Induction of variation in Gerbera (*Gerbera jamesonii* Bolus) and Chrysanthemum (*Chrysanthemum morifolium* Ramat) through gamma radiation and *in vitro* techniques

Ph.D. THESIS

BY

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Induction of variation in Gerbera (*Gerbera jamesonii* Bolus) and Chrysanthemum (*Chrysanthemum morifolium* Ramat) through gamma radiation and *in vitro* techniques

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Dedicated
To
My Respected Parents
And
Beloved Family

CERTIFICATE

This is to certify that the thesis entitled "**Induction of variation in Gerbera (*Gerbera jamesonii* Bolus) and Chrysanthemum (*Chrysanthemum morifolium* Ramat) through gamma radiation and *in vitro* techniques"** submitted by Jebunnesa Chowdhury has been carried out under our supervision in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. It is further to certify that it is an original work and suitable for submission for the award of Ph.D. in Botany (Plant Breeding and Biotechnology).

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-The Author

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ABSTRACT

Globally, the floral industry has experienced significant growth over the last few decades. Chrysanthemum (*Chrysanthemum morifolium* Ramat) and Gerbera (*Gerbera jamesonii* Bolus) are two very popular ornamental plants and widely used as cut flowers for decorative purposes all over the world. Gerbera and Chrysanthemums are popular because they have a lengthy vase life and a wide variety of colors and shapes.

Creation of variation in colour and shape of the flowers in case of both the ornamental plants are very important for commercialization. Both plants are primarily reproduced vegetatively, which restricts the development of variants. Therefore, gamma radiation coupling with techniques of *in vitro* micropropagation was applied to create variation in flower characters of these two economically important ornamental plants. Specifically, the objectives of the present investigation were to develop elite mutant lines of Chrysanthemum and Gerbera through gamma radiation and *in vitro* techniques. For this purpose, an efficient *in vitro* regeneration system was established for both locally grown Chrysanthemum and Gerbera varieties, namely, BARI Chrysanthemum-1, BARI Chrysanthemum-2, BARI Gerbera-1 and BARI Gerbera-2. Three different types of explants, namely, shoot tip, internode (IN) and leaf (L) segments of two varieties of Chrysanthemum were used for *in vitro* regeneration. Maximum responses of multiple shoot regeneration (94.73% for BARI Chrysanthemum-1 and 90% for BARI Chrysanthemum-2) in both the varieties were achieved when the leaf explants were cultured on MS medium supplemented with 2.0 mg/l IAA and 0.5 mg/l BAP (T6), followed by sub-culturing on hormone free MS medium. It took about six weeks for the regeneration of multiple shoots.

On the other hand, three different types of Gerbera explants, namely, flower bud (FB), flower stalk (FS) and leaf segments (LS) were used for *in vitro* regeneration of both the varieties. Highest percentage of multiple shoot regeneration (70.00% for Gerbera-1 and 73.30% for Gerbera-2) was achieved in both Gerbera varieties when the flower bud explants were cultured on MS medium supplemented with 6.0 mg/l BAP and 1.0 mg/l NAA (T6), followed by two subsequent cultures on half the strength MS medium with 2.0 mg/l BAP. It took about 7-8 weeks for the regeneration of multiple shoots in this case.

During elongation more than 90-95% of the shoots from both the varieties of Chrysanthemum produced healthy roots on hormone free MS medium within 6 to 7 weeks of culture. While the best responses towards root induction in two varieties of Gerbera was recoded on half strength of MS medium containing 0.5 mg/l of IAA. Fully developed *in vitro* regenerated plantlets of both Chrysanthemum and Gerbera were successfully established in soil for further growth and development of flowers.

For the induction of desired mutation in both Chrysanthemum and Gerbera five doses of gamma radiation (5Gy, 10Gy, 15Gy, 20Gy and 25Gy) were applied to the *in vitro* grown micro shoots. The survivability of the irradiated shoots (60 days after irradiation) was evaluated following determination of LD_{50} (50% lethal dose). The highest survival percentage was observed for 5 Gy irradiated shoots for both Chrysanthemum varieties (57.29% for BARI Chrysanthemum-1 and 64.08% for BARI Chrysanthemum-2). LD_{50} (50% lethal dose) was found at 9.25 Gy for BARI Chrysanthemum-1 and 11.19 Gy for BARI Chrysanthemum-2 variety. In case of Gerbera the highest survival percentage was observed for 5 Gy irradiated shoots for both varieties (72.77% for BARI Gerbera-1 and 68.09% for BARI Gerbera-2). The LD_{50} for BARI Gerbera -1 and BARI Gerbera-2 variety was found at 11.17 Gy and 9.32 Gy respectively. Among the five irradiation doses used, 15 Gy produced highest percentage of mutation frequency (75-80%) regarding the changes in leaf shape, nature of pigmentation, and internode size and plant height in BARI Chrysanthemum-1. Whereas in case of BARI Chrysanthemum-2, three different doses (5Gy, 10 Gy and 15 Gy) produced high frequency of mutation regarding the same factors as discussed for BARI Chrysanthemum-1.

In the case of BARI Gerbera-1, irradiation doses of 5 Gy and 20 Gy caused 20% and 30%, respectively, of mutation frequency in the case of the flower's shape and color. On the other hand, in the instance of BARI Gerbera-2, irradiation doses of 5 Gy and 10 Gy respectively caused a mutation frequency of 45% and 20% for flower morphologies and color.

Following the morphological studies, it was recorded that BARI Chrysanthemum-1 produced four mutant lines (YM1, Y1, Y5 and Y6) while BARI Chrysanthemum-2 produced three mutant lines (M1, M2 and M6) finally. Based on morphological differences BARI Gerbera-1 produced three mutant lines (WV1, WV2 and WV3) and BARI Gerbera-2 produced three mutant lines (RV1, RV2 and RV3). No variation was

recorded on the plant developed from non-irradiated *in-vitro* culture of both Chrysanthemum and Gerbera varieties used.

Further characterization of induced mutants was confirmed through ISSR (Inter-simple sequence repeats) molecular marker analysis. High levels of polymorphism (90.90% for chrysanthemum and 89.06% for Gerbera) were recorded among the mutants developed in these two plants. Moreover, this study revealed that mutants exhibited a broad range of diversity among themselves as well as different from their control plants. The mutants that developed through this study can be cultivated as new variants of Chrysanthemum and Gerbera.

1. INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium* Ramat) and Gerbera (*Gerbera jamesonii* Bolus) are two popular cut-flowers, successfully grown under different agro-ecological conditions in several areas of the world to meet huge demand. Chrysanthemum is 3rd and Gerbera is the 5th most used cut flower around the world. The reason for the popularity of these two flowers is due to the wide range of color and shape as well as the long vase life. Global boom in floriculture business for many countries, especially the cut flowers have become one of the leading areas for production of export quality flowers.

Floriculture is one of the newest rapidly growing agricultural sectors having a huge economic impact. Rose, carnation, Chrysanthemum, gladiolus, gerbera, orchids, lilies, tulip, etc. are the important floriculture crops in international flower trade.

In Bangladesh, floriculture has increased national attention since the late 1970s, with the small-scale cultivation of tuberose. Later, in the middle of the 1980s, Jhikargacha Upazila in the Jashore area began commercially producing a variety of flowers and flowering plants (Sultana 2003). Now, Jashore, Savar, Chuandanga, Mymensingh, and Gazipur are regarded as primary flower production hubs of our country. Four types of flowers, including roses, tuberoses, marigolds, and gladioli, account for almost 90% of the domestic flower industry's profits. But to meet local demand various flowers, such as Chrysanthemum, tuberose and gladiolus are being imported from India and orchids, gerbera, anthurium and Thai rose from Thailand every year. According to Dadlani (2003), Growing flowers can give 3-5 times more income instead of grains and 1.5-2.0 times more income than growing vegetables, respectively.

Every year in February, Bangladeshi flower growers and traders earn huge income. The number of sales is highest in this month as we Bangladeshi celebrate three major events – “Pohela Falgun” (Spring Festival), Valentine’s Day and International Mother Language Day. According to Mohiuddin (2016), every year flowers of about USD 54 crores were produced in Godkhali alone and a total of USD 100 crores money was earned. According to the report of Chowdhury *et al*. (2021), the wholesale flower business in Bangladesh is led by hundreds of enlisted traders. Flowers worth roughly taka 400 million were sold in these 40 retail outlets, under the "Shahbagh Battala Small flower Merchants Cooperative Association" in February 2020 before the

Coronavirus pandemic restrictions were enacted. Before the pandemic restrictions, each shop used to sell flowers worth at least Tk 40,000 per day, according to Md. Shamim Ahmed, general secretary of the Small Flower Merchants Cooperative Society. Flowers from Jibannagar and Gadkhali in Chuadanga arrive in big quantities in Shahbagh. The majority of the roses are grown in "Golap Gram" in Savar and Ashulia, Dhaka and supplied to Shahbagh.

According to Yearbook of Agriculture Statistics 2021of Bangladesh Bureau of Statistics (BBS), during the year 2020-21, the flower production area of Bangladesh was 3548.53 acres and the production was 32121.21 MT.

World Floriculture Map 2016 reported that the cut flower production area measures only 4380 ha (both covered and in the open field). The Netherlands is a major junction of international floriculture trade. Netherlands exports the largest amount of cut flower around 43% while other top ten cut flower exporter countries are Colombia (15%), Kenya (11%), Ecuador (9%), Malaysia (1%), China (1%), Italy (1%), Belgium (1%) and Germany (1%).

Since the 1990s, flower production centers have been shifting from Europe and North America to the countries where climatic conditions are more adaptable, and cost of production are lower. Now new promising centers of production are typically developing countries such as Colombia, Kenya, Ecuador and Ethiopia (Khan 2013), where Bangladesh can be a new addition. According to Export Promotion Bureau (EPB), the export earning of Bangladesh from cut flowers and foliage for the July to January period of the fiscal year 2018-19 touched $3.98 million, which was $0.02 million in the 2017-18 fiscal year.

Recently, Chrysanthemum (*Chrysanthemum morifolium* Ramat) and Gerbera (*Gerbera jamesonii* Bolus) are becoming very popular ornamental plants and are widely used for decorative purposes in Bangladesh. Based on the market demand, the ranks of cut flowers are rose, carnation, Chrysanthemum, tulip and gerbera. Both Chrysanthemum and gerbera flowers belong to the family Asteraceae.

Chrysanthemum and gerberas are great flowers for adding color to any room or garden. The eye-catching flower attracts bees and butterflies. They are used as model plants in studying flower formation. They also used in the preparation of traditional

Chinese medicines as they are rich in Asteraceae-type secondary metabolites. Moreover, different Chrysanthemum and gerbera varieties show an amazing spectrum of color, at all floral parts (important morphological levels i.e flower type, flower organ and within organs) during developmental stages.

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) flower is popularly known as 'Autumn Queen'. The word Chrysanthemum is derived from two Greek words '*chrysos*' means golden and '*anthos*' means flower (Gortzing and Gillow 1964). Most cultivated greenhouse or garden Chrysanthemums are classified as *Dendranthema* x *grandiflora* Tzvelv. (Kitamura) (Ramatuelle) (=*Chrysanthemum* x *morifolium* Ramat.; =*C.* x *hortorum* Ramat.), cultivars are hexaploids, with 2n = 6x = 54 chromosomes (Shinoyama *et al*. 2006).

The flower head of Chrysanthemum is composed of many florets borne on a receptacle. The outer ray florets are pistillate and infertile. They are developed around the outer rim of the receptacle. The inner disc florets are inconspicuous and fertile. In large–flowered Chrysanthemum varieties, the outer ray florets are prominent whereas the inner disc florets are either absent or inconspicuous. Many variations are found in Chrysanthemum cultivars based on the growth habit, size, color and shape of the bloom. According to the blooming pattern two types of Chrysanthemums (Standard and Spray) are available. Standard types possessing single large flower blooming at the top with long sturdy stems are suitable for cut flowers, whereas cultivars with more numbers of sprays per plant are recommended for cut flower production as well as garden decoration and exhibition. Shapes and sizes of Chrysanthemum bloom vary from pompon to daisy-like and button to anemone. Each type has its own distinctive characteristics, but all are hardy and long lasting.

Chrysanthemum is a cool climate crop and requires 20-28^{0}C (day) and 15-20^{0}C (night) temperature for flower bud initiation (Van Der Ploeg and Heuvelink 2006). However, the crop is well adapted to different climatic conditions. It is a qualitative short-day plant that requires 14.5 hrs photoperiod for flower initiation and 13.5 hrs light period for bud development (Runkle and Fisher 2004).

Gerbera is native to Transvaal and Natal province of South Africa commonly known as Transvaal Daisy, Barberton Daisy or African Daisy. The name Gerbera was coined

according to the name of German botanist and naturalist Traugott Gerbera (1743). J. D. Hooker in 1889 made the first scientific description of a gerbera in Curtis's Botanical Magazine when he described *Gerbera jamesonii*, as a south African species (Hooker 1912). It is perfect for growing in beds, borders, pots, and rock gardens. Cut gerberas have a long vase life and are suitable for different floral arrangements. Gerbera plant is a stemless, perennial herb (Bose *et al.* 2003). The flowers may be single, double or semi double. A wide range of variations is available in gerbera color, shape and types. The flower head is called capitulum. It consists of thin, long and conspicuous ray florets arranged in one or two outer whorls. Short and sometimes tubular disc florets are arranged in the inner rows of capitulum. Flower stalks are thin, long, slender, and leafless. The chromosome number of gerbera is 2n=50. Gerbera needs mild weather conditions, temperature 16-20°C during daytime and 12°C at night is suitable for its vegetative growth and flowering. Extremely cold or hot temperatures are not tolerable for gerbera. Therefore, under tropical and subtropical climate gerbera is grown in open or in unheated plastic houses.

Both gerberas and Chrysanthemums can be reproduced sexually and asexually. Gerberas cannot be grown from seed due to several factors, including their extreme heterozygous nature, low multiplication rate during asexual propagation, lengthy flowering times, limited yield, and strict weather conditions. Chrysanthemum cultivation also faces almost similar limitations adding more complex factors like high level of ploidy, heterozygosity, larger genome (hexaploids relative to diploids) and self-incompatibility.

Vegetative propagation of these two flowering plants is possible through divisions, but using this method, plant multiplication is very slow to be commercially feasible. Only a 50 to100 fold increase per year of a selected gerbera plant can be obtained by division (Murashige *et al.* 1974). But by tissue culture multiplication, a desirable plant might be grown a million times per year without any abnormalities. Many studies on the *in vitro* culture of Chrysanthemum and gerbera in recent years have proved that this technology is a feasible alternative for the quick multiplication of a particular plant species (Murashige *et al.* 1974; Nagatomi *et al.* 1997; Aswath and Choudhary 2002; Aswath *et al.* 2003; Teixeira da Silva 2003a, 2003b; Akter *et al*. 2012; Datta 2015; Teixeira da Silva *et al.* 2015; Chowdhury *et al.* 2021). Thus, *in vitro* culture has

been considered helpful for large-scale production and necessary for creating new Chrysanthemum or gerbera cultivars. Different explants of gerbera were used in micropropagation, namely, shoot tip, flower bud, flower, leaf, peduncle, petiole (Cardoso and Silva 2013). Gerbera plants were produced from explants of capitulum in red flower gerbera (Pierik *et al.* 1973; Pierik *et al.* 1975), leaves (Hedtrich 1979; Barbosa *et al.* 1994; Rahman *et al*. 2018), floral buds (Posada *et al.* 1999; Kanwar and Kumar 2007; Talla *et al.* 2018; Gantait and Mahanta 2021), floral bracts (Maia *et al.* 1983), torus (Zhang 2002) and inflorescence (Schum and Busold 1985). In Bangladesh, three varieties of gerbera (*Gerbera jamesonii* Bolus.) were used to develop an *in vitro* regeneration protocol using flower bud and flower stalk explants (Akter *et al*. 2012). The success of micropropagation was found to be better using shoot tip and flower bud than other explants.

Similarly, different explants, namely, shoot tip, leaf, leaf with petiole, leaf segment, internode, single node, ray florets, petals, petiole, etc. were used for *in vitro* culture of Chrysanthemum (Jaramillo *et al.*2008; Waseem *et al.* 2011; Zalewska *et al.* 2011; Lim *et al.* 2012; Miller and Malgorzata 2014; Naing *et al.* 2014; Miler and Kulus 2018; Nasri *et al.* 2018; Tymoszuk and Kulus 2020).

According to some studies, producing regenerated plants through somatic embryogenesis and direct or indirect organogenesis is not only useful for propagating plant material but a way to increase the genetic diversity of Chrysanthemum species to add new cultivars or introduce useful traits (Miler and Malgorzata 2014; Lema-Rumińska and Mellem 2017; Huang *et al.* 2018). A few reports are available in Bangladesh on *in vitro* regeneration protocol development through nodal segments and internode explants using imported Chrysanthemum varieties (Yesmin *et al.* 2014). Chowdhury *et al* (2021) developed an efficient regeneration protocol for Bangladeshi Chrysanthemum varieties using leaf and internode explants. Most of the aforementioned research studies reported leaves as the most suitable explant for Chrysanthemum.

Large quantities of genetically stable plants can be produced quickly by micropropagation. Somaclonal variation may occasionally add a small amount of diversity, but it is insufficient to meet customer demand.

In the US, Japan, Germany, and the UK, Chrysanthemum and gerbera have high market values. In Asia, Indonesia and Malaysia are the two most important suppliers, whereas the Netherlands dominate in Europe. The popularity of Chrysanthemum and gerbera among flower lovers has steadily risen over the years and many commercial growers had to increase their production of these two colorful flowers to meet the market demand.

In Bangladesh, they have also become widely used as ornamental plants, and there is a growing demand for these flowers, especially in metropolitan areas. Yet, there isn't a sufficient supply of Chrysanthemum and gerbera plantlets to satisfy local demand. Besides, the available color of the flower is also very limited. Under these circumstances, the local flower markets are looking for new variations.

Mutation breeding is regarded as one of the important techniques for producing additional beneficial variation in horticultural crops that are grown vegetatively. By mutation breeding, a great number of variations have been created for ornamentals. According to the FAO/IAEA (2021) mutant variety database, the majority of the 465 mutants that were developed among the vegetatively propagated plants, were in the plants used in floriculture.

There has been considerable success with both physical and chemical mutagens in breeding ornamental plants, as they have produced quite a large number of new varieties (Datta *et al.* 2005). The induced mutation process has created several new and novel cultivars of Chrysanthemums and gerberas. In vegetatively propagated plants, chemical mutagens are not widely used due to their low penetration levels. Physical mutagens are used to induce mutations in Chrysanthemum and gerbera because they are hexaploid plants propagated vegetatively, which makes them difficult to hybridize (Dwimahyani and Widiarsih 2010; Patil *et al*. 2017).

Physical mutagens like radiation cause mutations in plant cells when certain dosages are applied. Morphological changes were noticed in intact and *in vitro* plants after exposure to the radiation (Hasbullah *et al.* 2012). Ionizing radiation's gamma rays interact with atoms or molecules to create free radicals in living cells. Depending on the amount of radiation a plant receives, these radicals have been shown to influence

the morphology, anatomy, biochemistry, and physiology of plants differently. These impacts include modifications to the plant's cellular structure and metabolism, such as thylakoid membrane dilatation, altered photosynthesis, oxidative system modulation, and phenolic compound accumulation (Kovacs and Keresztes 2002; Wi *et al*. 2005; Kim *et al*. 2016).

Moreover, agricultural items are sterilized with ionizing radiation to lengthen their shelf life or to reduce germs when being traded internationally (Melki and Salami 2008). Moreover, gamma rays do not pose a threat to humankind and environment, and they are easily obtained. So, there has been a strong consideration that application of gamma rays should be preferred (Ulukapi and Nasircilar 2015).

The ornamentals have been improved using a variety of treatment methods, including direct dosage, split dose, recurring irradiation, and combined treatment. The large variety of propagules made it possible to determine radiosensitivity in ornamentals, which improved fundamental understanding. For the practical application of applied mutagenesis, it is also crucial to determine the radiosensitivity of various cultivars of the same crop. Gamma radiation mutation frequency varies depending on cultivar and dose. Certain cultivars are slightly more sensitive to mutagens than others, while others are resistant to them altogether. All experimental ornamentals have a precise effective dose that has been determined.

The genetic makeup of Chrysanthemum and gerbera is extremely heterozygous, making them excellent study materials for induced physical mutagenesis. Many researchers have investigated how physical mutagens affect Chrysanthemum and gerbera.

In an experiment, Walther and Sauer (1985) examined radio sensitivity, a prerequisite for *in vitro* somatic mutagenesis in *Geberea jamesonii*. Axillary shoots from two lines (A26 and 82/19/6) that were created as microshoots *in vitro* were exposed to X-ray dosages ranging from 10 to 25 Gy. Explant survival rate, the number of developing shoots on the first cutoff date (27 days after irradiation), and the total number of axillary shoots on four successive dates were evaluated for their utility in determining radiosensitivity during the 16 weeks of post-irradiation culture. Regeneration rates were dose dependent, the higher X-ray doses resulting in greater inhibition of shoot

regeneration. Irradiation-induced damage was higher in A26 than in 82/19/6. The increase in the number of regenerated shoots on each of the four subsequent assessment dates demonstrated the ability of the cells to repair a certain amount of the irradiation-induced damage. The results indicated that inhibition of shoot development on the first cut-off date could be used to estimate the radiosensitivity of gerbera idiotypes.

The first vegetative generation following irradiation in *G. jamesonii* produced a significant number of chimeras, which significantly raised the number of mutants with uniformly altered inflorescence color in the second vegetative generation. Twelve new cultivars of *D. grandiflora* were developed from cv. Richmond by this technique. Of which six cultivars entered the Polish cultivar Register in 1993.After irradiation with 15 Gy X-rays, Lady Antee (golden yellow), Lady Bronze (reddish brown), Lady Rosy (creamy pink) was obtained and after 15 Gy gamma radiation Lady Pink (pink), Lady Salmon and Lady Yellow were obtained. Bronze Wonder and Red Wonder were obtained from the violet pink Lilac Wonder by application of 15 Gy gamma radiation and were submitted to the Research Centre of Cultivar Testing in 1993. Also in 1993, the red *G. jamesonii* mutant cultivar Red Raisa (obtained after applying 25 Gy gamma radiations to violet- pink cv. Raisa) entered the Register (Jerzy and Lubomski 1992).

Laneri *et al.* (1990) collected shoots from *in vitro*-raised plantlets of gerbera (pink cv. Rebecca) and were irradiated with a single dose of (20 Gy) gamma radiation. Then they were micropropagated for two cycles. After rooting plantlets were grown in the greenhouse and examined for morphological changes. The propagation rate was reduced by 25% in each cycle through the induction of radiation.

According to Hasbullah *et al*. (2012), gamma radiation had a remarkable impact on the amount of soluble protein and chlorophyll present in the irradiated callus tissues of *Gerbera jamesonii.* This study proves that radiation effects could yield mutants with better quality, showing useful and applicable somaclonal changes in the morphological features of *G. jamesonii*. Hence, more economically viable varieties could be developed.

Colchicine was used by Gantait *et al*. (2011) to induce *in vitro* mutation, and several polyploids (tetraploids) were retrieved from *Gerbera jamesonii* Bolus cv. Sciella.

Due to the high level of ploidy, heterozygosity, bigger genome (hexaploids compared to diploids), and self-incompatibility of Chrysanthemum, mutant breeding may be the most common kind of chrysanthemum breeding. Exposure to physical or chemical mutagens can result in mutagenesis and somaclonal variation leading to create new colored plants with unusual leaf shapes or habits (Nagatomi *et al.* 1997; Datta 2015; Teixeira da Silva *et al.* 2015)

A lot of work has been done on *C. morifolium* to improve it through induced mutation by a variety of researchers in different places of globe. A wide range of physical mutagens like X-ray (Broertjes *et al*. 1980), gamma rays (Datta and Gupta 1980; Datta *et al*. 2005), fast neutrons (Broertjes *et al*. 1980), thermal neutrons (Bowen 1965), radioactive phosphorus (Drelow and Widmer 1971) and chemical mutagen like Ethylene Imine (Bowen 1965), ethyl methane sulphonate (Bowen 1965) and colchicine (Weedle 1941) have been used for such studies. Many ornamental mutant types have already been released for sale (Datta 2020).

Datta (2015) reported induced mutation of floral color variation in early and late blooming cultivars using gamma irradiation. Using one early blooming var. Somatic mutations in the color of the flowers were found in the 'Ajay' (Amaranth pink florets) and three late blooming varieties, 'Gouri' (white), 'Maghi' (mauve), and 'Puja' (pink). Two mutants (light pink and dark pink) were developed from ‘Ajay’, ‘Gouri’ developed two (cream and yellow), ‘Puja’ three (lighter, yellow and Terracotta) and ‘Maghi’ developed four (lighter, white, yellow, dark yellow) mutants. Maghi is a very demanding late blooming variety that blooms in the subtropical Lucknow climate in India at the end of January when no other chrysanthemum flowers are available. The isolated white and yellow varieties are quite impressive and will benefit the floriculture sector (Datta 2015).

A gamma ray induced mutant named Batik with yellow stripes on a red background of florets that was created from a chrysanthemum cv. "Flirt" with double Korean red and purple flowers. One late blooming small forward pompon type cv. 'Maghi' with mauve flowers has been developed with one white flower color mutant ('Maghi White'). Further gamma irradiation of "Maghi White" led to the development of a striped mutant (the "Maghi stripe") with yellow stripes on a white background (Datta 2015).

The choice of an appropriate genotype would be beneficial to produce flower color variety in chrysanthemums through mutation. Many reports on induced mutations in chrysanthemum would support this (Lamseejan *et al.* 2000). Pink chrysanthemums are particularly vulnerable to mutation and have a maximum number of dominant genes for floral color. Hence, they are more likely to produce recessive mutations that may be seen in M1V1 (Bowen *et al.* 1962; Bowen 1965; Broertjes 1978).

Yellow-flowering chrysanthemum cultivars have been reported to be very stable (Bowen 1965; Broertjes 1978; Gupta 1979). After gamma irradiation Datta and Gupta (1980) detected the yellow sector in white flowered cultivar 'Lilith'. After irradiating an E-13, a mauve-colored, small-flowered chrysanthemum of the pompon type, Datta (1985) successfully developed a Canary yellow flower color mutation. Gupta (1979) and Jank (1957) also reported induction of flower color mutations in bronze cultivars. Heterozygosity allows chimeric sports to be easily seen in ornamental germplasm. This area of floriculture trade is still unexplored. This method has successfully assisted in the isolation of spontaneous mutations and enriched the ornamental germplasm. This *in vitro* technique has been utilized to overcome chimera formation and development of solid mutants (Mandal *et al*. 2000; Mandal and Datta 2005).

The prominent chrysanthemum cultivars "Lalima," "Flirt," "Puja," "Maghi," and "Sunil" were used in a series of *in vitro* mutagenesis tests. Ray florets were exposed to 0.5 and 1.0 krad of gamma rays and regenerated following standard tissue culture techniques. Mutations were induced in all the tested varieties and the developed mutants (flower color and shape) were mostly solid. These mutants have generated true-to-type florets after being vegetatively propagated. Both induced mutation and natural mutation can be successfully treated with the standardized technique for the isolation of flower color chimeras. This method is useful for other ornamental breeders as well as chrysanthemum cultivation (Misra *et al.* 2003; Datta and Mandal 2005).

Kumari *et al.* (2013) found 10 Gy and 15 Gy gamma radiation doses were good for induction of color and shape mutations in chrysanthemum flowers. However, they found that most of the mutations took the form of chimeras, suggesting that creating chimeras using tissue culture techniques may be advantageous.

Through 0.5 Gy gamma irradiation Misra *et al.* (2003) developed two yellow-colored mutants of *Chrysanthemum morifolium* Ramat cv. Lalima.

The effect of various rates of gamma radiation on the frequency of mutation in inflorescence color and type of chimerism in chrysanthemum cv. 'Cherry Dark' was studied by Boersen *et al*. (2006) and a linear decrease in plant height and a quadratic tendency in survival percentage were observed with the increase in mutagen doses.

Kaul *et al.* (2011) reported, 10 Gy of gamma irradiation was the most effective in inducing mutations in flower color through direct *in vitro* mutagenesis. The original flower color of the cv. Snowball was white with incurve ray florets, whereas one branch of variant V9 produced yellow-colored flowers with incurve ray florets.

Kumar *et al.* (2012) found 20 Gy as optimal dose of gamma radiation for *in vitro* mutations and selected resistant plants of chrysanthemum toward *Septoria obesa*, a leaf spot pathogen.

The recommended doses for mutation induction in chrysanthemum are 10–20 Gy for *in vitro* cultures and 10–25 Gy for rooted cuttings (Broerties and Van Harten1988; Tien *et al.* 2000).

In vitro technology has made it possible to rapidly reproduce newly developed mutants, including chimeras made up of cells with multiple unique genotypes (Tymoszuk and Kulus 2020). The enormous breeding potential of tissue culture is still underutilized. Metal nano-colloids can be added to the culture media (for explant regeneration) in an affordable, simple, and efficient approach to encourage genetic variety (Tymoszuk and Kulus 2020).

The main problem with vegetatively propagated plants is that the mutation appears as a chimera after treatment with physical and/or chemical mutagens, despite mutation breeding being thought of as a useful method for adding new and novel ornamental cultivars (Mandal *et al.* 2000). Mutated cells coexist in chimeric tissues with normal cells. The mutant sector might be as small as a thin streak on a petal or as large as the entire flower or branch, depending on its size. The mutant tissue can be isolated when a branch, or a section of it, is altered. On the other hand, using the currently known traditional propagation techniques, a small portion of a mutant branch or flower

cannot be isolated. Therefore, many new flower color/shape mutants induced by mutagens are lost due to the lack of a regeneration system from small, mutated sectors either *in vivo* or *in vitro*. Recent developments in tissue culture and molecular genetics brought new tools for generating mutations. Hence the urgent need has been felt to optimize tissue culture techniques to regenerate plants from such mutated sectors and establish mutated chimeric tissues. This *in vitro* approach may open new windows to produce a wide range of new flower color/type mutants for the floriculture trade.

