months after planting

The data recorded for different parameters are provided in Table 12.

The number of leaves was recorded at two months stage for the plants derived from different gamma radiation and EMS treatments. There was no significant difference with respect to number of leaves of control, 5 Gy, 10 Gy and 0.1 per cent EMS treatments. Treatments at 15 Gy were significantly superior with respect to number of leaves. Treatment at 5 Gy were significant with respect to plant height. There were no significant differences in leaf length and width of different treatments; they behaved almost similar in all the treatments.

4.3.2 Four months after planting

The data recorded for different parameters are provided in Table 13.

The number of leaves recorded at four months derived from different treatments ranged between 7.48 and 6.9. There was no significant difference between treatments with respect to number of leaves. Plants derived from 5 Gy (7.56) were superior to the rest.

The plant height recorded at four months stage ranged between 6.83 and 5.20 and they differed significantly with respect to different treatments. Plants derived from 5 Gy (6.83) were significantly superior and plants from 15 Gy and 0.1 per cent EMS treatments were almost similar. Plants from 10 Gy (5.20) were inferior from others.

The leaf length of top three leaves recorded at four months stage ranged between 5.13 and 3.20cm. There were no significant differences in leaf length of

a- Control plants

b- 5 Gy plants

c- 10 Gy plants

d- 15 Gy plant

e- 0.1 per cent EMS treated plants

Plate 7: Six month old gamma and EMS treated plants

the three leaves for different treatments. Leaf length of first leaves derived from 5 Gy (5.13) were superior.

The leaf width of top three leaves ranged between 2.90 and 1.91. The widths of first leaves were differed significantly with respect to different treatments. Leaf width of 5 Gy (2.90) and 15 Gy (2.33) treated plants were varied significantly and no significant difference were found with respect to width of 2nd and 3rd leaves.

4.3.3 Six months after planting

The data recorded for different parameters are provided in Table 14.

The number of leaves derived from different treatments recorded at six months ranged between 8.59 and 7.45. Plants derived from 5 Gy (8.59) were significantly superior with respect to number of leaves. Plants from control, 10 Gy and 15 Gy were almost similar and plants of 0.1 per cent EMS treatment were significant.

The plant height recorded at six months stage ranged between 7.96 and 6.39cm. Plants derived from 5 Gy (7.96) were significantly superior with respect to height. The leaf length of top three leaves recorded at six months stage ranged between 6.86 and 4.25cm. Plants derived from 5 Gy (6.86) of 1st leaf and 5.92 of 2nd leaf were superior.

The leaf width of top three leaves ranged between 3.89 and 2.22cm. Significant difference was there with respect to width of 1st and 3rd leaves. Plants of 5 Gy (3.89) of 1st leaf and 2.80 of 2nd leaf were significantly superior.

Table 12. Morphological parameters of *in vitro* mutated anthurium (Tropical) two months after planting

Treatment	No. of leaves	Plant height (cm)	Leaf length (cm)			Leaf width (cm)		
			1st leaf	2nd leaf	3rd leaf	1st leaf	2nd leaf	3rd leaf
Control	6.56 ab	5.24 a	3.44 a	2.98 a	2.40 a	1.96 a	1.71 a	1.36 a
5 Gy	6.75 ab	5.32 a	3.60 a	3.01 a	2.49 a	2.03 a	1.74 a	1.38 a
10 Gy	6.61 ab	4.03 b	3.39 a	2.95 a	2.37 a	1.93 a	1.65 a	1.35 a
15 Gy	7.14 a	4.95 ab	3.43 a	3.05 a	2.46 a	1.79 a	1.59 a	1.33 a
20 Gy	6.00 b	4.48 ab	3.22 a	2.94 a	2.74 a	1.82 a	1.64 a	1.44 a
0.1% EMS	6.36 ab	5.04 a	3.43 a	2.81 a	2.50 a	1.96 a	1.58 a	1.50 a

Table 13. Morphological parameters of *in vitro* mutated anthurium (Tropical) four months after planting

Treatment	No. of leaves	Plant height (cm)	Leaf length (cm)			Leaf width (cm)		
			1st leaf	2nd leaf	3rd leaf	1st leaf	2nd leaf	3rd leaf
Control	7.28 a	5.34 b	4.80 a	4.08 a	3.45 a	2.67 ab	2.52 a	2.01 a
5 Gy	7.56 a	6.83 a	5.13 a	4.47 a	3.63 a	2.90 a	2.54 a	2.11 a
10 Gy	7.48 a	5.20 b	4.83 a	4.05 a	3.31 a	2.61 ab	2.29 a	1.84 a
15 Gy	7.53 a	6.23 ab	4.39 a	3.98 a	3.31 a	2.33 b	2.13 a	1.75 a
0.1% EMS	6.90 a	6.50 ab	4.71 a	4.22 a	3.20 a	2.57 ab	2.45 a	1.91 a

Table 14. Morphological parameters of *in vitro* mutated anthurium (Tropical) six months after planting

Treatment	No. of leaves	Plant height (cm)	Leaf length (cm)			Leaf width (cm)		
			1st leaf	2nd leaf	3rd leaf	1st leaf	2nd leaf	3rd leaf
Control	8.28 ab	7.16 ab	6.67 a	5.66 ab	4.71 a	3.85 ab	3.49 a	2.68 a
5 Gy	8.59 a	7.96 a	6.86 a	5.92 b	4.98 a	3.89 a	3.30 a	2.80 a
10 Gy	8.11 ab	7.03 ab	6.13 ab	5.27 ab	4.53 a	3.31 bc	2.87 a	2.49 ab
15 Gy	8.17 ab	6.39 b	5.56 b	4.86 a	4.25 a	3.02 c	2.61 a	2.22 b
0.1% EMS	7.45 b	7.27 ab	6.41 ab	5.27 ab	4.29 a	3.57 abc	3.00 a	2.62 a

Table 15. Flowering pattern in anthurium nine months after planting

Treatments	No. of plants Observed	No. flowered	Flowering (%)	Nature of flower	
				Normal (%)	Variants (%)
Control	135	77	57.03	100.00	-
5 Gy	115	84	73.04	84.53	15.47
10 Gy	90	41	45.55	63.42	36.58
15 Gy	25	8	32.00	82.50	17.50
0.1 % EMS	11	7	63.63	78.43	21.57

4.4 Flowering

Flowering observations were recorded nine months after planting. Among the plants evaluated, the maximum flowering were recorded in 5 Gy plants (73.04%) followed by 0.1 per cent EMS (63.63%). Maximum number of variants was observed in 10 Gy plants (36.58%), in 5 Gy (15.47%) and 100 per cent normal flowers were observed in untreated control plants. The details are provided in Table 15 and Plate 8.

4.5 Molecular analysis

4.5.1 Isolation, purification and quantification of DNA

Genomic DNA isolated through the CTAB method reported by Roger and Bendich (1994) had slight RNA contamination (Plate 9). There was no browning of the extract when β-mercaptoethanol was added. The agarose gel electrophoresis indicated clear discrete band without RNA contamination and spectrophotometric analysis gave ratio of UV absorbance ($A_{260/280}$) between 1.8 and 2.0.

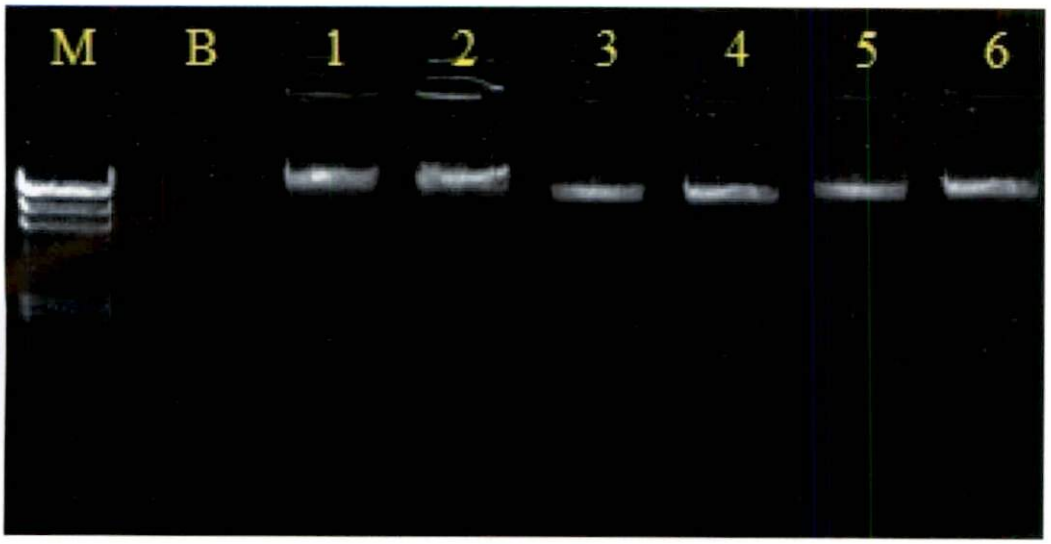
4.5.2 RAPD analysis

Seven RAPD primers reported by Khan and Pankajasan, (2010) were selected. After initial screening out of seven, five primers were selected. List and sequence information of selected RAPD primers is given in Table 16. RAPD analysis was carried out with five controls and five *in vitro* mutagenized plants to characterize the mutants.

4.5.3 Regenerants from *in vitro* mutants

The amplification pattern obtained for each of the primers with respect to plants derived from different doses of mutagen is provided in Plates 10 to 14. Details are as follows.

Plate 8: Variation in floral characters in *in vitro* mutated plants nine months after plant out



M: ladder/Marker (λDNA/*Eco*RI + *Hind*III)
1 control, 2-6 mutant plants

Plate 9: Intact DNA isolated through CTAB method from *in vitro* mutants derived from different doses

Table 16. Details of primers selected for RAPD assay

Sl. No.	Primer	Nucleotide Sequence
1	OPB 6	5'- GTGATCGCAG -3'
2	OPA 10	5'- CCACAGCAGT -3'
3	OPB 15	5'- GGACCCTTAC -3'
4	OPB 18	5'- GTCCACACGG -3'
5	OPB 20	5'- GGAGGTGTT -3'

OPB 6 with 5 Gy:

A total of seven amplicons were obtained after DNA amplification with the primer OPB 6 with 5 Gy treatments. The pattern of amplification is shown in Plate 10a. All amplicons obtained with this primer were monomorphic for the selected variants of 5 Gy. The molecular weight of the bands varied from 0.750 to 1.815 kb.

OPB 6 with 10 Gy:

Amplification with 10 Gy treatments with primer OPB 6 generated seven amplicons of which two were polymorphic. The pattern of amplification is shown in Plate 10b. The variants showed two polymorphic amplicons in each. The molecular weight of the bands varied from 0.350 to 2.300 kb.

OPB 6 with 15 Gy:

A total of seven amplicons were obtained after DNA amplification with the primer OPB 6 with 15 Gy treatments. The pattern of amplification is shown in Plate 10c. All amplicons obtained with this primer were monomorphic for the selected variants of 15 Gy. The molecular weight of the bands varied from 0.600 to 1.815 kb.

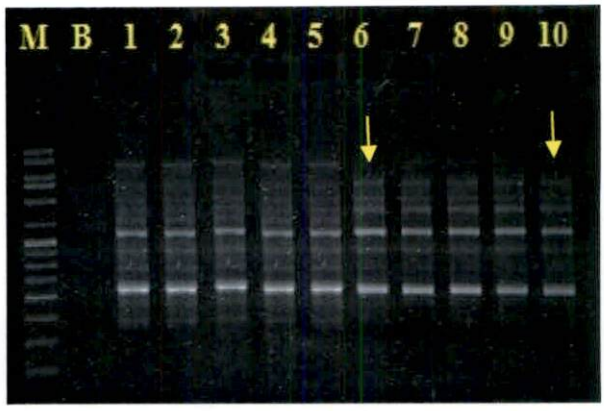
OPB 6 with 0.1 per cent EMS:

A total of seven amplicons were obtained after DNA amplification with the primer OPB 6 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 10d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.625 to 1.815 kb.

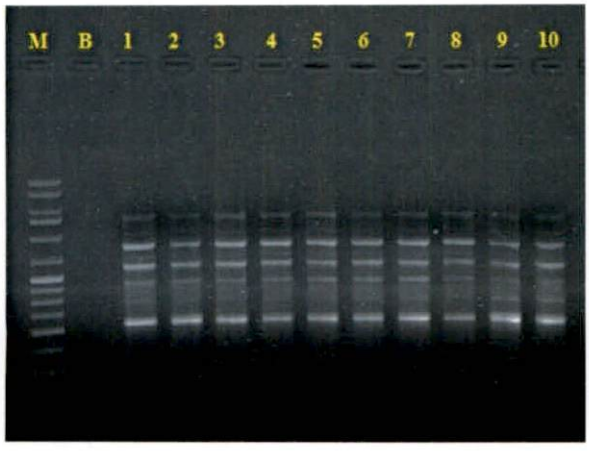
OPA 10 with 5 Gy:

Seven clear amplicons were obtained after DNA amplification with the primer OPA 10 with 5 Gy treatments. The pattern of amplification is shown in Plate 11a. All amplicons obtained with this primer were monomorphic for the

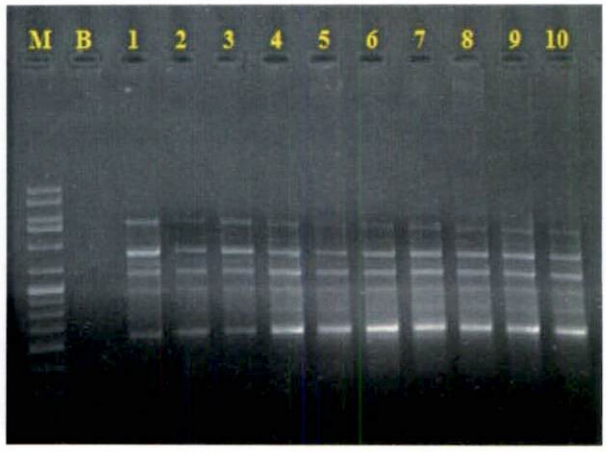
a. Amplification of 5 Gy plants with primer OPB 6



b. Amplification of 10 Gy plants with primer OPB 6



c. Amplification of 15 Gy plants with primer OPB 6



d. Amplification of 0.1% EMS plants with primer OPB 6

M: 1Kb ladder/Marker, B: Control ; 1- 5 control, 6-10 mutant plants

Plate 10: RAPD amplification pattern of *in vitro* mutants of anthurium with primer OPB 6

selected variants of 5 Gy. The molecular weight of the bands varied from 0.500 to 1750 kb.

OPA 10 with 10 Gy:

A total of seven amplicons were obtained after DNA amplification with the primer OPA 10 with 10 Gy treatments. The pattern of amplification is shown in Plate 11b. All amplicons obtained with this primer were monomorphic for the selected variants of 10 Gy. The molecular weight of the bands varied from 0.500 to 1815 kb.

OPA 10 with 15 Gy:

Amplification with the primer OPA 10 with 15 Gy treatments generated seven amplicons. The pattern of amplification is shown in Plate 11c. All amplicons obtained with this primer were monomorphic for the selected variants of 15 Gy. The molecular weight of the bands varied from 0.600 to 1.800 kb.

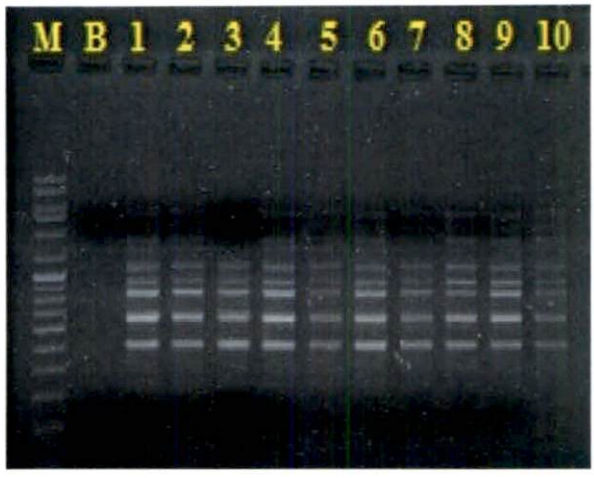
OPA 10 with 0.1 per cent EMS:

A total of seven amplicons were obtained after DNA amplification with the primer OPA 10 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 11d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.150 to 1.500 kb.

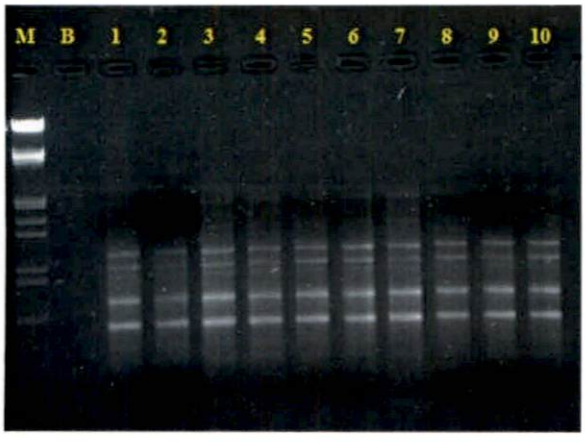
OPB 15 with 5 Gy:

Seven clear amplicons were obtained after DNA amplification with the primer OPB 15 with 5 Gy treatments. The pattern of amplification is shown in Plate 12a. All amplicons obtained with this primer were monomorphic for the selected variants of 5 Gy. The molecular weight of the bands varied from 0.250 to 1.350 kb.

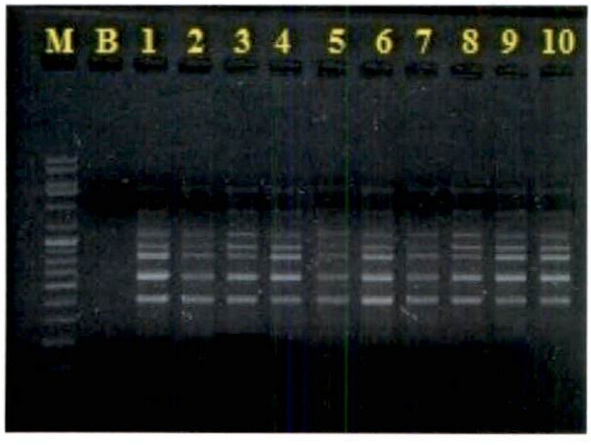
a. Amplification of 5 Gy plants with primer OPA 10



b. Amplification of 10 Gy plants with primer OPA 10



c. Amplification of 15 Gy plants with primer OPA 10



d. Amplification of 0.1% EMS plants with primer OPA 10

M: 1Kb ladder/Marker, B: Control; 1- 5 control, 6-10 mutant plants

Plate 11: RAPD amplification pattern of *in vitro* mutants of anthurium with primer OPA 10

OPB 15 with 10 Gy:

A total of seven amplicons were obtained after DNA amplification with the primer OPB 15 with 10 Gy treatments. The pattern of amplification is shown in Plate 12b. All amplicons obtained with this primer were monomorphic for the selected variants of 12 Gy. The molecular weight of the bands varied from 0.250 to 1.350 kb.