During evolution, genetic variability accumulates in a random fashion, after which morphological, biochemical, or physiological changes are induced and stabilized by environmental pressure. Besides, genetic variability can also be obtained through the use of mutation induction as mentioned above. Phenotypic markers such as morphological or anatomical characters are influenced by environmental changes. Therefore, molecular markers are needed which are not directly influenced by environmental changes. Thus, molecular markers are very useful for studies of genetic characterization and identification of mutants.

Using methods like map-based cloning and Marker-assisted Plant (MAP) Breeding, the idea of DNA-based markers has improved our capacity to track tiny portions of chromosomes and their changes. As the field of plant breeding developed, many methods were employed to address the issue that plant breeders were facing. The creation of marker systems, which was started with mutations at the loci determining plant morphology, is one of these strategies. The changed plant phenotype caused by these mutations ranges from pigment differences and drastic changes in growth (like vernalization habit or dwarf vs. tall habit) to an increased response to diseases.

The restricted availability of many mutants and the fact that morphological markers were not neutral in their impact on agronomic phenotype. This prevented morphological markers from being widely used in practical plant breeding (Worland *et al*.1987). These markers were also not thought to be trustworthy. Because the complex interactions between the trait's relevant genotype and environment could lead to incorrect phenotypic identification. It is challenging to effectively use the dominant phenotypes in plant breeding since they do express, but only at very low frequency in

a particular species. Protein and isozyme markers were developed as a remedy to some of these issues (Markert and Moller 1959).

Most mutants are often screened using their phenotypic qualities; however, this form of selection is time-consuming and labor-intensive, and the traits of interest may be influenced by environmental factors (Xi *et al.* 2012). Moreover, mutagenesis studies require large plant populations as mutation is a low-frequency event in plant cells and most regenerants are not affected by mutagenic treatment. This reveals the significance of keeping the potential regenerated lines up until the final selection stage of desired mutants that raise the price of breeding programs (Akhar *et al.* 2016).

Currently, molecular markers are successfully utilized in mutagenesis breeding during the early stages of the experiment to eliminate non-mutated plants and accurately identify true mutants (Xi *et al.* 2012). In addition, molecular markers can reveal the genetic relationship between the mutants and original mother plants (Kang *et al.* 2013).

Conventional morphological indicators are helpful, but molecular markers can effectively validate this for precise identification and placement of such genotypes in more appropriate positions based on their genetic relationships (Das *et al.* 2009). For their low cost, simplicity, reproducibility, and lack of prerequisite information, ISSR markers have been shown to be the most effective method for genetic diversity study. Due to the high degree of polymorphic nature, ISSR markers are extensively used (Venkatesan *et al.* 2021).

In contrast to other marker systems like short sequence repeats (SSR), amplified fragment length polymorphism (AFLP), and random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) markers are genomic fragments between adjacent, oppositely oriented microsatellite regions that range in size from 100 to 3000 bp (SSR). ISSRs do not require previous information of the genome, variable primer length, motif, and anchor are available, and they are highly polymorphic and informative (Reddy *et al*. 2002; Gholami *et al.* 2021).

Inter-simple sequence repeats (ISSRs) are a multilocus, dominant, repeatable, and highly polymorphic marker that is effective for genetic diversity research (Reddy *et*

al. 2002). It is thought that ISSR repeats are primarily found in non-coding chromosomal regions and certain stretches of inactive DNA (Azhar *et al.* 2013).

If the ISSR primers are sufficiently specific, high repeatability may be observed (Tsumura *et al*. 1996; Luz *et al*. 2020). Many ISSR markers' use for assessing the genetic diversity of chrysanthemums have also been found in some research (Shao *et al.* 2010; Zhang *et al.* 2010; Samarina *et al.* 2021). ISSR is distinct and useful for DNA fingerprinting due to its high frequency among normal coding genes and its presence on some chromosomes as satellite bodies. For identifying the new variety, the Inter Simple Sequence Repeat method is very useful (Das *et al*. 2009; Nasri *et al.* 2018). A lot of markers may be produced quickly, and the method is reliable and reproducible (Das *et al*. 2009; Wang *et al*. 2017). According to Palai and Rout (2011), ISSR markers were used to distinguish new variants from existing types that had been established through sports.

Polymorphism among the mother plants and irradiated plants of chrysanthemum using ISSR marker has been successfully presented by many researchers (Palai and Rout 2011; Tymoszuk and Kulus 2020; Wang *et al*. 2020; Nasri *et al.* 2021).

To study the clonal fidelity and polymorphism of gerbera several researchers have chosen the ISSR marker (Bhatia *et al.* 2010; Gantait *et al.* 2011; Gantait and Mahanta 2021).

To date no study has been done on chrysanthemum and gerbera for validation of the successful mutation using ISSR or with any other molecular markers in Bangladesh. Identification of mother plants, tissue cultured plants or mutated plants using ISSR molecular markers in Bangladesh is important for enrichment of the existing germplasm. To the best of our knowledge nobody from Bangladesh has reported any flower mutant developed from chrysanthemum and gerbera through *in vitro* and gamma radiation techniques.

Variation of attractive colors and long vase life of both chrysanthemum and gerbera attracted interest in Bangladesh. The diversity of inflorescence patterns and colors in chrysanthemum and gerbera are evidenced for the beauty of these flowers. Beside this, Bangladesh has a favorable climatic condition and is capable of producing a wide array of these two flowers of international standard.

Based on circumstances and considering the need of variation in color and shape of these two ornamental plants, the present investigation was undertaken to achieve the following objectives –

1. Development of *in vitro* regeneration protocols for the selected chrysanthemum and gerbera varieties.
2. Application of tissue culture techniques for induction of mutation using gamma irradiation in selected chrysanthemum and gerbera varieties to induce genetic variability in the existing gene pool through mutation.
3. Study of the morphological changes in chrysanthemum and gerbera after exposure to different doses of gamma irradiation under *in vitro*, field and greenhouse conditions to select and identify the promising mutant lines.
4. Selection and identification of the mutant through the analysis of vegetative and floral characteristics.
5. Finally, analysis of the mutant was carried out through the study of polymorphism using inter simple sequence repeat (ISSR) molecular.

2. MATERIALS

2.1 Plant materials

Chrysanthemum (*Chrysanthemum morifolium* Ramat):

The following two varieties of Chrysanthemum (*Chrysanthemum morifolium* Ramat**)** were used in the present investigation:

i. BARI Chrysanthemum-1 (yellow)
ii. BARI Chrysanthemum-2 (white)

Gerbera (*Gerbera jamesonii* Bolus):

The following two varieties of Gerbera (*Gerbera jamesonii* Bolus) were used in the present investigation:

i. BARI Gerbera-1 (white)
ii. BARI Gerbera-2 (red)

2.1.1. Source

Plantlets of two varieties of Gerbera (*Gerbera jamesonii*) and Chrysanthemum (*Chrysanthemum morifolium)* were collected from Ornamental and Floriculture Division of the Horticulture Research Center (HRC), Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Bangladesh. All the mother plants were maintained in the garden of Department of Botany, university of Dhaka, Bangladesh for six months before and after starting the experiment to see the nature of the plants.

2.1.2. Description of two varieties of *Chrysanthemum morifolium* and *Gerbera jamesonii*

The origin and important characteristics about these two varieties are described below:

(i) *Chrysanthemum morifolium* var. BARI Chrysanthemum-1 (yellow)

Morphological characters

Habit and habitat: Herbaceous and perennial herb.

Stem: These plants may have multiple flowered stems (spray type), 74-84 cm long, branched.

Flowers: Nice yellow colored spoon type flowers. They are same as the semi-double, except the ray florets are like spoons at the tips. The center disk is round and visible. Flower size 8 - 10 cm in diameter (Fig. 1a)

Leaves: Deep green in color. Leaves are simple, alternately arranged and with no petiole.

Flowering habit: It is an early variety. Flowers in between 110 and120 days, highly suitable for pot culture.

(ii) *Chrysanthemum morifolium* var. BARI Chrysanthemum-2 (white)

Morphological characters

Habit and habitat: Herbaceous and perennial herb.

Stem: These plants formed multiple- flowered stems (spray type). About 40 - 45 cm long, branched.

Flowers: Attractive white color anemone type flowers. Flower Size: 5-7 cm in diameter (Fig. 1b).

Leaves: Light green in color. Leaves are simple, alternately arranged with no petiole.

Flowering habit: It is an early variety mostly flowering takes place from early December to early February. Flowers in between 90and100 days, highly suitable for pot culture.

(iii) *Gerbera jamesonii* var. BARI Gerbera-1 (white)

Morphological characters

Habit and habitat: Herbaceous and perennial herb.

Stem: Cylindrical, smooth with full green pigmentation, 40 - 50 cm long, unbranched.

Flowers: Plants are come in a white floral color covered with silky hairs arising from a crown. Flower size varies from 8.5 - 9.5 cm in diameter (Fig. 1c).

Leaves: Leaves occur in basal rosettes, petioled, oblong-spatulate and deeply lobed by half as wide. Light green in color.

Fig. 1 (a-d): Two varieties of Chrysanthemum and Gerbera used in study. (a) Flower of BARI Chrysanthemum-1 (yellow colour); (b) Flower of BARI Chrysanthemum-2 (white colour) ; (c) Flower of BARI Gerbera-1 (white colour); (d) Flower of BARI Gerbera -2 (red flower).

Flowering habit: It takes about 110-130 days to flower depending on sowing time and
soil conditions

(iv) *Gerbera jamesonii* var. BARI Gerbera-2 (red)

Morphological characters

Habit and habitat: Herbaceous and perennial herb.
Stem: Cylindrical, smooth with full green pigmentation, 35 - 45 cm long, unbranched.
Flowers: Plants are come in a red floral color covered with silky hairs arising from a crown. Flower size varies from 06 - 09 cm in diameter ((Fig. 1d)
Leaves: Leaves occur in basal rosettes, petioled, oblong-spatulate and deeply lobed by
half as wide. Deep green in color.
Flowering habit: Flowers in between 120 - 150 days depending on the date of sowing and soil conditions.

2.1.3. Explants

Two types of *in vitro* grown Chrysanthemum explants, namely, young leaf and internode were used in this experiment. Two weeks old shoot tip (Fig. 2a) cultured on MS medium were the source of young leaves (Fig.2b) (4^{th} -5^{th} position) and internode (about 0.5-1.0 cm long) to see different regeneration responses (Fig.2c, f).
Three types of Gerbera explants, namely, leaf (3^{rd} position from the top of the plant) (Fig.27a, c), flower bud (7 - 8 days old, closed, 0.5-1.0 cm in diameter) (Fig. 27b,d) and flower stalk (7 - 8 days old, 0.5 cm long) (Fig.27b,e) were used in this experiment. Young leaf explants of 2-3 weeks old, closed flower bud with flower stalk (about 2.5-4.0 cm long) were collected from the field grown plants. Following surface sterilization flower buds were dissected into 4 - 6 pieces, leaf explants were cut into small pieces with mid-rib and flower stalks were cut into about 0.5 - 1.0 cm long segments to use as explant for *in vitro* regeneration (Fig. 27).

2.2 Gamma radiation (Co60 source) machine

For induction of mutation Co_{60} (cobalt $_{60}$) used as a source of gamma radiation. This experiment was done in the Institute of Food and Radiation Biology situated in Atomic Energy Research Establishment (AERE), Savar, Dhaka. Induction of mutation was done by the machine named as 50 KCi (1850 TBq) Cobalt $_{60}$ ($C0_{60}$ degree) Gamma irradiator (Made in India) (Fig. 7b).

3. METHODS

Experiments were carried out in the Plant Breeding and Biotechnology Laboratory of the Department of Botany, University of Dhaka. The experimental plant materials were raised in the Departmental Botanical Gardens as well as in the green house.

The different procedures followed in the present study are described under the following headlines.

3.1 Media used in the study

Different culture media used in the present investigation for various purposes were as follows:

3.1.1 Callus induction and maintenance

For the induction and maintenance of callus from various explants, MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of BAP and NAA were used.

3.1.2 Shoot differentiation and elongation

For initiation and development of shoot, MS medium supplemented with various combinations and concentrations of hormones e.g. BAP (6- benzyl aminopurine), NAA (α-napthalene acetic acid) and IAA (indole-3 acetic acid) were used.

3.1.3. Root induction

For induction of roots from the base of the *in vitro* grown shoots, half the strength of MS medium (half the strength of macro- and micro-nutrients of MS) and half strength of MS medium containing various concentrations and combinations of different auxins, namely, IBA and IAA were used.

3.1.4. Media for regeneration of radiated plants

For initiation and development of shoot, MS medium supplemented with various combinations and concentrations of hormones e.g. BAP (6- benzyl aminopurine), NAA (α-napthalene acetic acid) and IAA (indole-3 acetic acid) were used.

3.2 Preparation of stock solutions

3.2.1. Preparation of stock solutions for MS medium

Different stock solutions were prepared as the first step for the preparation of medium. The stock solutions of various constituents of the medium were prepared for ready use during the preparation of medium. As different constituents were required in different concentrations, separate stock solutions for macro- and micro-nutrients, vitamins, plant growth regulators, etc, were prepared.

3.2.1.1. Stock solution A (Macro nutrients) for MS medium

This stock solution was made in such a way that its strength was 10 times more than the final strength of the medium in 500 ml distilled water. For this purpose, 10 times the weight of different salts required for 1 liter of medium were weighed accurately and were sequentially dissolved one after another in a 500 ml volumetric flask with 350 ml of distilled water. The final volume of the solution was made up to 500 ml by further addition of distilled water. The solution was filtered through Whatman No.1 filter paper to remove all the solid contaminants like dust, cotton, etc. and was poured into a clean plastic container. After labeling, the solution was stored in a refrigerator at 4°C for several weeks.

3.2.1.2. Stock solution B (Micronutrients) for MS medium

For this constituent of the medium two separate stock solutions were prepared:

***(i)* Stock solution B_1 (all micro-nutrients except iron)**

This part of the stock solution was made with all the micro-nutrients except $Fe_2O4.7H_20$ and Na_2-EDTA. This was made 100 limes the final strength of necessary components in 500 ml of distilled water as described for the stock solution A. The solution was filtered and stored at 4°C for future use.

(*ii*) Stock solution B_2 (iron chelate solution)

The second solution was made 100 times the final strength of FeSO.7H.0 Na EDTA in 500 ml distilled water in a conical flask and heated slowly at high temperature until the salts were dissolved completely. Finally, the solution was filtered and stored in a refrigerator at 4'C for several weeks.

3.2.1.3 Stock solution C (organic constituents) for MS medium

It was also made 100 times the final strength of the medium in 500 ml of distilled water as described for stock solution B. This solution was also filtered and stored at 4°C for future use.

3.2.2 Stock solutions for growth regulators

The growth regulators are naturally synthesized in higher plants. For the growth, differentiation and organogenesis of tissue, the growth regulators (auxins, cytokinins and additives) are important in tissue culture. The following different supplements and growth regulators were used in the present investigation:

(i) Auxins

Auxins induce cell division and formation of callus. It causes cell division, cell elongation, swelling of tissues and the formation of adventitious roots. The auxins are:

Indole-3-acetic acid (IAA)

Indole-3-butyric acid (IBA)

α- naphthalene acetic acid (NAA)

(ii) Cytokinins

Cytokinins concerned with cell division and modification of shoot differentiation in tissue culture. It induces adventitious shoot formation. They have also been shown to activate RNA synthesis and to stimulate protein and enzyme activity in certain tissues. The most frequently used cytokinins are:

6-benzyl amino purine (BAP)

6-furfuryl amino purine (Kinetin/Kn)

The ratio of auxin and cytokinin is important with morphogenesis in the culture system. For embryogenesis, callus and root initiation, the ratio of auxin and cytokinin are high. On the other hand, for auxiliary shoot formation and shoot proliferation, the ratio of Auxin and cytokinin is low (Razdan 1993).

The growth regulators, their solvents, and molecular weight are listed below (Sigma Plant Cell Culture Catalogue):

The growth regulators, their solvents, and molecular weight

Growth regulator	Solvent	Molecular weight
IAA	1N NaOH	175.2
IBA	1N NaOH	203.2
NAA	1N NaOH	186.2
BAP	1N NaOH	225.3
Kn	1N NaOH	215.2

To prepare any one of the previously mentioned hormonal stock solution, 50 mg of the hormone was placed on a clean-plastic weighing boat and dissolved in 1 to 2 ml of appropriate solvent (mentioned above). The mixture was then washed off with distilled water and collected in a 250 ml measuring cylinder. It was then made up to 250 ml with the addition of distilled water. The solution was then filtered and poured into clean plastic container and stored in a refrigerator at 4'C for several weeks.

3.3 Preparation of one liter MS medium

To prepare one liter of medium the following steps were followed:

i. For the preparation of MS medium, 30 gm (at 3%) of sucrose was dissolved in 500 ml of distilled water in a liter volumetric flask.
ii. 50 ml of stock solution A, 5 ml of stock solution B and 5 ml of stock solution C were added to this 500 ml distilled water and mixed well.
iii. 100 gm of myo-inositol (Sigma, USA) was added to this solution and dissolved completely.
iv. To obtain different concentrations of hormonal supplements and additives required amounts were taken from stock solutions of hormones additives and added to the medium either individually or in combinations and were mixed thoroughly. Since each of the hormonal stock solution contained 20 mg of the chemical in 20 ml of solution, the addition of 10 ml of any hormonal stock solution into 1 liter of medium will result in 1 mg/l

concentrations of that hormonal supplement. Different concentrations of hormonal supplements were prepared by adding required amount of stock solution to the medium following the similar producer described earlier.

v. The whole mixture was then made up to 1 liter with further addition of distilled water.

vi. P^H of the medium was adjusted to 5.8 with a digital P^H meter (TOA, Japan) with the help of IN NAOH and IN HCl whichever was required. Before that, the P^H meter was calibrated with two buffer solutions having P^H 4.0 and 7.0, respectively.

vii. To solidify either 8.0 gm (at 0.8%) of Phyto agar (Duchefa Biocheme) or 2.0 gm (at 0.2%) of phytagel (Sigma, USA) was added to the desired medium. To dissolve solidifying agent, the whole mixture was heated in a microwave oven (National, Japan).

3.4 Preparation of MS medium using powdered MS salts

Occasionally commercially available dry powdered MS medium (Duchefa Biocheme) containing all the constituents of MS medium (inorganic salts, vitamins, and amino acids) except sucrose and agar were used to prepare medium for plant regeneration. Required amount of powdered was dissolved in appropriate volume of distilled water (10% less than the final volume of the medium), and after adding sucrose (3%), the P^H was adjusted to 5.8. To prepare solid media, agar (0.8%) was added.

3.5 Sterilization of medium

Fixed volume of the medium was dispensed into culture vessels, (i.e. test tubes, bottles, or conical flasks) or 500 ml screw capped bottles. The culture vessels were plugged with cotton plug made of non-absorbent cotton or covered with aluminum foil and marked with the help of a marker to indicate the specific media with hormonal supplements. The culture vessels were then autoclaved (Hirayama, Japan) at 15 lbs/sq. inch pressure at 121^oC temperature for 20 mins. In some cases, test tubes with medium were allowed to cool as slants after sterilization.

3.6 Precaution to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar airflow cabinet (Labcon Co. and Forma Scientific, USA). The cabinet was switched 'on' for at least half an hour before use and cleaned with 70% alcohol to make it free from surface contaminants. The instruments like scalpels, forceps, inoculation loop, petri dishes and materials like cotton wool, filter papers, micropipette tips, eppendorf tubes, etc, were sterilized by steam sterilization methods. During the entire period of work in the cabinet, the scalpels, forceps, and inoculation loop were kept immersed into absolute alcohol containing in 2 glass jar inside the cabinet. At the time of inoculation and subculture these were sterilized by flaming methods inside the cabinet. To ensure complete aseptic conditions, both the hands were rinsed with 70% alcohol and dried in air. All measures were taken to obtain maximum contamination free condition during the preparation of explants.

3.7 Culture techniques

The following culture techniques were employed in the present investigation:

i. Axenic culture
ii. Explant culture (Inoculation)
iii. Subculture
iv. Rooting
v. Transplantation

3.7.1 Axenic culture

3.7.1.1 Surface Sterilization of shoot tip explants of Chrysanthemum

Shoot tips were excised from garden grown mother plants and washed under running tap water for 30 minutes. Then they were soaked in detergent (‘Jet’ a product of Kohinor Chemical Industries Limited, Bangladesh) water for 15 minutes and washed under tap water followed by 3-4 times washing with distilled water. Further steps were done in laminar air flow cabinet where clean explants then immersed in 0.8% (v/v) sodium hypochlorite solution for 10 minutes followed by 3-4 times rinsing with distilled water (each 30 s) for surface sterilization. In rainy season an extra step was

introduced to minimize contamination rate by immersing the explants in 70% ethanol for 1 minute followed by 3-4 times wishing with distilled water.

3.7.1.2 Surface Sterilization of leaf, flower bud and flower stalk explants of Gerbera

For surface sterilization the explants (leaf, flower bud and stalk) collected from the garden were first washed under running tap water for 30 minutes. Then the surface of the explants was cleaned softly with a soft small paint brush and washed the explants with detergent 'Jet' (Kohinor Chemical Industries Limited, Bangladesh) under running tap water until the detergent washed out completely. The explants were then deepened in 20% (W/V) Savlon, an antiseptic dis-infectant containing chlorhexidine gluconate and cetrimide (Novartis Consumer Health UK Ltd) for 5 minutes and agitate constantly. Following washing with tap water explants were washed 3 times with distilled water. Further steps were done in laminar air flow cabinet where clean explants were immersed in 0.1% (w/v) $HgCl_2$ solution for 8-10 minutes and was shake continuously by hand. It was followed by 3 - 4 times rinsing with sterilized distilled water to remove every trace of $HgCl_2$ Finally, explants were immersed in 70 % ethanol for 30 secs followed by 3 - 4 times washed with sterilized distilled water.

3.7.2 Explant culture (inoculation)

3.7.2.1 Inoculation of Chrysanthemum explants

Sterilized shoot tips were cultured on preculture medium (MS) for explant collection and repeated subculture was performed at two weeks interval to ensure supply of sterilized explants. Two weeks old shoot tip cultured on MS medium were the source of young leaves (4^{th} - 5^{th} position) and internode (about 0.5 - 1.0 cm long). *In vitro* grown leaf explants with midrib (about 0.5 - 1.0 cm) and internode (about 0.5 cm) were inoculated on different regeneration media to see the responses regeneration.

3.7.2.2 Inoculation of Gerbera explants

Young leaf explants of 2 - 3 weeks old, closed flower bud with flower stalk (about 2.5 - 4.0 cm long) were collected from the field grown plants. Following surface sterilization flower buds were dissected into 4 - 6 pieces, leaf explants were cut into

small pieces with mid-rib and flower stalks were cut into about 0.5 -1.0 cm long segments. The explants were put on sterile filter paper to absorb extra water after surface sterilization.

3.7.3 Precaution for aseptic manipulation

During culturing all the dissecting instruments (which were autoclaved earlier), namely, blades, scalpels and forceps were dipped in 70% alcohol and flamed over a spirit lamp before each time of use. The floor of the cabinet and hands were rinsed with 70% alcohol before starting the procedure.

3.7.4 Incubation of inoculated culture vessels

The culture tube and the conical flasks containing explants were incubated under fluorescent light of 20,000 lux intensity. The light period of the culture room was maintained at 16/8 (light/dark) hours and temperature was 25 ± 2°C.

3.7.5 Induction of callus

The calli were regenerated on MS media containing different concentration of BAP and NAA in different combinations. The induced calli were kept on the same media combination up to 8 weeks until the shoot proliferated.

3.7.6 Subculture

After two weeks, the regenerated callus was transferred to conical flasks or small bottles containing fresh media. Cultures were sub-cultured regularly at an interval of 12-15 days for maintenance and were routinely examined.

3.7.7 Subculture for multiple shoot regeneration

Explants showing the sign of initiation of multiple shoot regeneration from any explants, then they were sub-cultured on the fresh MS media (containing different hormone or hormone free) contained in the conical flasks. When shoots were produced in higher number, elongated shoots were separated and cultured on the rooting medium for root formation. The rest of the small shoots were separated into 2 - 3 pieces and were sub-cultured on the freshly prepared same medium for further development.

3.7.8. Rooting

About 3 - 4 cm long shoots were separated and cultured on freshly prepared rooting medium containing full and half the strength MS with different combinations and concentrations of hormonal supplements (IBA, IAA) for root induction.

3.7.9 Transplantation

The plantlets with sufficient root system were taken out of the culture vessels and the roots were washed under running tap water. The plantlets were then transplanted to small pots containing sterilized soil. Pots were then covered with transparent perforated polythene bags. The inner side of these bags was moistened with water to prevent desiccation. To reduce sudden shock, the pots were kept in growth room for two weeks. But after the first week the polythene covers were removed. These plantlets were exposed to natural environment for 2 - 8 hours daily and again placed in growth room for another week. Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were then transferred to larger pots for further growth and then these plants were transferred to the field. A similar method of transplantation was also maintained for irradiated plants.

3.8 Induction of mutation through gamma radiation

3.8.1 Induction of mutation through gamma radiation in Chrysanthemum

About 3- 4 weeks old culture from leaf explants of both Chrysanthemum varieties were used for gamma irradiation using different exposure time and doses (Fig.12A). Five different doses, namely, 5Gy (1 minute 35 seconds), 10 Gy (3-minute 30 sec), 15 Gy (5 min 0 sec), 20 Gy (6 min 36 sec) and 25 Gy (8 min 15 sec) were applied at a rate of 3.03 Gy/min from 152 cm distance of Co_{60} radiation source. For each variety 3 replicates were used for each dose along with the control. The samples were placed at north side of the source for different time interval to irradiate accordingly. During the experiment temperature was 25^0C and the applied doses were calculated using "Fricke Dosimeter" (by the research personnel from Institute of Food and Radiation Biology situated in Atomic Energy Research Establishment (AERE), Savar). Three lots of experimental materials of both chrysanthemum and gerbera were irradiated in this method in various time. Finally, they were micro-propagated following the method as mentioned earlier.

3.8.2 Induction of mutation through gamma radiation in Gerbera

About 7 - 8 wks old culture of shoot primordial clumps (each containing about 20 microshoots) derived from flower bud explants of both Gerbera varieties were used for gamma irradiation using different exposure time and doses (Fig.12A). Five different doses, namely, 5Gy (35 seconds), 10 Gy (1 minute 10 sec), 15 Gy (1 min 44 sec), 20 Gy (2 min 19 sec) and 25 Gy (2 min 54 sec) were applied at a rate of 8.618 Gy/min from 91 cm distance of Co_{60} radiation source. For each variety 3 replicates were used for each dose along with the control. During the experiment temperature was maintained 25^0C and the applied doses were calculated using "Fricke Dosimeter" (by the personal from Institute of Food and Radiation Biology situated in Atomic Energy Research Establishment (AERE), Savar). In the same way we irradiated three lots of explants of both chrysanthemum and gerbera. The samples were placed at north side of the source for different time interval to irradiate accordingly. Finally, they were micro propagated following the method as mentioned earlier.

3.9 Performance analyses of irradiated explants and plants

3.9.1 Phenotypic study

3.9.1.1 Observations of vegetative characters (*in vitro*)

(i) **Per cent survival (%)**

Number of plants survived 60 days after transplanting were calculated. The plant survival (%) was calculated by following formulae:

$$\text{Plant survival (\%)} = \frac{\text{Number of plants survived 60 days after transplanting}}{\text{Total no.of plants planted}} \times 100$$

(ii) **LD_{50} Dose**

LD_{50} dose was calculated based on the survivability of irradiated explants after 8-10 weeks. After 60 days of irradiation, the survival rate of irradiated micro shoots was measured. After radiation, data of measurements were recorded for 8 - 10 weeks at an interval of 3 weeks.

(iii) **Observation of variation in growth room**

Two weeks after culture initiation, the frequency of morphological variation at different radiation doses was measured as the index reflecting the effect of radiation on plant morphological changes. According to the formula (Walther 1969), variation frequency (%) = (No. of variations/No. of treated plants) × 100, frequency of morphological variation was calculated.

3.9.1.2 Observations of vegetative characters (*in vivo*)

(i) **Plant height (cm)**

Height of randomly selected three plants and their mean was calculated from each replication was recorded at the time of first bud appearance and plant height was recorded from the ground level to the apical portion of longest shoot with the help of a meter scale.

(ii) **Number of branches per plant**

The number of primary branches per plant were counted from three randomly selected plants from each replication at the time of full bloom and their means were calculated.

(iii) **Internodal length (cm)**

The intermodal length of plants was recorded from three randomly selected plants from each replication at the time of full bloom and their means were calculated. Internodal length was recorded between the second intermodal length with the help of meter scale.

(iv) **Leaf size (cm)**

The leaf size (length and width) of three randomly selected plants from each replication was measured with the help of scale and their means were calculated.

(v) **Leaf color**

The visual observations were recorded on leaf color i.e. dark green, green, light green compared to normal plant. The color of the leaf was compared with the help of R.H.S color chart.

(vi) **Leaf abnormalities**

It was calculated by counting the number of abnormal leaves i.e. leaves with variation in shape and size from randomly selected three plant from each replication and their means were calculated.

(vii) **Plant spread (cm)**

The plant spread from top of the plant (length and width) of three randomly selected plants from each replication was measured with the help of meter scale at the time of peak flowering and their means were calculated.

3.9.1.3 Observations of flowering characters

(i) **Days required for bud initiation**

The total number of days from date of planting to first bud appearance was calculated for three randomly selected plants from each replication and their means were worked out.

(ii) **Days required for Color break stage**

The total number of days from date of first bud appearance to colour break stage was calculated for three randomly selected plants from each replication and their means were calculated.

(iii) **Days required for flower opening**

The total number of days taken to flower from date of planting was calculated for three randomly selected plants per replication and means were worked out.

(iv) **Number of flowers per plant**

The total number of flowers were counted at the time of full bloom of three randomly selected plants per replication and means were calculated.

(v) **Flower size (cm) in diameter**

The flower size (length and width) of three randomly selected flowers per replication was measured with the help of scale at the time of peak flowering and their means were calculated.

(vi) **Flower weight (gm)**

The flower weight of three randomly selected plants per replication was calculated with the help of electronic weighing machine at the time of peak flowering and their means were calculated.

(vii) **Number of ray florets**

The number of ray florets of three randomly selected plants per replication was counted at the time of peak flowering and their means were calculated.

(viii) **Duration of flowering (days)**

The number of days in which flowers remain in full bloom is longevity. The three plants are randomly selected per replication and days are counted from flower opening till 50 % of flowers dry.

(ix) **Flower color variation**

Any change in hue or shade of flower color over the color of control was recorded with the help of R.H.S color chart from (RHSC) colors of the Royal Horticulture Society Color Charts Eddition V (Royal Horticultural Society Colour Charts Edition V (orgfree.com) & RHS Fan 3 - Azalea Society of America). During the color analysis study, CIE Lab system with a CIE Lab colorimeter was also used to determine the color of the mutants and their parental control.

(x) **Flower form variation**

The plants showing change in form of flowers were critically observed and the type of flower form other than normal were recorded and classified.

3.9.2 Molecular study (ISSR Analysis of the mutants along with the mother plants)

3.9.2.1 Collection of leaf sample for genomic DNA isolation

To extract genomic DNA, young, fresh leaves were collected from greenhouse about three months old pot grown plants of all the seven mutants of chrysanthemum and two mother varieties. Six gerbera mutant and two mother varieties of gerbera young fresh leaves was also collected from greenhouse at same growth stage. The leaves were washed in distilled water and ethanol and dried on fresh tissue paper to remove spores of microorganisms and any other source of foreign DNA.

3.9.2.2 Preparation of Stock and Working Solutions used for genomic DNA Isolation

For conducting the isolation procedures, the following stock solutions and working solutions were prepared.

3.9.2.2.1 M Stock Solution of Tris-HCl pH 8.0 (100 ml)

12.14 g of Trizma base (MW=121.14) was dissolved in 75 ml of distilled water. The pH of this solution was adjusted to 8.0 by adding about 5 ml of concentrated HCl. The volume of the solution was adjusted to a total of 100 ml with de-ionized distilled water. Then it was sterilized by autoclaving and stored at 4ºC.

3.9.2.2.2 0.5 M Stock Solution of EDTA pH 8.0 (100 ml)

18.61 g of EDTA (EDTA.2H2O, MW = 372.24) was added to 75 ml of distilled water and stirred vigorously with a magnetic stirrer. Approximately 2 g of NaOH pellets was added 21 to adjust the final pH to 8.0. The final volume of the solution was adjusted to 100 ml by adding sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4ºC.

3.9.2.2.3 5 M Stock Solution of NaCl (100 ml)

29.22 g of sodium chloride (NaCl, MW = 58.44) was dissolved in 75 ml of distilled water. The total volume of the solution was adjusted to 100 ml with distilled water. The solution was heated in an oven for 15 seconds and stirred vigorously on a magnetic stirrer to dissolve NaCl. It was then sterilized by autoclaving and stored at 4ºC. NaCl was added in small amount at once to be dissolved in solution.

3.9.2.2.4 β-Mercaptoethanol

β-Mercaptoethanol was prepared as a 14.4 M solution and stored in a dark bottle at room temperature.

3.9.2.2.5 Ribonuclease A stock solution

10 mg RNase A was dissolved in 1 ml of deionized distilled water and stored in -20 ºC.

3.9.2.2.6 Tris-HCl Saturated Phenol

It was prepared following procedure described below,

1. The crystal phenol was melted in a water bath at 65ºC for 30 minutes.
2. Then 100 ml of melted phenol was taken and the same volume of Tris-HCl (pH 8.0) was added.

3. It was mixed with a magnetic stirrer for 10 minutes and then was left in rest for 5 minutes.
4. At this stage, two distinct phases were visible, colorless upper phase and colored lower phase.
5. The upper phase was removed with the help of a dropper.

This step was performed six times which took about 3.5 hours to obtain pH 7.75. After saturation, the phenol became half of the initial volume. As phenol is very corrosive and highly toxic, protective measures like wearing of Apron, Gloves and Musk were adopted during the whole process.

3.9.2.2.7 Phenol: Chloroform: Isoamyl alcohol (25 : 24 : 1) (100 ml)

50 ml of Phenol, 48 ml of Chloroform and 2 ml of Isoamyl alcohol were added and mixed properly using vortex mixture. Mixing was done under fume hood to ensure safety. The solution was then stored at 4^{o}C and was shaken before every use. The Phenol:Chloroform:Isoamyl alcohol mixture is caustic and produces fumes. So, this solution was used only within the fume hood wearing gloves and eye protection.

3.9.2.2.8 70% Ethanol (100 ml)

30 ml double distilled water was added in 70 ml absolute ethanol to prepare 100 ml 70% ethanol.

3.9.2.2.9 Stock Solution of TE (Tris-HCl EDTA) Buffer pH 8.0 (100 ml)

1 ml of 1 M Tris-HCl was added with 0.2 ml (200 µl) of 0.5 M EDTA. The final volume was adjusted to 100 ml with sterile de-ionized distilled water. The solution was sterilized by autoclaving and stored at 4^{0}C. 3.3.12 3M Sodium acetate pH 5.2 (100 ml) 40.824 g of sodium acetate with 70 ml of ddH2O and adjusted the final volume to 100 ml with ddH2O and pH was adjusted to 5.2. Then it was sterilized by autoclaving.

3.9.2.2.10 Preparation of Extraction Buffer

To prepare extraction buffer the following components and concentrations were used. Considering the economic use of chemicals, different volume of solutions was prepared as mentioned in bellow:

Estimation of solutions required for the preparation of extraction buffer

Chemical Names	Molecular Weight	Stock Con.	Reference Con./Working Con.	Working Volume 100 ml	1000 ml
CTAB			3%	2 g	20 g
NaCl	58.44	5 M	1.4 M	28 ml	280 ml
EDTA (p^H 8.0)	372.24	0.5 M	20 mM	4 ml	40 ml
Tris-HCl (p^H 8.0)	121.1	1 M	100 mM	10 ml	100 ml
β-Mercaptoethanol		14.4 M	100 mM	700 µl	7 ml

Steps of Extraction Buffer Preparation (100 ml)

Extraction Buffer was Prepared following the steps below (100 ml):

1. 2.5 ml of 1 M Tris-HCl (pH 8.0) was taken in a 250 ml conical flask.
2. 28 ml of 5 M NaCl was added to it.
3. 4 ml of 0.5 M EDTA (pH 8.0) was added next.
4. The solution was then autoclaved.
5. After autoclaving 30 ml CTAB was added and was stirred on the magnetic stirrer.
6. 700 µl β-mercaptoethanol was added prior to use and was mixed by glass rod.
7. The pH of the solution was adjusted to pH 5.0 with HCl and was made up to 100 ml by adding sterile de-ionized distilled water.

Freshly prepared extraction buffer solution was used, β-mercaptoethanol was added to the solution prior to use. The whole task was performed in the fume hood.

3.9.2.3 Genomic DNA isolation protocol

DNA was isolated using the modified CTAB method of Islam (2005). The method is described below:

1. 2.0 g freshly harvested leaf tissue was taken in liquid nitrogen in ice cold mortar and grinded to fine powder with the help of pestle. 5 ml extraction buffer was added to it until it became homogenous paste.
2. The paste was then transferred to a 1.5 ml centrifuge tube and allowed to incubate at 65ºC preheated water bath with occasional mild shaking.
3. After incubation the homogenate was centrifuged at 13,000 rpm for 10 minutes at room temperature to remove non soluble debris.

4. The supernatants were then transferred to fresh tubes and an equal volume of Phenol: Chloroform: Isoamyl alcohol (25: 24:1) was added and mixed well by slow inversion followed by centrifuging at 13,000 rpm for 5 mins. This step was repeated for three times.
5. The supernatants were then transferred to fresh tubes and an equal volume of Chloroform:Isoamyl alcohol (24:1) was added and mixed well by slow inversion followed by centrifugation at 13,000 rpm for 5 minutes. This step was repeated three times.
6. The supernatants were then transferred to fresh tubes and an equal volume of Chloroform was added and mixed well by slow inversion followed by centrifuging at 13,000 rpm for 5 minutes. This step was repeated two times.
7. The supernatants were transferred to fresh tubes and 0.1 vol. of 3 M Sodium acetate (p^H 5.2) followed by 0.6 vol. of 100% chilled Isopropanol was added and mixed slowly. In this step DNA became visible as whitish thread like substance in the solution.
8. These mixtures were then centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatants were discarded carefully by using adjustable micropipettes.
9. The pellets were washed with 70% ethanol. This washing step was repeated at least 2 -3 times. The pellets were air dried on towel paper for about one hour.
10. The dried DNAs were resuspended in 500 µl of TE buffer and treated with RNAase A for 30 minutes at 37ºC and stored at 4ºC.

3.9.2.4 Estimation of quality and quantity of isolated DNA samples

Before PCR amplification it is important to know the quality and quantity of genomic DNA because different DNA extraction methods produced DNA of different purity. It is necessary to optimize the amount of DNA to achieve reproducibility and strong signal in PCR assay.

3.9.2.5 Quantification of DNA

Isolated DNA was diluted 10 times. Then DNA was quantified by Nano drop meter (Qubit® 2.0 Fluorometer, catalog no. Q32866).

3.9.2.6 Preparation of stock solutions used for gel electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared.

3.9.2.6.1 50 × TAE Buffer (pH 8.3) (1 liter)

242 g Trizma base (MW=121.14) was dissolved into 900 ml of sterile de-ionized distilled water. Then 57 ml glacial acetic acid was added to the solution. Finally, 100 ml 0.5 EDTA (pH 8.0) was added in it. They were mixed well. The pH of the solution was adjusted by mixing concentrated HCl at pH 8.3. The final volume of the solution was adjusted to 1000 ml.

3.9.2.6.2 6x Loading Dye

This is required to load samples in gel electrophoresis for further visualization.

Preparation of stock solutions

i. 10 ml of 2% bromophenol blue stock solution
ii. 10 ml of 2% xylene cyanol stock solution
iii. 50% glycerol solution

The stock solution was diluted to prepare 10 ml of the final 6x loading dye with the following component concentrations-

i. 30% Glycerol
ii. 0.3% bromophenol blue
iii. 0.3% xylene cyanol

The 6x loading dye solution can be stored indefinitely in the refrigerator. The bromophenol blue, xylene cyanole and glycerol stock solutions can be stored at room temperature indefinitely. The 6x loading dye is added to the DNA samples to achieve the final dye concentration of 1x.

3.9.2.6.3. Ethidium Bromide Solution

For the preparation of 1ml solution, 10 mg of Ethidium Bromide was dissolved in 1 ml of sterile de-ionized distilled water. It was then mixed by gentle shaking with hand. The solution was transferred to a dark bottle and was stored at room temperature. Stock solution of 10 mg/ml can be purchased directly from companies.

3.9.2.6.4. Agarose gel electrophoresis

The standard method used to separate, identify, and purify DNA fragments through electrophoresis was followed according to the method described by Sharp et al. (1973).

i. 1.0 g of agarose was heated to melt into 100 ml of TAE buffer, ethidium bromide was added (10 µg/ml) and poured into gel-tray fixed with appropriate combs.
ii. After solidification the gel was placed into gel-running kit containing 1 x TAE buffer.
iii. The required plant DNA solutions were loaded with 6x gel loading dye and electrophoresis was continued until DNA fragments were separated well.

3.9.2.6.5 Documentation of the DNA sample

i. After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed in gel documentation system (Biosens SC Series-645) for checking the DNA bands.
ii. The DNA was observed as multiple bands in each lane and photographed using Gel Documentation system (Bio.Sci. Tech.Gelsecan,6.0, Professional, Germany).

3.9.2.7 ISSR analysis using polymerase Chain Reaction

To perform the ISSR analysis, a single oligonucleotide of arbitrary DNA sequence was mixed with genomic DNA in the presence of a thermos stable DNA polymerase, dNTPs, H2O and suitable buffer and then subjected to thermal cycling conditions typical to the polymerase chain reaction (PCR).

3.9.2.7.1 Preparation of working solution (30 ng/µl) of DNA samples for PCR Reaction

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (30 ng/µl) using the following formula:

$$S_1 \times V_1 = S_2 \times V_2$$

$$V_1 = S_2 \times V_2 / S_1$$

Where, S_1= stock DNA concentration (ng/µl)

V_1= required volume (µl)

S_2= working DNA concentration (ng/µl)

V_2= working volume of DNA solution (µl)

Original stock DNA (2 µl) was taken in a 1.5 ml Eppendorf tube and required amount of TE buffer calculated from the above formula was added to it.

3.9.2.7.2 Primers used in ISSR analysis

Primarily, thirteen random ISSR primers were tested for ISSR amplification. Finally, primers exhibiting good quality banding patterns and sufficient variability were selected for further analysis. The details of the primers used in this study as mentioned bellow:

Random primers for screening used in the present study

Sl. No	Primer code	Primer sequence (5´- 3´)	G+C content (%)	Annealing Temp. °C
1	HB_09	GTGTGTGTGTGTGG	57.14	44.0
2	HB_13	GACGACGACGC	72.73	38.0
3	UBC 812	GAG AGA GAG AGA GAG AA	47.059	45.0
4	UBC 816	CAC ACA CAC ACA CAC AT	47.059	45.0
5	UBC 818	CAC ACA CAC ACA CAC AG	52.94	52.4
6	UBC 835	AGAGAGAGAGAGAGAGYC	50.0	48.0
7	UBC 841	GAG AGA GAG AGA GAG AYC	50.0	53.9
8	UBC 843	CTCTCTCTCTCTCTCTRA	44.44	51.6
9	UBC 847	CACACACACACACACARC	50.0	53.9
10	UBC 857	CACACACACACACACARC	50.0	48.0
11	UBC 878	GGA TGG ATG GAT GGA T	50.0	48.0
12	UBC 889	DBD ACA CAC ACA CAC AC	41.176	42.0
13	UBC 828	TGTGTGTGTGTGTGTGA	-	45

3.9.2.7.3 Preparation of Primers

The supplied primers were dissolved to 100 pM concentrations by adding the volume of water indicated in the supplied vial. All primers were diluted to 10 times i.e. 10 µM to make a working solution for use.

3.9.2.8 PCR reaction for ISSR analysis

3.9.2.8.1 Preparation of purified taq DNA polymerase

1. 2µl of purified Taq DNA Polymerase was transferred to an Eppendorf (fresh and autoclaved).
2. 4µl of storage buffer was added.
3. Dilution of the enzyme was 50-fold.
4. 0.0174g of PMSF was first dissolved in minimal volume of isopropanol (~l ml). Then 1.2I g of Tris, 0.745g of KCL, 0.0074g of EDTA and 0.03084 g of DTT were added and mixed thoroughly with de-ionized water after adjusting the p^H to 7.9, the final volume was made 75 ml with ddH_2O and filter sterilized through 0.2/µm Millipore filter.
5. Meanwhile, 80 % glycerol was prepared from commercially available glycerol (98%) and autoclaved. 125 ml of the 80 % glycerol was added to 75 ml of the mixture of storage buffer and stored at 4°C.

Storage Buffer components

	Components	MW	Concentration
a.	Tris	121.14	50.0 mM
b.	KCL	74.50	50.0 mM
c.	EDTA	372.20	0.1 mM
d.	DTT	154.20	1.0 mM
e.	PMSF	174.20	0.5 mM
f.	Glycerol	----------	50.0%

3.9.2.8.2 Preparation of dNTPs mixture

100µl each of dATP, dGTP, dCTP, dTTP (their concentrations being l0 mM each) were mixed in a fresh autoclaved Eppendorf tube and the final volume was made up to l000µl by adding 600µl of TE solutions (l0mM Tris-HCI, 0.lmM EDTA, pH 8.0)

and dispensed as aliquots in tubes and stored at -20°C. The final concentration of each of the nucleotide in the above mixture was 1.0 mM.

3.9.2.8.3 Preparation of PCR Reaction Mixture/PCR Cocktail

The following components were used to prepare PCR cocktail:

Component of PCR reaction mixture and the amount used for each reaction

Sl. No.	Reagents	Amount per sample
1	Sterile de-ionized distilled water	17.3 µl
2	*Taq* Buffer A 10×	2.5 µl
3	$MgCl_2$	1.5 µl
4	Primer	1.0 µl
5	dNTPs 2.5 mM	0.5 µl
6	*Taq* DNA Polymerase 5U/µl	0.2 µl
7	Template DNA 30 ng/µl	2.0 µl
	Total	**25.0 µl**

During the experiment, PCR buffer, dNTPs, Primers, and DNA sample solution were thawed from frozen stocks, mixed well by vortexing and kept on ice.

3.9.2.8.4 PCR Amplification for ISSR:

PCR amplification was done in an oil-free thermal cycler (Biometra UNO II). The optimum amplification cycle was as follows:

	Initial denaturation	94º C	for	3 minutes
35 Cycles	Denaturation at	94º C	for	30 second
	Annealing at	38-54º C	for	1minutes
	Extension at	72º C	for	2 minutes
	Final extension at	72º C	for	7 minutes

After completion of cycling programmed, the reactions were held at 4ºC.

3.9.2.9 Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 2 % agarose gel. The gel was prepared using 2.0 g agarose powder containing ethidium bromide and 100 ml 1xTAE buffer. Agarose gel electrophoresis was conducted in 1x TAE buffer at 100 Volts and 100 mA for 1.0 hour. 1kb plus DNA ladder was electrophoresed alongside the products of ISSR reactions. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system (Bio.Sci. Tech.Gelsecan,6.0, Professional, Germany).

3.9.2.10 Analysis of ISSR Data

Cluster Analysis and Tree Diagram (Dendrogram) Construction

Following agarose gel electrophoresis, the size of amplification products was estimated by comparing the migration of each amplified fragment with that of a known size fragments 30 of 1 kb or 1kb plus molecular weight marker. All distinct bands or fragments (ISSR marker) were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual germplasm (mutant or mother plants of chrysanthemum or gerbera tested two varieties) and each primer. The scores obtained using all primers in the ISSR analysis were then combined to create a single data matrix. This was used for estimating genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendrogram among the germplasm using computer programme “Popgene 32, version 1.32”. Genetic distances were computed from frequencies of polymorphic markers to estimate genetic relationship between the studied mutants of both varieties of both chrysanthemum and gerbera germplasm using UPGMA (Nei’s 1972). The Dendrogram tree was constructed using the computer software “Popgene 32, version 1.32”.

3.10 Statistical Analysis

Statistical analysis was carried out for the observations recorded in experiment to find out whether there exist any significant differences among the regeneration medium used toward different explants through 2-way ANOVA. The same experiment was performed for analysis of rooting responses. Statistical analysis was also carried out for the observations recorded in experiment to find out whether there exists any significant variation for various parameters among different gamma ray treatments (doses) and find out LD_{50} through regression analysis. All these data were subjected to statistical analysis by using Prism GraphPad 8.0.

4. RESULTS

The current study was conducted during 2017-2020 in the Plant Breeding and Biotechnology laboratory as well as in the greenhouse and in the field of the Department of Botany, University of Dhaka. Two varieties of Chrysanthemum (*Chrysanthemum morifolium* Ramat**),** namely, BARI Chrysanthemum-1 (yellow) local and BARI Chrysanthemum-2(white) and two varieties of Gerbera (*Gerbera jamesonii* Bolus), namely, BARI Gerbera- 1 (white) and BARI Gerbera -2 (red) were used in the present investigation. The result has been divided into three parts for both Chrysanthemum and Gerbera varieties used and has been presented in this chapter. The first part of the experiments was carried out to establish an efficient protocol for *in vitro* plant regeneration. The second part of the study was to induce variation using gamma radiation in *in-vitro* grown micro shoots and isolation of the mutants following different morphological evaluation at both *in vitro* and field conditions. The third and final part of the study was conducted for studying the molecular status of the selected mutants derived from both Chrysanthemum and Gerbera varieties. In this chapter firstly results of all the experiments done for Chrysanthemum will be presented which will be followed with the results of the experiments carried out with Gerbera.

4.1 Development of *in vitro* regeneration protocol and induction of mutation through gamma radiation in Chrysanthemum

This study was carried out in three phases. In the first phase, several experiments were conducted to obtain an efficient *in vitro* regeneration system for the above mentioned two varieties of Chrysanthemum using various explants, namely, shoot tip, internode (IN) and leaf (L) segments. In the second phase, a series of experiments were conducted for *in vitro* mutation induction using five different gamma radiation doses, namely, 5 Gy, 10 Gy, 15 Gy, 20 Gy and 25 Gy. Afterward experiments were conducted for isolation of morphological mutants both *in vitro* and *in vivo* conditions during plant development. In the last phage promising mutants were isolated following different morphological studies and final mutant lines were characterized through molecular study using ISSR molecular markers.

4.1.1 *In vitro* regeneration of Chrysanthemum plantlet

In this study, experiments were conducted to regenerate shoots and roots from various explants of two Chrysanthemum varieties. Shoot tip, internode (IN) and leaf (L) segments of two varieties were used as explants. Internode (IN) and leaf (L) explants were obtained from aseptically grown seedlings developed from shoot tip (collected from field grown plant materials) cultured on MS medium following sterilization. Shoots were successfully developed on MS medium supplemented with suitable phytohormonal combinations. These shoots were transferred to the rooting medium. Following successful root formation, the regenerated plantlets were acclimatized in soil following proper hardening and allowed to grow under field condition. Results of these experiments are presented under the following heads:

4.1.1.1 Surface sterilization and preparation of explants used for *in vitro* regeneration

Shoot tips were excised from field grown mother plants and washed under running tap water for 30 minutes. Then they were treated with a locally available mild detergent (Jet powder solution) for 15 minutes and were washed thoroughly under tap water followed by 3-4 times washing with distilled water. Further steps of sterilization of explants were carried out in a laminar air flow cabinet where previously cleaned explants were immersed in 0.8% (v/v) sodium hypochlorite solution for 2-10 minutes followed by 3-4 times rinsing with sterile distilled water. The effect of sodium hypochlorite solution was examined to obtain contamination free explants. For this purpose, sterilized shoot tips were inoculated on hormone free MS medium. The percentages of contaminated explants as well as the effect of various sterilization periods (2-10 mins) on shoot regeneration were examined and data for both varieties are presented in Table 1. It has been observed from the Table that in case of BARI Chrysanthemum-1 variety the percentage of contamination was recorded to be 90 (winter season) and 95 (summer season) when the duration of treatment was 2 minutes. On the other hand, when duration of sterilization treatment was 10 minutes the percentage of contamination was only 20 (winter season) and 40 (summer season). More or less similar results were observed in the case of BARI Chry-2 variety. However, in both the varieties, the rate of shoot tip regeneration was low when the sterilization treatment was 10 minutes. On the other hand, although the rate of contamination was high when sterilization period was 2 minutes, the percentage of

shoot regeneration was comparatively higher in both the varieties irrespective of seasons. It was found that a 10 mins sterilization period reduced contamination rate considerably during winter, 20% for BARI Chrysanthemum-1 and 25% for white variety. But in summer the same treatment produced 40% and 38% contamination for BARI Chrysanthemum-1 and white varieties respectively. During the rainy season an extra step was needed to minimize contamination rate of the explants by immersing them in 70% ethanol for 1 minute followed by 3-4 times washing with distilled water. It was also seen that extended sterilization treatment has a negative effect on regeneration rate (Table 1). Sterilized shoot tips were then cultured on hormone free MS (Murashige and Skoog 1962) medium for explant collection and repeated subcultures were performed at two weeks interval to ensure supply of adequate contamination free leaf and internode explants (Fig. 2 a,b,c and f). Two weeks old shoot tips cultured on MS medium were the source of young leaves (4^{th} and 5^{th} position) (Fig. 2 b). Leaf explants of about 0.5-1.0 cm long and about 0.5-1.0 cm long internode (from mid part) of the plant were inoculated on MS medium supplemented with different concentration and combinations of hormones to examine their regeneration responses (Figs. 2 c, d and f).

Table 1. Effect of surface sterilization periods using 0.8% (v/v) sodium hypochlorite in controlling contamination of shoot tip explants of both white and BARI Chrysanthemum-1 varieties of Chrysanthemum

Variety	Sterilization period (minutes)	No. of shoot tips inoculated	% of Contamination		% of shoot Regeneration
			Winter	Summer	
BARI Chrysanthemum-1	2	30	90	95	95
	4	30	70	77	80
	6	30	66	70	66
	8	30	50	60	50
	10	30	**20**	40	40
BARI Chrysanthemum-2	2	30	92	95	90
	4	30	60	70	85
	6	30	56	66	75
	8	30	50	54	60
	10	30	**25**	38	55

4.1.1.2 Determination of suitable medium and explant for *in vitro* regeneration of shoots

Several experiments were performed to select suitable concentrations and combinations of hormonal supplements as well as suitable explants for *in vitro* shoot regeneration using MS medium. Two types of explants, namely, leaf (L) and internode (IN) of both varieties of Chrysanthemum were cultured on MS medium supplemented with various concentrations of BAP and IAA for shoot regeneration. The regenerated shoots were routinely sub-cultured for further multiplication of shoots and for their elongation.

4.1.1.2.1 Effect of culture medium, explant type and hormonal supplements towards regeneration of shoots using different explants

Each type of explants requires specific hormonal supplements for the initiation of organogenesis from *in vitro* grown cultures. Composition of culture medium is always considered as an important factor for the successful *in vitro* regeneration from any explant. In these set of experiments, the effects of various combinations of hormonal supplements in MS medium on organogenesis using leaf and internode explants were tested following the development of shoots of both varieties (Tables 2-5). MS medium supplemented with different concentrations of BAP (0.5 &1.0 mg/l) and IAA (0.5, 1.0 & 2.0 mg/l) were employed to examine their effects on initiation of regeneration and development of shoots from the tested explants of BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties. Nine treatments were formulated using cytokinin and auxin in MS medium for regeneration response study. Analysis of variance (ANOVA) for various responses of regeneration with these nine treatments in two varieties is shown in Figs 2-6. Statistical analysis showed significant difference in the percentages of regeneration responses, mean number of shoot/explant as well as the mean shoot length for the nine treatments for both tested varieties. Interaction between hormonal combinations in MS medium (treatments), type of explant and the varieties were also found significant Figs. 2-6.

4.1.1.2.1.1 Effect of different concentrations and combinations of IAA and BAP towards shoot regeneration from leaf explants in case of BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties

In this experiment firstly, we studied the effect of auxin (0.5,1.0 & 2.0 mg/l IAA) supplementation in MS medium to induced regeneration responses in leaf explants

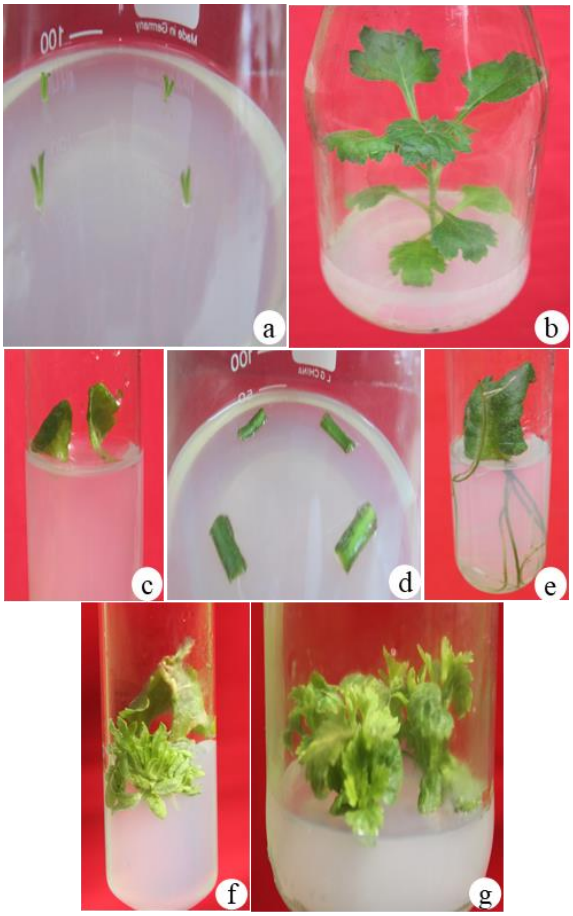

Fig. 2 (a-g): Various explants of Chrysanthemum used in the present study and their response toward PGRs on MS medium. (a) Garden grown shoot tip explant cultured on hormone free MS medium; (b) *In vitro* grown plantlet developed from shoot tip used as the source of explants used for *in vitro* multiple shoot regeneration; (c) *In vitro* grown leaf explants cultured on hormone supplemented MS medium; (d) *In vitro* grown inter node explants cultured on hormone supplemented MS medium; (e) *In vitro* grown leaves cultured only on MS hormone free medium after 6 wks; (f) *In vitro* grown leaves cultured on regeneration medium (T6) after 6 wks; (g) *In vitro* grown inter node cultured on regeneration medium (T6) after 6 wks.