OPB 15 with 15 Gy:

A total of seven amplicons were obtained after DNA amplification with the primer OPB 15 with 15 Gy treatments. The pattern of amplification is shown in Plate 12c. All amplicons obtained with this primer were monomorphic for the selected variants of 15 Gy. The molecular weight of the bands varied from 0.250 to 1.350 kb.

OPB 15 with 0.1 per cent EMS:

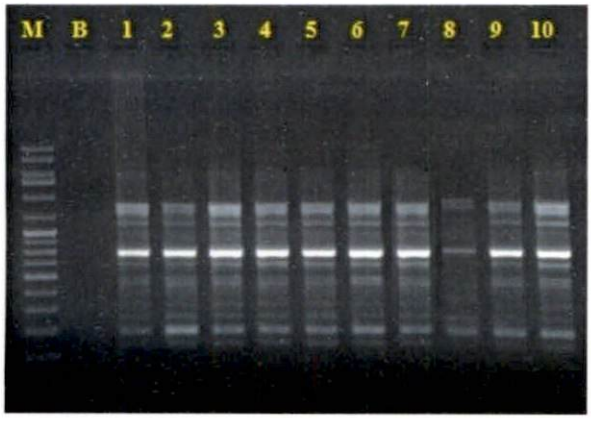
A total of seven amplicons were obtained after DNA amplification with the primer OPB 15 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 12d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.150 to 1.350 kb.

OPB 18 with 5 Gy:

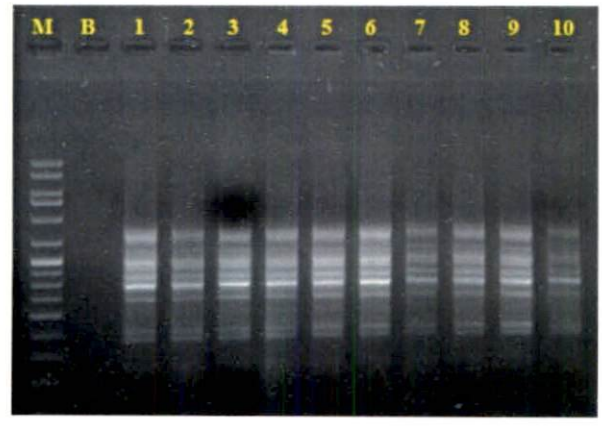
Amplification with the primer OPB 18 with 5 Gy treatments generated eight amplicons. The pattern of amplification is shown in Plate 13a. Only plant no. 8 showing polymorphism; rest was monomorphic for the selected variants of 5 Gy. The molecular weight of the bands varied from 0.200 to 1.815 kb.

OPB 18 with 10 Gy:

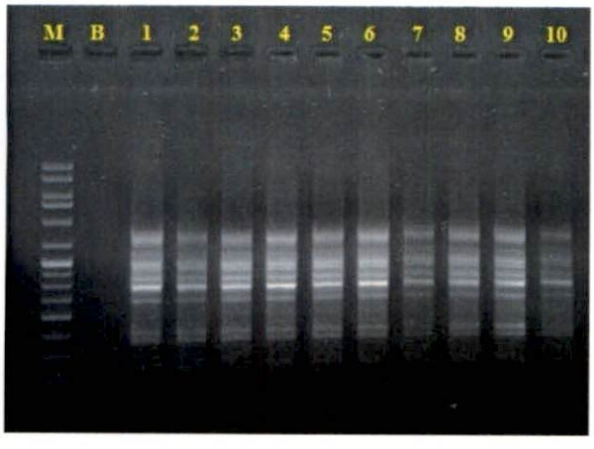
Eight clear amplicons were obtained after DNA amplification with the primer OPB 18 with 10 Gy treatments. The pattern of amplification is shown in Plate 13b. All amplicons obtained with this primer were monomorphic for the



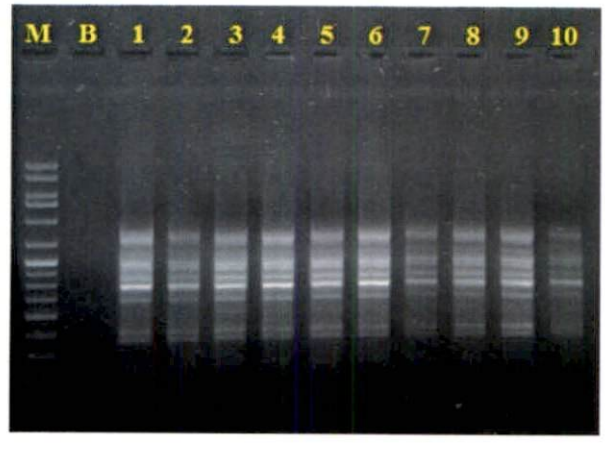
a. **Amplification of 5 Gy plants with primer OPB 15**



b. **Amplification of 10 Gy plants with primer OPB 15**



c. **Amplification of 15 Gy plants primer OPB 15**



d. **Amplification of 0.1% EMS plants with with primer OPB 15**

M: 1Kb ladder/Marker, B: Control ; 1- 5 control, 6-10 mutant plants

Plate 12: RAPD amplification pattern of *in vitro* mutants of anthurium with primer OPB 15

selected variants of 10 Gy. The molecular weight of the bands varied from 0.200 to 1.815 kb.

OPB 18 with 15 Gy:

A total of eight amplicons were obtained after DNA amplification with the primer OPB 18 with 15 Gy treatments. The pattern of amplification is shown in Plate 13c. All amplicons obtained with this primer were monomorphic for the selected variants of 15 Gy. The molecular weight of the bands varied from 0.200 to 1.815 kb.

OPB 18 with 0.1 per cent EMS:

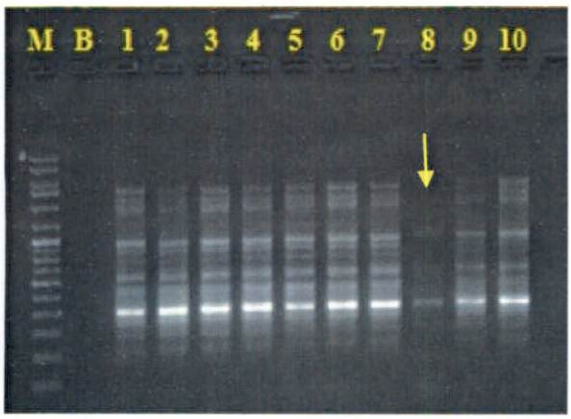
Eight clear amplicons were obtained after DNA amplification with the primer OPB 18 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 13d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.200 to 1.815 kb.

OPB 20 with 5 Gy:

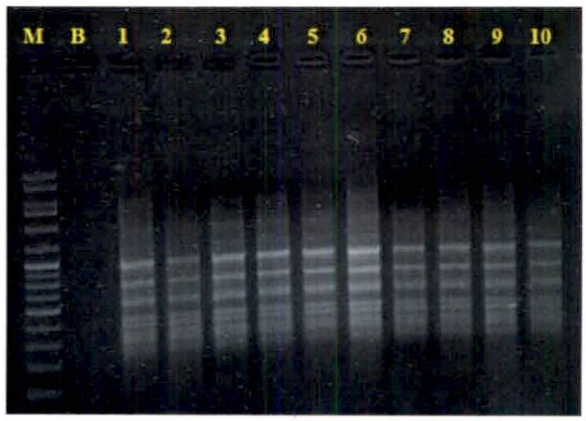
Amplification with the primer OPB 20 with 5 Gy treatments generated nine amplicons. The pattern of amplification is shown in Plate 16a. The pattern of amplification is shown in Plate 14a. Only one band was polymorphic; rest was monomorphic for the selected variants of 5 Gy. The molecular weight of the bands varied from 0.250 to 2.250 kb.

OPB 20 with 10 Gy:

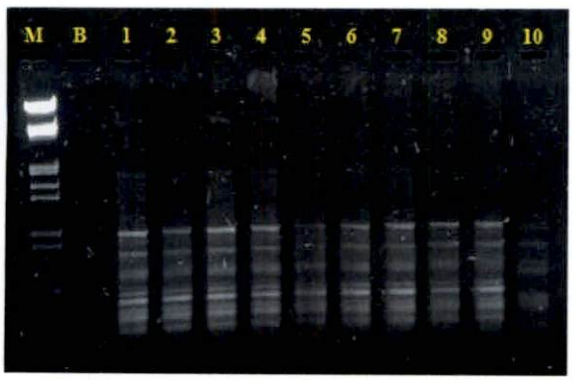
A total of nine amplicons were obtained after DNA amplification with the primer OPB 20 with 10 Gy treatments. The pattern of amplification is shown in Plate 14b. All amplicons obtained with this primer were monomorphic for the selected variants of 10 Gy except plnt no. 6. The molecular weight of the bands varied from 0.250 to 2.250 kb.



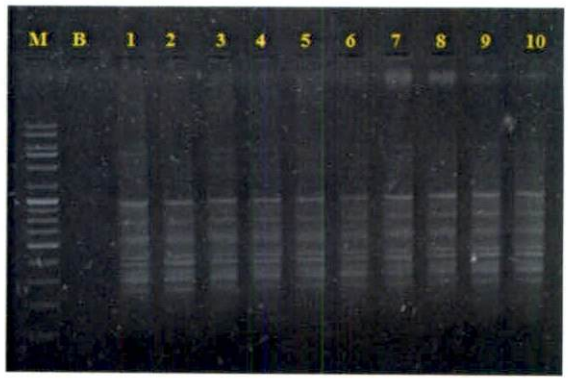
a. Amplification of 5 Gy plants with primer OPB 18



b. Amplification of 10 Gy plants with primer OPB 18



c. Amplification of 15 Gy plants primer OPB 15



d. Amplification of 0.1% EMS plants with with primer OPB 15

M: 1Kb ladder/Marker, B: Control ; 1- 5 control, 6-10 mutant plants

Plate 13: RAPD amplification pattern of *in vitro* mutants of anthurium with primer OPB 18

OPB 20 with 15 Gy:

A total of nine amplicons were obtained after DNA amplification with the primer OPB 20 with 15 Gy treatments. The pattern of amplification is shown in Plate 14c. All amplicons obtained with this primer were monomorphic for the selected variants of 15 Gy. The molecular weight of the bands varied from 0.250 to 2.250 kb.

OPB 20 with 0.1 per cent EMS:

A total of nine amplicons were obtained after DNA amplification with the primer OPB 20 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 14d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.250 to 2.250 kb.

4.5.4 ISSR analysis

Eight ISSR primers reported by Buldewo *et al.* (2012) were selected. After initial screening out of eight five primers were selected. List and sequence information of selected ISSR primers is given in Table 17.

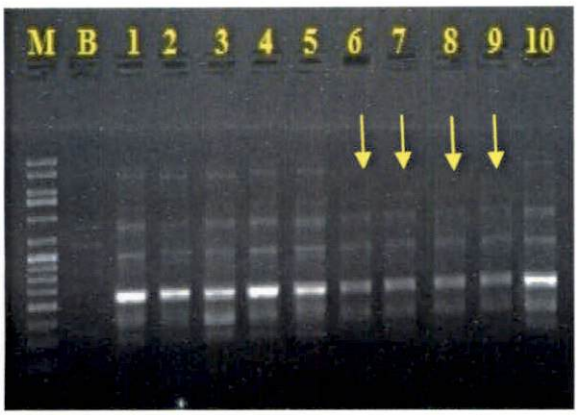
ISSR analysis was carried out with three controls and six *in vitro* mutagenized plants to characterize the mutants.

4.5.5 Regenerants from *in vitro* mutants

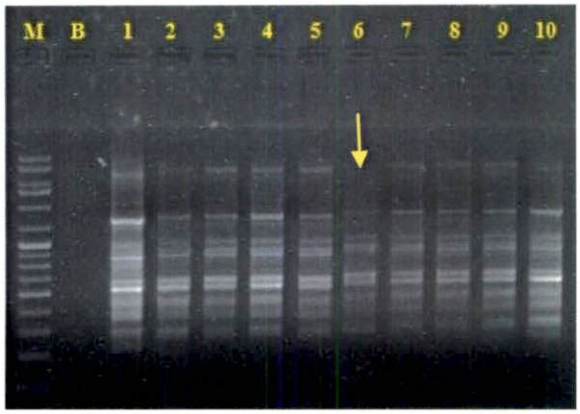
The amplification pattern obtained for each of the primer with respect to plants derived from different doses of mutagen is provided in Plates 15 to 19. Details are as follows.

B2 with 5 Gy:

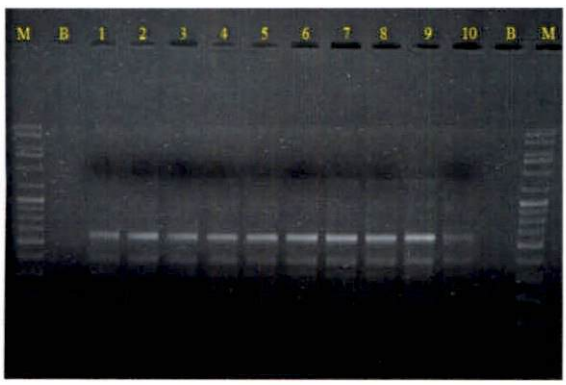
Amplification with 5 Gy treatments with primer B2 generated ten clear amplicons of which two were polymorphic. The pattern of amplification is shown in Plate 15a.



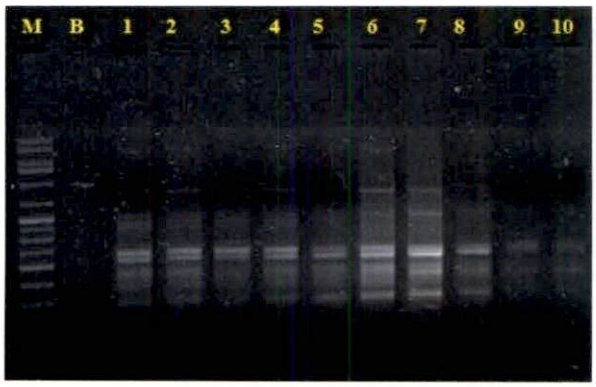
a. Amplification of 5 Gy plants with primer OPB 20



b. Amplification of 10 Gy plants with primer OPB 20



c. Amplification of 15 Gy plants primer OPB 20



d. Amplification of 0.1% EMS plants with with primer OPB 20

M: 1Kb ladder/Marker, B: Control ; 1- 5 control, 6-10 mutant plants

Plate 14: RAPD amplification pattern of *in vitro* mutants of anthurium with primer OPB 20

Table 17. Details of primers selected for ISSR assay

Sl. No.	Primer	Nucleotide Sequence (5'-3')
1	B2	5'- GGCGTCGGTTTCCATTAT -3'
2	B11	5'- TGTGCCGACGATGTTGATGCAAT -3'
3	AN3	5'- ACTTCATGCTATGTGGCGACT -3'
4	ISSR2	$(CTGA)_4$
5	ISSR3	$(CCA)_5$

The variants showed two polymorphic amplicons in each except plant 2. The molecular weight of the bands varied from 0.300 to 2.350 kb.

B2 with 10 Gy:

Ten clear amplicons were observed on the agarose gel for the DNA amplified with the primer B2 with 10 Gy treated plants (Plate 15b). Three bands were polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.275 to 2.350 Kb.

B2 with 15 Gy:

Amplification with 15 Gy treatments with primer B2 generated ten clear amplicons of which two to three bands were polymorphic. The pattern of amplification is shown in Plate 15c. The molecular weight of the bands varied from 0.275 to 2.350 Kb.

B2 with 0.1 per cent EMS:

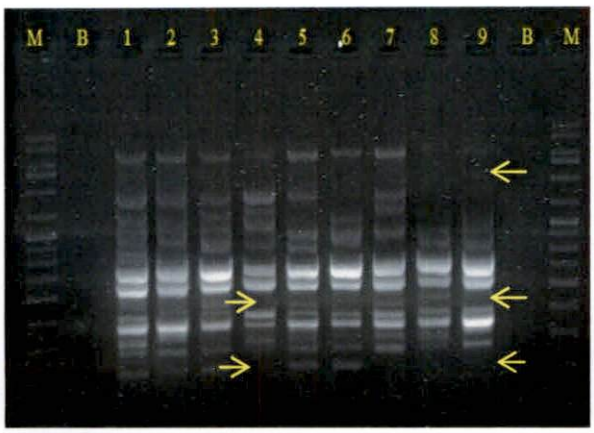
Ten clear amplicons were observed on the agarose gel for the DNA amplified with the primer B2 with 0.1 per cent EMS treated plants (Plate 15d). Three bands were polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.275 to 2.350 Kb.

B11 with 5 Gy:

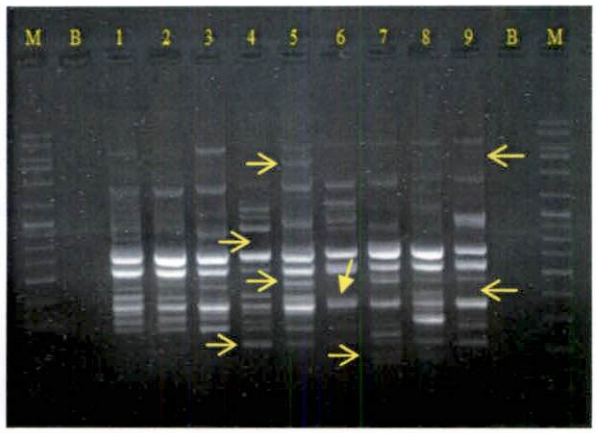
Seven clear amplicons were observed on the agarose gel for the DNA amplified with the primer B11 with 5 Gy treated plants (Plate 16a). Only one band was polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.150 to 1.000 Kb.