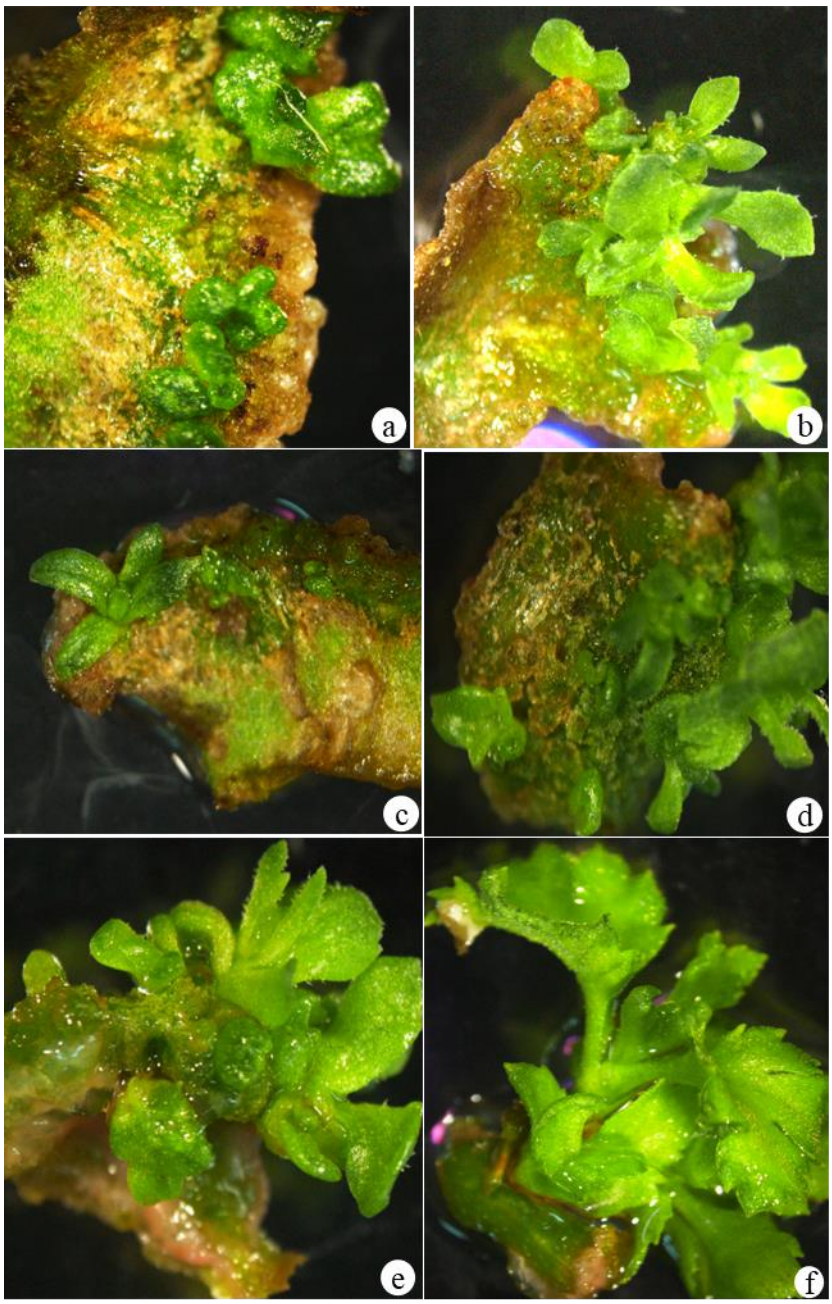

Fig. 3 (a-f): Adventitious shoot bud initiation and multiple shoot induction in BARI Chrysanthemum-2 variety from leaf and internode explants on T6. (a) Early stages of apical shoot (globular shape) bud formation from leaf explant after 2 weeks of culture; (b) Formation of multiple shoots after 4 weeks of culture from leaf explant; (c) Early stages of apical shoot (globular shape) bud formation from internode explant after 2 weeks of culture; (d) Formation of multiple shoots from internode after 4 weeks of culture from internode explant; (e) Formation of multiple shoots after 5 weeks of culture from leaf; (f) Same as (e) from internode explant

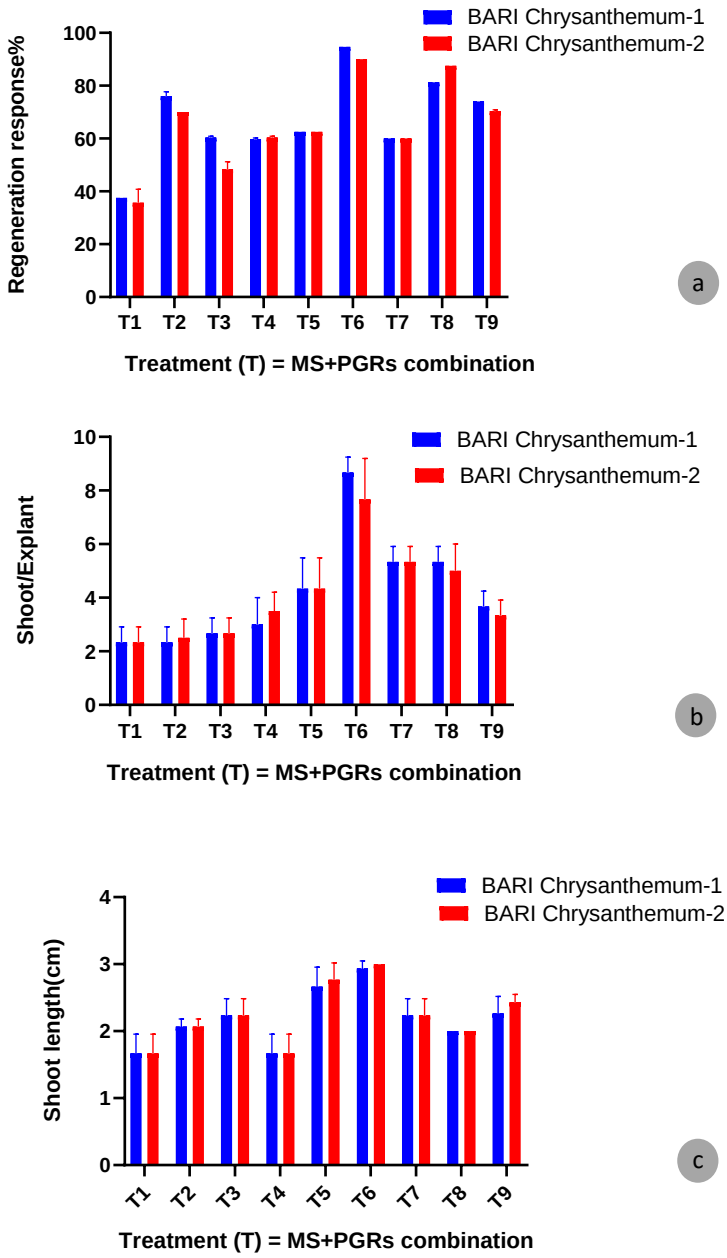

Fig. 4 (a-c): Regeneration response of leaf explant for both varieties towards MS medium with different PGRs combinations in the two varieties after six weeks. (a) Effects of different treatments on regeneration %; (b) Same for shoot/explant; (c) Same for shoot lengths (cm). Data are mean of three replicates with bars representing SEM.

and formulated three treatments namely, T1= MS+0.5 mg/l IAA, T2= MS+1.0 mg/l IAA, T3= MS+2.0 mg/l IAA). It was found that T1= MS + 0.5 mg/l IAA and T2= MS + 1.0 mg/l IAA produced a gradual increase in regeneration response (37.50 to 75.50 % for BARI Chrysanthemum-1 and 35.60 to 70% for BARI Chrysanthemum-2 respectively) in leaf explants of both varieties. Further increase in IAA concentration (T3= MS+2.0 mg/l IAA) decreased the regeneration percentages for both the varieties (60.33% for BARI Chrysanthemum-1 and 48.33% for BARI Chrysanthemum-2) (Tables 2 and 3)

Fig. 4a. As the percentage of regeneration obtained was not satisfactory, we used two concentrations (0.5&1.0 mg/l) of BAP in the previously used three treatments and formulated six more treatments. Interesting results were found when we used a combination of cytokinin and auxin in MS medium. Addition of 0.5 mg/l BAP in T1 (MS+0.5 mg/l IAA medium) treatment increased regeneration around 60.00% for both varieties. And addition of 0.5 mg/l BAP in T2 (MS+2.0 mg/l IAA medium) treatment increased regeneration even more (94.73% for BARI Chrysanthemum-1 variety and 90.00% for BARI Chrysanthemum-2). But addition of 1.0 mg/l BAP in all three treatments (T1, T2&T3) again decreased the regeneration response for both varieties (Table 2, Fig. 4a.).

Table 2. Response of leaf explants towards multiple shoot regeneration using different combinations and concentrations of cytokinins and auxins in MS media for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 chrysanthemum varieties

Variety	Treatment		Hormonal supplements (mg/l)		% of responsive explants	Days required to get response	Mean no. of shoot buds/ explants after 6 wks	Mean length in 6 wks (cm)
			BAP	IAA				
BARI Chrysanthemum-1	T1	MS	0	0.5	37.50	15	2.33	1.67
	T2	MS	0	1.0	75.50	14	2.33	2.10
	T3	MS	0	2.0	60.33	16	2.67	2.23
	T4	MS	0.5	0.5	59.67	15	3.00	1.67
	T5	MS	0.5	1.0	62.50	15	4.33	2.67
	T6	**MS**	**0.5**	**2.0**	**94.73**	**14**	**8.67**	**2.93**
	T7	MS	1.0	0.5	60.00	18	5.33	2.23
	T8	MS	1.0	1.0	81.25	17	5.33	2.00
	T9	MS	1.0	2.0	74.00	17	3.67	2.27
BARI Chrysanthemum- 2	T1	MS	0	0.5	35.60	15	2.33	1.67
	T2	MS	0	1.0	70.00	14	2.50	2.07
	T3	MS	0	2.0	48.33	16	2.67	2.23
	T4	MS	0.5	0.5	60.00	15	3.50	1.67
	T5	MS	0.5	1.0	62.50	15	4.33	2.77
	T6	**MS**	**0.5**	**2.0**	**90.00**	**14**	**7.67**	**3.00**
	T7	MS	1.0	0.5	60.00	18	5.33	2.23
	T8	MS	1.0	1.0	87.50	17	5.00	2.00
	T9	MS	1.0	2.0	70.00	17	3.33	2.43

Among the nine treatments tested, the highest mean number of shoot/explants was found (8.67 and 7.67) for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 variety, respectively when explants were cultured on T6 (MS+ 2 mg/l IAA+ 0.5 mg/l BAP) treatment. Minimum shoot/explant (mean number) were produced from T1, T2 and T3 (2.33-2.67) treatment for both varieties (Fig. 4b).

Highest mean shoot length for both varieties were found to be approximately 3.00 cm after six weeks of culture initiated from the T6 (MS+ 2 mg/l IAA+ 0.5 mg/l BAP) treatment and minimum mean shoot length approximately 1.6 cm were found in case of treatments T1 and T3 derived shoots. Treatment T7 (MS + 0.5 mg/l IAA + 1.0 mg/l BAP) and T8 (MS + 2.0 mg/l IAA+ 1.0 mg/l BAP) produced approximately 5.00-5.33 shoots/ explant (mean number) and mean shoot length was 2.00-2.23 cm for both varieties (Fig. 2-3).

Present study revealed that MS medium supplemented with 0.5 mg/l BAP and 2.0 mg/l IAA showed the best response regarding the parameters tested. It produced the highest percentage of responsive explants (94.73% for BARI Chrysanthemum-1 and 90% for BARI Chrysanthemum-2), average no. of shoot buds/explants after 6 wks (8.67 for BARI Chrysanthemum-1 and 7.67 for BARI Chrysanthemum-2) and highest length of the shoot (2.93 cm for BARI Chrysanthemum-1 and 3.00 cm for BARI Chrysanthemum-2) where the days required to get response was less (14 days for both varieties).

4.1.1.2.1.2 Effect of different concentrations and combinations of IAA and BAP towards shoot regeneration from internode explants in case of BARI Chrysanthemum-1 and white BARI chrysanthemum-2 varieties

In this experiment firstly, we studied the effect of only auxin IAA (0.5,1.0 & 2.0 mg/l) supplementation in MS medium to induced regeneration responses in internode explants and formulated three treatments (T1= MS+0.5 mg/l IAA, T2= MS+1.0 mg/l IAA, T3= MS+2.0 mg/l IAA). It was observed that, addition of IAA (0.5 mg/l) in MS media induced regeneration responses (37.50 % and 36.67% for BARI Chrysanthemum-1 and BARI chrysanthemum-2 respectively) in internode explants. Significant increase in regeneration percentage (74.67% for BARI Chrysanthemum-1 variety and 67.00% for BARI Chrysanthemm-2 variety) was recorded for the T2 (MS+1.0 mg/l IAA) treatment regarding internode explants. Further increased concentration of auxin, 2 mg/l IAA in MS (T3) medium showed a negative effect on regeneration percentages for both the varieties (Table 2) Fig. 4d. Interesting results

like leaf explants were found when a combination of cytokinin and auxin were used to study regeneration response. Increased concentration of IAA (2 mg/l) along with low BAP (0.5 mg/l) in MS medium (T6) increased the regeneration response for both the varieties (90.90% for BARI Chrysanthemum-1 and 86.07% for BARI Chry-2). But addition of double amount of cytokinin T9 treatment (MS + 2.0 mg/l IAA+ 1.0 mg/l BAP) dropped (72.00% for BARI Chrysanthemum-1 and 67.67% for BARI Chrysanthemum-2) the regeneration rate (Table 3) Fig. 4d.

Among the nine treatments tested, the highest mean number of shoot/explants was found (6.67 and 7.33) for BARI Chrysanthemum-1 variety and BARI Chrysanthemum-2 variety, respectively when internode explants were cultured on T6 (MS+ 2 mg/l IAA+ 0.5 mg/l BAP) treatment. Minimum shoot/explant (mean number) were produced from T1, T2 and T3 (1.67-2.67) treatment for both varieties (Table 3).

Table 3. Response of Internode explants towards multiple shoot regeneration using different combinations and concentrations of cytokinins and auxins in MS media for BARI Chrysanthemum-1 and BARI Chrysanthemum- 2 Chrysanthemum varieties

Varieties	Treatment	Hormonal supplements (mg/l)		% of responsive explants	Days required to get response	Mean no. of shoot buds/ explants after 6 wks	Mean length in 6 wks (cm)
		BAP	IAA				
BARI Chrysanthemum-1	T1	0	0.5	37.50	15	2.00	1.67
	T2	0	1.0	74.67	14	2.00	2.23
	T3	0	2.0	69.00	16	2.33	2.17
	T4	0.5	0.5	59.33	15	4.00	1.90
	T5	0.5	1.0	69.33	15	5.00	2.70
	T6	**0.5**	**2.0**	**90.90**	**14**	**6.67**	**3.07**
	T7	1.0	0.5	65.66	18	4.33	2.30
	T8	1.0	1.0	83.10	17	5.33	2.07
	T9	1.0	2.0	72.00	17	4.00	2.67
BARI Chrysanthemum- 2	T1	0	0.5	36.67	15	1.67	1.67
	T2	0	1.0	67.00	14	2.00	2.07
	T3	0	2.0	60.83	16	2.67	2.17
	T4	0.5	0.5	53.00	15	3.33	1.77
	T5	0.5	1.0	67.67	15	4.00	2.77
	T6	**0.5**	**2.0**	**86.07**	**14**	**7.33**	**3.23**
	T7	1.0	0.5	63.67	18	4.00	2.30
	T8	1.0	1.0	77.67	17	4.67	2.13
	T9	1.0	2.0	67.67	17	3.33	2.67

Present study revealed that MS medium supplemented with 0.5 mg/l BAP and 2.0 mg/l IAA showed the best response regarding the parameters tested. It produced the highest percentage of responsive explants (90.90% for BARI Chrysanthemum-1 and 87.07% for BARI Chrysanthemum-2), average no. of shoot buds/explants after 6 wks (6.67 for BARI Chrysanthemum-1 and 7.33 for BARI Chrysanthemum-2) and highest length of the shoot (3.07 cm for BARI Chrysanthemum-1 and 3.23 cm for BARI Chrysanthemum-2) where the days required to get response was less (14 days for both varieties).

4.1.1.2.1.3 Comparison of regeneration response of two types of explants towards different concentrations of cytokinins and auxins in MS media for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 Chrysanthemum variety

A set of experiments were conducted using leaf and internode explants from the BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties on MS medium with different concentrations of cytokinin (0.5 & 1.0 mg/l BAP) and auxin (0.5, 1.0 & 2.0 mg/l IAA) in MS medium. These results have been presented in Table 4. In these experiments, T6 (MS+1.0 mg/l BAP+2.0 mg/l IAA) showed the best response regardless of the explant types and variety. In this best treatment (T6) leaf explants showed the highest shoots regeneration percentage 94.73% and 90.00% for BARI

Table 4. Comparison of regeneration response of two types of explants towards different concentrations of BAP and IAA in MS media for BARI Chrysanthemum-1 variety.

Variety		**% of responsive explants**		**Mean no. of shoot buds/ explants after 6 wks**	
BARI Chrysanthemum-1	Treatment	Leaf	Internode	Leaf	Internode
	T4	59.67	59.33	3.00	4.00
	T5	62.50	69.33	4.33	5.00
	T6	**94.73**	**90.90**	**8.67**	**6.67**
	T7	60.00	65.66	5.33	4.33
	T8	81.25	83.10	5.33	5.33
	T9	74.00	72.00	3.67	4.00
BARI Chrysanthemum- 2	T4	60.00	53.00	3.50	3.33
	T5	62.50	67.67	4.33	4.00
	T6	**90.00**	**86.07**	**7.67**	**7.33**
	T7	60.00	63.67	5.33	4.00
	T8	87.50	77.67	5.00	4.67
	T9	70.00	67.67	3.33	3.33

Chrysanthemum-1 and BARI Chrysanthemum-2 variety respectively. Whereas internode explants in the same treatment (T6) produced 90.90% and 86.07% regeneration response. The mean no of shoots/explant was found the highest 8.67 and 7.67 for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 variety respectively in T6 with leaf explants. Using internode explants in the same treatment it was found 6.67 and 7.33 for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 variety respectively. Present results indicate that both explants were able to produce more than 85% regeneration response and suitable for use. Whereas we can consider leaf explants as better responsive explants (Table 4).

4.1.1.2.1.4 Response of leaf and internode explants towards organogenesis of multiple shoots on MS medium for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties

To study the process of organogenesis leaf containing midrib and internode explants were inoculated on T6 (MS+ 2 mg/l IAA+ 0.5 mg/l BAP) treatment. Considering all investigating factors of regeneration, this treatment was found to be the best for the varieties tested (Figs. 4 and 5). The leaf and internode explants became enlarged and curved within 7-10 days of culture after inoculation (Figs. 3a and 3c) and adventitious shoot buds were developed within 2 weeks (Figs. 3b, and 3d). Clusters of multiple shoots with well-developed leaves were formed by the end of 4 weeks of culture (Figs. 3e and 3f). Adventitious shoot bud induction and developmental sequence of shoot primordia has been illustrated for BARI Chry-2 variety in Fig. 6.

4.1.1.2.2 Subculture of regenerated shoots and elongation

The present findings revealed that subsequent subculture of the explants after 4 weeks in a hormone free MS medium enhanced the production of multiple shoots (Fig. 6a). Therefore, MS medium (hormone free) were used for further shoot proliferation and shoot elongation for 5-6 weeks till the regenerated shoots achieved the optimum (3.0-3.5 cm) length (Fig. 6c). A similar response was found for shoots derived from both types of explants using selected best regeneration treatment T6 (MS+ 2 mg/l IAA+ 0.5 mg/l BAP). But subculture in the same treatment T6 produced the same response while taking almost double the time. It can be mentioned that both tested varieties produced almost the same result during this study.

4.1.1.3 Induction of roots on regenerated shoots

In the present investigation spontaneous rooting was observed when consequent subcultures of the regenerated shoots were done in hormone free MS medium (Figs. 6d and 6e). In both the varieties more than 90-95% shoots were found to produce healthy roots within 6 to 7 weeks of culture while healthy shoot elongation was observed. Almost all excised elongated shoots developed into complete rooted plantlets within 2 months.

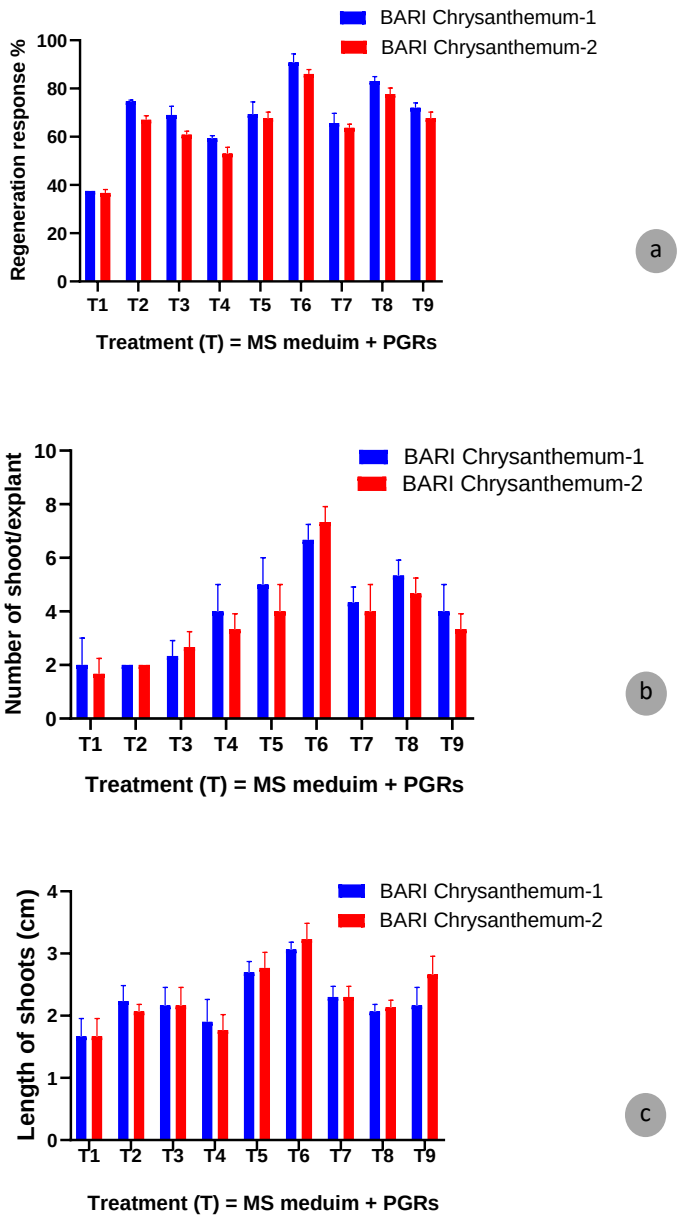

Fig. 5 (a-c): Regeneration response of internode explant for both varieties towards MS medium with different PGRs combinations in the two varieties after six weeks. (a) Effects of different treatments on regeneration %; (b) Same for shoot/explant; (c) Same for shoot lengths (cm). Data are mean of three replicates with bars representing SEM.

Fig. 6 (a-f): Development of plantlets from leaf explants and hardening of plantlets of BARI Chrysanthemum-2. (a) Shoot induction after 4 weeks of culture in T6 treatment; (b-c) Shoot multiplication and elongation after 5-6 weeks in hormone free MS medium; (d) Spontaneous rooting of the regenerated shoot during elongation and multiplication after 6-7 weeks of culture; (e) Healthy rooted plantlet ready to transfer in soil after 8 weeks; (f) Acclimatized healthy plants ready to transplant in the field.

4.1.1.4 Establishment of the plantlets in natural environment

The plantlets with sufficient root system were taken out from the culture vessels after 7-8 weeks of culture and transplanted to small pots containing soil, sand, and organic fertilizer in the ratio 1: 2: 1 (Fig. 6f) and were allowed to be acclimatized adequately.

Table 5. Field performance of transplanted plants of BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties during anthesis

Variety	Plant height during anthesis (cm)	Days required for bud initiation	Days required to full bloom (day)	No. of flower/Plant	Flower diameter (cm)
BARI Chrysanthemum-1	65±3	150±10	18±5	55±5	8±0.5
BARI Chrysanthemum-2	45±2	140±10	20±5	40±5	6±0.5

Note# Mean± SEM of the five factors discussed.

Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were then transferred to the field. All the surviving plants were maintained until flowering and the flowers showed normal morphology without any variation. During the flowering time height of the plant, days required for bud initiation, days required to full bloom, no. of flower/plant and flower diameter were noted (Table 5). It was found that BARI Chrysanthemum-2 (45±2 cm) is shorter than the BARI Chrysanthemum-1 (65±3 cm) variety. They produced lesser number of flowers/plant and diameter of the flower heads were smaller than the BARI Chrysanthemum-1 variety.

4.1.2 Induction of mutation through gamma radiation in BARI Chrysanthemum-1 and BARI Chrysanthemum-2 and selection of promising mutant lines through morphological study

In this phase of investigation, a series of experiments were carried out to induce mutation in two varieties of *Chrysanthemum morifolium* Ramat namely, BARI Chrysanthemum-1 and BARI Chrysanthemum-2 through gamma radiation using $Cobalt_{60}$ as a source for creating variant lines.

Five different gamma radiation doses namely, 5, 10, 15, 20 and 25 Gray (Gy) were applied. Evaluation of the effect of radiation doses and expected effect of creating variation among the plant genotype towards radiation are important steps in the development of an elite mutant line. For induction of mutation 3-4 weeks old leaf explants derived cultures were used for gamma irradiation (Figs.7a-c). MS media

supplemented with 0.5 mg/l BAP and 2.0 mg/l IAA was used for multiple shoot induction and for their development (before and after irradiation). Subsequent subculture was then carried out at a 3-weeks interval to develop M_1V_1 to M_1V_3 shoots. Several shoots died in each subculture (Fig. 7d). During these experiments the survivability of the irradiated shoots M_1V_3 (60 days after irradiation) were evaluated following determination of LD_{50}. After 20 days *in-vitro* regenerated M_1V_4 irradiated shoots (80 days after irradiation) were developed on MS hormone free medium and formed healthy rooted M_1V_5 plants which were transplanted in soil. During this study, different mutant lines were selected based on visual observation through morphologic study. Finally, these M_1V_5 mutant lines were grown in the field till flowering for successive two vegetative generations (M_1V_5 - M_1V_6) to observe stability of the developed mutants. Ultimately four mutant lines from BARI Chrysanthemum-1 and three mutant lines from BARI Chrysanthemum-2 were confirmed after field evaluation. These series of results of this part of the study have been discussed in the following heads:

4.1.2.1 Induction of mutation through gamma radiation in local BARI Chrysanthemum-1 and BARI Chrysanthemum-2

Evaluation of radiation doses and expected effect of creating variation among the plant genotype towards radiation are important steps in the development of an elite mutant line. In the present study, 3-4 weeks old culture from leaf explants were used for gamma irradiation (Fig.7c). MS media supplemented with 0.5 mg/l BAP and 2.0 mg/l IAA was used for shoot induction and development, both before and after irradiation. Subsequent subculture was then carried out at 3 weeks interval to develop M_1V_1 to M_1V_3 shoots (Section 3). Several shoots died in each subculture (Fig. 7d). During this period, the percentage of *in vitro* survival of Chrysanthemum shoots after 60 days of gamma irradiation were recorded (Fig. 8). Using the percentage of *in vitro* survival of Chrysanthemum shoots, LD50 dose for both varieties were determined (Fig. 9). Finally, the surviving M_1V_4 shoots were rooted in MS hormone free medium during elongation and developed M_1V_5 plantlets (Section 3). These M_1V_5 Plantlet (rooted shoots) were transplanted to soil and kept in the growth room for 2 weeks to acclimatize (procedure has been described in the method section). Using this method, the survival rate of the transplanted plantlets in the growth room was found to be about 99%. Variation in leaf structure (Fig. 10 a), chlorophyll (Fig. 10 b) and height of the plant (Fig. 10c) were

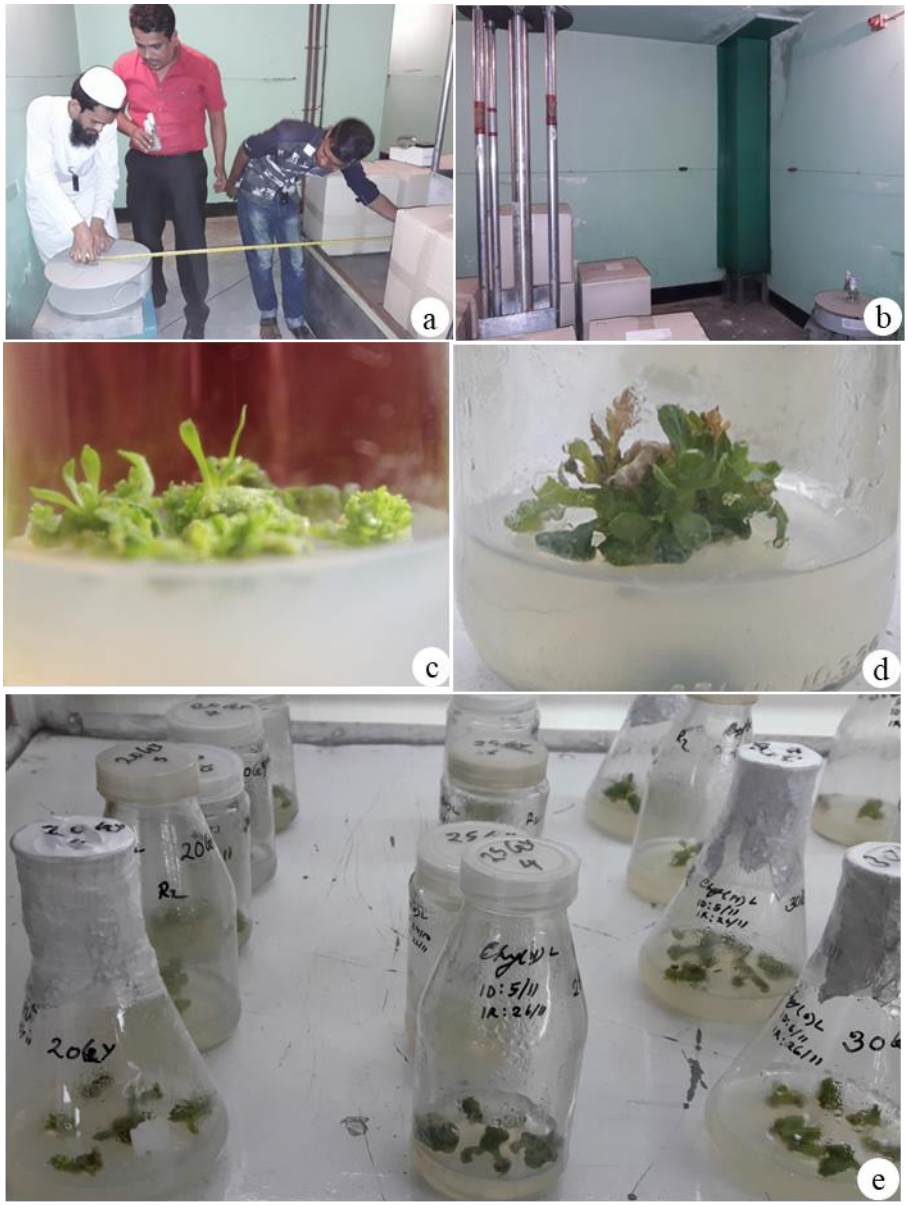

Fig. 7 (a-e): Induction of mutation and its effect on *in vitro* shoot development. (a) Measuring the distance of 90 kCi Cobalt-60 Gamma source at IFRB, AERE, Savar, Dhaka and the plant sample containing flasks; (b) Placement of sample toward the gamma source; (c) 3-4 weeks old culture developed from leaf explants used for gamma irradiation; (d-e) Irradiated shoots died according to the doses of the irradiation after 60 days of irradiation.