B11 with 10 Gy:

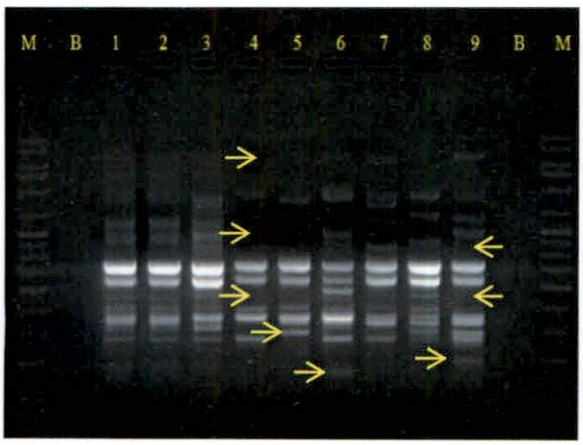
Seven clear amplicons were observed on the agarose gel for the DNA amplified with the primer B11 with 10 Gy treated plants (Plate 16b). Two bands



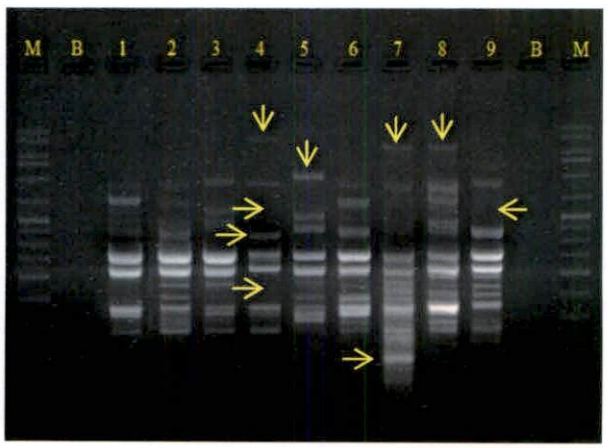
a. Amplification of 5 Gy plants with primer B2



b. Amplification of 10 Gy plants with primer B2



c. Amplification of 15 Gy plants primer B2



d. Amplification of 0.1% EMS plants with with primer B2

M: 1Kb ladder/Marker, B: Control ; 1- 3 control, 6-9 mutant plants

Plate 15: ISSR amplification pattern of *in vitro* mutants of anthurium with primer B2

were polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.175 to 0.650 Kb.

B11 with 15 Gy:

Seven clear amplicons were observed on the agarose gel for the DNA amplified with the primer B11 with 15 Gy treated plants (Plate 16c). Only one band was polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.150 to 1.000 Kb.

B11 with 0.1 per cent EMS:

A total of seven amplicons were obtained after DNA amplification with the primer B 11 with 0.1 per cent EMS treatments. The pattern of amplification is shown in Plate 16d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.150 to 1.000 Kb.

AN3 with 5 Gy:

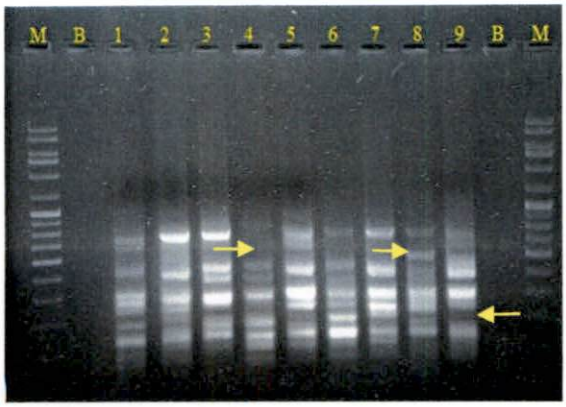
Amplification with 5 Gy treatments with primer AN3 generated fourteen clear amplicons. All amplicons obtained with this primer were monomorphic for the selected variants. The pattern of amplification is shown in Plate 17a. The molecular weight of the bands varied from 0.100 to 2.800 kb.

AN3 with 10 Gy:

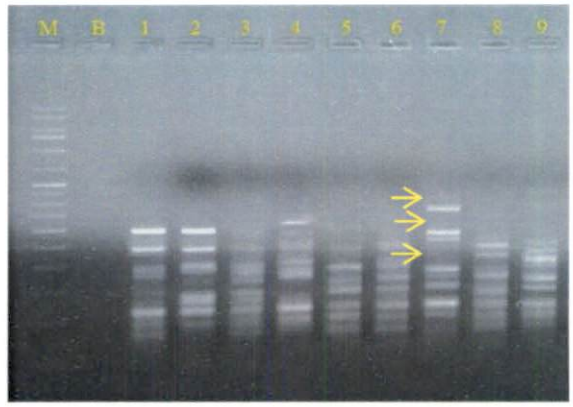
Fourteen clear amplicons were observed on the agarose gel for the DNA amplified with the primer AN3 with 10 Gy treated plants (Plate 17b). Two bands were polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.275 to 1.750 Kb.

AN3 with 15 Gy:

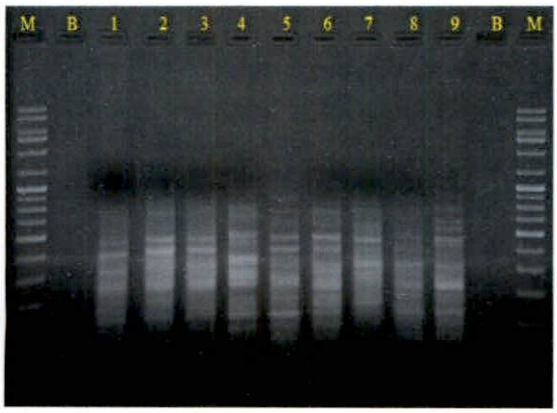
A total of fourteen amplicons were obtained after DNA amplification with the primer AN3 with 15 Gy treated plants. The pattern of amplification is shown



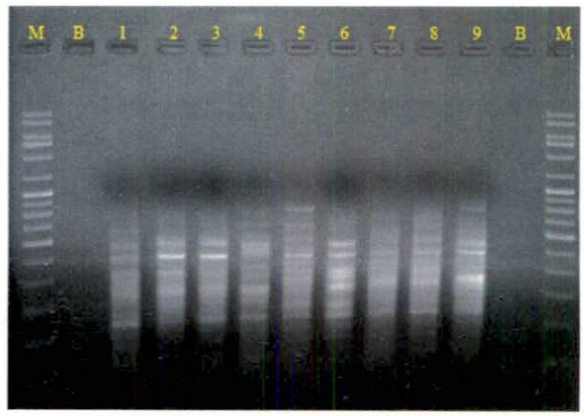
a. Amplification of 5 Gy plants with primer B11



b. Amplification of 10 Gy plants with primer B11



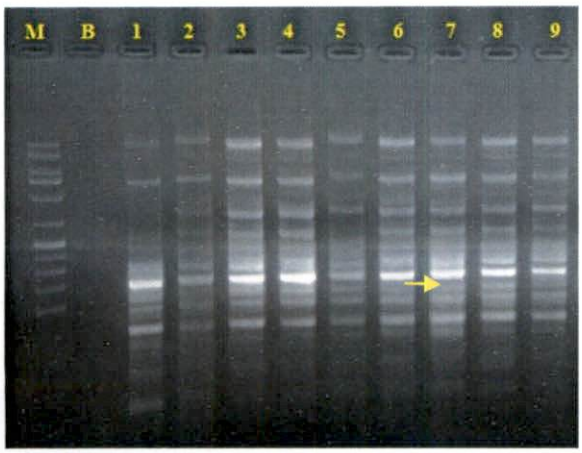
c. Amplification of 15 Gy plants primer B11



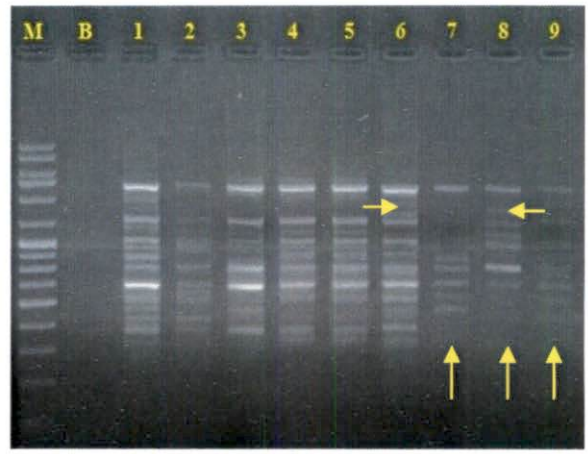
d. Amplification of 0.1% EMS plants with with primer B11

M: 1Kb ladder/Marker, B: Control ; 1- 3 control, 6-9 mutant plants

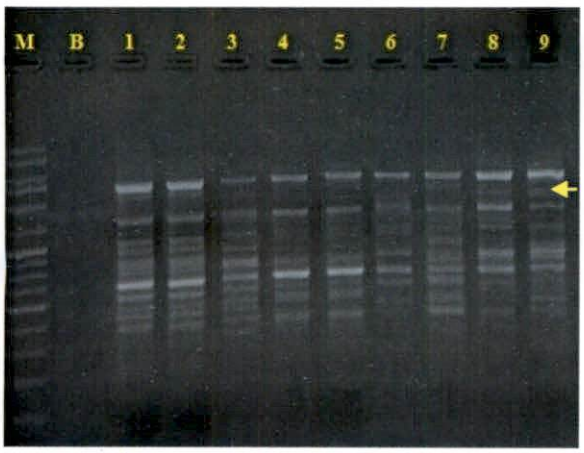
Plate 16: ISSR amplification pattern of *in vitro* mutants of anthurium with primer B11



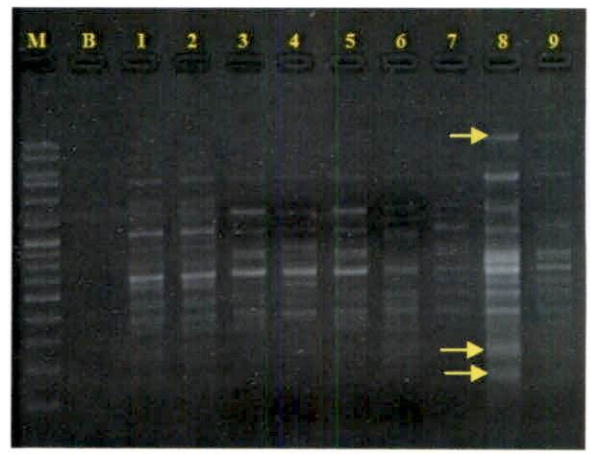
a. Amplification of 5 Gy plants with primer AN3



b. Amplification of 10 Gy plants with primer AN3



c. Amplification of 15 Gy plants primer AN3



d. Amplification of 0.1% EMS plants with with primer AN3

M: 1Kb ladder/Marker, B: Control ; 1- 3 control, 6-9 mutant plants

Plate 17: ISSR amplification pattern of *in vitro* mutants of anthurium with primer AN3

in Plate 17c. Only one band was polymorphic; rest was monomorphic. The molecular weight of the bands varied from 0.275 to 1.750 Kb.

AN3 with 0.1 per cent EMS:

Fourteen clear amplicons were observed on the agarose gel for the DNA amplified with the primer AN3 with 0.1 per cent EMS treated plants (Plate 17d). Two bands were polymorphic; rest was monomorphic. The molecular weight of the products ranged between 0.275 to 1.750 Kb.

ISSR 2 with 5 Gy:

A total of eleven amplicons were obtained after DNA amplification with the primer ISSR 2 with 5 Gy treatments. The pattern of amplification is shown in Plate 18a. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.425 to 2.250 kb

ISSR 2 with 10 Gy:

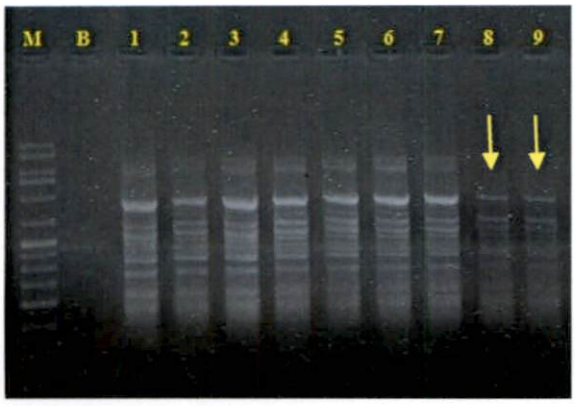
Amplification with 10 Gy treatments with primer ISSR 2 generated eleven clear amplicons. All amplicons obtained with this primer were monomorphic for the selected variants. The pattern of amplification is shown in Plate 18b. The molecular weight of the bands varied from 0.425 to 2.250 kb.

ISSR 2 with 15 Gy:

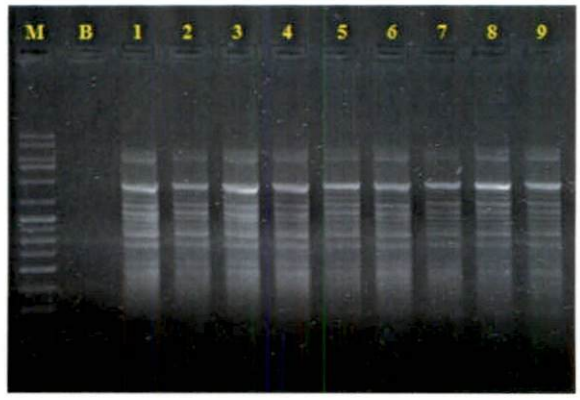
Eleven clear amplicons were observed on the agarose gel for the DNA amplified with the primer ISSR 2 with 15 Gy treated plants (Plate 18c). All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the products ranged between 0.425 to 2.250 kb.

ISSR 2 with 0.1 per cent EMS:

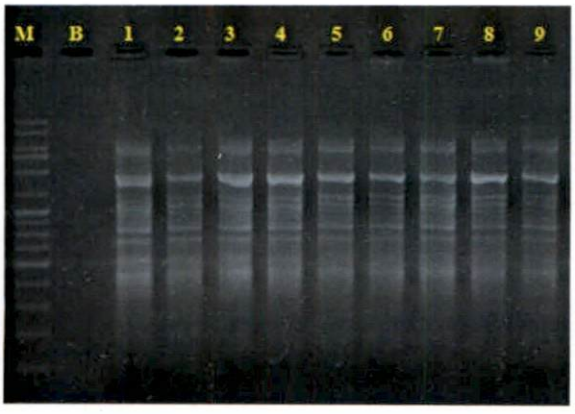
A total of eleven amplicons were obtained after DNA amplification with the primer ISSR 2 with 0.1 per cent treatments. The pattern of amplification is



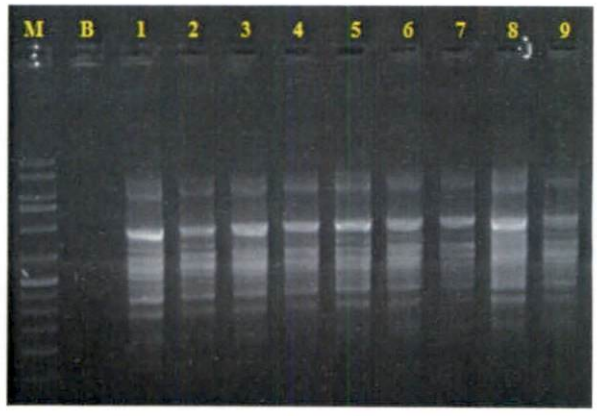
a. Amplification of 5 Gy plants with primer ISSR 2



b. Amplification of 10 Gy plants with primer ISSR 3



c. Amplification of 15 Gy plants primer ISSR 2



d. Amplification of 0.1% EMS plants with with primer ISSR 2

M: 1Kb ladder/Marker, B: Control ; 1- 3 control, 6-9 mutant plants

Plate 18: ISSR amplification pattern of *in vitro* mutants of anthurium with primer ISSR 2

shown in Plate 18d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.425 to 2.250 kb.

ISSR 3 with 5 Gy:

Seven clear amplicons were observed on the agarose gel for the DNA amplified with the primer ISSR 3 with 5 Gy treated plants (Plate 19a). All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the products ranged between 0.325 to 1.500 kb.

ISSR 3 with 10 Gy:

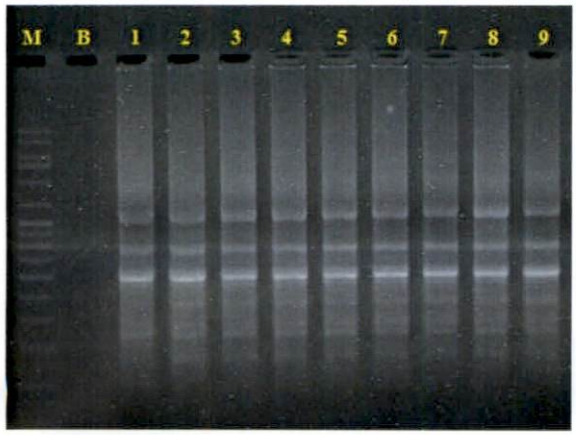
Amplification with 10 Gy treatments with primer ISSR 3 generated seven clear amplicons. All amplicons obtained with this primer were monomorphic for the selected variants. The pattern of amplification is shown in Plate 19b. The molecular weight of the bands varied from 0.325 to 1.500 kb.

ISSR 3 with 15 Gy:

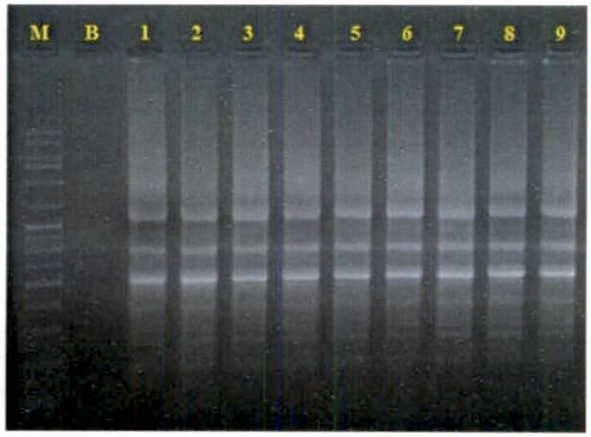
A total of seven amplicons were obtained after DNA amplification with the primer ISSR 3 with 15 Gy treatments. The pattern of amplification is shown in Plate 19c. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.325 to 1.500 kb.