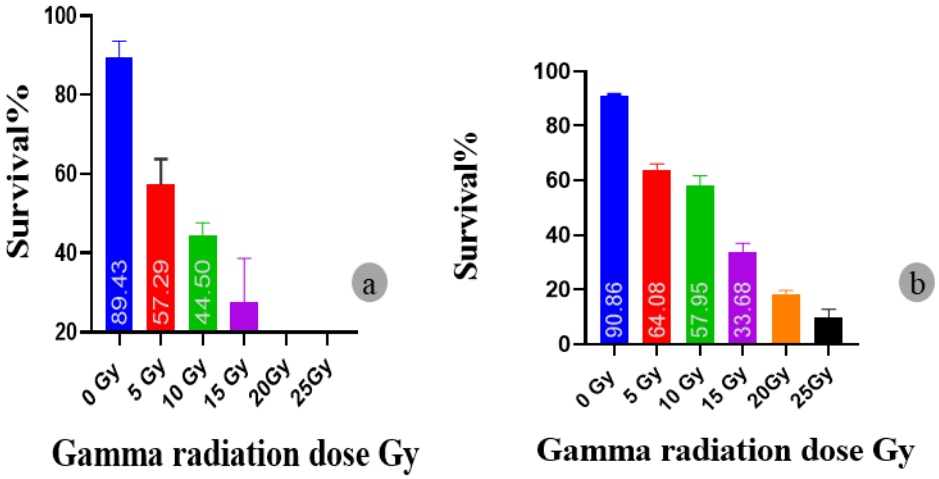

Fig. 8 (a-b). *In vitro* survival % of Chrysanthemum shoots after 60 days of gamma irradiation on Chrysanthemum. (a) *In vitro* survival % of Chrysanthemum-1; (b) *In vitro* survival % of BARI Chrysanthemum-2.

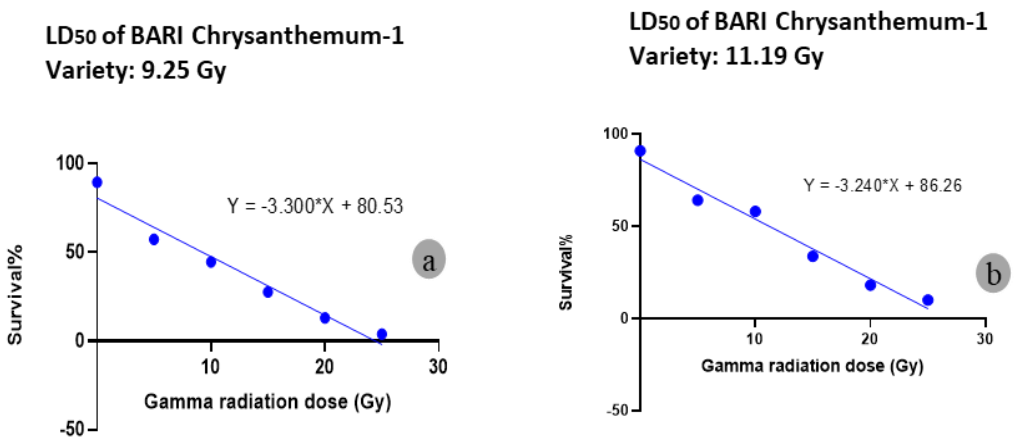

Fig. 9 (a-b): Determination of LD50 dose for both varieties of Chrysanthemum. (a) LD50 dose for Yellow variety; (b) LD50 dose for BARI Chrysanthemum-2.

observed in M_1V_5 plants. Following proper acclimatization, the plantlets were transferred to the field for their further growth. The irradiated plants flowered during the flowering season and were used for evaluation of different morphological factor analysis to identify mutant lines.

4.1.2.1.1 Effect of gamma irradiation on *in vitro* grown shoots survival (60 days after irradiation) in two Chrysanthemum varieties

During optimization of regulatory factors for induction of mutation, it was found that the increase in radiation dose has a direct effect on the percentage of *in vitro* shoot regeneration. In this experiment percentage of lethality was tried to find out following the five doses of gamma radiation on 3-4 weeks old culture derived from leaf explants of both Chrysanthemum varieties. Data for percentage of *in vitro* plant survival were recorded after 60 days of irradiation (Table 6). A comparative study on the effects of five radiation doses (5,10,15,20 and 25 Gy) on the percentage of shoot survival of two varieties of Chrysanthemum indicated that highest dose (25Gy) showed highest lethality as the percentage of survival for both varieties is low (3.90% and 9.93% for BARI Chrysanthemum-1 and BARI Chrysanthemum-2). However, the highest percentage of survival was observed for 5 Gy irradiated shoots for both varieties (57.29% for BARI Chrysanthemum-1 and 64.08% for BARI Chrysanthemum-2) (Fig. 8).

4.1.2.1.2 Determination of LD_{50} for two Chrysanthemum varieties

LD_{50} dose is the dose of gamma radiation which would kill 50% of the treated individuals because recombination will lead to generation of new variability that will

Table 6. Comparison of the effect of gamma irradiation on *in vitro* plant survival (60 days after irradiation) in BARI Chrysanthemum-1 and BARI Chrysanthemum-2 variety

Dose of gamma rays (Gy)	Plant survival (%)	
	BARI Chrysanthemum-1	**BARI Chrysanthemum-2**
0	89.43	90.86
5	**57.29**	**64.08**
10	44.50	57.95
15	27.56	33.68
20	12.97	18.05
25	**3.90**	**9.93**

be difficult to separate from effects of mutation. LD_{50} is important because the rate of mutation is high at this dose and in most of the mutation induction studies found successful mutation induction around this dose. During this study, the effects of gamma radiation doses on the micro shoots survival and plant regeneration from irradiated (explant) were determined as percentages. The LD_{50} for both varieties were calculated and recorded after evaluation of 60 days of irradiation of the micro shoots. To obtain the LD_{50} (50% lethal dose) the data presented in Table 6 were plotted as shown in Fig. 9 following regression analysis. The LD_{50} obtained from regression analysis was found at 9.25 Gy for BARI Chrysanthemum-1 and 11.19 for BARI Chrysanthemum-2 variety (Fig. 9).

4.1.2.1.3 Response of the *in-vitro* grown irradiated shoots of two Chrysanthemum varieties towards variation induction

In this experiment, regeneration response as well as morphological changes of surviving M_1V_4 to M_1V_5 shoots were recorded while growing on MS hormone free

Table 7. Percentage of variations observed in growth room during hardening of BARI Chrysanthemum-1

Dose Gy	Variation frequency % of BARI Chrysanthemum-1			
	Leaf shape	Chlorophyl	Internode size %	Plant height %
Control (0)	0	0	10	25
5	5	0	10	5
10	25	15	10	5
15	80	80	75	75
20	0	0	0	0
25	10	10	10	10

[According to Walther, 1963, Formula for physiological variation frequency % = No. of plants showing variation / No. of treated plants * 100%] Here n=20]

Table 8. Variations (%) observed in the growth room during hardening

Dose Gy	Variation frequency % of BARI Chry-2			
	Leaf shape change %	Chlorophyl change %	Internode size %	Plant height %
0	0	0	0	0
5	35	90	75	80
10	50	60	50	50
15	40	60	75	50
20	0	0	0	0
25	0	0	0	0

[According to Walther, 1963, Formula for physiological variation frequency % = No. of plants showing variation / No. of treated plants * 100%, Here n=20]

medium (during elongation and developed M_1V_5 plantlets). These M_1V_5 Plantlet (rooted shoots) were transplanted to soil and kept in the growth room for 2 weeks to acclimatize and variation in leaf structure, chlorophyll and height of the plant were observed (Fig.10). The frequency of morphological variation at different radiation doses was measured as the index reflecting the effect of radiation on plant morphological changes. According to the formula (Walther, 1969), variation frequency (%) = (No. of variations/No. of treated plants) × 100%, frequency of morphological variation was calculated (Tables 7 and 8). Highest variation frequency% (75-80%) regarding Leaf shape change %, Chlorophyll change %, Internode size% and Plant height% were found for BARI Chrysanthemum-1 from dose 15 Gy. Whereas dose 5Gy, 10 Gy and 15 Gy produced remarkable variation frequency % for BARI chry-2. None of the 20 Gy treated shoots could survive in the elongation or rooting phage, so variation frequency regarding all four measured aspects were 0% for both varieties. It was also noticed that variation frequency is not directly increasing with the increased radiation doses.

4.1.2.1.4 Establishment of plantlets from selected variants M_1V_5 produced in growth room

After sufficient development of roots the selected variant plantlets (M_1V_5) of both Chrysanthemum varieties were successfully transplanted into small plastic pots (Fig. 10). The transplantation procedure has been described in the method section. Using this method, the survival rate of the transplanted plantlets was found to be about 99%. Following proper acclimatization, the plantlets were transferred to the field for their further growth and flowered during flowering season for both varieties (Figs. 11-12.).

4.1.2.2 Establishment of M_1V_5 mutant lines in field and morphological evaluation for selection of variant lines

During these series of experiments, plants showing vegetative variations were grown in the field till flowering and noted performance in field condition along with the control plants. Observation and selection were made for desirable variants at flowering time (Figs. 11-12). Changes in flower color, form and shape were observed in plants treated with gamma rays of 5, 10, 15, 20 and 25 Gy for both Chrysanthemum varieties. Mutation frequency of flower form and flower color among the irradiated plants with 5, 10, 15, 20 and 25 Gy was 45%; 0%,15%; 15%, 20%;

Fig. 10 (a-c): Variations in leaf shape and plant height observed in growth room during hardening. (a) Variation observed in leaves in yellow Chrysanthemum (Left variant developed from 25 Gy irradiated microshoots and right one is nonirradiated plant); (b) Chlorophyll variant developed from 15 Gy irradiated micro shoots (c) Difference in plant height and leaf shape observed in 10 Gy dose treated yellow Chrysanthemum.

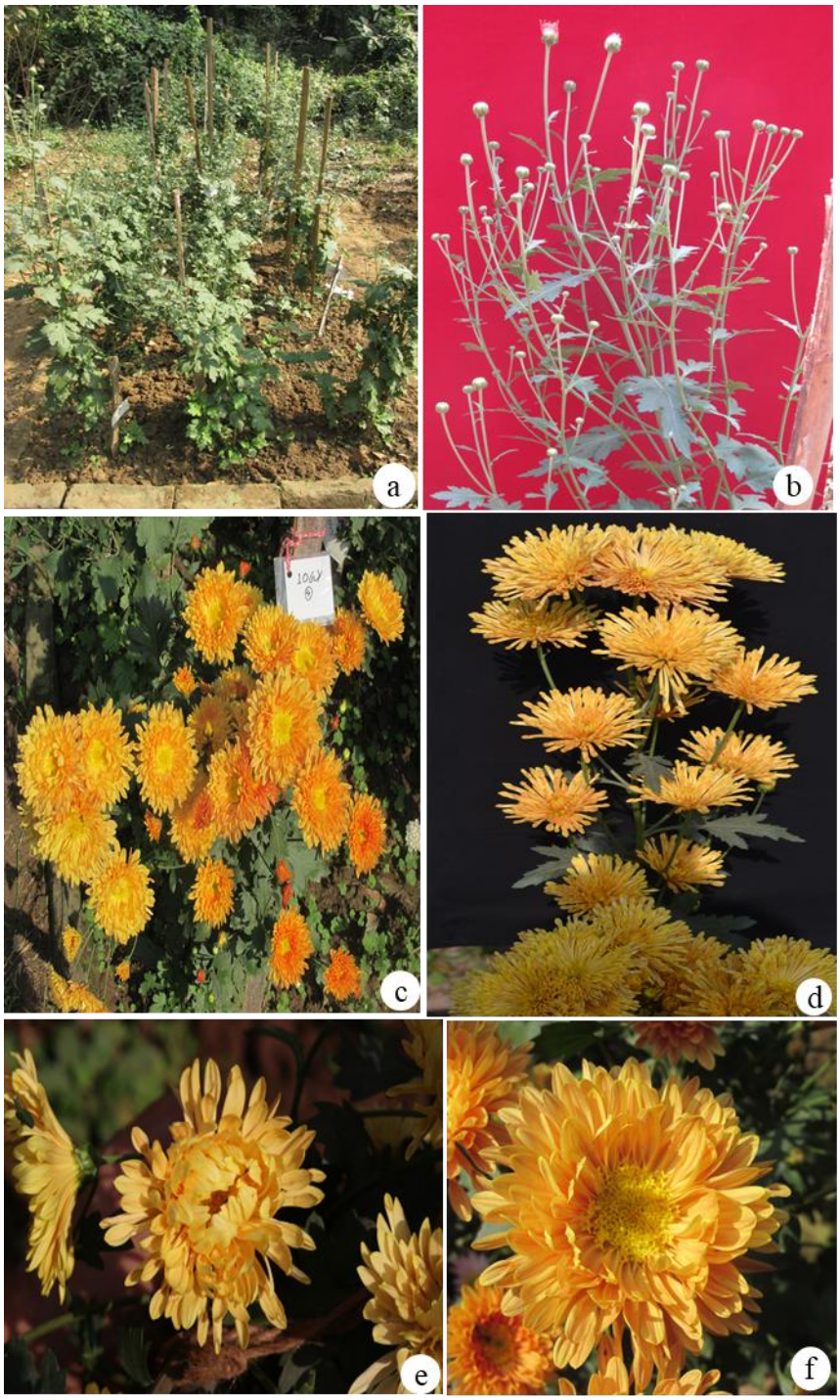

Fig. 11 (a-d): The full bloom of Yellow Chrysanthemum mutants: (a) Yellow Chrysanthemum plants growing in field; (b) Bud initiation in Yellow Chrysanthemum (c-f) Four promising mutants of yellow Chrysanthemum in field. during full bloom.

Fig. 12 (a-d): The full bloom of M_1V_5 BARI Chry-2 mutants plants grown in field showing changes in flower color, form and shape. (a) M_1V_5 BARI Chry-2 plants during budding; (b-d) M_1V_5 BARI Chry-2 plants showing variation in color, form and shape.

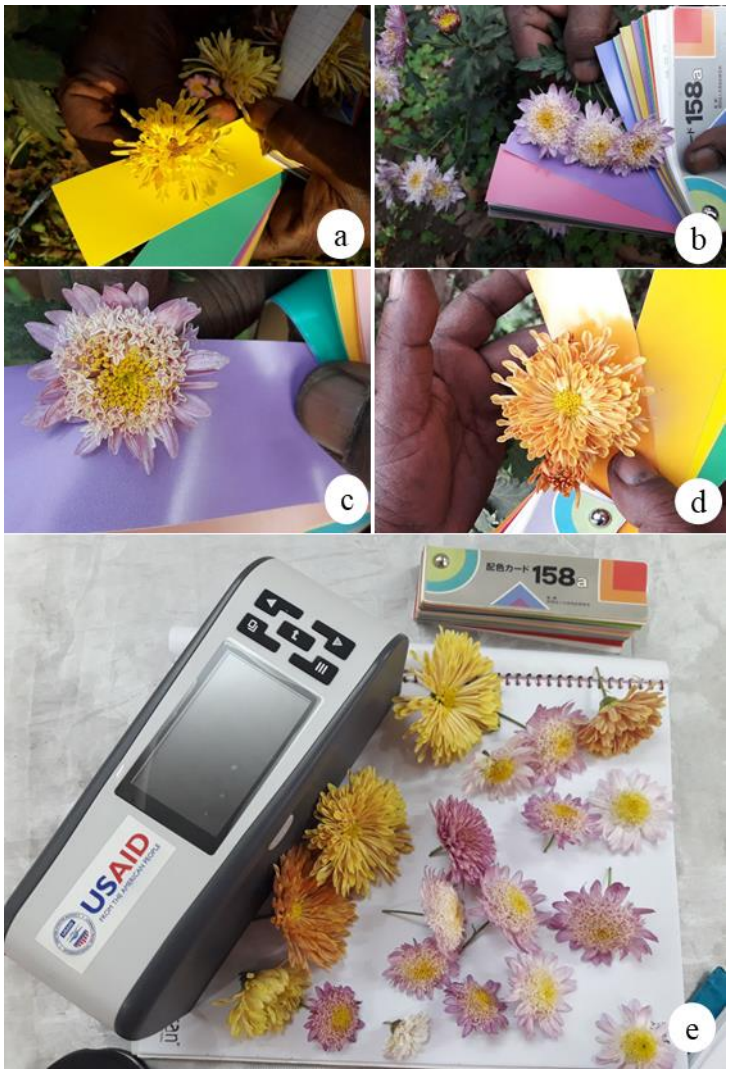

Fig. 13 (a-e): Identification of flower color through color coding and LAP colorimeter. (a-d) Flower colour determination using color code; (e) Flower color determination using CIBLAP colorimeter.

Fig. 14 (a-e): The full bloom of BARI Chrysanthemum-1 mother plant along with four promising mutants in field. (a) YM1 mutant grown in field during its full bloom condition; (b) Y1 mutant grown in field during its full bloom condition; (c) Y6 mutant grown in field during its full bloom condition; (d) CY (mother plant) grown in field during its full bloom condition; (e) Y5 mutant grown in field during its full bloom condition.

10%, 0%;0% and 0%;10%, respectively for BARI chry-2 and BARI Chrysanthemum-1 variety (Table 9). As none of the plants survived due to the contamination from 20 Gy treated explants, we could not get the actual mutation frequency of this dose. So in Table 9 the mutation frequency of this dose is zero.

During this screening several variant lines from both BARI Chrysanthemum-1 and BARI Chry-2 were identified. Screening was made for the 1st vegetative generation (field) and identified four mutant lines from BARI Chrysanthemum-1 variety and six mutant lines from BARI Chrysanthemum-2. Finally, in the 2nd vegetative generation (field) four types of mutant lines from BARI Chrysanthemum-1variety regarding flower shape and color were identified (Figs. 11 and 13). From the BARI Chrysanthemum-2 variety three types of variants regarding flower shape and color were identified (Fig. 12 and 13) and considered as solid mutants. Phenotypic evaluation of these promising mutants for two vegetative generations along with the flower morphology has been discussed in the following heads.

Table 9. Flower form and color mutation frequency in M1V5 of BARI Chrysanthemum-1 and BARI Chrysanthemum-2 chrysanthemum variety in field during flowering

Radiation dose (Gy)	No. of plant Investigated	Flower form and color mutation frequency (%)	
		BARI Chrysanthemum-1	BARI Chrysanthemum-2
5	20	0	45
10	20	15	15
15	20	10	20
20	0	0	0
25	20	10	0

4.1.2.2.1 Phenotypic evaluation of promising mutants (M1V6) developed from M1V5 variant lines (1st vegetative generation in field)

During this study, both BARI Chrysanthemum-1 and BARI Chrysanthemum-2 Chrysanthemum plants grown in field (M1V5) were multiplied through vegetative cutting and their (M1V6) field performance (till flowering) were evaluated. M1V6 plants grown in the field, only the data regarding flowers has been listed in Tables 10 -11 as the flowers are the focal point in the present study.

Table 10. Color and size of the selected variants of BARI Chrysanthemum-1 (M1V6)

Selected Variants	Radiation Dose (Gy)	Diameter of the flower (cm)	Flower color	Flower/petal shape
YM1	10	6±0.41	Yellowish orange	Daisy like flower
Y1	10	7.77±0.25	Bright yellow	Spoon shape
Y5	25	8.33±0.14	Bright Yellow	Twisted/curved
Y6	10	7.38±0.24	Orangish yellow	Daisy like
Control	0	7±0.82	Orangish yellow	Spoon

Primarily four variant lines from BARI Chrysanthemum-1 and six variant lines from BARI Chrysanthemum-2 were identified based on flower morphology. After analyzing their flower morphology (based on flower architecture and stability of the flowers in successive generation) all four BARI Chrysanthemum-1 (YM1, Y1, Y5 and Y6) and three variants of BARI Chrysanthemum-2 (M1, M2 and M6) were selected as promising mutant lines for further study to identify solid mutant lines (Figs. 14 and 15).

Table 11. Flower color and size of the selected variants of BARA Chry-2 (M1V6)

Selected Variants	Radiation Dose (Gy)	Diameter of the flower (cm)	Flower color	Flower shape
M1	5	3.23± 0.21	Dark purple	Anemone
M2	5	4.33±0.15	Light purple	Anemone
M3	5	4.77±2	purple	Anemone
M4	10	3.77±0.21	White	Anemone
M5	10	5.43±0.40	Light purple	Anemone
M6	15	5.77±0.25	Light purple	Anemone
Control	0	5.43±0.33	White	Irregular/ Pompon

4.1.2.2.2 Cultivation of 2nd vegetative generation of promising M1V6 variant lines and phenotypic evaluation of developed promising mutants (M1V7) lines

In this experiment, selected four variant lines from BARI Chrysanthemum-1 variety and six variant lines from BARI Chrysanthemum-2 were showing constant flower variations in the field were multiplied by conventional cutting during the month of July- August 2019. Observation was made and selected desirable variants at flowering time for the second vegetative generation (M1V7) to isolated solid mutant lines. After

Fig. 15 (a-d): The full bloom of BARI Chrysanthemum-2 (White) along with three promising mutants in field. (a) M1 mutants grown in field during its full bloom condition; (b) M2 mutants grown in field during its full bloom condition; (c) M6 mutants grown in field during its full bloom condition; (d) CW (mother plant) grown in field during its full bloom condition.

Fig. 16 (a-c): The comparison of the mutants derived from BARI Chrysanthemum-1 along with mother plant. (a) Leaf, mature flower and different types of florets of YM1 mutants; (b) Leaf, mature flower and different types of florets of Y1 mutants; (c) Leaf, mature flower and different types of florets of CY (mother plant).

Fig. 17(a-c): The comparison of the mutants derived from BARI Chrysanthemum-1 along with mother plant. (a) Leaf, mature flower and different types of florets of Y5 mutants; (b) Leaf, mature flower and different types of florets of Y6 mutants; (c) Leaf, mature flower and different types of florets of CY (mother plant).

Fig. 18 (a-e): The changes of different types of florets in yellow mutants. (a) Tubal and spoon shape florets of CY (mother) plant; (b) Tubal and spoon shaped opening containing ray florets and disk florets of Y1; (c) flat and curved ray florets of YM1 and disk florets; (d) Flat ray and trans florets with tubal but star shaped opening of Y5; (e) Flat ray and trans florets with tubal but star shaped opening of Y6. Scale bar = 1 cm

the study four promising mutant lines from BARI Chrysanthemum-1and three promising lines from BARI Chry-2 were confirmed as solid mutant lines (Tables 12-13). Data were recorded on plant height, primary branch number, days required for bud initiation, days required for bloom, number of flowers per plant and diameter of flower in field condition. These data were recorded on an individual plant basis from five randomly selected plants per mutant type (genotype). Their performance stability and uniformity were evaluated through comparison with their mother plants where significant differences among them were observed (Tables 14 and 15).

4.1.2.3 Description of the selected promising mutant lines and taxonomy

4.1.2.3.1 Description of the mutant lines

Table 12. Classification of BARI Chrysanthemum- 1 and its mutants:

Serial no.	Plant ID	Class Name	Class characteristics	Flower Characteristics	Plant height
1	YM1	**Class 7 Single and Semi-Double**	A daisy-like flower with a center disk and one or more rows of ray florets.	disbud or spray	medium
2	Y1	**Class 9 Spoon**	A daisy-like flower with a center disk and one or more rows of ray florets. The ray florets are like spoons at the tips. The center disk is round and visible.	disbud or spray	tall
3	Y5	**Class 13 Unclassified or Exotic**	Those blooms which fit in none of the other classes. They are often exotic, with twisted florets. They may also exhibit characteristics of more than one bloom class.	disbud or spray	medium
4	Y6	**Class 13 Unclassified or Exotic**	Those blooms which fit in none of the other classes. They are often exotic, with twisted florets. They may also exhibit characteristics of more than one bloom class.	disbud or spray	medium
5	CY	**Class 9 Spoon**	A daisy-like flower with a center disk and one or more rows of ray florets. The ray florets are like spoons at the tips. The center disk is round and visible.	disbud or spray	tall

Finally, seven mutants were developed from BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties after gamma irradiation. Four mutants from BARI

Chrysanthemum-1 (YM1, Y1, Y5 and Y6) and three mutants from white variety (M1, M2 and M6) through gamma radiation were developed. No variation was seen on the in-vitro cultured both Chrysanthemum variety and control white and yellow flowers were labelled as CW and CY respectively. These new cultivars consist of different flower forms, shapes, and colors. To describe and characterize them, all the new cultivars were evaluated into the respective classes of National Chrysanthemum Society Classification system where they belonged to (Tables 12-13). Following visual observation, color of the flowers of M1V7 generation of both varieties were confirmed through (RHSC) colors of the Royal Horticulture Society Colour Charts Eddition V (Royal Horticultural Society Colour Charts Edition V (orgfree.com) & RHS Fan 3 - Azalea Society of America).During the color analysis study, CIELab system with a CIELab colorimeter were used to determine the color of the mutants and the mother flowers. The obtained result has been presented in Table 16.

Table 13. Classification of BARI Chrysanthemum- 2 and its mutants:

Serial no.	Plant ID	Class Name	Class characteristics	Flower Characteristics	Plant height
1	M1	**Class 8 Anemone**	A daisy-like flower with a center disk and one or more rows of ray florets (similar to the semi-doubles) but have a raised cushion-like center.	Grown as a disbud or spray	Medium
2	M2	**Class 8 Anemone**	,,	,,	,,
3	M6	**Class 8 Anemone**	,,	,,	,,
4	CW	**Class 6 Pompon**	A small globular bloom, somewhat flat when young but fully round when mature. Size ranges from small button types to large, disbudded blooms. The florets incurve or reflex in a regular manner and fully conceal the center.	,,	Tall

Fig. 19 (a-l): The comparison of leaf and flower between three mutants derived from BARI Chrysanthemum-2 (White) along with mother plant. (a-c) Leaf, mature flower and different types of florets of M1 mutants; (d-f) Leaf, mature flower and different types of florets of M2 mutants; (g-i) Leaf, mature flower and different types of florets of M6 mutants; (j-l) Leaf, mature flower and different types of florets of CW mother plant. Scale bar = 1 cm

Fig. 20 (a-d): A close look at the florets of BARI Chrysanthemum-2 (White) mutants along with mother florets. (a) Different types of florets of M1 mutants; (b) Different types of florets of M2 mutants; (c) Different types of florets of M6 mutants; (d) Different types of florets of control flower. Scale bar = 1 cm

4.1.2.3.1.1 YM1: These daisy-like vivid yellowish orange (17A) color (same as the control flower color) flowers have a visible center disk with three to five or more rows of ray florets. Outer most rows of ray florets are flat and the innermost two to three rows of ray florets are inward curved or loosely incurved. Tubular disk florets are yellow colored, and the size of the disk is less than 1/3 of the flower diameter (Figs. 16a and 18c). This mutant was derived from BARI Chrysanthemum-1 using 10 Gy treatment.

4.1.2.3.1.2 Y1: These daisy-like bright yellow (6B) color flowers have a large center disk with three to four or more rows of tubal but spoon shaped tip ray florets. The size of the ray florets is almost the same for each layer from outside to inside. Tubular disk florets are yellow colored, and the size of the disk is less than ¼ of the flower diameter (Figs. 16b and 18b). This mutant was derived from BARI Chrysanthemum-1 using 25 Gy treatment.

4.1.2.3.1.3 Y5: These daisy- like flowers are very fascinating vivid yellowish orange (N25C) color and have a large center disk with five to six or more rows of semi flat ray florets. The size of the ray florets is decreasing from outside to inside of the flower. Tubular disk florets are yellow colored, and the size of the disk is less than ¼ of the flower diameter (Figs.17a and 18d). This mutant was derived from BARI Chrysanthemum-1 using 10 Gy treatment.

4.1.2.3.1.4 Y6: These daisy- like flowers are very decorative vivid yellowish orange (N25B) color and have a very small center disk with six to seven or more rows of spoon shaped ray florets. The size of the ray florets is decreasing drastically from outside to inside of the flower. Tubular disk florets are yellow colored, and the size of the disk is less than 1/6 of the flower diameter (Figs. 17b and 18 e). This mutant was derived from BARI Chrysanthemum-1using 10 Gy treatment.

4.1.2.3.1.5 M1: These daisy- like flowers are very attractive bright purple (84B) colored and have a raised cushion-like center. The size of the cushion is more than ¾ of the flower diameter which is composed of 180-200 disk florets. Young tubular disk florets are yellow colored but the matured one is purple colored with a star shaped opening. The purple colored 30-35 ray florets are flat and arranged in 1-2 layers (Figs. 19 a-c and Figs. 20 a). This mutant was derived from BARI Chrysanthemum-2 (white) using 5Gy treatment.

Fig. 21 (a-d): The changes of different florets in mutants. (a) Flat and tubal ray florets of CW (mother) plant; (b) Tubal but star shaped opening containing trans florets of different mutants of CW (c) Ray florets of different mutants of CW; (d) Disk florets of different mutants of CW. Scale bar = 1 cm

Fig. 22 (a-c): Color change before fall off. (a) mother flower before fall off; (b) same for M1; (c) same for M2 and M6

4.1.2.3.1.6 M2: These daisy- like flowers are nice looking light purple (76C) to white (W) colored and have a heavily raised cushion-like center. The size of the cushion is more than ¾ of the flower diameter which is composed of 180-200 disk florets. Young tubular disk florets are yellow colored but the matured one is white colored with a star shaped opening. Light pink colored 30-35 ray florets are flat and arranged in 1-2 layers (Figs. 19d-f and Figs. 20 b). This mutant was also derived from BARI Chrysanthemum-2 (white) using 5Gy treatment.

4.1.2.3.1.7 M6: These daisy- like flowers are very fascinating light purple (76D) to white (W) colored and have a heavily raised cushion-like yellow center. The size of the cushion is ½ of the flower diameter which is composed of 100-150 disk florets. Young tubular disk florets are yellow colored but the matured one is white colored with a star shaped opening. Light pink colored 30-35 ray florets are flat and arranged in 1-2 layers (Figs. 19g- and Figs. 20c). This mutant was derived from BARI Chrysanthemum-2 (white) using 15Gy treatment.