ISSR 3 with 0.1 per cent EMS:

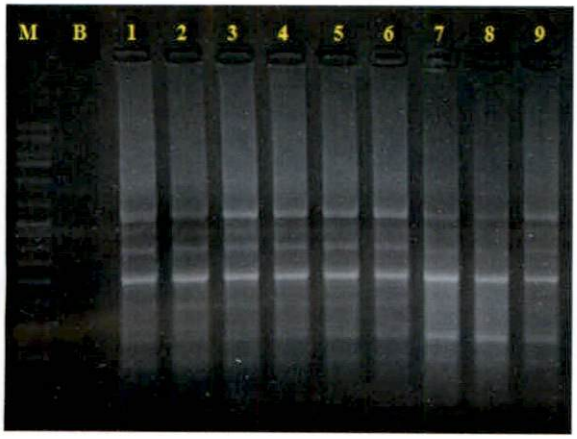
A total of seven amplicons were obtained after DNA amplification with the primer ISSR 3 with 0.1 per cent treatments. The pattern of amplification is shown in Plate 19d. All amplicons obtained with this primer were monomorphic for the selected variants. The molecular weight of the bands varied from 0.325 to 1.500 kb.



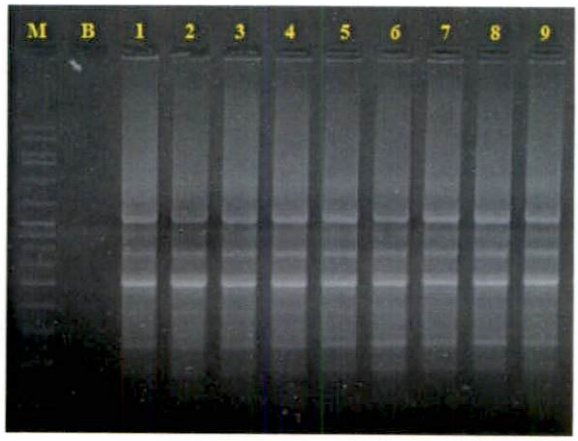
a. Amplification of 5 Gy plants with primer ISSR 3



b. Amplification of 10 Gy plants with primer ISSR 3



c. Amplification of 15 Gy plants primer ISSR 3



d. Amplification of 0.1% EMS plants with with primer ISSR 3

M: 1Kb ladder/Marker, B: Control ; 1- 3 control, 6-9 mutant plants

Plate 19: ISSR amplification pattern of *in vitro* mutants of anthurium with primer ISSR 3

Discussion

5. DISCUSSION

In vitro mutagenesis in anthurium (*Anthurium andreanum* Lind.) var. Tropical was attempted in order to create variability and widen the genetic base. Plantlets regenerated at different subcultures of *in vitro* mutated cultures were hardened and evaluated for morphological and molecular parameters. The results obtained in different aspects of the study are discussed in this chapter.

Anthurium is one of the most popular of the mostly sub tropical 18- 28 ^{0}C range cut flowers which are being grown commercially for export as well as for the local market. It belongs to the complex family Araceae. Within the family Araceae, anthurium is the largest genus, which comprises of over 900 species, including well known cultivated varieties *Anthurium andreanum* and *Anthurium scherzerianurn.* They are cultivated for its attractive long lasting 'flower' which is not really a flower but an inflorescence subtended long a colourful bract. Majority of anthurium species have 2n = 30 chromosomes. Some are polyploidy with 2n = 60, while a few species have 2n = 20 to 124 chromosomes (Petersen, 1989).

Anthurium is conventionally propagated through vegetative methods such as stem cuttings and suckers which are tedious and not practical when carried out on a large scale. Though the flowers rarely set seeds, viability and germination percentage are very low. Seeds are viable only for two-three days and germination is low as 20 to 30 per cent. Hence, there is a need to standardize a quicker method of propagation which may be achieved through *in vitro* techniques (Jahan *et al.*, 2009). Tissue culture greatly increases the normal multiplication rate of plants and can provide a source for *in vitro* mutagenesis the best option for inducing variability in vegetatively propagated plants.

The method for *in vitro* production of plantlets of *Anthurium andreanum* was first developed by Pierik *et al.* (1974). The production of *in vitro* plants directly from proliferating axillary buds (Kunisaki, 1980), adventitious buds (Cen *et al.*, 1993), leaf or petiole derived callus (Finnie and Van Staden, 1986 and Kuehnle and Sugii, 1991 and Kuehnle *et al.*, 1992) etc. have been reported.

All the workers have reported that there was great variation in the in *vitro* requirements of different genotypes. Mutagenic agents have been used to induce useful phenotypic variations in plants for more than 70 years (Foster and Twell, 1996). For anthurium, new techniques are needed for further improving crop cultivars apart from the traditional plant breeding since they have a narrow genetic base. Mutation breeding is therefore being proposed as a means to create additional variation. The applications of ionizing radiation and chemical mutagens as well as use of somaclonal variation from tissue culture are some options for creating genetic variation.

5.1 *In vitro* multiplication

The *in vitro* multiplying cultures supplied from the Centre for Plant Biotechnology and Molecular Biology when inoculated in full MS media supplemented with 2 mg 2ip gave good multiplication and plant development. In same media healthy shoots started producing normal roots. Hence no separate rooting media was tried for rooting. Rooted plantlets were successfully planted out and hardened.

Pierik *et al.* (1979) highlighted that good callus induction in anthurium could be obtained with 10 mg/l of 2ip to the medium. Viegas *et al.* (2007) statistically analysed the importance of 2ip for callus induction, they used media with and without 1 mg/l 2ip. After the incubation they found that 2ip supplemented medium was significantly superior to the 2ip free medium.

Gabryszewska and Rudnicki (1997) developed a micropropagation protocol for *F. benjamina* by using shoot meristems; shoot numbers increased on MS medium supplemented with 15 mg/l 2ip by red light treatment.

Most of the studies related to callus formation in Anthuriums have been performed on leaf explants using 1 mg/l BA or BAP and 0.1 mg/l 2,4-D (Puchooa, 2005). Jahan *et al.* (2009) obtained the highest proliferation of Anthuriums on medium enriched with BAP. The combination of BA and NAA improved the

shoot proliferation in *Anthurium andreanum*. The combination of 1 mg/l BA + 0.01 mg/l NAA with 22.83 shoots per explant was found to be the most suitable growth regulator. Rooting is usually induced by auxins and IBA and NAA are reported to be more effective for rooting in anthurium compared with other auxins (Malhotra *et al.*, 1998; Puchooa and Sookun, 2003).

5.2 *In vitro* mutagenesis

In the early years of the breeding technology for inducing mutations, whole plants, seeds and bulbs were often used as receptor materials. However, problems such as low mutation efficiency and chimera often occur. Combining conventional breeding technology with tissue culture to induce mutations has been reported to have apparent advantages (Lee *et al.* 2003; Velmurugan *et al.*, 2010).

5.2.1 Gamma irradiation

Induced mutation combined with *in vitro* culture techniques are often used to make variation in crops with narrow genetic base. Gamma irradiation is the common method employed for physical mutagenesis.

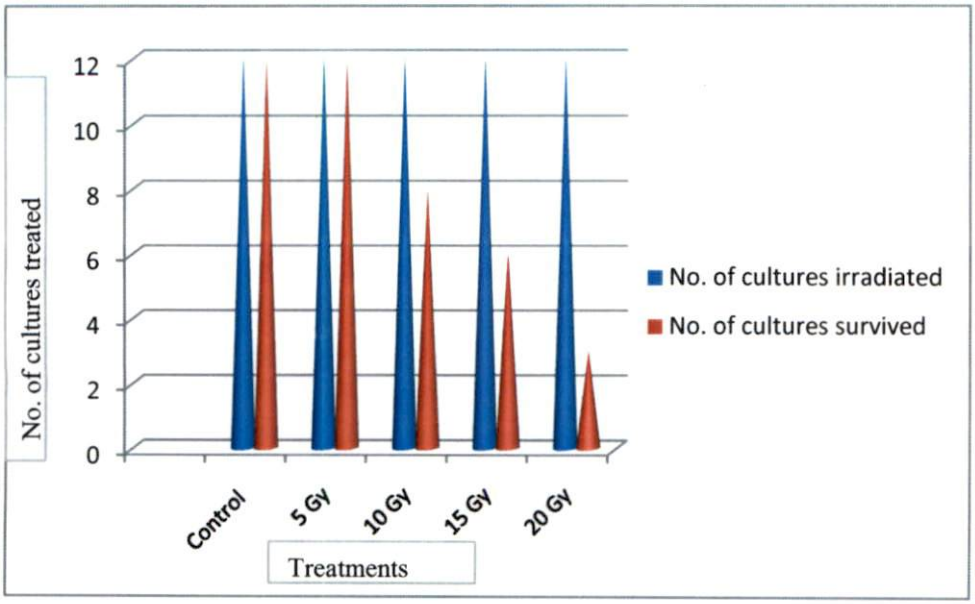
Irradiation by this physical mutagenic agent leads to DNA break via direct and indirect detrimental effects. In direct interactions, the radiation energy is transferred to the targets; in indirect interactions, energy is absorbed by the water present in the external medium. After hydrolysis of water, seconder messenger molecules (H_2O_2, O_2- OH) affect the biomolecules (Esnault *et al.*, 2010). The ionizing gamma rays can produce free radicles in cells which inturned can damage or modify important component of plant cell. This can affect morphology, anatomy, biochemistry and physiology of plants at varying level depending on irradiation level. Such effects will also include changes in cellular structure and metabolism of plants (Kim *et al.*, 2004; Girija *et al.*, 2013).

The free radicles can result in breakage of chemical bonds or oxidation of affected molecules. The major effect in cell is DNA breakage, since DNA consist

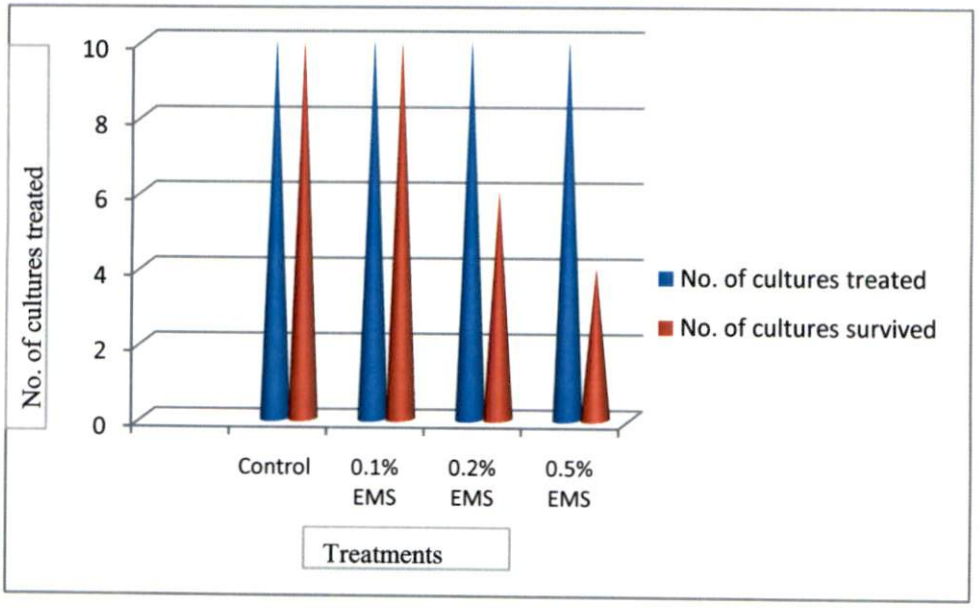
of a pair of complementary double strands, break of either a single strand or both strands can occur. Most single strand break can be repaired. In the case of double strand break repair is more difficult and erroneous rejoining of broken ends may occur. These so called misrepairs result in induction of mutation, chromosome aberration and cell death. Deletion of DNA segment is the predominant form of irradiation damage in cells. It may be caused by misrepairs of two separate double strand break in DNA molecule with joining of the two outer ends and loss of the fragment between the breaks (Yamaguchi, 2005).

Ethyl methane sulphonate (EMS) is an alkylating agent inducing chemical modification of nucleotides, which result in mispairing and base changes. Strong biased alkylation of guanine (G) residue results, forming O6-ethyl guanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A/T. Ninety-nine per cent of mutations from alkylation of guanine induced by EMS are reported as G/C-to-A/T transitions (Greene *et al.*, 2003).

In the present study lethal effects of gamma irradiation were observed at doses higher than 15 Gy while the survival rate was above 50 per cent at lower doses. Irradiation at 5 Gy did not influence the survival of cultures as it was comparable with the untreated control (Table 4 and Fig. 1). Though 50 per cent cultures survived when treated with 15 Gy, the callus were weak with very few shoot initials. The number of shoot initials per culture even after three sub culture were only 8 in such treatment whereas, it was three fold (24) in control plants (Table 5). It was also obvious to observe better performance at cultures treated with 5 Gy, which gave more number of shoot initials in 1st and subsequent subcultures. The higher dose of 20 Gy was lethal to the cultures and was evident from morphology of cultures (Table 5 and Plate 2). Outer cells of such cultures became brown and appeared as if they were decayed within one month. The emergence of shoot initials from survived cultures of high dose was very slow and might be from inner healthy cells in the mass which was less affected by the



a- Effect of gamma irradiation on *in vitro* cultures of anthurium var. Tropical



b- Effect of EMS treatment on *in vitro* cultures of anthurium var. Tropical

Fig. 1. Effect of gamma and EMS treatment on *in vitro* cultures of anthurium var. Tropical

strong dose. The variation observed for *in vitro* response of irradiated cultures might be due to the intensity of radiation effect on DNA breakage.

Mini, 1998 also attempted the *in vitro* mutagenesis in anthurium in the range of 25 to 400 Gy. She has found that doses above 150 Gy were lethal to callus as well as shoot tips. Maximum response in terms of plant height, plant spread and leaf area was recorded at lower doses of 50 Gy. Where as Leena, 2003 found that irradiation at 0.5 to 10 Gy was effective. Lower doses induced pale green leaves and clustered appearance while higher doses induced browning of callus.

In vitro mutation in anthurium through gamma irradiation was also reported by Puchooa (2005) from Mauritius. He has also reported the beneficial effects when treated with 5 Gy and lethal effects at 15 Gy.

Kahrizi *et al.* (2011) had shown the effect of irradiation on survival rates of axillary buds in two cultivars of rose ('Maurossia' and 'Apollo'). Higher dosage (100 Gy) of radiation resulted in higher death rates and there was no significant difference between the control plants and plants irradiated with low dosage, i.e., 20 Gy. However, the survival rate was reported to get reduced to 20 per cent at 100 Gy.

5.2.2 Ethyl methane sulphonate (EMS) treatment

EMS treatment was observed to have more drastic effect than gamma irradiation on anthurium cultures. Though the cultures were found to survive even after 0.5 per cent treatment, the survival rate was low and number and number of healthy shoot initials produced were poor at doses above 0.1 per cent (Table 8 and 9). *In vitro* mutagenesis with EMS has been attempted earlier in anthurium by Te-chato and Susanon (2005) in a variety with pink spadix. They have treated the callus and have reported to survival even at 0.75 per cent. The deviation in results in the present study might be due to the difference in the source material used for treatment. The *in vitro* cultures with tender shoot initials with tender multiplying

shoot initials utilised in the present study might be more sensitive to the treatment than undifferentiated callus reported earlier.

Liu *et al.* (2006) and Xu *et al.* (2007) have also reported sensitivity of different plant tissue to chemical mutagen, they have reported plant pollen and seeds to thrive over higher doses of EMS.

EMS induces DNA damage or variations by chemical modification of nucleotides, which result in mispairing and base changes. Strong biased alkylation of guanine (G) residue results, forming O6-ethyl guanine, which can pair with thymine (T) but not with cytosine (C). Through subsequent DNA repair, the original G/C pair can then be replaced with A/T. Most of the mutations induced by EMS are reported as G/C-to-A/T transitions (Greene *et al.*, 2003).

Stimulatory effects of lower concentrations of chemical mutagens have also been reported by Zargar *et al.* (1994). Increase in the dose of chemical mutagens caused damaging effects on biological activities of plants which may be due to inactivation of cells because of mitotic disturbances and chromosomal aberrations at higher doses of EMS, leading to poor growth of the plants.

Singh *et al.* (2000) observed moderate doses of EMS to have stimulatory effect on growth of some vegetative as well as floral characters in carnation. Maximum abnormalities including chlorosis in leaves were observed at 1.00 per cent concentration of EMS.

Hofmann *et al.* (2004) subjected soybean embryogenic culture to ethyl methane sulphonate (EMS) (1to 30 mM), and found a decrease in survival rate of embryogenic culture with increasing concentrations of EMS.

Jong, (2011) reported direct diffusion of EMS into *Saintpaulia* leaves as evidenced by the gradual loss of explant viability with increasing EMS exposure and concentration.

5.3 Morphological characterization

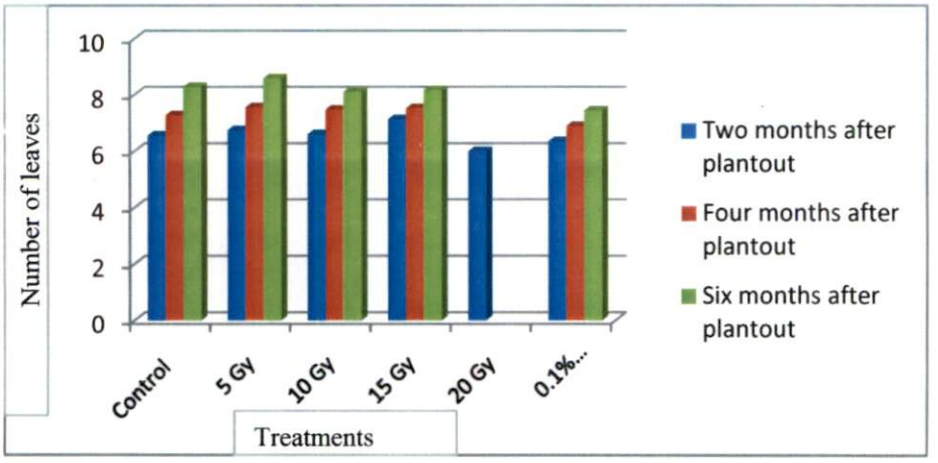
The mutant plants derived from *in vitro* culture of anthurium obtained from different subculture intervals were hardened successfully. The morphological data were recorded at every two months interval for six months.

The number of leaves varied significantly with respect to different irradiation doses. Highest number of leaves was observed in 5 Gy (6.75) (Table 12 and Fig. 2). Number of leaves was reduced with increasing dose of irradiation and in 15 Gy plants leaves were short and narrow as compared to control (Plate 7). Abnormal plant growth induced by radiation has been reported by number of workers and has been variously interpreted (Hewawasam *et al,* 2004).

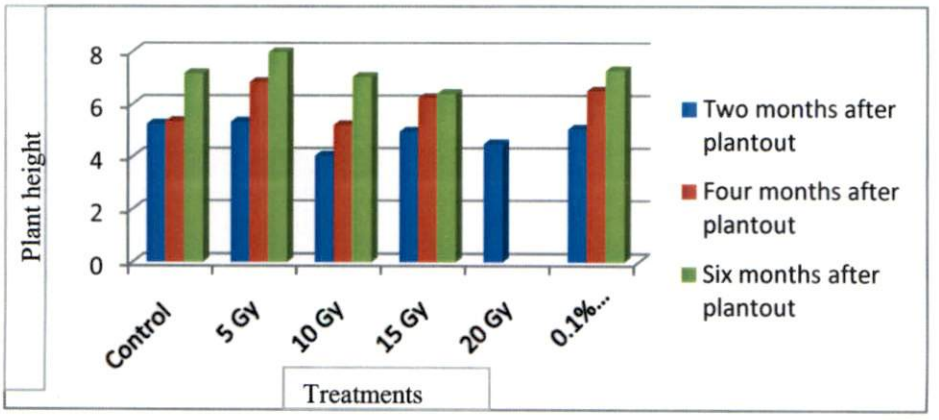
It has been suggested that chromosome breakage, reduction in the auxin level, change in enzyme activity and variation in ascorbic acid concentration are some of the factors which lead to the development of abnormal leaves (Datta, 1997).

The plant height of mutants varied significantly, the highest plant height observed in 5 Gy (5.32) and lowest in 15 and 20 Gy plants. Similar result was reported by Barakat *et al.* (2010) who found that the variant of *Chrysanthemum* treated with the 0.5 Gy dose gave highest mean value of plant height (93.30 cm) compared with the control (85.0 cm) and 1.0 Gy dose; which could be attributed to change in metabolic levels in mutated cultures.

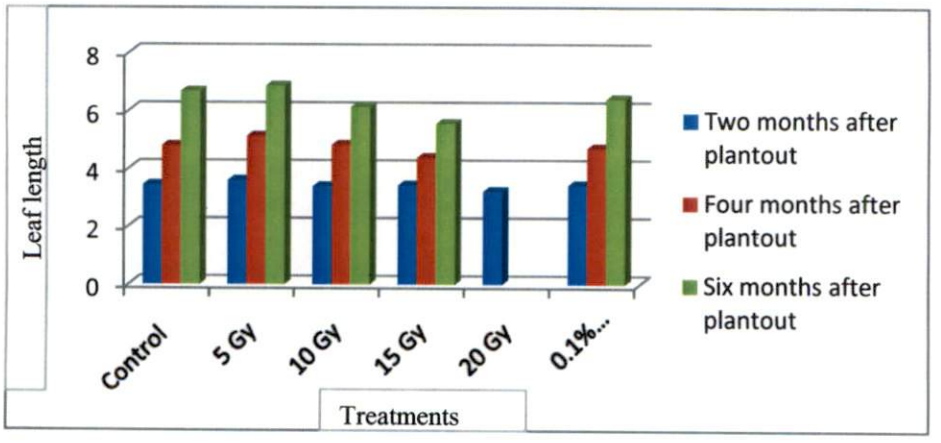
Dhakshanamoorthy *et al.* (2010) observed the maximum plant height at maturity at lower doses of 5 Kr and 1 per cent EMS treatment as compared with higher doses of 25 Kr and 4 per cent EMS in *Jatropha*. Reduction in plant height after exposure to different mutagenic agents has also been reported by Mackey (1956) and Kahrizi *et al.* (2011).



a- No. of leaves



b- Plant height



c- Leaf length of 1st leaf

Fig. 2. Variation in no. of leaves, plant height and leaf length recorded at two months interval

Bharathi *et al.* (2013) observed the maximum number of leaves at lower doses of 5KR in Ashwagandha (*Withania somnifera* (L.). while it was minimum with 50kr gamma rays.

As the dose of gamma rays increased, the shoot length of the regenerated plants was found to decrease (Plate 3). Ndzana *et al.* (2008) and Barakat *et al.* (2010) have also reported that increasing the gamma ray exposure, significantly decreased the plant height.

Similar result was also reported by Bharathi *et al.* (2013) who observed that all the mutagenic treatments caused a reduction in the plant height compared with control in ashwagandha.

5.4 Flower characters

The results indicate that the gamma irradiated plants started flowering within seven months after planting and lot of variations were observed with respect to flower size, colour, length of flower stalk, arrangement of spathe and spadix (Plate 8). The EMS treated plants at 0.1 per cent produced erect spathe. *In vitro* mutagenesis with EMS has been attempted earlier in anthurium by Te-chato and Susanon (2005) in a variety with pink spadix. They have reported smaller size of spathe compared with the control having 14.2 per cent yellow spadix at 0.75 per cent EMS treatment. EMS at 1 per cent gave 60 per cent yellow spadix, whereas the control treatment gave pink spadix. Moreover, spadices from those treated with EMS were shorter with more erect angle (45-90°) than the control treatment (25°).

Laneri *et al.* (1990) obtained several flower mutants in gerbera by gamma irradiation. Jain *et al.* (1998a) reported that gamma irradiation (10 and 20 Gy doses) of two gerbera cultivars produced mutants showing variations in flower colour, flower morphology, and plant morphology.

A red colour mutant with white stripes along with petal length was induced in carnation at 0.075 and 0.100 per cent EMS. Another colour variant (pink with white stripes) was also isolated from the population treated with the doses of 0.75 and 1.00 per cent EMS (Singh *et al.*, 2000).

Lamseejan *et al.* (2003) used chrysanthemum var. 'Taihei' for mutation induction with chronic and acute gamma irradiation treatment, and obtained mutants with different traits such as flower colour, form and size.

Barakat *et al.* (2010) have reported that the irradiation dose 0.5 Gy was the most effective dose in inducing mutations in ray floret shape and number of florets/head and conversion from tubular florets to spoon shaped florets was observed in *Chrysanthemum morifolium*. Xi *et al.* (2012) have also reported the induced variation in flowers with respect to changes in the shape and size in lily.

5.5 Molecular characterization

5.5.1 DNA isolation

The DNA was isolated from young tender leaves of anthurium plants derived from different subcultures. The protocols reported by Rogers and Bendich (1994) with 4x CTAB extraction buffer yielded 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. Liquid nitrogen was used for the homogenisation of the leaf tissue. Liquid nitrogen helps 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 disrupts the protein disulphide bond and is thereby capable of initiating protein degradation. Poly vinyl pyrrolidone (PVP) removes polyphenols

and inhibits co-precipitation of polysaccharides which resulted in good quality DNA. This was confirmed by Matasyoh *et al.* (2008).

The detergent present in the extraction buffer, CTAB helps in the release of nucleic acids into buffer disruption of the cell membrane. The released DNA is 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 freed from chlorophyll by using the chloroform: isoamyl alcohol which aids 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 ranged from 430.2 ng/µl to 3236 ng/µl. The purity (A260/ A280) ranged from 1.82 to 2.15. This could be due to the interference of various compounds in the plant tissue during the procedure.

5.5.2 Molecular marker analysis

In the present study, two PCR- based techniques, RAPD and ISSR were adopted to characterise the variants.

5.5.3 RAPD assay

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. RAPD among other molecular marker methods has considerable advantages because it is fast, not expensive, applicability to any organism without prior information on the nucleotide sequence and in the potential detection of

DNA damage and mutation (Ahloowalia and Maluszynski, 2001; Atienzar *et al.*, 2002).

In the present study five variant plants of each treatment were selected for amplification; all the five controls and treatment plants were amplified with all selected five primers. Out of the five primers OPA 10 and OPB 15 gave monomorphic amplicons while others OPB 6, 18 and OPB 20 gave polymorphic amplicons when amplified with 5 and 10 Gy plants. The polymorphism was obtained in the terms of disappearance of amplicons (Plate 9 and 15).

Details of genetic variations detected in *in vitro* mutated anthurium plants through RAPD assay with five selected primers are presented in Table 18. The primer OPB 6, OPB 18 and OPB 20 have shown polymorphism (11.11 to 14.28%) in 5 and 10 Gy irradiated plants; confirming the genetic variation in the mutants.

Atanassov *et al.* (1996) used gamma rays and sodium azide (SA) to create mutation in barley. They suggested that RAPD assay is a sensitive and representative approach to distinguish the variability created by tissue culture and mutagenesis.

Sandhu *et al.* (2002) irradiated seeds of 21 rice lines with different doses of gamma irradiation and the variation between resistant and susceptible lines for glufosate were analysed with RAPD method. Lema-Rumińska *et al.* (2004) have reported the genetic variations of *Dendranthema grandiflora* Tzvelev irradiated with 5 and 15 Gy gamma radiation dose. They obtained polymorphic bands from the mutant plants with 8 RAPD primers.

Table 18. Genetic variation in *in vitro* mutated anthurium plants detected through RAPD assay

Sl. No.	Primer	Treatment	Total no. of amplicons	No. of polymorphic amplicons	Polymorphism (%)
1	OPB 6	Control	7	-	-
		5 Gy	7	1	14.28
		10 Gy	7	0	0
		15 Gy	7	0	0
		0.1% EMS	7	0	0
2	OPA 10	Control	7	-	-
		5 Gy	7	0	0
		10 Gy	7	0	0
		15 Gy	7	0	0
		0.1% EMS	7	0	0
3	OPB 15	Control	7	-	-
		5 Gy	7	0	0
		10 Gy	7	0	0
		15 Gy	7	0	0
		0.1% EMS	7	0	0
4	OPB 18	Control	8	-	-
		5 Gy	8	1	12.50
		10 Gy	8	0	0
		15 Gy	8	0	0
		0.1% EMS	8	0	0
5	OPB 20	Control	9	-	-
		5 Gy	9	1	11.11
		10 Gy	9	1	11.11
		15 Gy	9	0	0
		0.1% EMS	9	0	0

Puchooa (2005) observed no difference in RAPD profiles between mother *Anthurium* plant and mutant plants irradiated with 5 Gy dose which showed phenotypic differences, where as Lu *et al.* (2007) detected mutagenesis in narcissus, through RAPD marker (8.33%) and AFLP marker (15.48%).

The variation in band intensity and disappearance of some bands may correlate with the level of changes in DNA template after radiation, which can alter the number of binding sites for Taq polymerase. Appearance of new bands can also be explained as a result of DNA structural changes (Breaks, transpositions, deletions, etc) (Danylchenko and Sorochinsky, 2005; Hegazi and Hamideldin, 2010).

Atienzar *et al.* (2000) reported that mutation could only be responsible for the appearance of new bands if they occur at the same locus in a sufficient number of cells (a minimum of 10% of mutations may be required for new PCR product visible in agarose gel). The new bands could be attributed to mutation, while the disappearance of bands could be attributed to DNA damage, DNA methylation, and chromosomal damage.

Kumar *et al.* (2006) detected genetic variability among chrysanthemum radiomutants with RAPD. The cluster analysis of chrysanthemum radiomutants separated then into different groups but the genetic distance, which was observed between them, was low, except in two mutants.

Khan *et al.* (2007) studied genetic variability in sugarcane with induced somatic mutations using gamma ray and their RAPD data showed that the similarity between mutants and parents decreased while the irradiation doses increased. Atak *et al.* (2011) have also reported RAPD analysis to detect genetic difference between *Rhododendron* varieties and mutants, which were exposed to the gamma radiation doses 5 and 10 Gy.

5.5.4 ISSR assay

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 molecular technique based on PCR that identifies insertions and deletions in DNA. It is highly sensitive, highly reproducible, provides Mendelian segregation, and has been successfully applied in genetic and evolutionary studies of many species (Godwin *et al.*, 1997; Zietkiewicz *et al.*, 1994).

In the present study six morphologically variant plants of each treatment and three controls were selected for amplification. All the three control and variant plants were amplified with five selected primers. Out of five primers ISSR 2 and ISSR 3 gave monomorphic amplicons while others B2, B11 and AN3 gave polymorphic amplicons in gamma irradiated and EMS treated variants. The polymorphism was obtained in terms of appearance and disappearance of amplicons (Plates 19-25).

Details of genetic variations detected through ISSR assay with five selected primers are presented in Table 19. The primers B2, B11, AN3 and ISSR 2 were good enough to detect polymorphism (7.14 to 28.57%) in all the treatments. ISSR assay was found to be better to detect genetic variation in the mutant generated.

Some of these changes appeared identical in different plants as represented by appearance of non-parental bands. The reason for such commonness of genetic variation in these plants could be because they were all derived from the same culture. However it could be suggested that mutations have induced varied amount of genetic changes in different regenerated plants.

Table 19. Genetic variation in *in vitro* mutated anthurium plants detected through ISSR assay

Sl. No.	Primer	Treatment	Total no. of amplicons	No. of polymorphic amplicons	Polymorphism (%)
1	B 2	Control	10	-	-
		5 Gy	10	2	20
		10 Gy	10	2	20
		15 Gy	10	3	30
		0.1% EMS	10	2	20
2	B 11	Control	7	-	-
		5 Gy	7	2	28.57
		10 Gy	7	2	28.57
		15 Gy	7	0	0
		0.1% EMS	7	0	0
3	AN 3	Control	14	-	-
		5 Gy	14	0	0
		10 Gy	14	2	14.28
		15 Gy	14	1	7.14
		0.1% EMS	14	2	14.28
4	ISSR 2	Control	11	-	-
		5 Gy	11	1	9.09
		10 Gy	11	0	0
		15 Gy	11	0	0
		0.1% EMS	11	0	0
5	ISSR 3	Control	7	-	-
		5 Gy	7	0	0
		10 Gy	7	0	0
		15 Gy	7	0	0
		0.1% EMS	7	0	0

The presence of specific bands/loci in the parental clones and loss of them in the regenerated parental plantlets indicates the loss of certain loci during tissue culture due to *in vitro* mutagenesis (Newbury *et al.*, 2000).

The absence of a band/ loci in one of the genotypes indicates genetic changes of the plants brought about during *in vitro* mutagenesis (Soniya *et al.*, 2001). Even single base change at the primer annealing site is manifested as appearance or disappearance of ISSR bands (Creste *et al.*, 2003).

Occurrence of specific bands/loci in the regenerated plants and their absence in mother plants may indicate the occurrence of genetic changes leading to formation of new binding sites in these plants. Such specific loci are of high importance in the genetic identification of the somaclones from each other (Uma *et al.*, 2006).

The variations observed in the ISSR pattern has been attributed to different causes including loss/ gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements in banana (Muhammad & Othman, 2005).

However, Xi *et al.* (2012) observed no difference compared with the control by using the 7 ISSR primers, indicating that these four lines have no variation at the DNA level in lily.

In vitro mutagenesis in anthurium was done by using physical (gamma irradiation) and chemical mutagen (EMS). Both morphological and molecular analysis confirmed variation among the plants derived through *in vitro* mutagenesis in anthurium var. Tropical. The present investigations reveal that induced mutation through tissue culture techniques is highly desirable for developing new variants in anthurium.

Summary

6. SUMMARY

The study entitled "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2012-2014, with the objective to induce variation in anthurium var. Tropical through *in vitro* mutagenesis and to characterise the variability through morphological and molecular assay.

The salient features of findings are as follows:

1. *In vitro* multiplying culture of anthurium var. Tropical obtained from Centre for Plant Biotechnology and Molecular Biology (CPBMB) were multiplied in large numbers in MS medium supplemented with 2ip (2 mg/l).

2. *In vitro* derived multiplying cultures of var. Tropical were utilized for *in vitro* mutagenesis at Radio Tracer Laboratory (RTL), College of Horticulture. Cultures were exposed to different levels of gamma radiation (0, 5, 10, 15 and 20 Gy) from ^{60}Co source.

3. Cultures exposed to higher doses lost their green appearance while those at lower doses (5 Gy) were better in multiplication and number of healthy shoots produced, compared to control. The cultures were sub cultured thrice at 45 days interval. Lower doses of 5 and 10 Gy gave healthy shoots which rooted in the 3rd subculture.

4. The *in vitro* response of irradiated cultures varied with respect to number of shoot initials, number of healthy shoots and *in vitro* rooting percentage.

5. The survival rate was poor at higher doses of 15 and 20 Gy. Though the cultures turned black and shoot initials were low up to the 2nd

subculture, it started regeneration from the surviving tissues by the 3rd subculture.

6. The chemical mutagen EMS had a drastic influence on anthurium cultures than gamma irradiation. Healthy shoots were produced only in cultures treated with 0.1 per cent EMS. Shoot initials produced at higher doses (0.2 and 0.5%) were very weak and failed to produce roots. Survival rate of such unhealthy shoots were nil at hardening stage. However, 0.1 per cent EMS treatment recorded 44 per cent survival at hardening stage.

7. The plants recovered from lower doses (5 Gy and 0.1% EMS) of mutagenesis were found superior with respect to plant height and leaf dimensions. Plants regenerated from cultures treated with higher doses of mutagen were stunted in appearance with narrow and smaller leaves.

8. Precocious flowering was observed in plants regenerated from 5 Gy treated cultures. Over 73 per cent flowering was observed in 5 Gy treated plants as against 57 per cent in control plants within nine months after planting.

9. Variations were observed in flower characters with respect to flower size, colour, length of flower stalk, spathe and spadix.

10. The selected variants were analysed at molecular level using RAPD and ISSR assay to confirm variation at genomic level.

11. The protocol suggested by Rogers and Bendich (1994) was used for extraction of genomic DNA from young and tender leaves of variants and control plants.

12. Primers already reported for anthurium were used for characterization. Fifteen primers were initially screened for the purpose and ten promising ones selected for characterizing the mutants. Out of five

RAPD primers, OPA 10 and OPB 15 did not detect any variation among regenerants. While others (OPB 6, 18 and OPB 20) gave polymorphic amplicons in some of the variants derived from 5 and 10 Gy treated cultures.

13. ISSR assay was found ideal to detect variation in mutant plants. Out of the five primers tested, B2, B11 and AN3 gave polymorphic amplicons in secondary hardened *in vitro* mutated plants.

14. The study confirmed variation at genomic level induced through *in vitro* mutagenesis in anthurium. Desirable mutants could be screened out after confirming the stability of phenotypic expression. Hence the variants are to be evaluated further for few more generations.