The drastic changes of different florets in mutants was observed in the case of BARI Chrysanthemum- 2 (Fig. 21). It was also observed that all these three mutant flowers turned into pinkish white color before fall regardless of their own color (Fig. 22).

4.1.2.3.2 Analysis of morphological features of the new Chrysanthemum mutant line M_1V_7 for both varieties

Seven different mutant lines were isolated from both yellow and white Chrysanthemum after *in-vitro* culture and irradiation of those cultures with gamma ray. *In-vitro* grown non-irradiated and irradiated plants brought up in the field condition as well as in the earthen pot after hardening.

Different morphological characteristics such as plant height, primary branch number per plant, size of internode, leaf length, leaf breadth, days required for bud initiation after plantation, days required for full bloom, diameter of the flower, flower number per plant, no. of ray floret, no. of disk floret etc. were observed for M1V7 plants. One-way ANOVA following Tukey's multiple comparisons test result of eleven tested characters were presented in Tables 14-15. The plant height of M6 (56.2±1.78 cm) was similar to the mother plant's CW height (55.4±1.50 cm). The plant's height of Ym1 (62.6±0.87 cm) and Y1 (77.4±2.51 cm) were too short compared to the mother

Table. 14 Mean± SEM performance levels of vegetative parameters in M1V7 plants of control and mutants of the two tested varieties during full bloom.

Genotype	Plant height (cm)	Primary branch number per plant	Size of internode (cm)	leaf length (cm)	leaf breadth (cm)
cv. BARI Chrysanthemum -2					
Control	55.4±1.50	22.8±0.86	2.32±0.08	7.18±0.36	4.28± 0.09
M1	50±3.54	22 ±0.95	1.57±0.15*	5.82±0.36	4.26±0.16
M2	43.8±0.97*	19.1± 0.4	1.26±0.11*	7.4±0.29	4.26±0.17
M6	56.2±1.78	23 ± 0.89	2.74±0.11	6.7±0.18	3.56±0.18
cv. BARI Chrysanthemum-1					
Control	92.2±2.91	32.6 ±1.12	3.06±0.17	9.1±0.33	5.7±0.20
Ym1	62.6±0.87*	27.6 ± 0.93*	1.76±0.12*	9 ±0.45	5.7±0.20
Y1	77.4±2.51*	26.8 ±0.86*	1.76±0.12*	8.9±0.40	5.58±0.18
Y5	87±1.85	32.4±1.03	3.06±0.17	10 ±0.45	6.96±0.32
Y6	86±1.70	33.2±0.91	3.12±0.13	9.96±0.32	5.7±0.12

* Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one-way ANOVA

plants CY (92.2±2.91 cm). The plant's height of M2 (43.8±0.97cm) was significantly shorter than the mother plant's CW height (55.4±1.50 cm) (Figs. 23a). It was observed that gamma Plant height of the mutants (M_1V_7 plants) was shorter than the mother plants for both varieties as M2, YM1 and Y1 mutant lines were significantly shorter. Therefore, these three mutants are suitable for culturing in tabs. So, radiation decreased the plant height of the mutant lines (Table 14).

The primary branch number per plant decreased or remained the same compared to the mother plants regardless of the variety (Table 14). Only in the case of YM1 and Y1 mutant lines, significant decreases in the primary branch number per plant were

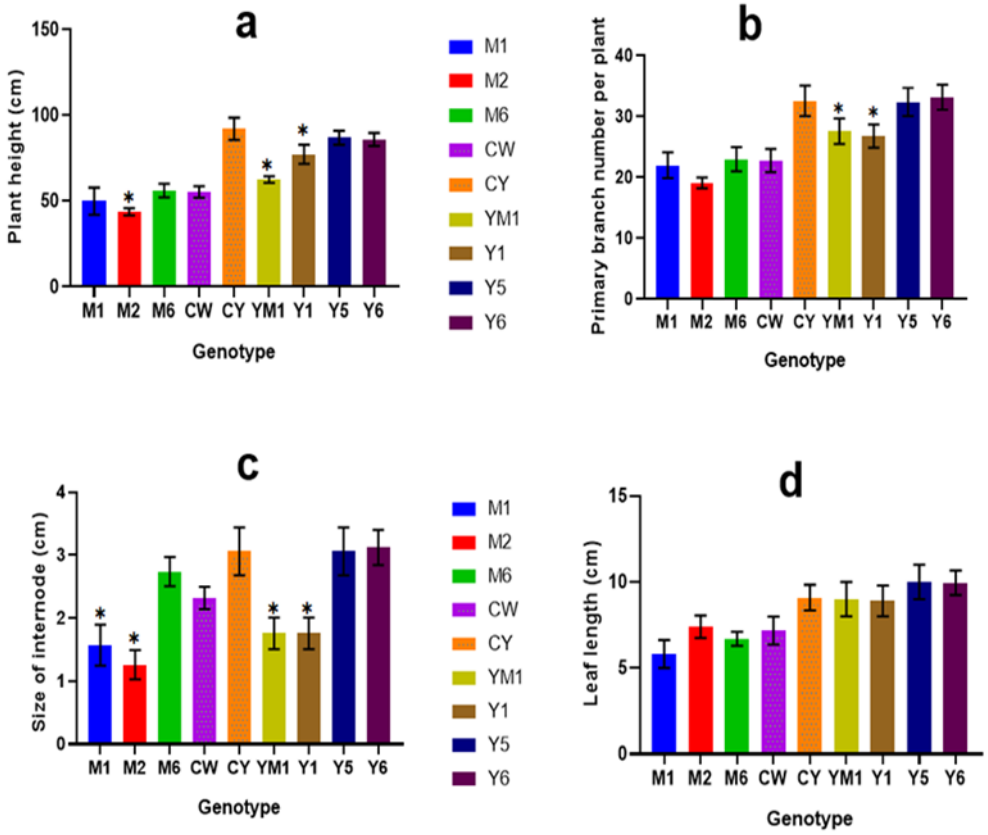

Fig 23 (a-d): Changes in morphological characteristics of mutant lines. (a) Changes in plant height (cm); (b) changes in primary branch numbers per plants; (c) change in size of internode (cm) and (d) change in leaf length (cm). Each bar represents mean ± SME (n=5). * Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one-way ANOVA.

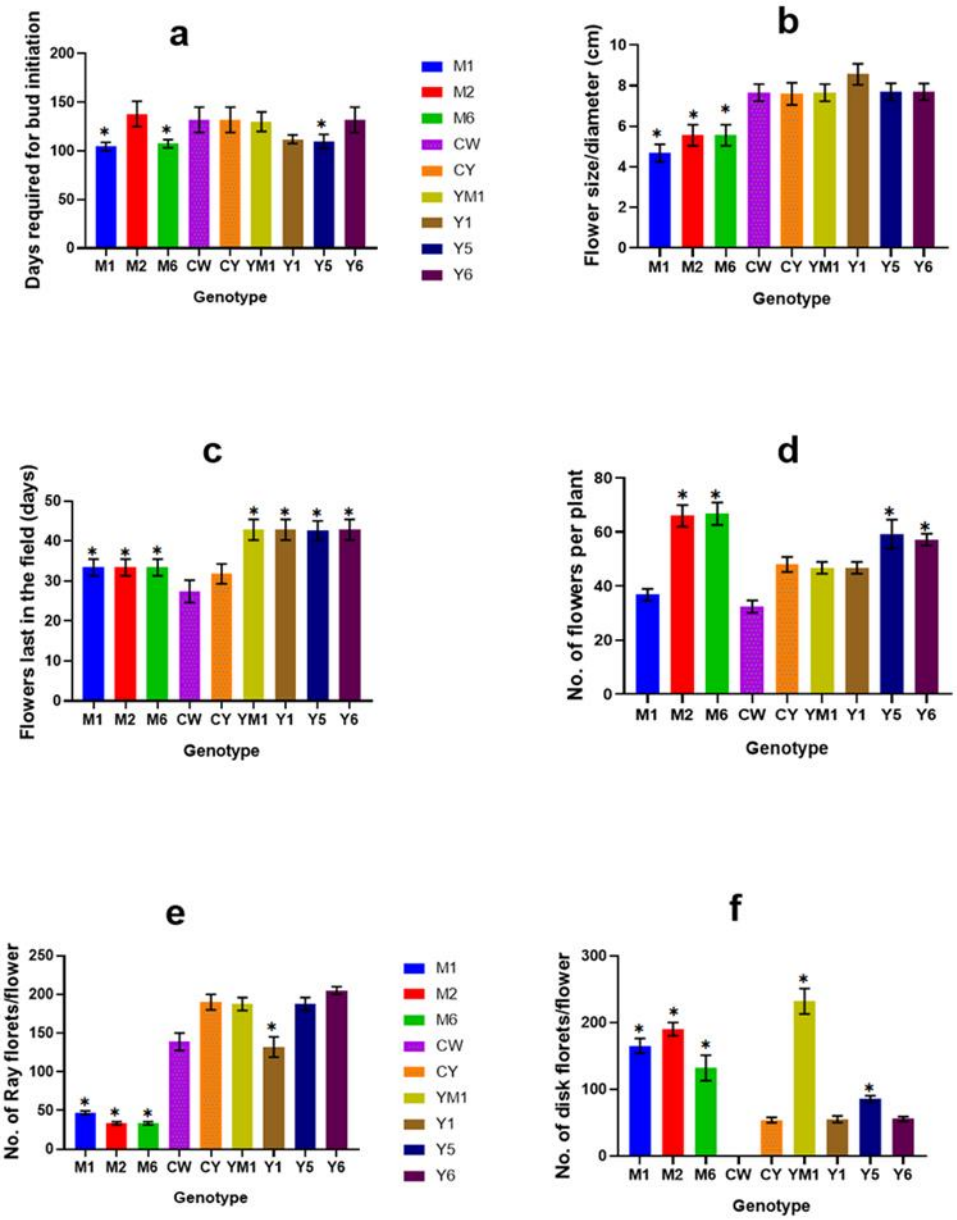

Fig 24 (a-f): Morphological parameters of flowers in M1V7 plants of control and mutants of two tested varieties. (a) Variation in days required for bud initiation on control and mutant lines; (b) Same in case of flower size/ diameter (cm); (c) Same in case of flowers longevity; (d) Same in case of no. of flower/ plant; (e) Same in case of no. of ray floret/ flower; (f) Same in case of no. of disk floret/ flower. (Each bar represents mean ± SME (n=5). *Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one way ANOVA

observed (Fig. 23b). The size of the internode significantly decreased in M1, M2 (developed from CW) and YM1 and Y1(developed from CY) mutant lines (Fig 23c). The leaf length and leaf breadth remained almost unchanged in all the mutant lines compared to the mother plants regardless of the variety (Table 14 and Fig. 23d).

The days required for bud initiation after plantation have been decreased in case of M1, M6, Y1 and Y5 mutant line. So, these four mutant lines can be considered as early varieties. Other mutant lines took almost similar time for bud initiation while comparing with the mother plants (Fig. 24a).

Table. 15 Mean± SEM performance levels of morphological parameters of flowers in M1V7 plants of control and mutants of two tested varieties

Genotype	Days required for bud initiation after plantation	Flower diameter (cm)	No. of flower per plant	No. of Ray floret	No. of disk floret	Flowers last in the field (days)
cv. BARI Chry-2						
Control	107.4± 1.87	7.66± 0.1887	32.4±1.03	138.8±5.044	0	27.4±1.249
M1	104.4 ± 1.97	4.68± 0.1934	36.8±0.9695	47.2±1.02	165.2±5.00	33.4±0.927
M2	138 ± 5.83	5.56± 0.2315	66± 1.761	33.4±0.9274	190±4.472	33.4±0.927
M6	107.4 ± 1.87	5.56± 0.2315	66.8±1.855	33.4±0.9274	132±8.602	33.4±0.927
cv. BARI Chrysanthemum-1						
Control	132 ± 5.831	7.6± 0.2449	48± 1.225	190±4.472	54±1.871	31.8±1.114
Ym1	130± 4.472	7.66± 0.1887	46.8±0.9695	187.6±3.709	232±8.602	42.8±1.158
Y1	112 ± 2	8.56± 0.2315	46.8±0.9695	132±5.831	55±2.236	42.8±1.158
Y5	110 ± 3.162	7.7± 0.1897	59.2± 2.396	187.6±3.709	85.4±2.227	42.6±1.077
Y6	132 ± 5.831	7.7± 0.1844	57.2±0.9695	205±2.236	55.6±1.631	42.8±1.158

*Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one-way ANOVA

Tukey's multiple comparisons test showed all three mutant lines M1 (4.68± 0.1934 cm), M2 (5.56± 0.2315 cm) and M6 (5.56± 0.2315 cm) derived from BARI Chry-2 produced significantly smaller flowers compared to the control flowers CW (7.66± 0.1887 cm). While all the mutant lines developed from BARI Chrysanthemum-1produced larger flowers compared to the controls (Fig. 24b). Flowers of all the seven mutant lines last significantly longer than the mother varieties which can be considered as a remarkable agronomic trait for all of them (Fig.24c). So, it can be assumed that gamma radiation influences days required for bud initiation and flower diameter of the mutants.

Y5 and Y6 mutant lines of BARI Chrysanthemum-1 and M2 and M6 mutant lines of BARI Chry-2 showed significantly increased no. of flowers per plant (Fig. 24d). Significant changes in no. of ray and disk florets per flower were found in most of the mutants accept Y1 and Y6 (Fig. 24e-f). However, the radiation dose is not directly related to the characters tested (Tables 12,13,14,15,16 and Fig.24a-f). It was also noted that the dose of radiation did not directly affect any characteristics analyzed during the present study.

During the study Comparison of color and flower diameter of selected M1V7 mutants derived from both BARI Chrysanthemum-1 and BARI Chrysanthemum -2 varieties were done. It has been presented in Table 16.

Finally, the promising seven mutant lines derived from both Chrysanthemum varieties were confirmed through molecular study using ISSR molecular markers.

Table 16. Comparison of color and flower diameter of selected M1V7 mutants derived from both BARI Chrysanthemum-1 and BARI Chrysanthemum -2 varieties

Selected Variants	Radiation dose (Gy)	Diameter of flower (cm)	Length of ray florets (cm)	Length of disk florets (cm)	Flower color code
M1	5	4.71± 0.06	2.22±0.21	1±0.22	84B
M2	5	5.82 ± 0.31	2.55±0.02	1.2±0.21	76C
M6	15	6.41± 0.15	3.15±0.31	1.2±0.32	76D
Ym1	10	7.88± 0.26	3.23±0.41	0.88±0.12	17A
Y1	25	8.87±0.34	4.32±0.33	0.91±0.21	6B
Y5	10	7.77±0.55	3.21±0.45	0.8±0.16	N25C
Y6	10	7.89±0.42	7.88±0.07	0.9±0.15	N25B

4.1.3 Molecular analysis of the Chrysanthemum mutants

4.1.3.1 Analysis of genetic diversity among the seven promising mutants and two mother variety of Chrysanthemum plants using ISSR markers

Molecular marker technology provides information that can help to define the differences of regenerated mutants and their status according to the number of close relatives and their phylogenetic position. DNA marker technology is a new approach based on DNA polymorphism among tested genotypes and thus applicable to biological research. Among several molecular markers ISSR (Inter-simple sequence repeats) is the widely used most accountable molecular marker. In this experiment, 13 primers were used to find out the variation among 7 mutants generated from two varieties of Chrysanthemum and mother plant of both varieties. Among all the primer tested 5 primers produced clearly scorable and reproducible bands in all these tested 9 groups (7 mutants and both BARI Chrysanthemum-1 as well as BARI Chrysanthemum-2 mother plants). Results of these experiments are presented under the following heads:

4.1.3.2 Optimization of PCR amplification conditions and selection of primers for ISSR analysis

PCR amplification conditions play a crucial role in amplification of DNA. In this part of the study, PCR amplification conditions were optimized by analyzing several factors. For this reason, various concentrations of the components of the reaction mix as well as variable amplification conditions were tested in obtaining most reproducible results. Template DNA concentration over the range of 20-60 ng/ µl gave a constant banding pattern but higher concentrations resulted in smear in gel in most cases. At lower template concentrations, there was a tendency for appearance of non-reproducible low molecular weight bands. Template DNA concentrations of 30 ng/µl was found to be the most suitable for getting reproducible banding patterns. Primers were selected carefully as some primers produced non-reproducible bands and some others could not amplify the DNA in all the investigating plants. These primers used in the present study are 10-18 nucleotides long and have the possibility of annealing at a random number of locations in the genome. However, all the primers do not produce reproducible and polymorphic banding patterns in all plant species. Initially, 13 primers were used. Among them 5 primers gave reproducible and scorable bands across the investigated samples. Therefore, these 5 primers were used for further analysis and mostly bright, prominent bands were scored. The bands amplified with ISSR primers were in the range of 307 to 2665 bp (Table 17).

4.1.3.3 Analysis of ISSR profiles

The selected 5 primers produced highly reproducible banding patterns in all the developed mutants as well as the two mother plant varieties investigated. However, the primers varied considerably in case of number of bands produced, intra-plants polymorphism and discriminating capacity. A particular band has been described by the primer name by which it was amplified suffixed with the molecular weight of the band (for example, the band UBC 83_{1265} denotes that it was amplified with the primer UBC 835 and its molecular weight was 1265 bp). Compilation of five ISSR primer profiles during the analysis of the six Chrysanthemum mutants along with the two mother plants has been presented in Table 17. The number of bands produced by primers ranged from two fourteen, with an average of 6.6 bands per primer obtained. During this study, 90.90% polymorphism was recorded for the two cultivated Chrysanthemum varieties and for the mutants developed from them. This broad range of polymorphism revealed wide diversity among the mutants developed through gamma radiation. The diversifications would be useful for different breeding program of floriculture and can contribute to the global floral market. The primer wise ISSR profiles and banding patterns of 9 tested groups (7 mutants and both BARI Chrysanthemum-1 as well as BARI Chrysanthemum-2 mother plants) of plants are given below:

4.1.3.3.1 ISSR profile using primer UBC 835

The primer UBC 835 produced a total of 14 bands within both mother varieties of Chrysanthemum and mutants. This primer produced highest bands among all the primers used in the present investigation. The lowest number of bands was produced by Y1 BARI Chrysanthemum-1 mutant (2 bands) and rest of the investigated materials produced bands that ranged from 3 to 13. The highest numbers of bands (13 bands) with one unique (UBC 83_{1265}) band were produced by M1 mutant developed from BARI Chrysanthemum-2. It produced 2 monomorphic band and showed 78.57% polymorphism (Table 17). The investigated mutants belong to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig.25a).

4.1.3.3.2 ISSR profile using primer UBC 857

The primer UBC 857 produced a total of 10 bands within both mother varieties of Chrysanthemum and mutants. This primer produced 2nd highest bands among all the

primers used in the present investigation. The lowest number of bands was produced by Y1 BARI Chrysanthemum-1 mutant (2 bands) and rest of the investigated materials produced bands that ranged from 3 to 10. The highest numbers of bands (10 bands) were produced by M1 and M2 mutants developed from BARI Chrysanthemum-2. It produced no monomorphic as well as no unique band and showed 100% polymorphism (Table 17). The investigated mutants belong to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 25b).

4.1.3.3.3 ISSR profile using primer UBC 878

The primer UBC 878 produced only 2 bands within both mother varieties of Chrysanthemum and mutants. This primer produced lowest bands among all the primers used in the present investigation. It produced one unique band (UBC 878_{1544}) and one polymorphic band. No band were produced by Ym1 and Y1 mutants developed from BARI Chrysanthemum-1. It produced no monomorphic showing 100% polymorphism (Table 17). The investigated mutants belong to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 26a).

4.1.3.3.4 ISSR profile using primer UBC 889

The primer UBC 857 produced a total of 4 bands within both mother varieties of Chrysanthemum and mutants. Both M1 and M2 produced 4 bands while Ym1, Y6 and Y5 produced one band. Y1 produced no band and showed 100% polymorphism with no monomorphic band (Table 17). The investigated mutants belonging to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 26b).

4.1.3.3.5 ISSR profile using primer Hb_9

The primer Hb_9 produced only 3 bands within both mother varieties of Chrysanthemum and mutants. This primer produced 2nd lowest bands among all the primers used in the present investigation. No band was produced by the CY BARI Chrysanthemum-1 mother plant. It produced no monomorphic band showing 100% polymorphism (Table 17). The investigated mutants belong to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 27).

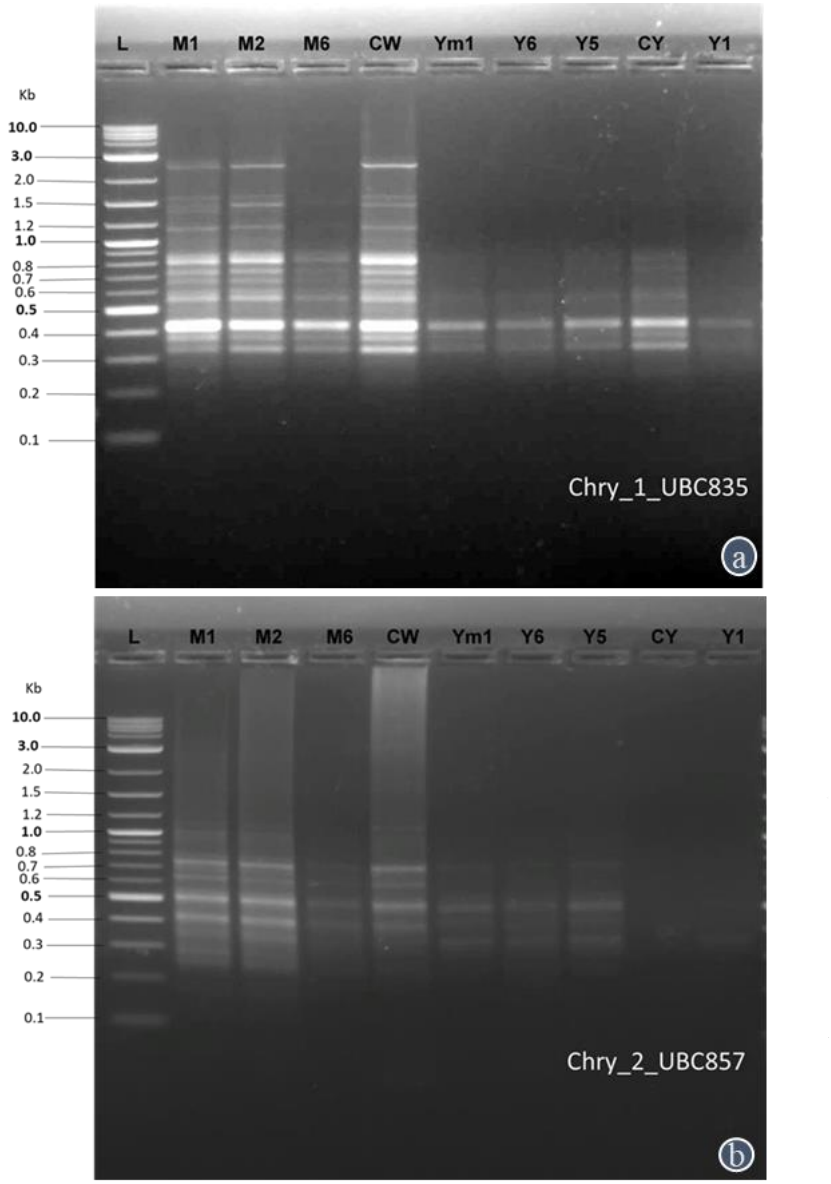

Fig. 25 (a-b): ISSR profiles of both varieties of Chrysanthemum mothers and 7 mutants derived from gamma irradiation obtained with the primer UBC835 and UBC857. (a) Amplification of bands with UBC 835 primer. Lane L: Ladder (1 Kb plus); Lane 2,3,4 &5: mutants M1, M2,M6 and mother plant of BARI chry-2 variety CW; Lane (6-10): mutants Ym1, Y6, Y5, CY (mother plant) and Y1; (b) Same as (a) but with UBC 857 primer and 1Kb + Ladder.

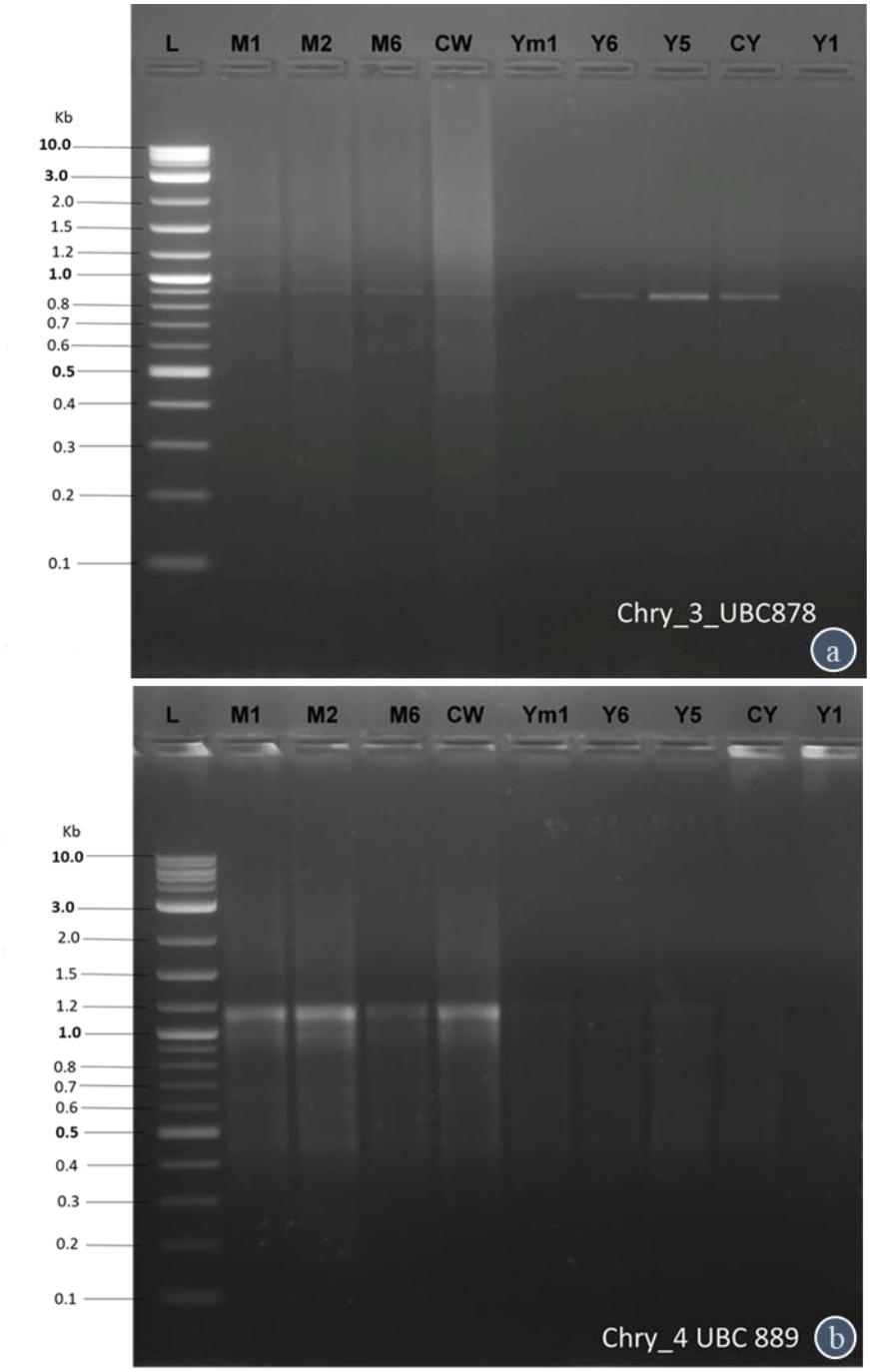

Fig. 26. (a-b) ISSR profiles of both varieties of Chrysanthemum mothers and 7 mutants derived from gamma irradiation obtained with the primer UBC878 and UBC889. (a) Amplification of bands with UBC 878 primer. Lane L: Ladder (1 Kb plus); Lane 2,3,4 &5: mutants M1, M2,M6 and mother plant of BARI chry-2 variety CW; Lane (6-10): mutants Ym1, Y6, Y5, CY (mother plant) and Y1; (b) Same as (a) but with UBC 889 primer and 1Kb plus Ladder.

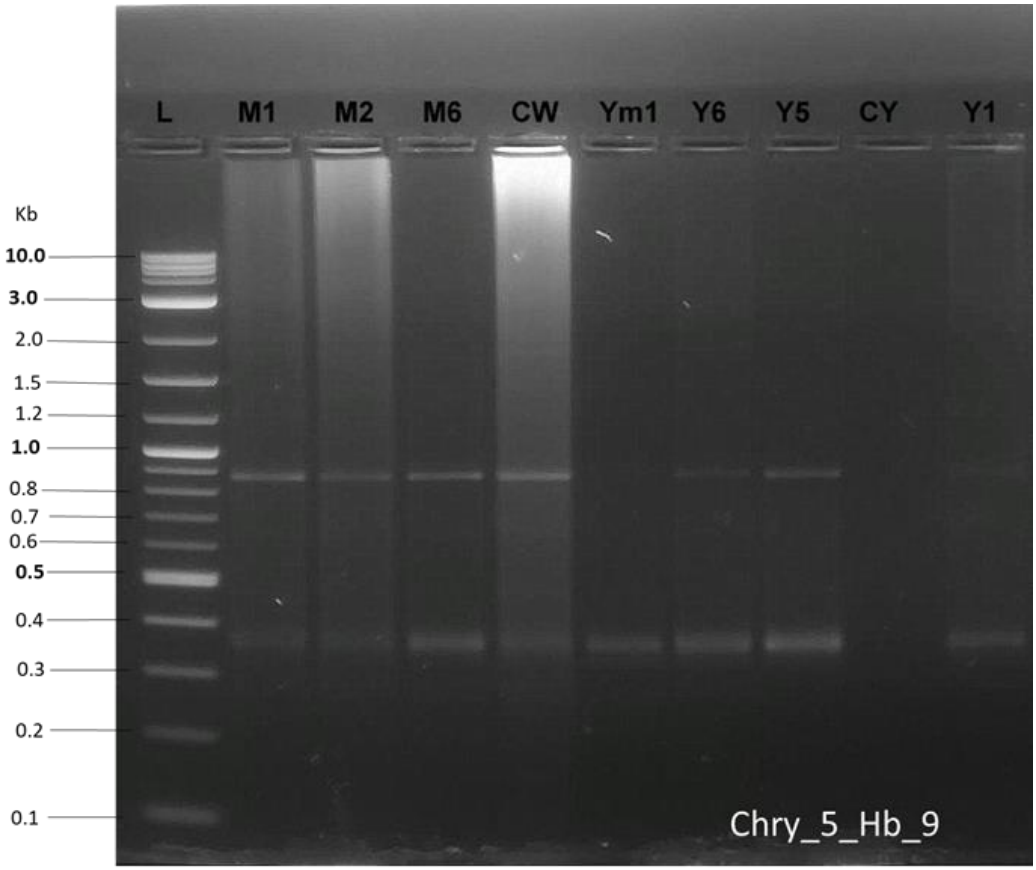

Fig. 27 ISSR profiles of both varieties of Chrysanthemum mothers and 7 mutants derived from gamma irradiation obtained with the primer Hb_9. (a) Amplification of bands with Hb_9 primer. Lane L : Ladder (1 Kb plus); Lane 2,3,4 &5: mutants M1, M2,M6 and mother plant of BARI chry-2 variety CW; Lane (6-10): mutants Ym1, Y6, Y5, CY (mother plant) and Y1.

Table 17. Compilation of five ISSR primer profile during the analysis of the six Chrysanthemum mutants along with the two mother plants

Primer codes	Total loci	Size ranges (bp)	Number of Polymorphic loci	Number and size (bp) of unique bands	Polymorphism (%)
UBC 835	14	307-2665	11	M1 (1265)	78.57
UBC 857	10	198-1120	10	-	100
UBC 878	2	1028-1544	2	M1 (1544)	100
UBC 889	4	450-1212	4	-	100
Hb_9	3	346-862	3	-	100
Grand Total	33	307-2665	30	2	90.90

4.1.3.4 Analysis of genetic diversity

The Dendrogram of the materials investigated was constructed using Unweighted Pair-Group Mean Average (UPGMA) cluster analysis supported by distance linkage mapping along with the percentage of polymorphism present in each material investigated. From all this information it has been revealed that a very high level of polymorphism is present among the mother and mutant lines developed through gamma irradiation involving tissue culture techniques.

Table 18. Summary of Nei's (1972) genetic distances of ISSR analysis of the seven Chrysanthemum mutants along with the two mother plants

pop ID	M1	M2	M6	CW	Ym1	Y6	Y5	CY	Y1
M1	****								
M2	0.0953	****							
M6	0.5008	0.4520	****						
CW	0.3185	0.2007	0.2007	****					
Ym1	0.9316	0.8575	0.3185	0.6061	****				
Y6	0.8575	0.7885	0.2776	0.5521	0.0953	****			
Y5	0.7885	0.7239	0.2384	0.5008	0.1292	0.0308	****		
CY	0.9316	0.8575	0.2384	0.5008	0.3610	0.3185	0.2776	****	
Y1	1.2993	1.1939	0.5008	0.8575	0.2007	0.1643	0.2007	0.3610	****

Genetic variation was high among all the materials investigated of Chrysanthemum as revealed by the genetic distance (Table 18). In this study, the UPGMA analysis of dendrograms and distance matrix coefficients revealed good relationships between some mutants (Table 18). Primarily, the genetic distance matrix was calculated according to Nei (1972) for all the 9 investigated Chrysanthemums considering ISSR

banding pattern (Table 18). Genetic variation among the germplasm is usually revealed by the genetic distance matrix. In this study, the lowest genetic distance was found between Y5 and Y6 (0.0308). On the other hand, the highest genetic distance was found between M2 and M6 (0.4520); Y1 and CY (0.3610).

The phylogenetic relationships among 9 tested Chrysanthemum germplasm were analyzed by the UPGMA method (Fig.28). The cluster result indicated that ISSR markers could distinguish all the mutants as well as the mother plants. Grouping of the investigated plants into each cluster or branch correlated with similarities in their ISSR DNA patterns. For example, the mutant that produced the same DNA banding patterns were also recognized as being similar from phylogenetic analysis. Those germplasms belonging to the same cluster have been presented in the resulting phylogenetic tree (Fig. 28).

Dendrogram produced by the Unweighted Pair-Group Mean Average (UPGMA) method was distributed among 7 mutants along with the two mother varieties. There was a total of 8 clusters (1 -8) present in the dendrogram (Fig. 28). All the germplasm was grouped into two major branches (B1, 7 and B2, 6) which included BARI Chrysantheum-2 and BARI Chrysanthemum-1 varieties along with the mutants developed from them respectively.

The branch B1 (7) were divided into two subbranches and the BARI Chry-2 mother variety (CW) were in one subbranch (SB1) (5) along with the M6 mutant line. Another subbranch (SB1) (2) included M1 and M2 mutant lines. Then branch B2 (6) were further separated into two subbranches and the BARI Chrysanthemum-1mother variety (CY) formed a separate subbranch (SB1), which was totally different from the other 4 mutants developed in this study through mutation induction. Then subbranch (SB2) (4) was showing diversity/genetic distance by separating Y1(SSB1) in a sub - subbranch and included Ym1, Y6 and Y5 mutant lines in another sub-subbranch (SSB2) (3). The sub-subbranch (SSB2) (3) was further divided into two groups, which included Ym1 in one group and in the other group Y6 and Y5 mutant lines (SSB2G2) (1). Among all these 4 mutants, Y6 and Y5 are mostly similar whereas Ym1 is mostly different then Y1, Y5, Y7 and Ym1 mutant lines (Fig. 28). Thus, there was a clear difference between the mutants and the mother varieties.

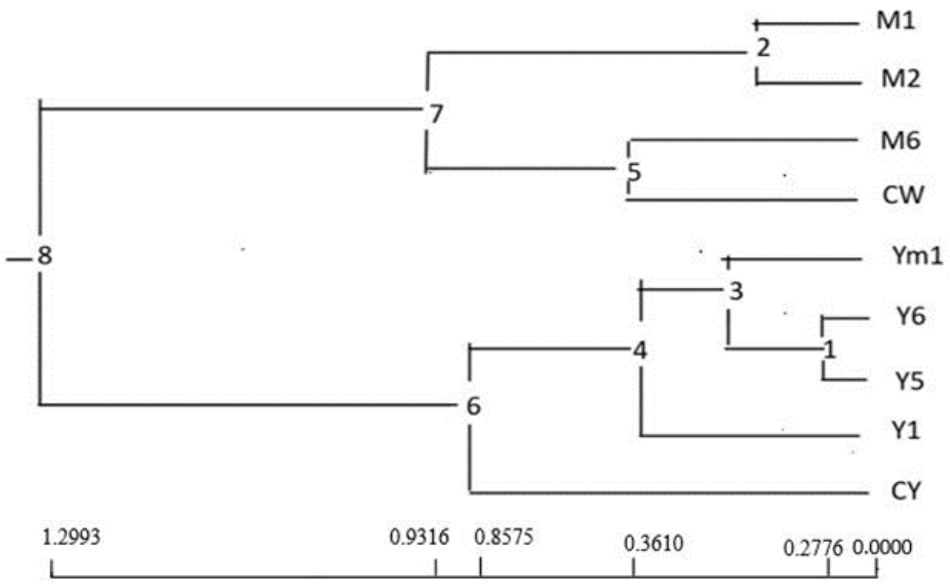

Fig. 28 UPGMA dendrogram constructed based on Nei's genetic distance summarizing the data on differentiation among the mother varieties of chrysanthemum and the developed mutants by ISSR analysis.

4.2 Development of *in vitro* regeneration protocol and induction of mutation through gamma radiation in Gerbera

This study was carried out in three phases. In the first phase, several experiments were conducted to obtain an efficient *in vitro* regeneration system for the above mentioned two varieties of Gerbera using various explants, namely, flower bud (FB), flower stalk (FS) and leaf segments (LS). In the second phase, experiments were conducted for *in vitro* mutation induction using five different gamma radiation doses, namely, 5, 10, 15, 20 and 25 Gy. Afterward experiments were conducted for isolation of morphological mutants in both *in vitro* and *in vivo* conditions during plant development. In the last phage promising mutants were isolated following different morphological studies and finally mutant lines were selected through molecular study using ISSR molecular markers.

4.2.1 *In vitro* regeneration of Gerbera plantlet

In this study, experiments were conducted to regenerate shoot and root from various explants of two Gerbera varieties. Flower bud (FB), flower stalk (FS) and leaf segments (LS) of two varieties were used as explants.

Following sterilization of the explants successful shoots were developed using suitable phytohormonal supplementation in MS medium. Elongated shoots were transferred to the suitable rooting medium. After successful root formation, the regenerated plantlets were acclimatized in soil following proper hardening and allowed to grow under greenhouse condition. Results of these experiments are presented under the following heads:

4.2.1.1 Surface sterilization and preparation of explants used for *in vitro* regeneration

The three types of explants were excised from field grown mother plants and washed under running tap water for 30 minutes. Then they were treated with a locally available mild detergent (Jet powder solution) in water for 15 minutes and were washed thoroughly under tap water followed by 3-4 times washing with distilled water. Further steps of sterilization of explants were carried out in a laminar air flow cabinet where previously cleaned explants were treated in 0.1% (v/v) $HgCl_2$ solution for 8-10 minutes and was slowly shaken continuously by hand. Then they were rinsed with sterile distilled water for 3-4 times. The effect of $HgCl_2$ solution was examined to obtain contamination free explants. For this purpose, surface sterilized explants

were inoculated on MS medium supplemented with phytohormones. The percentage of contaminated explants as well as the effect of various sterilization periods (2-10 mins) on shoot regeneration were examined during winter and summer seasons. Data for both varieties are presented in Table 19. It has been observed from the Table that in case of the BARI Gerbera-1 variety the percentage of contamination was recorded to be 80 for flower bud (winter season) and 93 (summer season) when the duration of treatment was 2 minutes. During this sterilization treatment, in winter the percentage of contamination was recorded to be 60 for both leaf segment (LS) and flower stalk (FS) explants. Whereas in summer the percentage of contamination was 65 for LS and 67 for FS explants. On the other hand, when duration of sterilization treatment was 10 minutes the percentage of contamination was only 35 (winter season) and 40 (summer season) for flower bud (FB) explant. During this sterilization treatment the percentage of contamination was recorded to be 25 for leaf segment (LS) and 30 for flower stalk (FS) in winter. Whereas in summer the percentage of contamination was 30 for LS and 33 for FS explants. More or less similar results were observed in the case of BARI Gerbera-2 variety. It was also found that, in both the varieties the percentage of contamination was higher during the summer season. However, among the three explants tested, flower bud (FB) showed the highest percentage of contamination towards all five sterilization treatments in both varieties. It was found that a 10 mins sterilization period reduced contamination rate considerably during winter, 25% (LS), 35(FB) &30% (FS) for BARI Gerbera-1 and 25% (LS), 29% (FB) & 25% (FS) for BARI Gerbera-2 variety. Sterilized young leaves (3^{rd} and 4^{th} position), flower bud and flower stalk were cut into small pieces (about 0.5-1.0 cm) as mentioned in method section. These three types of explants were inoculated on MS medium supplemented with different concentrations and combinations of hormones to examine their regeneration responses.

4.2.1.2 Determination of suitable medium for *in vitro* regeneration of shoots

Several experiments were performed to select suitable concentrations and combinations of hormonal supplements as well as suitable explants for *in vitro* shoot regeneration using MS medium. Three types of explants, namely, leaf segments (LS), flower bud (FB) and flower stalk (FS) of both varieties of Gerbera were cultured on MS medium supplemented with various concentrations of BAP and NAA for shoot regeneration. The regenerated shoots were routinely sub-cultured for further multiplication of shoots and elongation.

Table 19. Efficiency of the sterilization procedure in *Gerbera Jamesonii* with different explants of BARI Gerbera-1 (white) and BARI Gerbera-2 (red)

Variety	Sterilization period (minutes)	Number of explants inoculated	% of contamination in winter			% of contamination in summer		
-	-	-	LS	FB	FS	LS	FB	FS
BARI Gerbera-1 (White)	2	30	**60**	**80**	**60**	**65**	**93**	**67**
	4	30	60	78	48	60	88	50
	6	30	48	75	45	50	75	50
	8	30	40	55	35	40	55	40
	10	30	**25**	**35**	**30**	**30**	**40**	**33**
BARI Gerbera-2 (Red)	2	30	**60**	**85**	**60**	**70**	**95**	**70**
	4	30	56	82	55	60	85	53
	6	30	45	72	50	48	75	50
	8	30	30	43	35	40	55	42
	10	30	**25**	**29**	**25**	**30**	**35**	**30**

Note # LS= leaf segment, FB= flower bud and FS= flower stalk.

4.2.1.2.1 Effect of culture medium, explant type and hormonal supplements towards regeneration of shoots using different explants

In this set of experiment, the effects of various combinations of hormonal supplements in MS medium on organogenesis using flower bud (FB), flower stalk (FS) and leaf segments (LS), explants were tested following the development of shoots of both varieties (Tables 20-21 and Fig. 29). MS medium supplemented with different concentrations of BAP (1.0-7.0 mg/l) and NAA (0.5 & 1.0 mg/l) were employed to examine their effects on initiation of regeneration and development of shoots from the tested explants of BARI Gerbera-1 (white) and BARI Gerbera-2 (red) varieties. Seven different treatments were formulated using cytokinin and auxin in MS medium to see their effect on *in vitro* regeneration. Analysis of variance (ANOVA) for various responses of regeneration following these seven treatments in two varieties is shown in Figs. 30-33. Statistical analysis showed significant difference in the percentages of regeneration responses, mean number of shoot/explant as well as the mean shoot length for the seven treatments for both tested varieties. Interaction between hormonal combinations in MS medium, type of explant and the varieties were also found significant (Figs. 31-33).

4.2.1.2.1.1 Effect of different concentrations and combinations of BAP and NAA on MS medium towards shoot regeneration from flower bud explants in case of BARI Gerbera- 1 (white) and BARI Gerbera-2 (red) varieties

In this experiment seven different concentrations of BAP (1.0 – 7.0 mg/l) in combinations with two different concentrations of NAA (0.5 and 1.0 mg/l) in MS medium were used to see their effects on shoot regeneration from flower bud explants of two varieties of Gerbera. A total of seven different combinations (T1 – T7) were formulated for the above study.

Table 20. Effects of different concentration of BAP and NAA on MS medium towards shoot regeneration from flower bud explants of BARI Gerbera 1

Treatment	Hormonal Supplementation (mg/l)		Days required for callus induction	No. of responsive explants which produced shoots	Regeneration %	Days to shoot initiation	Mean no. of shoot/ explants after 7-8 wks	Mean length of shoot after 7-8 wks (cm)
	BAP	NAA						
T1	1.0	0.5	25-35	Only callus	0	-	-	0
T2	2.0	0.5	25-35	Only callus	0	-	-	0
T3	3.0	0.5	20-30	8	26.7	55-60	3.55	1.1
T4	4.0	1.0	20-30	10	33.3	55-60	4.08	1.5
T5	5.0	1.0	20-30	16	53.3	50-55	7.66	1.9
T6	6.0	1.0	20-30	21	**70.0**	**40-45**	**10.33**	**3**
T7	7.0	1.0	25-35	10	33.3	40-45	3.22	2.1

Note # Number of explants inoculated for each experiment = 30

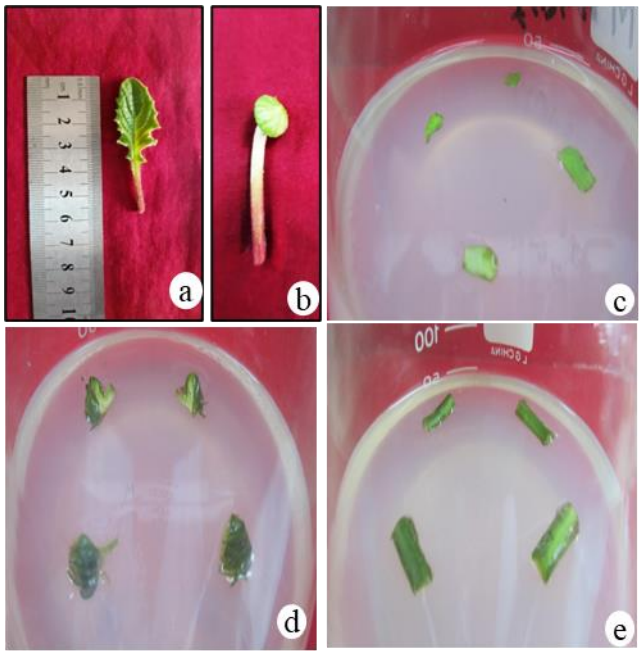

Fig. 29 (a-e): Various Gerbera explants used in this study. (a) leaf collected from garden; (b) unopened flower bud with stalk collected from garden; (c) Segmented leaf explants on regeneration medium; (d) Segmented flower bud explants on regeneration medium; (e) Segmented flower stalk explants on regeneration medium.

Fig.30 (a-k): Different stages of Regeneration (organogenesis) from different explants of white variety in MS+6 mg/l BAP+1 mg/l NAA. (a) 2-3 wks old culture; (b) 3- 4 wks old culture; (c) 4-5 wks old culture; (d) 7-8 wks shoot primordial clump; (e) 9-10 wks old clump containing multiple shoots for flower bud; (f-h) for flower stalk response for b-e culture condition; (i-k) for leaf response for b-e culture condition.

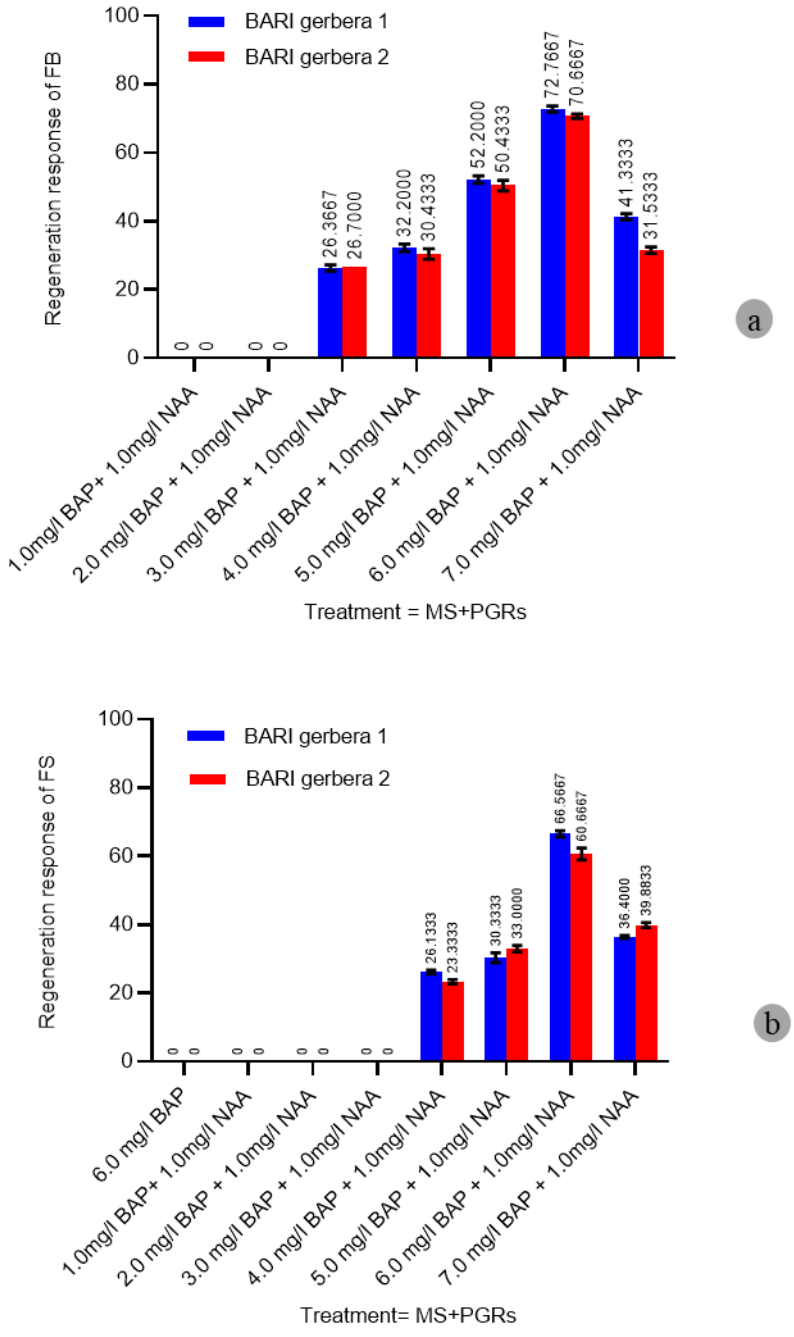

Fig. 31. (a-b): Regeneration response of flower bud and flower stalk explants for both varieties towards MS medium with different PGRs combinations in the two varieties after six weeks. (a) Effects of different treatments on percentage of regeneration of flower bud explant; (b) Same for flower stalk explants. Data are mean of three replicates with bars representing SEM.

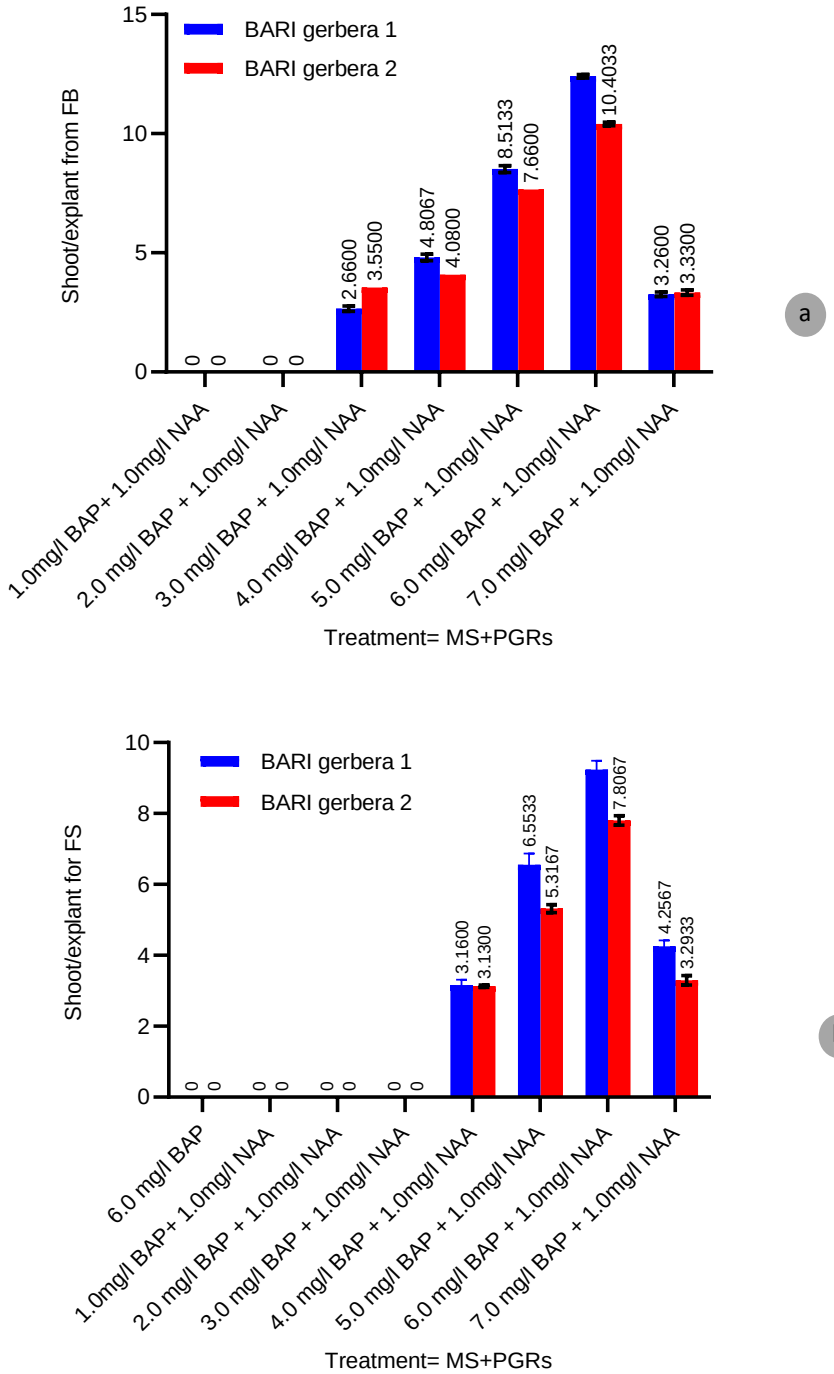

Fig. 32. (a-b): Number of shoot/explant developed from flower bud and flower stalk explants for both varieties towards MS medium with different PGRs combinations in the two varieties after six weeks. (a) Effects of different treatments on. of shoot/explant in case of flower bud explant; (b) Same for flower stalk explants. Data are mean of three replicates with bars representing SEM.

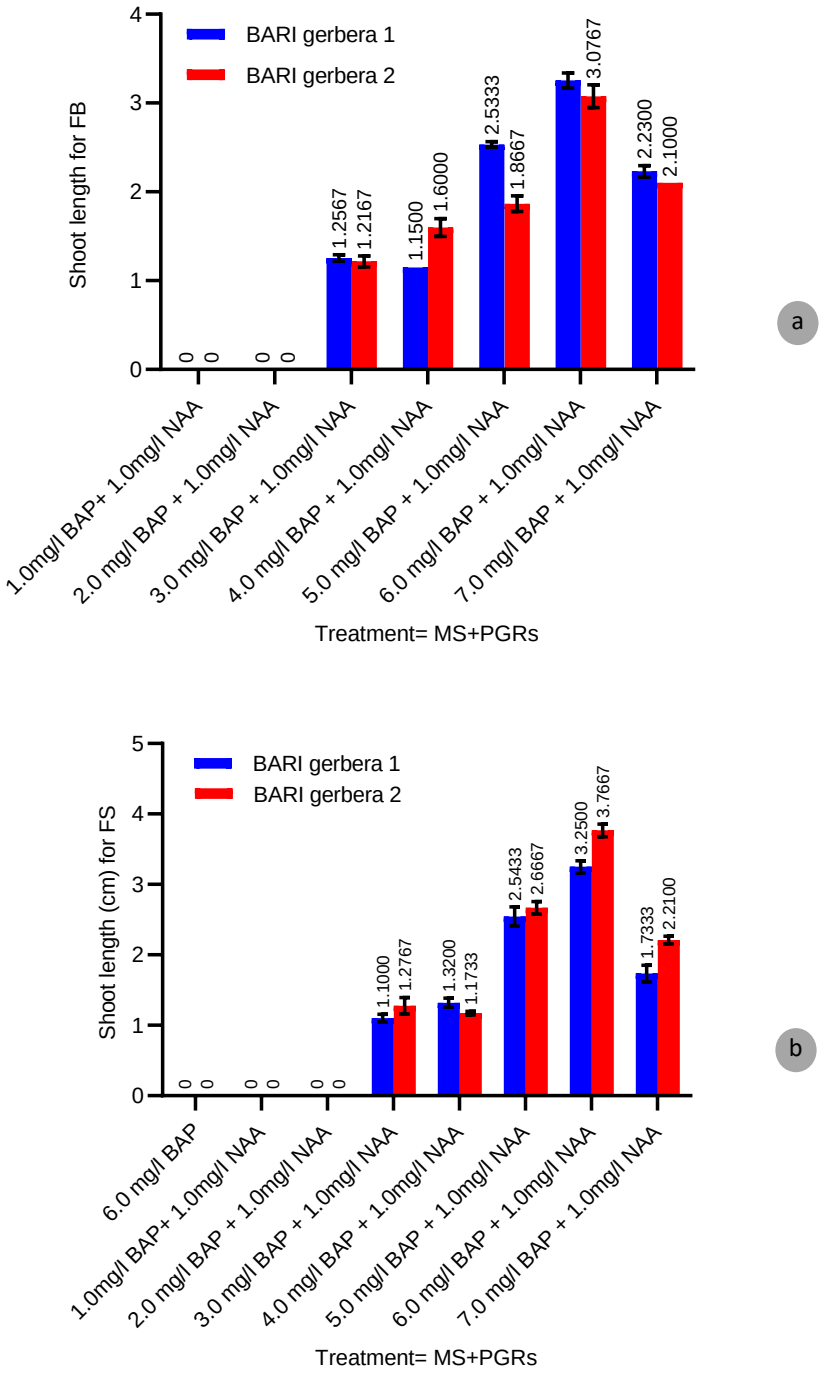

Fig. 33. (a-b): Length of the shoots developed from flower bud and flower stalk explants for both varieties towards MS medium with different PGRs combinations in the two varieties after six weeks. (a) Effects of different treatments on length of the shoots (cm) in case of flower bud explant; (b) Same for flower stalk explants. Data are mean of three replicates with bars representing SEM.

Results of this investigation have been presented in Table 20&21. It has been observed from the Table that maximum shoot regeneration in both the varieties was achieved when the flower bud explants were cultured on MS medium supplemented with 6.0 mg/l BAP and 1.0 mg/l NAA (T6). On this medium composition 70.00% explants of BARI Gerbera-1 variety and 73.30% explants of BARI Gerbera-2 variety showed shoot regeneration within three weeks of culture. In the case of BARI Gerbera-1 variety the mean number of shoot buds/explants after 8 wks was recorded to be 10.33 and mean shoot length was 3.00 cm within the same time period.