References

REFERENCES

Abdullah, T. L., Endan, J., Nazir, B. M. 2009. Changes in flower development, chlorophyll mutation and alteration in plant morphology of *Curcuma alismatifolia* by gamma irradiation. *American J. Appl. Sci.* **6**(7): 1436-1439.

Agasimani, A. D., Harish, D. K., Imamsaheb, S. J. and Patil, V. S. 2011. Anthurium varieties performance in rainy and winter seasons under greenhouse. *Res. J. Agri. Sci.* **2**(2): 337-339.

Ahloowalia, B. S. and Maluszynski, M. 2001. Induced mutations - A new paradigm in plant breeding. *Euphytica* ***118***(2): 167-173.

Ahmad, Z., Abu Hassan, A., Salleh, S., Ariffin, S., Shamsudin, S. and Basiran, M. N. 2012. Improvement of Malaysian Ornamental Plants through induced mutation. *Pertanika J. Trop. Agric. Sci.* **35** (3): 631-636.

Akhar, K. F., Bagheri, A., Moshtaghi, N. and Nezami, A. 2011. The effect of gamma radiation on freezing tolerance of chickpea (*Cicer aretinum* L.) at *in vitro* culture. *J. Biol. Environ. Sci.* **5**(14): 63-70.

Alonso, J. M. and Ecker, J. R. 2006. Moving forward in reverse: Genetic technologies to enable genome-wide phenomic screens in *Arabidopsis*. *Nat. Rev. Genet. 7*: 524-536.

Alonso-Blanco, C., Peeters, A. J., Koornneef, M., Lister, C., Dean, C., Bosch N. V. D., Pot, J. and Kuiper, M. T. 1998. Development of an AFLP based linkage map of *Arabidopsis thaliana* and construction of recombinant inbred line population. *Plant J.* **14**: 259-271.

Anbarasan, K. R. and Rajendran. 2013. Studies on the mutagenic effect of EMS on seed germination and seedling characters of Sesame (*Sesamum indicum* (L.) Var. TMV3. *In. J. Res. Biol.* Sci. **3**(2): 27-29.

Anu, K. G. 1997. Improvement of propagation efficiency of *Anthurium andreanum* Andre. by *in vivo* and in *vitro*. M. Sc. thesis, Kerala Agricultural University, Thrissur, India. 83p.

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

Arunyanart, S. and Soontronyatara, S. 2002. Mutation induction by γ and X-ray irradiation in tissue cultured lotus. *Plant Cell Tiss. Org. Cult.***70** (1): 119-122.

Aslam, M., Ashfaq, M., Saeed, T., Allah, S. U. and Sajjad, M. 2009. Development and evaluation of a new high yielding and better fibre quality mutant niab-824 of cotton through pollen irradiation. *Am.-Eurasian J. Sustain. Agric.* **3**(4): 715-720.

Atak, C., Alikamanoğlu, S. Açık, L. and Canbolat, Y. 2004. Induced of plastid mutations in soybean plant (*Glycine max*. L. Merrill) with gamma radiations and determination with RAPD. *Mutat. Res.* **556**: 35-44.

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

Atak, C., Celik, O. and Acik, L. 2011. Genetic analysis of *rhododendron* mutants using random amplified polymorphic DNA (RAPD). *Pak. J. Bot.* **43**(2): 1173-1182.

Atanassov, A., Todorovska, E., Trifonova, A., Petrova, M., Marinova, E., Gramatikova, M., Valcheva, D., Zaprianov, S., and Mersinkov, N. 1996. Investigation of the somaclonal and mutagen induced variability in barely by the application of protein and markers. In: Application of DNA Based Marker Mutations for Improvement of Cereals and other Sexually

Reproduced Crop Plants. Proceeding of a Final Research Co-ordination Meeting organized by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. pp. 21-31, IAEA, Veinna.

Atienzar, F. A., Cordi, B., Donkin, M. E., Evenden, A. J., Jha, A. N. and Depledge, M. H. 2000. Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmaria palmate*. *Aquat. Toxicol.* **50**: 1-12.

Atienzar, F. A., Venier, P., Jha, A. N. and Depledge, M. H. 2002. Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. *Mutat. Res.* **521**: 151-163.

Atta, A. H., McAlister, B. G. and Staden, V. J. 1998. *In vitro* culture and establishment of *Anthurium parvispathum*. *South Afr. J. Bot.* **64**(5): 296-298.

Azzam, Clara R. and El-Sawy, W. 2005. Variation induced via gamma radiation in peanut (*Arachis hypogaea* L.) and its assessment by protein finger printing. *Egypt. J. Plant Breed.* 9(2): 237-256.

Bailey, L. H. 1963. *The Standard Cyclopedia of Horticulture*, the Macmillan Co., New York.

Barakat M.N. and El-Sammak, H. 2011. *In vitro* mutagenesis, plant regeneration and characterization of mutants via RAPD analysis in Baby's breath *Gypsophila paniculata* L. *A. J. C. Sci.* **5**(2): 214-222.

Barakat, M. N., Rania S., Fattah, A., Badr, M. and El-Torky, M. G. 2010. *In vitro* mutagenesis and identification of new variants via RAPD markers for improving *Chrysanthemum morifolium*. *Afr. J. Agri. Res.* **5**(8): 748-757.

Bautista, R., Del, N., Peñalver, A. D., Rodríguez, B. R., Chiu, C. W. and Terry, J. F. 2005. Effects of the application of IAA and sucrose in the *in vitro* rooting of Sonate and Lambada cultivars of *Anthurium andraeanum* Lind. *Biotecnologia Vegetal* **5**(2): 89-94.

Bejoy, M., Sumitha, V. R. and Anish, N. P. 2008. Foliar regeneration in *Anthurium andraeanum* Hort cv. Agnihorti. *Biotechnology* 7 (1): 134-138.

Beneteka, V. 1995. Some experience of methodology with the isolation of somatic mutants in the rose cultivar 'Sonia'. *Acta Prubon.* **50**: 9-25.

Bhajantri, A. and Patil, V. S. 2013a. Response of gamma rays and ethyl methane sulphonate (EMS) on seedling characters of gladiolus cultivars. *Bioinfolet* **10**(1A): 115 -116.

Bhajantri, A. and Patil, V. S. 2013b. Studies on ethyl methane sulphonate (EMS) induced mutation for enhancing variability of gladiolus varieties (*Gladiolus hybridus* HORT) in M_1V_1 generation. *Karnataka J. Agric. Sci.* **26**(3): 403-407.

Bharathi, T., Gnanamurthy, S., Dhanavel, D., Murugan, S. and Ariraman, M. 2013. Induced Physical mutagenesis on seed germination, lethal dosage and morphological mutants of Ashwagandha (*Withania somnifera* (L.) Dunal). *Int. J. Adv. Res.* **1**: 136-141.

Borevitz, J. O., Liang, D., Plouffe, D., Chang, H. S., Zhu, T., Weigel, D., Berry, C. C., Winzeler, E. and Chory, J. 2003. Large-scale identification of single-feature polymorphisms in complex genomes. *Genome Res.* **13**(3): 513-523.

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.

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

Buldewo, S. and Jaufeerally, F. Y. F. 2002. Isolation of clean and PCR amplifiable DNA from *Anthurium andreanum. Plant Mol. Bio. Rep.* **20**: 71a-71.

Buldewo, S. Pillay, M. and Jaufeerally-Fakim, Y. 2012. Genetic diversity in *Anthurium andraeanum* cultivars in Mauritius. *Afr. J. Biotechnol.* **11**(103): 16737-16744.

Cen, Y. Q., Jiang, R. M., Deng, Z. L. and Ni, D. X. 1993. *In vitro* propagation of *Anthurium andreanum.* Morphogehesis and effects of physical and chemical factors. *Acta Hort. Sinica.* **20**: 187-92.

Charbaji, T. and Nabulsi, I. 1999. Effect of low doses of gamma irradiation on in vitro growth of grapevine. *Plant Cell Tiss. Org. Cult.* **57**: 129-132.

Chen, C. L., Wang, H. H., Jeng, T. L., Chuang, S. J., Wei, M. L. and Min, S. J. 2011. Genetic diversity in NaN3-induced common bean mutants and commercial varieties detected by AFLP. *Crop Breed. Appl. Biotechnol.* **11**: 365-369.

Chen, F. C., Kuehnle, A. R. and Sugaii, N. 1997. *Anthurium* roots for micropropagation and *Agrobacterium tumefaciens*-mediated gene transfer. *Plant Cell Tiss. Org. Cult.* **49**: 71-74.

Colbert, T., Till, B. J., Tompa, R., Reynolds, S., Steine, M. N., Yeung, A. T., McCallum, C. M., Comai, L. and Henikoff, S. 2001. Highthroughput screening for induced point mutations. *Plant Physiol.* **126**: 480-484.

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

Creste, S., Neto, A. T., Silva, S. O. and Figueira, A. 2003. Genetic characterization of banana cultivars (*Musa* spp.) from Brazil using microsatellite markers. *Euphytica* **132**: 259- 268.

Croat, T. B. 1988. Ecology and life forms of Araceae. *Aroideana* **11**: 4-56.

Danylchenko, O. and Sorochinsky, B. 2005. Use of RAPD assay for the detection of mutation changes in plant DNA induced by UV-B and gamma rays BMC *plant. Bid.* **5**(1): 59.

Das, A., Gosal, S. S., Shidhu, J. S. and Dhaliwal, H. S. 2000. Induced of mutations for heat tolerance in potato by using *in vitro* culture and radiation. *Euphytica* **114***: 205-209.*

Datta, S. K. 1997. Ornamental plants-role of mutation. Daya Publishing House, Delhi, India.

Datta, S. K. and Chakrabarty, D. 2005. Classical mutation breeding and molecular methods for genetic improvement of ornamentals. In: S.K. Datta (ed.), Role of classical mutation breeding in crop improvement, Daya Publishing House, Delhi, pp. 260-310.

Datta, S. K., Misra, P. and Mandal, A. K. A. 2005. *In vitro* mutagenesis - a quick method for establishment of solid mutant in chrysanthemum. *Curr. Sci.* **88**: 155-158.

Dhakshanamoorthy, D., Selvaraj, R. and Chidambaram, A. 2010. Physical and chemical mutagenesis in *Jatropha curcas* L. To induce variability in seed germination, growth and yield traits. *Rom. J. Biol.* **55**(2): 113-125.

Dhakshanamoorthy, D., Selvaraj, R. and Chidambaram, A. 2013. Induced Mutagenesis in *Jatropha curcas* L. Using Ethyl Methane sulphonate (EMS) and Assessment of DNA Polymorphism through RAPD Markers. *J. Crop Sci. Biotech.* **16**(3): 201-207.

Duong, T. N., Nguen, D., Nguen, N. H. V., Chau, D. K., Dinh, V. K. and Do, N. V. 2007. Impact of *Anthurium* spp. genotype on callus induction derived from leaf explants and shoot and root regeneration capacity from callus. *J Appl. Hort.* **9**: 135-137.

Duron, M. 1992. Induced mutagens through EMS treatment after adventitious bud formation on shoot internodes of Weigela cv. Bristol Ruby. *Acta Hort.* **320**: 113-18.

Dwimahyani, I. and Ishak. 2004. Induced mutation on Jatropha (*Jatropha curcas* L.) for improvement of agronomic characters variability. *Atom Indonesia* **30**(2): 53-60.

Edwards, K. J., Barker, J. H. A., Daly, A. Jones, C. and Karp, A. 1996. Microsatellite libraries enriched for several microsatellite sequences in plants. *Biotechniqes* **20**: 758-760.

Esnault, M. A., Legue, F. and Chenal, C. 2010. Ionizing radiation: Advances in plant response. *Environ. Exp. Bot.* **68**: 231-237.

Evans, H. J. 1962. Chromosome aberrations induced by ionizing radiations. *Int. Rev. of Cyt.* **13**: 221-308.

Fang, J. Y. 2011. *In Vitro* Mutation Induction of *Saintpaulia* Using Ethyl Methane sulfonate. *Hort. Sci.* **46**(7): 981-984.

Finnie, J. F. and Van Staden, J. 1986. *In vitro* culture of *Anthurium andreanum S.A.J. Bot.* **52**: 343-346.

Foster, G. D. and Twell, D. 1996. *Plant Gene Isolation: Principles and Practice.* John Wiley and Sons Ltd. pp. 215-45.

Frary, A., Xu, Y., Lin, J., Mitchell, S., Tedeschi, E. and Tanksley, S. 2005. Development of a set of PCR-based anchor markers encompassing the

tomato genome and evaluation of their usefulness for genetics and breeding experiments. *Theor. Appl. Genet.* **111**: 291-312.

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.

Fuller, M. P., Metwali, E. M. R., Eed, M. H. and. Jellings, A. J. 2006. Evaluation of abiotic stress resistance in mutated populations of cauliflower (*Brassica oleracea* var. botrytis). *Plant Cell Tiss. Org. Cult.* **86**:239-248.

Gabryszewska, E. and Rudnicki, R. M. 1997. The effects of light quality on the growth and development of shoots and roots of Ficus benjamina *in vitro*. *Acta Hortic.* **418**: 163-167.

Gang, W. J., Ying, G., DaiDi, C., ShenKui, L., ChuanPin, Y. 2009. RAPD analysis of M_1 generation of *Gladiolus hybridus* hort treated by EMS. *J. NE. Agri. Uni.* **16**(1): 5-9.

Gantait, S. and Sinniah, U. R. 2011. Morphology, flow cytometry and molecular assessment of *ex-vitro* grown micropropagated anthurium in comparison with seed germinated plants. *Afr. J. Biotechnol.* **10**(64): 13991-13998.

Gantait, S., Mandal, N., Bhattacharyya, S. and Das, P. K. 2008. *In vitro* Mass Multiplication with Pure Genetic Identity in *Anthurium andreanum* Lind. *Plant Tissue Cult. Biotech.* **18**(2): 113-122.

Geier, T., 1982. Morphogenesis and plant regeneration from spadix fragments of *Anthurium scherzeranium* cultivated *in vitro*. In: Fujiwara, A. (ed.) *Plant Tissue Culture*. Proceedings of the 5th International Congress of plant tissue culture, Tokyo, pp. 137-42.

Geier, T. 1986. Factors affecting plant regeneration from leaf segments of *Anthurium scherzerianum* Schott cultured *in vitro*. *Plant Cell Tiss. Org. Cult.* **6**:115-125.

Gesteira, A. S., Otoni, W. C., Barros, E. G. and Moreira, M. A. 2002. RAPD-based detection of genomic instability in soybean plants derived from somatic embryogenesis. *Plant Breed.* **121**: 269-271.

Ghani, M., Kumar, S. and Thakur, M. 2013. Induction of novel variants through physical and chemical mutagenesis in Barbeton daisy (*Gerbera jamesonii* Hook.). *J. Horti.Sci. Biotechnol.* **88**(5): 585-590.

Girija, M., Dhanavel, D. and Gnanamurthy, S. 2013a. Gamma rays and EMS induced flower color and seed mutants in cowpea (*Vigna unguiculata* L. Walp). *Adv. App.Sci. Res.* 4(2):134-139.

Girija, M., Gnanamurthy, S. and Dhanavel, D. 2013b. Genetic Diversity Analysis of Cowpea Mutant (*Vigna unguiculata* L. Walp) as revealed by RAPD Marker. *Int. J. Adv. Res.* **1**(4): 139-147.

Gnanamurthy, S., Dhanavel, D. and Girijam, M. 2013. Effect of gamma radiation on morphological characters of Cowpea (*Vigna unguiculata* (L.) Walp). *Int. J. Cur. Tr. Res.* **2**(1): 38- 43.

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.

Gottlieb, L. D. 1981. Electrophoretic evidence and plant populations. *Prog. Phytochem.*7: 1-46.

Greene, E. A., Codomo, C. A., Taylor, N. E., Henikoff, J. G., Till, B. J., Reynolds, S. H., Enns, L. C., Burtner, C., Johnson, J. E., Odden, A. R., Comai L. and Henikoff, S. 2003. Spectrum of chemically induced

mutations from a large-scale reverse-genetic screen in *Arabidopsis*. *Genetics* **164**(2): 731-740.

Hameed, A., Shah, T. M., Atta, B. M., Haq, M. A. and Sayeed, H. 2008. Gamma radiation effects on seed germination and growth, protein content, peroxidase and protease activity, lipid perixadation in desi and kabuli Chickpea. *Pak. J. Bot.* **40**(3): 1033-1041.

Hashmi, G., Huettel, R., Meyer, R., Krusberg, L. and Hammerschlag, F. 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. *Plant Cell Rep.* **16**: 624-627.

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

Hegazi, A. Z. and Hamideldin, N. 2010. The effect of gamma irradiation on enhancement of growth and seed yield of okra (*Abelmoschus esculentus* (L.) Moench) and associated molecular changes. *J. Horti and Forestry.* **2**(3): 38-51.

Hernandez, C. S. and Oyarzum, J. C. 2006. Two miniprepreration protocol to DNA extraction from plant with high polysaccharide and secondary metabolites. *Afr. J. Biotechnol.* **5**(20): 1864-1867.

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

Hewawasam, W. D. C. J., Aberathne, W. M. and Bandara, D. C. 2004. Novel Mutant Cultivar *Crossandra* "Savindi" by Gamma Ray Induced *in vitro* Mutagenesis. *Trop. Agric. Res.* **16**: 253-270

Hofmann, N. E., Raja, R., Nelson, R. L. and Korban, S. S. 2004. Mutagenesis of embryogenic cultures of soybean and detecting polymorphisms using RAPD markers. *Biol. Plantarum* **48**(2): 173-177.

Hossain, Z., Mandal, A. K., Datta, S. K. and Biswas, A. K. 2006. Isolation of a NaCl-tolerant mutant of *Chrysanthemum morifolium* by gamma radiation: *in vitro* mutagenesis and selection by salt stress. *Funct. Plant Bio.* **33**(1): 91-101.

Islam, M. S., Mehraj, h., Roni, M. Z. K., Shahrin, S. and Jamaluddin, A. F. M. *2013.* Varietal study of Anthurium (*Anthurium andraeanum*) as a cut flower in Bangladesh. *J. Bangladesh Acad. Sci.* 37(1): 103-107.

Jahan, M. T., Islam, M. R., Khan, R., Mamun, A. N. K., Ahmed, G. and Hakim, H. 2009. *In vitro* clonal propagation of Anthurium (*Anthurium andraeanum* Lind) using callus culture. *Plant Tiss. Cult. Biotech.* **19**(1): 61-69.

Islam, S. A., Dewan, M. M. R., Mukul, M. H. R., Hossen, M. A. and Khatun, F. 2010. *In vitro* regeneration of *Anthurium andreanum* cv. Nitta. *Bangladesh J. Agril. Res.* **35**(2): 217-226.

Jain, S. M. 2005. Major mutation-assisted plant breeding programs supported by FAO/IAEA. *Plant Cell Tiss. Org. Cult.* **82**: 113-123.

Jain, S. M. 2007. Mutation-assisted breeding for improving ornamental plants. *Acta Hortic. 714:* 85-98.

Jain, S. M. and Maluszynski, M. 2004. Induced mutations and biotechnology on improving crops. In: *In vitro* applications in crop improvement: Recent progress. In: A. Mujib, M. Cho, S. Predieri and S. Banerjee (eds.), IBH-Oxford, India, pp.169-202.

Jain, S. M. and Spencer, M. M. 2006. Biotechnology and mutagenesis in improving ornamental plants. In: J.A. Teixeira da sliva (eds). Floriculture,

ornamental and plant biotechnology: Advances and topical issues, 1st Edition, Global Sci. Books, UK, pp. 589-600.

Jain, S. M., Vitti, D., Tucci, M., Grassotti, A., Rugini, E. and Saccardo, F. 1998a. Biotechnology and agronomical aspects in gerbera improvement. *Adv. Hort. Sci.* **12**: 47-53.

Jau-yueh, W., Keng-Chang, C. and Ming-Jen, F. 2001. A comparative analysis of genetic similarity among A*nthurium andraeanum* cultivars by using ISSR and RAPD marker. *J. Agric. Res. China* **48**(4): 52-63.

Joseph, D., Martin, K. P., Madassery, J. and Philip, V. J. 2003. *In vitro* propagation of three commercial cut flower cultivars of *Anthurium andraeanum* Hort. *Indian J. Exp. Biol.* **41**: 154-159.

Kahrizi, A. Z., Kermani, J. M., and Amiri, M. 2012. Effect of gamma rays on nuclear DNA content in different rose genotypes. *Int. Res. J. App. Basic Sci.* 3(6): 1155-1160.

Kahrizi, Z. A., Jafarkhani, M., Kermani, M. J., Amiri, M. E. and Vedadi, S. 2011. Identifying the correct dose of gamma-rays for *in vitro* mutation of rose cultivars. *Acta Hort.* 923: 121- 128.

Kang, E. J., Lee, M. E., Sung, Y. S., Ha, B. K., Kim, S. H., Kim, D. S., Kim, J. B. and Kang, S. Y. 2013. Analysis of the Genetic Relationship of Gamma-irradiated in Vitro Mutants Derived from Standard-type Chrysanthemum cv. Migok. *Hort. Environ. Biotechnol.* **54**(1):76-81.

Kavita, P., Divya, H. Ruchi, B. Dinesh, C. P. and Gaur, A. K. 2011. Study of biochemical variability in the populations of *Aconitum balfourii* by soluble protein and isoenzyme electrophoretic patterns. *J. Chemical Pharmaceutical Res.* **3**: 295-301.

Khaleifa, M. M. A., Azzam, C. R. and Azer, S. A. 2006. Biochemical Markers Associated with Disease Resistance to Damping-Off and Root-Rot

Diseases of Peanut Mutants and their Productivity. *Egypt. J. Phytopathol.* **34**(2): 53-74.

Khan, A. Hassan, S., Tariq, M. and Khan, T. 2001. Haploidy breeding and mutagenesis for drought tolerance in wheat. *Euphytica 120: 409-414.*

Khan, I. A., Dahot, M. U. and Khatri, A. 2007. Study of genetic variability in sugarcane induced through mutation breeding. *Pak. J. Bot.,* **39**(5): 1489-1501.

Khan, M. R. and Goyal, S. 2009. Improvement of mungbean varieties through induced mutations. *Afr.J.Plant Sci.* **3**: 174-180.

Khan, Y. J. and Pankajaksan, M. 2010. Genetic diversity among commercial varieties of *Anthurium andraeanum* Linden using RAPD markers. *J. Plant Genet. Transgenics* **1**:11-15.

Khatri, A., Bibi, S., Dahot, M. U., Khan, I. A. and Nizamani, G. S. 2011. *In vitro* mutagenesis in banana and variant screening through ISSR. *Pak. J. Bot.* **43**: 2427–2431.

Kim, J. H., Baek, M. H., Chung, B.., Wi S. G. and Kim, J. S. 2004. Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. *J. Plant Biol.* **47**: 314-321.

Klarizze, A. M. P. 2005. Mathematical analysis of root growth in gamma-irradiated Cashew (*Anacardium occidentale* L.) and Mangosteen (*Garcinia mangostana* L.) using fractals. *Nat. Sci.* **3**(1): 59–64.

Kobayashi, R. S., Brewbaker, J. L. and Kamemoto, H. 1987. Identification of *Anthurium andraeanum* cultivars by gel electrophoresis. *J. Am. Soc. Hortic. Sci.* **112**: 164-167.

Kolar, F., Pawar, N. and Dixit. G. 2011. Induced chlorophyll mutations in *Delphinium malabaricum* (Huth) Munz. *J. Appl. Hortic.* **13**(1): 18-24.

Kuehnle, A. R. and Sugii, N. 1991. Callus induction and plantlet regeneration in tissue cultures of Hawaiian anthuriums. *Hort. Sci.* **26**: 919-21.

Kuehnle, A. R., Chen, F. C. and Sugii, N. 1992. Somatic embryogenesis and plant regeneration in *Anthurium andreanum* hybrids. *Plant Cell Rep.* **11**:438-42.

Kumar, S. S., Deth, S. K. and Seeni, S. 1992. Development of floricultural resources in Kerala- rapid micropropagation of anthuriums. In: Proceedings of the Forth Kerala Science Congress, Thrissur Kerala, pp 347-350.

Kumar, S., Prasad, K. V. and Choudhary, M. L. 2006. Detection of genetic variability among chrysanthemum radimutants using RAPD markers. *Curr. Sci.,* **90**(8): 1108-1113.

Kunisaki, J. T. 1980. *In vitro* propagation of *Anthurium andreanum* Lind. *Hort Sci.* **15**: 508-9.

Lamseejan, S., Jompuk, P. and Deeseepan, S. 2003. Improvement of chrysanthemum var 'taihei' through *in vitro* induced mutation with chronic and acute gamma rays. *J. Nuclear Soc.* **4**: 2-13.

Laneri, U., Franconi, F. and Altavista, P. 1990. Somatic mutagenesis of *Gerbera jamesonii* hybrid: irradiation and *in vitro* culture. *Hort. Sci.* **20**:137-139.

Lara, A., Mosqueda, O. and Gonzalez-Olmedo. 2004. Determination of the effect of Pectimorf and C-751 on shoot multiplication in *Anthurium andraeanum* propagated in temporary immersion bioreactors. *CIEBA* **45**: 121-128.

Latado, R. R., Adames, A. H. and Neto, A. T. 2004. *In vitro* mutation of chrysanthemum (*Dendranthema grandiflora* tzvelev) with ethylmethane

sulphonate (EMS) in immature floral pedicels. *Plant Cell Tiss. Org. Cult.* 77: 103-106.

Lee, S. Y., Cheong, J. I, and Kim, T. S. 2003. Production of doubled haploids through anther culture of M1 rice plants derived from mutagenized fertilized egg cells. *Plant Cell Rep.* **22**: 218-223.

Lee-Espinosa, H. E., Cruz-Castillo, J. G. and Garcia-Rosas, B. 2003. Multiple shoot proliferation and acclimatization of Midori and Kalapana Anthurium (*Anthurium andraeanum* Lind.) cultured *in vitro*. *Rev. Fitotec. Mex.* **26**: 301-307.

Lema-Rumińska, J., Zalewska, M. and Sadoch, Z. 2004. Radiomutants of chrysanthemum (*Dendrathema grandiflora* Tzvelev) of the Lady group: RAPD analysis of the genetic diversity. *Plant Breed.* **123**: 290-293.

Leena, R. 2003. Improvement of *Anthurium andreanum* Lind. By *in vivo* and *in vitro* methods. Ph. D. thesis, Kerala Agricultural University, Thrissur, India. 159p.

Liu, X. L., Yang, Z. and Li, G. 2006. Biological effect of M_1 generation by utilizing EMS mutagen on maize pollen. *J. Shenyang Agric. Uni.* **37**(6): 806-810.

Lu, G., Zhang, X. Y., Zou, Y. J., Zou, Q. C., Xiang, X. and Cao, J. S. 2007. Effect of radiation on regeneration of Chinese narcissus and analysis of genetic variation with AFLP and RAPD markers. *Plant Cell Tiss. Org. Cult.* **88**: 319-327.

Mackey, J. 1956. Mutation Breeding in Europe. *Brookhaven Symp. Biol.* **9**: 141-156.

Madinchey, L. and Kottliama, S. 2007. Production of mutants with high cold tolerance in spring canola. *Euphytica 10: 1-17.*

Mairo, O., Alexander, M. and Vargas, T. E. 2010. Micropropagation and organogenesis of *Anthurium andreanum* Lind cv Rubrun. Methods. *Mol. Biol.* **589**: 3-14.

Malhotra, S., Puchooa, D. and Goofoolye, K. 1998. Callus induction and plantlet regeneration in three varieties of *Anthurium andraeanum* Lind. *Revue Agricole et sucriere de L, lle Maurice* 77: 25-32.

Maluszynski, M., Sigurbjörnsson, B., Amano, E., Sitch, L. and Kamra, O. 1992. Mutant varieties-data bank, FAO, IAEA database. Part II. Mutation Breed News **39**: 14-17.

Mandal, A. K. A., Chakrabarty, D. and Datta, S. K. 2000a. Application of *in vitro* techniques in mutation breeding of chrysanthemum. *Plant Cell, Tiss. Org. Cult.* **60**: 33-38.

Mandal, A. K. A., Chakrabarty, D. and Datta, S. K. 2000b. *In vitro* selection of solid novel flower colour mutants from induced chimeric ray florets of chrysanthemum. *Euphytica* **114**: 9-12.

Martin, K. P., Joseph, D., Madassery, J. and Philip, V. J. 2003. Direct shoot regeneration from lamina explants of two commercial cut flower cultivars of *Anthurium andreanum* Hort. *In Vitro Cell Dev. Biol. Plant* **39**: 500-504.

Matasyoh, L. G., Wachira, F. N., Kinyua, M. G., Muigai, A. W. T. and Mukiama, T. K. 2008. Leaf storage condition and genomic DNA isolation efficiency in *Ocimum gratissimum* L. from Kenya. *Afr. J. Biotechnol.* 7(5): 557- 564.

McCallum, C.M., Comai, L., Greene, E.A. and Henikoff, S. 2000. Targeting induced local lesions in genomes (TILLING) for plant functional genomics. *Plant Physiol.* **123**(2): 439-442.

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.

Mekala, P., Kumar, N., Kannan, M. and Vijayakumar, G. 2010. Effect of physical and chemical mutagens on floral characters of *Jasminum sambac* cv. Gundumalli and isolation of important mutants. *J. Ornam. Hortic.* **13**(3): 200-206.

Melki, M. and Marouani, A. 2009. Effects of gamma rays irradiation on seed germination and growth of hard wheat. *Environ. Chem. Lett.* **8**(4): 307-310.

Mini, B. 1998. Improvement of *Anthurium andreanum* Lind. *in vitro*. Ph. D thesis, Kerala Agricultural University, Thrissur, India. 210p.

Misra, P., Datta, S. K. and Chakrabarty, D. 2003. Mutation in flower colour and shape of *Chrysanthemum morifolium* induced by gamma irradiation. *Biol. Plant* **47**: 153-156.

Mohan, M.; Mair, S.; Bahgwat, A.; Krishna, T. G. and Yana, M. 1997. Genomic mapping molecular markers and maker-assisted selection in crop plants. *Mole. Breed.* **3**: 87-103.

Montes S, Hernandes M. M. and Varela, M. 1999. Organogenesis in *Anthurium cubense*. *Cultivos Tropicales* **20**: 51-54.

Moussa, R. H. 2006. Gamma irradiation regulation of nitrate level in rocket (*Eruca vesicaria* subsp. *sativa*) plants. *J. New Seeds* **8**(1): 91-101.

Mudibu, J., Nkongolo, K.K.C., Mehes-Smith, M. and Kalonji-Mbuyi, A. 2011. Genetic analysis of a soybean genetic pool using ISSR marker: effect of gamma radiation on genetic variability. *Int. J. Plant Breed. Genet.* **5**: 235-245.

Muhammad, A. J. and Othman. R. Y. 2005. Characterization of *Fusarium* wilt-resistant and *Fusarium* wilt-susceptible somaclones of banana cultivar Rastali (Musa AAB) by random amplified polymorphic DNA and retrotransposon markers. *Plant Mol. Bio. Rep.* **23**: 241-249.

Mujaju, C., Sehic, J., Werlemark, G., Garkava-Gustavsson, L., Fatih, M. and Nybom, H. 2010. Genetic diversity in watermelon (*Citrullus lanatus*) landraces from Zimbabwe revealed by RAPD and SSR markers. *Hereditas* **147**: 142-153.

MuLan, C., Wei, Y., Xiong, L. Z., HuiMing, Z., Wei, S., ChangMing, C. and QiuPing, J. 2012. Rooting and transplanting of tissue culture plantlets of *Anthurium andraeanum*. *J. Fujian Agri. For. Uni.* **41**(3): 225-231.

Nagatomi, S., Tanaka, A., Kato, A., Watanabe, H. and Tano, S. 1995. Mutation induction on chrysanthemum plants regenerated from *in vitro* cultured explants irradiated with 12C ion beam. *JAERI*-Review **96**(17): 50-52.

Nagatomi, S., Yamaguchi, H., Degi, K. and Morishita, T. 2003. Six mutant varieties induced by ion beams in chrysanthemum. Institute of Radiation Breeding, Technical News No. 65. ISSN 0285-1962.

Ndzana, X., Zok, S. and Sama, A. E. 2008. Preliminary study on radiation sensitivity of in vitro cultures of *Xanthosoma* (Macabo) in Cameroon. Plant Mutation Reports 2(1).

Newbury, H. J., Howell, E. C., Crouch, J. H. and Lioyd. B. V. 2000. Natural and culture-induced genetic variation in plantains (*Musa* spp. AAB group). *Aust. J. Bot.* **48**: 493- 500.

Nhut, D. T., Duy, N., Vy, N. N. H., Khue, C. D., Khiem, D. V. and Vinh, D. N. 2006. Impact of *Anthurium* spp. genotype on callus induction derived from leaf explants, shoot and root regeneration capacity from callus. *J. Appl. Hort.* **8** (2): 135-137.

Nowbuth, P., Khittoo, G., Bahorun, T. and Venktasamy, S. 2005. Determination of genetic variation among some anthurium cut flower cultivars. *Afr. J. Biotech.* 4(10):1189-1194.

Okamura, M., Ohtsuka, M., Yasuno, N., Hirosawa, T., Tanaka, A., Shikazono, N., Hae, Y. and Tanase, M. 2001. Mutation generation in carnation plants regenerated from *in vitro* cultures irradiated with ion beams. *JAERI* **39**: 52-54.

Oleykowski, C. A, Bronson, M. C. R., Godwin, A. K. and Yeung, A. T. 1998. Mutation detection using a novel plant endonuclease. *Nucleic Acids Res.* **26**(20): 4597-4602.

Orlikowska, T., Sabala, I. and Nowak, E. 1995 Adventitious shoot regeneration on explants of *Anthurium*, Codiaeum, Dieffe- nbachia, Gerbera, Rosa and Spathiphyllum for breeding purposes. *Acta. Horti.* **420**: 115-117.

Pathiana, N. S. and Mishra, N. L. 2003. *In Vitro* Mutagenesis Studies in *Gladiolus* for Induction of Resistance to *Fusarium oxysporum* f.sp. *gladioli. Acta. Hort.* **624**: 487-494.

Perry, J. A., Wang, T. L., Welham, T. J., Gardner, S., Pike, J. M., Yoshida, S. and Parniske, M. 2003. A TILLING reverse genetics tool and a web-accessible collection of mutants of the legume *Lotus japonicus. Plant Physiol.* **131**(3): 866-871.

Pestanana, R.K.N., Amorim, E.P., Ferreira, C.F., Amorim, V.B.O., Oliveira, L.S., Ledo, C.A.S. and Silva, S.O. 2011a. Agronomic and molecular characterization of gamma ray induced banana (*Musa* sp.) mutants using a multivariate statistical algorithm. *Euphytica* **178**: 151-158.

Pestanana, R. K. N., Amorim, E. P., Ferreira, C. F., Amorim, V. B. O., Oliveira, L. S., Ledo, C. A. S. and Silva, S. O. 2011b. Genetic dissimilarity of putative gamma-ray-induced 'Preciosa – AAAB-Pome type' banana

(*Musa* sp) mutants based on multivariate statistical analysis. *Genet. Mol. Res.* **10:** 3976-3986.

Petersen, G. 1989. Cytology and systematics of Araceae. *Nord. J. Bot. 9*: 119-158.

Pierik, R. L. M. 1975. Callus multiplication of *Anthurium andreanum* Lind in liquid media. *Netherlands J. Agric Sci.* **23**: 299-302.

Pierik, R. L. M. 1976. *Anthurium andreanum* plantlets produced from callus tissues cultivated *in vitro*. *Physiol. Plant* **37**: 80–2.

Pierik, R. L. M., Leeuwen, P. V. and Rigter, G. C. C. M. 1979. Regeneration of leaf explants of *Anthurium andraeanum* Lind. *in vitro*. *Netherlands J. Agric. Sci.* **27**: 221-226.

Pierik, R. L. M., Steegmans, H. H. M. and Meys, V. D. 1974. Plantlet formation n callus tissues of *Anthurium andraeanum* Lind. *Scientia Hort.* **2**: 193-198.

Prakash, D., Choudhary, K. V., Prasad and Nagesh, N. 2001. Regeneration pf plantlets from petiole explants of *Anthurium andreanum* Lind. Cv. Mauritius Orange. *Phytomorph* **51**: 83-85.

Puchooa, D. 1996. Seminar on Induced Mutation and *In vitro* Culture of Anthurium. Programme Ph.D. Symposium. Auditorium De Vleesschauwer, Faculty of Agriculture, University of Mauritius. October 23rd 1996.

Puchooa, D. 2005 *In vitro* mutation breeding of *Anthurium* by gamma radiation. *Int. J. Agric. Biol.* 7 (1): 17-20.

Puchooa, D. and Sookun, D. 2003. Induced mutation and *in vitro* cultured of *Anthurium andraeanum*. AMAS, Food and Agricultural Research Council, Reduit, Mauritius 17-27.

Qassar, J., Narmeen, S. A. and Kadhim M. I. 2011. Application of Random Amplified Polymorphic Analysis for the Detection Genetic Variation in Tomato *(Lycopersicon Esculentum* Mill.). *J. Al-Nahrain Uni. Sci.* **14**(1): 122-133.

Raad, M. K., Zanjanil, S. B., Shoor, M., Hamidoghli, Y., Sayyad, A. R., Kharabian-Masouleh, A. and Kaviani, B. 2012. Callus induction and organogenesis capacity from lamina and petiole explants of *Anthurium andreanum* Linden (Casino and Antadra). *A. J. C. S.* **6**(5): 928-937.

Rajwant, K., Kalia, M. K., Rai, S., Kalia, R. S. and Dhawan, A. K. 2011. Microsatellite markers: an overview of the recent progress in plants. *Euphytica* **177**: 309-334.

Rakotoarisoa, N. V., Andrianjakan, A., Zapata-Arias, F. J., Afza, R. and Mba, C. 2008. Inducing cold tolerance in Malagasy rice varieties IR58614, Maladay, Rojofotsy through *in vitro* mutagenesis. *Plant Mutat. Rep. 2: 16-23.*

Ranamukhaarachchi, D. G., Henny, R. J., Guy, C. L. 2001. DNA fingerprinting to identify nine anthurium pot plant cultivars and examine their genetic relationships. *Horti. Sci.* **36**: 758 -760.

Reddy, J. H., Bopaiah, A. K. and Abhilash, M. 2011. *In vitro* micropropagation of *Anthurium digitatum*, using leaf as explants. *Asian J. Pharma. Health Sci.* **1**(2): 70-74.

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. and Jain, S. M. 2005. Micropropagation of floricultural crops. In: S.J. Murch and P.K. Saxena (eds.), Journey of a single cell to a plant, Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, India, pp. 310-365.

Roychowdhury, R. and Tah, J. 2011. Assessment of chemical mutagenic effects in mutation breeding programme for M1 generation of Carnation (*Dianthus caryophyllus*). *Res. Plant Biol.* 1(4): 23-32.

Saal, B. and Wricke, G. 2002. Clustering of amplified fragment length polymorphism markers in a linkage map of rye. *Plant Breed.* **121**: 117-123.

Sabaghi, H. R., Arab, M., Lotfi, M., Akbari, M. and Azizinia, S. 2013. Morphological characteristics evaluation of induced mutant lines of stock var. centum white. *Ann. Biol. Res.* 4(4): 152-157.

Sallem, M. Y., Mukhter, Z., Cheema, A. A. and Atta, B. M. 2005. Induced mutation and *in vitro* technique as a method to induce salt tolerance in Basmati rice. *Environment. Sci. Tech.* **2**: 141-145.

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

Sandhu, S. S., Bastos, C.R., Azini, L. E., Neto A. T. and Colombo, C. 2002. RAPD analysis of herbicide resistant Brasilian rice lines produced via mutagenesis. *Genet. Mol. Res.* **1**(4): 359-370.

Sarwar, G. and Ahmad. M. 2003. Development of a new high yielding mungbean variety "aem96" through induced mutation. *SAARC j. Agri.* **1**: 173-180.

Schum, A. 2003. Mutation breeding in ornamentals: an efficient breeding method *Acta Horti.* **612**: 47- 60.

Selvi, B. S., Ponnuswami, V. and Sumathi, T. 2007. Identification of DNA Polymorphism induced by gamma ray irradiation in Amla (*Emblica*

officinalis Gaertn.) Grafts of V1M1 and V2M1 generation. *J. Appl. Sci. Res.* **3**(12): 1933-1935.

Sen, A. and Alikamanoglu, S. 2014. Characterization of drought-tolerant sugar beet mutants induced with gamma radiation using biochemical analysis and isozyme variations. *J. Sci. Food Agric.* **94**: 367–372.

Senapati S. K. and Rout G. R. 2008. Mutagenesis in rose: Early selection through molecular marker. *Indian J. Hort. 65(4): 452-460.*

Sharabash, M. T. 1998. Radiation induced variation in potato for tolerance to salinity using tissue culture technique. *National Center for Research and Radiation Technology. Atomic Energy Authority, Nacr City, Cario, Egypt* **1227**: 83-86.

Sharma, S. C., Srivastava, R., Datta, S. K. and Roy, R. K. 2002. Gamma rays induced bract colour mutation in single bracted Bougainvillea 'Palekar'. *J. Nuc. Agric. Biol.* **31**: 206-208.

Shufang, G., Huijuan, F. U. and Jingang, W. 2010. ISSR Analysis of M_1 Generation of *Gladiolus hybridus* Hort Treated by EMS. *J.N.E. Agri. Uni.* **17**(2): 22-26.

Silva, J. A. T., Nagae, S. and Tanaka, M. 2005. Effect of physical factors on micropropagation of *Anthurium andraeanum. Plant Tiss. Cult.* **15** (1): 1-6.

Singh, K. P., Singh, B., Raghava, S. P. S. and Kaua, C. S. 2000. Induced flower colour mutations in carnation through in *vitro* application of chemical mutagen. *Indian J. Genet.* **60**(4): 535-539.

Singh, S., Dhyani, D. and Kumar, A. 2011. Expression of Floral Fasciation in Gamma-ray Induced *Gerbera jamesonii* Mutants. *J Cell Plant Sci.* **2**:7-11.

Slade, A.J. and Knauf, V.C. 2005. TILLING moves beyond functional genomics into crop improvement. *Transgenic Res.* **14**: 109-115.

Soniya, E. V., Banerjee, N. S. and Das. M. R. 2001. Genetic analysis of somaclonal variation among callus-derived plants of tomato. *Curr. Sci.* **80**: 1213-1215.

Sreelatha, U. 1992. Improvement of propagation efficiency of anthurium species *in vitro*. Ph. D thesis, Kerala Agricultural University, Thrissur. 119p.

Sung, S. Y., Chung, S. J., Kim, S. H., Kim, W. J., Lee, J. Y., Kim, D. S., Kim, J. B. Kim, H. G. and Kang, S. Y. 2010. Analysis of genetic diversity among spray-type chrysanthemum (*Dendranthema grandiflorum*) 'Argus' and its flower-colored mutants. *J. Rad. Ind.* **4**: 171-177.

Taheri, S., Abdullah, T. L. and Abdullah, N. A. P. 2013a. Use of Inter simple Sequence Repeat Assay for Detection of DNA Polymorphism Induced by Gamma Rays in *Curcuma alismatifolia. Hort. Sci.* **48**(11): 1346-1351.

Taheri, S., Abdullah, T. L., Ahmad, Z. and Abdullah, N. A. P. 2013b. Effect of acute gamma irradiation on *Curcuma alismatifolia* varieties and detection of DNA polymorphism through SSR marker. Biomed Research International (In press).

Tajuddin, E. and Prakash, R., 1996, Anthurium, the Directorate of Extension, Kerala Agricultural University, pp. 5-6.

Tanksley, S. D. and Rick, C. M. 1980. Isozyme gene linkage map of the tomato: applications in genetics and breeding. *Theor. Appl Genet.* **57**: 161-170.

Tantasawat, P., Trongchuen, J., Prajongjai, T., Seehalak, W. and Jittayasothorn, Y. 2010. Variety identification and comparative analysis of genetic diversity in yardlong bean (*Vigna unguiculata* spp. *sesquipedalis*) using morphological characters, SSR and ISSR analysis. *Sci. Hortic.* **124**: 204-216.

Te-chato, S. and Susanon, T. 2005. Floral mutation in anthurium cv. valentino after induction by ethyl methane sulfonate. *Songklanakarin J. Sci. Technol.* **27**(3): 675-682.

Te-Chato, S., Susanon, T. and Sontikun, Y. 2006. 20 medium influencing embryogenesis and organogenesis in *Anthurium* spp. cultivar, explants type and culture. S*ongklanakarin J. Sci. Technol.* **28** (4): 717-722.

Tejaswini, Chowdury, S., Paramesh, H. and Tripathi, N. 2006. *In vitro* approaches for chemical mutagenesis in carnation *(Dianthus caryophyllus). Indian J. Genet.* **66**(1): 71-72.

Thapa, C. B. 2004. Effect of acute exposure of gamma rays on seed germination and seedling growth of *Pinus kesiya* Gord and *P. wallichiana* A.B. Jacks. *Nature* **2**: 13-17.

Till, B. J., Colbert, T., Tompa, R., Enns, L. C., Codomo, C. A., Johnson, J. E., Reynolds, S. H., Henikoff, J. G., Greene, E. A., Steine, M. N., Comai, L. and Henikoff, S. 2003. High-throughput TILLING for functional genomics. *Methods Mol. Bio.* **236**: 205-220.

Till, B. J., Reynolds, S. H., Weil, C., Springer, N., Burtner, C., Young, K., Bowers, E., Codomo, C. A., Enns, L. C., Odden, A. R., Greene, E. A., Comai, L. and Henikoff, S. 2004. Discovery of induced point mutations in maize genes by TILLING. *BMC Plant Biol.* **2229**:4-12.

Trujillo, S., Concepcion-Laffitte, O., Daquinta-Gradaille, M., Napoles-Borrerro, L. and Balmaseda-Avila, M. 2000. *In vitro* plant propagationon *Anthurium andraeanum* Lind. var. Sonate. *Biotechnologia Vegetal* **1**: 33-38.

Uma, S., Siva, S. A., Saraswathi, M. S., Manickavasagam, M., Durai, P., Selvarajan, R. and Sathiamoorthy. S. 2006. Variation and intraspecific relationships in Indian wild *Musa balbisiana* (BB) population as

evidenced by random amplified polymorphic DNA. *Genet. Resour. Crop Evol.* **53**: 349-355.

Vargas, T. E., Mejías, A., Oropeza, M. and De-García, E.2004. Plant regeneration of *Anthurium andreanum* cv Rubrun. *Electra J. Biotech.* 7: 282-286.

Varshney, A., Mohapatra, T. and Sharma, R. P. 2004. Molecular mapping and marker assisted selection of traits for crop improvement. In: Srivastava, A. and Narula, S. (eds.), *Plant Biotechnology and Molecular Markers*, New Delhi, Anamaya. pp. 289-301.

Veasey, E. A., Cardin, D. C., Silva, R. M., Bressan, E.A. and Vencovsky, R. 2008. Assessing the genetic structure of *Oryza glumaepatula* populations with isozyme markers. *Brazilian Archives Bio. Technol.* **51**: 873-882.

Velmurugan, M., Rajamani, K., Paramaguru, P., Gnanam, R., Bapu, J. R., Harisudan, C. and Hemalatha, P. 2010. *In vitro* mutation in horticultural crops-a review. *Agric. Rev.* **31**: 63-67.

Viégas, J., Da Rocha, M. T. R., Ferreira-Moura, I., Da Rosa, D. L., De Souza, J. A., Corrêa, M. G. S. and Da Silva, J. A. T. 2007. *Anthurium andreanum* (Linden ex André) culture: *In vitro* and *Ex vitro*. *Floriculture Ornam. Biotechol.* **1**(1): 61-65.

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, J. Y. and Chuang, K. C. 2013. Development of novel microsatellite markers for effective applications in Anthurium cultivar identification. *Euphytica* **189**: 421-431.

Wang, Z., Weber, J. L., Zhong, G. and Tanksley, S. D. 1994. Survey of plant short tandem DNA repeats. *Theor. Appl. Genet.* **88**:1-6.

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

Wei, W. P., Jian, L. H. and Ping, Z. X. 1996. Studies on breeding of single cell mutagenesis in cut-flower chrysanthemum. *Acta Hortic. Sinica.* **23**(3): 285-288.

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

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., Sun, L., Qiu, S., Liu, J., Xu, J. and Shi, J. 2012. *In vitro* mutagenesis and identification of mutants via ISSR in lily (*Lilium longiflorum*). *Plant Cell Rep.* **31**: 1043-1051.

Xu, M. L., Ren, Z. L. 2007. Biological effect of EMS mutagenesis *in vitro* on Rieger Begonia. *J. Nucl. Agric.Sci.* **21**(6): 577-580.

Yamaguchi, H. 2005. Use of induced mutations for crop improvement: revisited. In: S.K. Datta (ed.), Role of classical mutation breeding in crop improvement, Daya Publishing House, Delhi, pp.1-20.

Yang, Y. H., Chen, F. C. and Tsai, C. T. 2000. Effect of cytokinins on plant regeneration from *in vitro* lamina of *Anthurium. J Chinese Soc. Hortic. Sci.* **48**: 371-377.

Yaycili, O. and Alikamanoglu, S. 2012. Induction of salt-tolerant potato (*Solanum tuberosum* L.) mutants with gamma irradiation and characterization of genetic variations via RAPD-PCR analysis. *Turk. J. Biol.* **36**: 405-412.

Yunus, M. F., Aziz, M. A., Kadir, M. A., Daud, S. K. and Rashid, A. A. 2013. *In vitro* mutagenesis of *Etlingera elatior* (Jack) and early detection of mutation using RAPD markers. *Turk. J. Biol.* **37**: 716-725.

Zaka, R., Chenal, C. and Misset, M. T. 2004. Effect of low doses of short-term gamma radiation on growth and development through two generations of *Pisum sativum. Sci. Total Environ.* **320**: 121-129.

Zalewska, M., Tymoszuk, A., Miler, N. 2011. New chrysanthemum cultivars as a result of *in vitro* mutagenesis with the application of different explant types. *Acta Sci. Pol.* **10**(2): 109-123.

Zargar, G. H., Zeerak, N. A., Ahanger, H. J. and Shafiq A.W. 1994. Mutagenic effect of ethyl methane sulphonate and their combinations in top onion *(Allium cepa var. viviparum). J. Nucl. Agric. Biol.* **23**: 249-250.

Zhang, G. H., Xu, B. Y., Peng, C. Z., Lu, L. 2001. Shoot cutting tissue and propagation *in vitro* of *Anthurium andreanum* Lind. *Acta Agric Shanghai* **17**: 13-16.

Zhang, L., Li, Z., Wang, Y., Jiang, Z., Wang, S. and Huang, H. 2010. Vitamin C, flower color and ploidy variation of hybrids from a ploidy-unbalanced Actinidia interspecific cross and SSR characterization. *Euphytica* **175**: 133-143.

Zhao, Y.P., Guo, W.M. and Wang, G.D. 2004. Aseptic plantlet hardening of *Anthurium andreanum in vitro* culture. *Plant Physiol. Comm.* **40**: 48-50.

Zhou, C.L. and Dai, S.L. 2002. AFLP analysis of some *Dendranthema* spp. *J. Beijing For. Univ.* **24**: 71-75.

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.

Zorrilla-Fontanesi, Y., Cabeza, A., Torres, M., Botella, M. A., Valpuesta, V., Monfort, A., Sanchez-Sevilla, J. F. and Amaya, I. 2011. Development and bin mapping of strawberry genic-SSRs in diploid Fragaria and their transferability across the Rosoideae subfamily. *Mol. Breed.* **27**: 137-156.

Annexures

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.

INDUCTION OF VARIABILITY IN ANTHURIUM (*Anthurium andreanum* Lind.) THROUGH *IN VITRO* MUTAGENESIS

By

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ABSTRACT OF THESIS

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ABSTRACT

Anthurium (*Anthurium andreanum* Lind.) of family Araceae, is highly valued for their exotic flowers and foliage. The attractive characteristics like vibrant inflorescence with straight spathe, candle-like spadix, exotic foliage and particularly the long lasting 'flower' of anthurium have ensured its great commercial importance.

Anthurium is conventionally propagated by traditional vegetative methods such as stem cuttings and suckers which are tedious and not practical when carried out on a large scale. Plants derived from seeds show marked variation in colour, quality, yield and time of first flowering. Seed viability and germination percentage are also low. Seed are viable only for two to three days and germination is as low as 20 to 30 per cent. Hence, there is a need to standardize a quicker method of propagation which may be achieved through *in vitro* techniques. Tissue culture greatly increases the normal multiplication rate of plants and can provide a source of clean planting material. Vegetative means of propagation and poor seed viability limits genetic variation in anthurium and this necessitate alternate means to wider the genetic base. *In vitro* mutation breeding is therefore being proposed as a means to create additional variation.

The present study entitled "Induction of variability in anthurium (*Anthurium andreanum* Lind.) through *in vitro* mutagenesis" was conducted with the objective to induce variation in anthurium var. Tropical and to characterise the variability through morphological and molecular assay.

In vitro mutagenesis in anthurium was carried out using *in vitro* cultures of var. Tropical as source material for treatments. Cultures were treated with gamma radiation as physical mutagen at different doses such as 5, 10, 15 and 20 Gy and chemical mutagen Ethyl Methane Sulphonate (EMS) at different concentrations such as 0.1, 0.2 and 0.5 per cent for 30 minutes.

The irradiation was carried out at Radio Tracer Laboratory, College of Horticulture at room temperature using a gamma chamber equipped with ^{60}Co source. After irradiation the cultures were kept in dark room for two days and later it was transferred to fresh media and incubated in light. The cultures were observed periodically for their response. Cultures exposed to higher doses lost their green appearance while the culture at lower doses (5 Gy) were good in multiplication and number of shoots compared to control. The cultures were sub cultured at one month interval; rooted plantlets planted out and hardened.

The cultures differed in their *in vitro* response with respect to plant height, number of leaves, leaf dimensions and flowering habit. Plantlets derived from low doses (5 Gy) of irradiation performed better than control while higher doses (15 to 20 Gy) gave stunted plants with more number of leaves. The cultures treated with EMS failed to regenerate except those with 0.1 per cent treatment. Mutated plants started flowering within seven months and lot of variations were observed with respect to flower size, colour, length of flower stalk, arrangement of spathe and spadix. The selected variants were analysed at molecular level using RAPD and ISSR assay and genetic variation was confirmed.

The desirable mutants are to be further evaluated for their stability with respect to the altered trait.