Table 21. Effects of different concentration of BAP and NAA on MS medium toward shoot regeneration from flower bud explants of BARI Gerbera-2

Treatment	Hormonal Supplementation (mg/l)		Days required for callus induction	No. of responsive explants Which produced shoots	Regeneration %	Days to shoot initiation	Mean no. of shoot/ explants after 7-8 wks	Mean length of shoot after 7-8 wks (cm)
	BAP	NAA						
T1	1.0	0.5	25-35	Only callus	0	-	-	0
T2	2.0	0.5	25-35	Only callus	0	-	-	0
T3	3.0	0.5	20-30	8	26.7	55-60	2.55	1.22
T4	4.0	1.0	20-30	10	33.3	55-60	4.88	1.15
T5	5.0	1.0	20-30	16	53.3	50-55	8.66	2.5
T6	6.0	1.0	20-30	22	**73.3**	**40-45**	**12.33**	**3.25**
T7	7.0	1.0	20-30	12	40.0	40-45	3.22	2.25

Note # Number of explants inoculated for each experiment = 30

On the other hand, in case of BARI Gerbera- 2 variety the mean number of shoot buds/explants after 8 wks was recorded to be 12.33 and mean shoot length was 3.25 cm and 21 & Figs. 32 and 33). During the study high concentrations of auxin (1.0 mg/l NAA) were found to have increased effect on shoot induction for both varieties. Whereas low auxin (0.5 mg/l NAA) formed only callus and failed to form shoots in both varieties respectively. The minimum response towards shoot regeneration in both the varieties was observed when the explants were cultured on MS medium supplemented with 3.0 mg/l BAP and 0.5 mg/l NAA (T3) (Fig. 31-33).

It was also observed that addition of higher concentration of BAP (4.0 - 6.0 mg/l) along with NAA (1.0 mg/l) in MS medium either of the combinations (T4-T6) increased the frequency of shoot regeneration (Tables 20 and Fig. 31).

4.2.1.2.1.2 Effect of different concentrations and combinations of BAP and NAA on MS medium towards shoot regeneration from flower stalk of BARI Gerbera -1 (white) and BARI Gerbera -2 (red) varieties

In this experiment seven different concentrations of BAP (1.0 – 7.0 mg/l) in combinations with two different concentrations of NAA (0.5 and 1.0 mg/l) in MS medium were used to see their effects on shoot regeneration from flower stalk explants of two varieties of Gerbera. A total of seven different combinations (T1 – T7) were formulated for the above study. Results of this investigation have been presented in Tables 22 and 23. It has been observed from the Table that maximum shoot regeneration in both the varieties was achieved when the flower stalk explants were cultured on MS medium supplemented with 6.0 mg/l BAP and 1.0 mg/l NAA (T6). On this medium composition 50.00% explants of BARI Gerbera-1 variety and 56.70% explants of BARI Gerbera-2 variety showed shoot regeneration within three weeks of culture. In the case of BARI Gerbera-1 variety the mean number of shoot buds/explants after 8 wks was recorded to be 7.88 and mean shoot length was 2.7 cm within the same time period. On the other hand, in the case of BARI Gerbera- 2 variety the mean number of shoot buds/explant after 8 wks was recorded to be 9.22 and mean shoot length was 2.08 cm respectively (Fig. 30). The treatments T1, T2 and T3 could not produce any shoot in both varieties, whereas T4 produced the lowest percentage of regeneration (23.33 for BARI Gerbera -1 and 26.70 for BARI Gerbera -2) with minimum no. of shoot/ explant.

Table 22. Effects of different concentration of BAP and NAA on MS medium toward shoot regeneration from flower stalk explants of BARI Gerbera -1 (White)

Treatment	Hormonal Supplementation (mg/l)		Days required for callus induction	No. of responsive explants Which produced shoots	Regeneration %	Days to shoot initiation	Mean no. of shoot/ explants after 7-8 Wks	Mean length of shoot after 7-8 wks (cm)
	BAP	NAA						
T1	1.0	0.5	20-18	Only callus	0	-	0	-
T2	2.0	0.5	20-18	Only callus	0	-	0	-
T3	3.0	0.5	20-18	Only callus	0	-	0	-
T4	4.0	1.0	20-18	7	23.3	55-65	3.08	1.08
T5	5.0	1.0	20-18	10	33.3	50-60	5.34	1.34
T6	6.0	1.0	20-18	18	**50.0**	**40-50**	**7.88**	**2.08**
T7	7.0	1.0	20-18	12	40.0	40-45	3.22	2.22

Note # Number of explants inoculated for each experiment = 30

Table 23. Effects of different concentration of BAP and NAA on MS medium towards shoot regeneration from flower stalk explants of BARI Gerbera -2 (red) variety

Treatment	Hormonal Supplementation (mg/l)		Days required for callus induction	No. of responsive explants Which produced shoots	Regeneration %	Days to shoot initiation	Mean no. of shoot/ explants after 7-8 wks	Mean length of shoot after 7-8 wks (cm)
	BAP	NAA						
T1	1.0	0.5	20-25	Only callus	0	-	0	-
T2	2.0	0.5	20-25	Only callus	0	-	0	-
T3	3.0	0.5	20-25	Only callus	0	-	0	-
T4	4.0	1.0	20-18	8	26.7	55-65	3.18	1.56
T5	5.0	1.0	20-18	9	30.0	50-60	6.66	2.20
T6	6.0	1.0	**20-18**	20	**56.7**	**40-50**	**9.22**	**2.70**
T7	7.0	1.0	20-18	11	36.7	40-50	4.22	2.33

Note # Number of explants inoculated for each experiment = 30; T1-T7= Treatment used for regeneration

4.2.1.2.1.3 Effect of different concentrations and combinations of BAP and NAA on MS medium towards shoot regeneration from leaf explants of BARI Gerbera -1 (white) and BARI Gerbera -2 (red) varieties

In this experiment seven different concentrations of BAP (1.0 – 7.0 mg/l) in combinations with two different concentrations of NAA (0.5 and 1.0 mg/l) in MS medium were used to see their effects on shoot regeneration from leaf explants of two varieties of Gerbera. A total of seven different combinations (T1 – T7) were formulated for the study. Among the treatments used T7 (MS medium supplemented with 7.0 mg/l BAP and 1.0 mg/l NAA) showed considerable results in both callus induction and shoot formation. Within 25-35 days 50% of the explants produced callus in case of both varieties on this medium combination. It was observed that the time required for shoot initiation from leaf explants was very high. About 60-85 days were required for shoot initiation from the callus on this medium combination containing BAP and NAA (T7) and mean number of shoots/explants was recorded to be 2.8 (Fig. 30 i-k). Regeneration percentage was very poor in other treatments (T1-T6) and induced calli failed to produce shoots. It was also noticed that the result was not consistent in the replicates. So, for both varieties further data for this explant was not recorded.

4.1.1.2.1.4 Comparison of regeneration response of two types of explants towards different concentrations of cytokinins and auxins in MS media for BARI Gerbera -1 (white) and BARI Gerbera -2 (red) varieties

A set of experiments were conducted using flower bud and flower stalk explants from the BARI Gerbera-1 and BARI Gerbera -2 varieties in MS medium with different concentrations of BAP (1.00 - 7.00 mg/l) and NAA (0.5& 1.0 mg/l). Results of the above experiments have been presented in Tables 20-23. In these experiments, T6 (MS+6.0 mg/l BAP+1.0 mg/l NAA) showed the best response regardless of the explant types and variety. On this medium composition (T6) flower bud explants showed the highest percentage of shoot regeneration (70% for BARI Gerbera-1 and 73.3% for BARI Gerbera-2). Whereas flower stalk explants on the same treatment (T6) produced the highest percentage of shoot regeneration 50.00% for BARI Gerbera-1 and 56.70% for BARI Gerbera-2. Again, the mean no of shoots/explant was also found the highest (10.33 for BARI Gerbera-1 and 12.33 for BARI Gerbera-2) using T6 treatment. Using flower stalk explants of both varieties in the same treatment, the mean no of shoots/explant was found 7.88 for BARI Gerbera-1 and 9.22 for BARI Gerbera-2.

In the present experiment it was noticed that flower stalk explants of both varieties showed early response towards callus induction, but percentage of shoot formation was low in all the combinations of BAP and NAA compared to flower bud explants. About 18 - 25 days were required for callus induction at various concentrations of BAP and NAA. Results indicated that both explants were able to produce more than 50% regeneration response and suitable for future experiments. However, flower bud explant has been considered as better responsive explants (Tables 20-23).

4.2.1.2.1.5 Response of flower bud and flower stalk explants towards organogenesis of multiple shoots on MS medium for BARI Gerbera -1 (white) and BARI Gerbera -2 (red) varieties

To study the process of organogenesis flower bud and flower stalk explants were inoculated on MS medium supplemented with BAP 6.0 mg/l and NAA 1.0 mg/l. Considering all investigating factors of regeneration response, this treatment was found to be the best for the varieties tested (Figs. 30 a-h). After inoculation both explants became enlarged and curved within 12-21 days of culture and adventitious shoot buds were developed by 7-8 weeks of culture (Figs. 30 a-h). Clusters of multiple shoots with well-developed leaves were formed by the end of 9-10 weeks of culture (Figs. 30e, 28h). Adventitious shoot bud induction and developmental sequence of shoot primordia has been illustrated for the BARI Gerbera-1 variety in Fig. 30.

4.2.1.2.2 Subculture of regenerated shoots, multiplication and elongation

The present findings revealed that subculture had a vital role for further multiplication of shoots developed from various explants. It was observed that multiplication and elongation of shoots was not satisfactory when the callus of flower buds was subcultured on the same regeneration media (treatment). Micro shoots isolated for further multiplication from a 7-8 weeks old shoot primordial clump developed on MS+6.0 mg/l BAP +1.0 mg/l NAA medium (T6) was transferred to ½ MS+2.0 mg/l BAP medium. It (micro shoots on ½ MS+2.0 mg/l BAP medium) produced new healthy shoot clumps within 20 days, whereas on MS+6.0 mg/l BAP +1.0 mg/l NAA medium (T6) would produce similar shoot clump in 40 days. So, each micro shoots subcultured on ½ MS medium supplemented with 2.0 mg/l BAP produced a new shoot clump having a huge number of shoots (uniform and rapidly developed) within a short period of time (20-25 days). All these microshoots are ready for transferring in the rooting media (Figs. 34 a-d). Table 24 and Figs. 35a-d showed the elastration of the effect of subculture medium on multiple shoot development. It can be mentioned that both tested varieties produced almost the same result during this study.

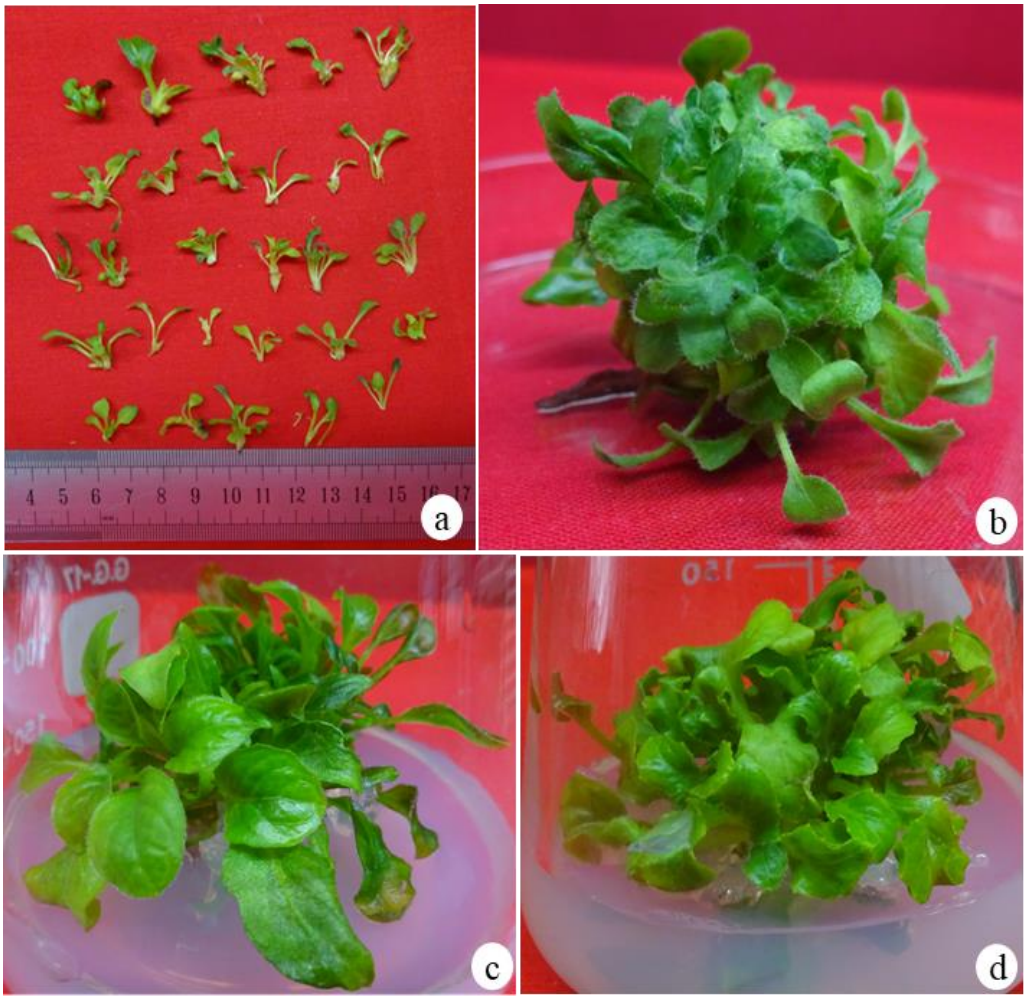

Fig. 34 (a-d): Micro shoots isolation and elongation from a clamp developed from flower bud explant of white variety in MS+6 mg/l BAP +1 mg/l NAA. (a) Separated micro shoots; (b) About 60-70 days old shoot primordial clump which is the source of micro shoots; (c-d) New shoot clump developed from each micro shoot within 20-25 days in ½ MS+2 mg/l BAP for both varieties white and red respectively.

Table 24: Effect of subculture medium (treatment) on multiple shoot development in *Gerbera Jamesonii* with different explants

Explant	Treatment	(%) of responsive clumps	Mean no. of shoots/sub culturing clumps	Mean length of shoot (cm)
Flower bud	TS1= MS+1.0 mg/l BAP	71.20	18.75	3.10
	TS2= MS+ 2.0 mg/l BAP	78.60	22.61	3.80
	TS3= ½ MS+1.0 mg/l BAP	85.84	23.88	4.11
	TS4= ½ MS+2.0 mg/l BAP	**96.20**	**34.22**	**4.50**
Flower stalk	TS1= MS+1.0 mg/l BAP	68.00	12.50	2.22
	TS2= MS+ 2.0 mg/l BAP	70.15	15.33	3.40
	TS3= ½ MS+1.0 mg/l BAP	78.33	17.11	3.58
	TS4= ½ MS+2.0 mg/l BAP	**93.57**	**29.20**	**4.10**

Note # Number of explants inoculated for each experiment = 30; TS1-TS4= Treatment used for subculture

4.2.1.3 Effect of different growth regulators on induction of roots from regenerated shoots

Root formation is an indispensable step to produce plantlets. Therefore, 3.5 – 4.5 cm long shoots were excised and cultured on half strengths of MS medium for root induction. Various auxins, namely, IBA (0.2 and 0.5 mg/l) and IAA (0.2 and 0.5 mg/l) with half strength MS medium were used to see their effects on root induction from the *in vitro* grown shoots (Fig. 36). Among the different auxins used on ½ MS medium 0.5 mg/l IAA showed the best response towards root induction regarding root formation %, number of root/shoot and root length (Fig. 37). Rooting on this medium was found to initiate within 12-15 days. On the other hand, IBA supplemented media produces large callii and the percentage of root formation was found to be low. Figs. 36a-b shows the rooting on five different treatments tested. It was found that the maximum number of shoots produced healthy roots on IAA supplemented rooting medium. Best root formation was observed on ½ MS + 0.5 mg/l IAA medium within a short time. Roots induced on this medium were well developed and rooted plantlets were suitable for subsequent transplantation (Figs. 36c-d, Table 25).

Fig. 35 (a-d): Different stages of new shoot clump developed from each micro shoot within 20-25 days on ½ MS+2 mg/l BAP of both BARI Gerbera-1 and BARI Gerbera-2 varieties. (a) Separated micro shoots of BARI Gerbera-1 cultured on ½ MS+2 mg/l BAP; (b) About 10-12 days old shoot primordial clump in elongation; (c) New shoot clump developed from each micro shoot within 20-25 days in ½ MS+2 mg/l BAP; (d) Same as 'a' but for BARI Gerbera-2; (e) Same as 'b' but for BARI Gerbera-2; (f) same as 'c' but for BARI Gerbera-2.

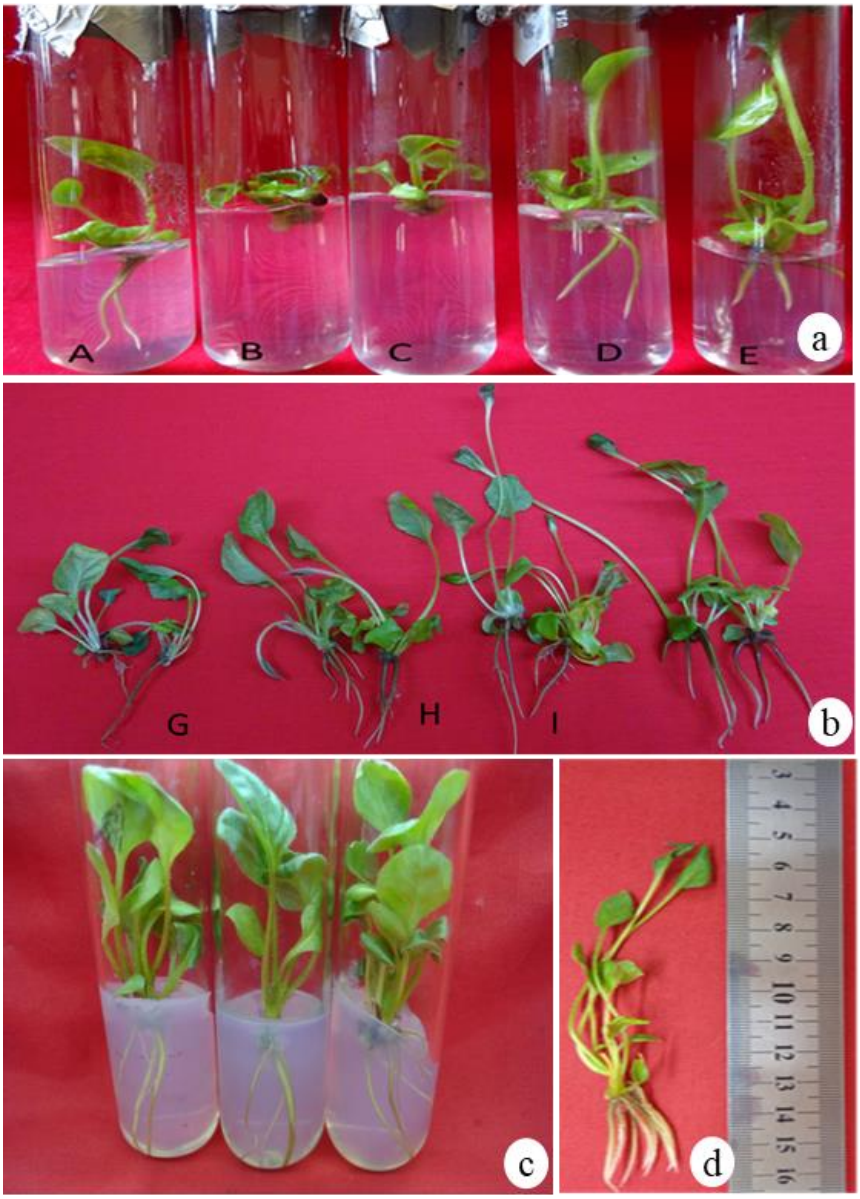

Fig. 36 (a-d): Response towards different hormonal combinations on ½ MS medium during rooting of BARI Gerbera-1. (a) A= Early stage of rooting on ½ MS+ 0.5 mg/l IBA medium, B= Same in case of ½ MS medium, C= Same in case of ½ MS+ 0.2 mg/l IBA medium, D= Rooting on ½ MS+ 0.2 mg/l IAA and E= Same on ½ MS+ 0.2 mg/l IAA medium; (b) Final stage of rooting, G= Rooting on ½ MS+ 0.2 mg/l IBA medium, H= Same in case of ½ MS+ 0.5 mg/l IBA medium, I= Same in case of ½ MS+ 0.2 mg/l IAA medium, J= Rooting on ½ MS+ 0.5 mg/l IAA medium; (c-d) Final stage of rooting on ½ MS+ 0.5 mg/l IAA medium; (d) Plantlet ready to transplant in the soil.

4.2.1.3.1 Effect of different concentrations of IBA and IAA on half strength of MS for the induction of roots in two varieties of Gerbera

Two concentrations of IBA (0.2 and 0.5 mg/l) and IAA (0.2 and 0.5 mg/l) with half strength of MS were used to examine their effects on induction of roots in two varieties of Gerbera. has the Effect of those concentrations of IBA and IAA has been presented in Table 25.

In the case of BARI Gerbera-1 the best root induction was observed on half strength of MS medium containing 0.5 mg/l of IAA. Higher percentage (96.66) of regenerated shoots showed initiation of roots within 8-12 days of culture while it took about 15-18 days to get fully developed roots (Figs. 36 and 37). Number of roots/shoot was 5-8 when the shoots were cultured on treatment, TR5= ½ MS+0.5 mg/l IAA (Fig. 36c-d).

In the case of BARI Gerbera-2 similar results were observed. Here also the best induction of roots (92.08%) was observed on half strength of MS medium containing 0.5 mg/l IAA within 9-12 days. This treatment (TR5) produced well developed roots within 18-20 days by forming 5-7 roots/shoot without the intervention of callus (Figs. 36c and d). It was also noticed that in ½ MS or various combinations of IBA supplemented ½ MS medium callus like structure produced from the cut end of shoots. Formation of roots on various combinations of IBA and IAA on half strength of MS in three varieties is shown in Figs. 36 a and b.

4.2.1.4 Establishment of the plantlets in natural environment

After sufficient development of roots, the plantlets obtained from both varieties of *Gerbera* were successfully transplanted into small plastic pots Fig. 38a. The transplantation procedure has been described in the method section. Using this method, the survival rate of the transplanted plantlets was found to be about 99%. Following proper acclimatization, the plantlets were transferred to greenhouse for their further growth Figs. 38b-d. The plantlets of both varieties flowered within 12-16 weeks (Figs. 38b-d).

Table 25. Effect of different concentration of IBA and IAA on half strength of MS towards formation of roots from regenerated shoots in BARI Gerbera-1 and

Variety	Treatment	Hormonal supplementation (mg/l)		% of shoots forming roots	Days required to initiate roots	Formation of callus	Days required to gets well developed roots	No. of roots /shoot	Length of the roots (cm)
		IBA	IAA						
BARI Gerbera -1	TR1	0	0	25.66	10-20	+	30-40	3-4	1.5
	TR2	0.2	0	41.00	10-20	+	30-40	3-5	2
	TR3	0.5	0	70.33	10-18	+	20-25	2-3	2.8
	TR4	0	0.2	79.33	10-15	-	22-28	3-4	2.5
	TR5	**0**	0.5	**96.66**	**8-12**	**-**	**15-18**	**5-8**	**3.5**
BARI Gerbera -2	TR1	0	0	20.00	10-20	+	30-40	3-4	1.5
	TR2	0.2	0	50.87	10-20	+	28-35	3-4	2.2
	TR3	0.5	0	58.77	10-18	+	28-30	2-3	2.5
	TR4	0	0.2	78.66	10-15	-	18-22	3-5	2.7
	TR5	**0**	0.5	**92.08**	**9-12**	**-**	**18-20**	**5-7**	**3.4**

Note # Number of shoots inoculated for each experiment = 30; TR1-TR5= Treatment used for rooting

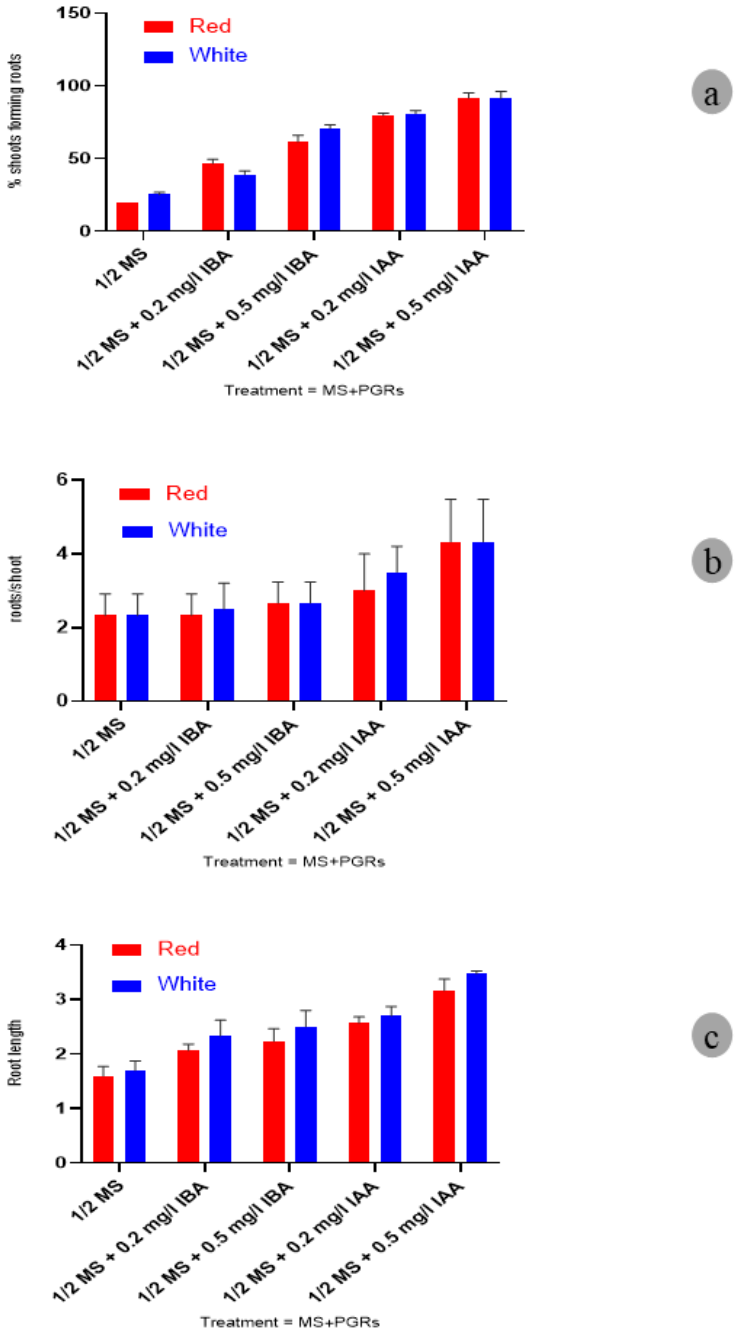

Fig. 37 (a-c) Effect of different auxins with different concentrations on root on ½ strength of MS medium of both Gerbera varieties. (a) Root formation % towards different concentrations of different auxins after 3 weeks; (b) Root/ explant produced towards different concentrations of different auxins after 3 weeks; (c) Root length produced after 3 weeks towards different concentrations of different auxins.

Fig. 38 (a-d): Transplantation after sufficient rooting and green house performance till flowering of red variety. (a) Plant removed from best rooting media is in hardening after transplantation; (b) Plantation of red Gerbera plants in the greenhouse; (c) Vegetative growth of the plant inside greenhouse; (d) Flowering of the plant inside greenhouse.

Table 26: Transplantation and evaluation of tissue culture derived plants growth in greenhouse (Evaluation of greenhouse performance of transplanted plants)

Variety	Mean plant height during anthesis (cm) ± SEM	Days required for bud initiation ± SEM (cm)	Days required to full bloom ± SEM (day)	No. of flower/Plant	Flower diameter ± SEM (cm)
BARI Gerbera-1 (Tissue culture)	24.32 ±1.21	120±0.77	28±1.05	50±0.11	8.1±0.35
BARI Gerbera-1 (Mother plant)	25.32 ±2.01	122±0.41	30±0.12	50±0.75	7.8±0.61
BARI Gerbera–2 (Tissue culture)	22.78 ±1.78	110±0.32	25±1.31	41±0.68	6.2±0.51
BARI Gerbera–2 (Mother plant)	20.78 ±0.22	111±0.4	24±2.09	42±1.02	6.0±0.15

Note # Number of plants used for each experiment = 30

Three weeks after transplantation, when the regenerated plants were fully established in the small pots, they were then transferred to the greenhouse. All the surviving plants were maintained there until flowering and the flowers showed normal morphology without a huge variation or difference compared to the mother plants (Table 26). Days required to bud initiation was less in tissue culture derived plants then the mother plants. It was also observed that the flower diameter and the no. of flowers/ plant were high in case of tissue culture derived plants. Therefore, the regeneration protocol developed during the study can be considered as highly efficient, rapid and genotype independent and commercially viable.

4.2.2 Induction of mutation through gamma radiation in BARI Gerbera-1 and BARI Gerbera-2 and selection of promising mutant lines through morphological study

In this phase of investigation, a series of experiments were carried out to induce mutation in two varieties of *Gerbera jamesonii* Bolus namely, BARI Gerbera-1 and BARI Gerbera-2 through gamma radiation using $Cobalt_{60}$ as a source for creating variant lines. Five doses of gamma radiation, 5,10, 15, 20 and 25 Gy were applied to the *in vitro* grown micro shoots of both varieties for the above purpose (Section 3.8.2). Following irradiation of *in vitro* shoot primordia, routinely subculture was done on MS medium supplemented with 6.0 mg/l BAP+ 1.0 mg/l NAA for elongation after 60 days of irradiation (Section 4.2.1.2.1.4). During these experiments the survivability of the irradiated shoots M_1V_3 (60 days after irradiation) were evaluated following determination of LD_{50}.

After another 21-28 days *in-vitro* regenerated M1V4 irradiated shoots (80-88 days after irradiation) were developed on ½ MS+2 mg/l BAP medium (Section 4.2.1.2.2). Healthy shoots M1V5 were rooted on ½ MS + 0.5 mg/l IAA medium (Section 4.2.1.3). During this study, different mutant lines were selected based on visual observation through morphologic study. Finally, these M1V5 mutant lines were grown in the greenhouse till flowering for successive two vegetative generations (M1V5-M1V6) to observe stability of the developed mutants. Ultimately three mutant lines from each type of variety were developed and were confirmed after evaluation. These series of results of this part of the study have been discussed in the following heads:

4.2.2.1 Induction of mutation through gamma radiation in BARI Gerbera-1 and BARI Gerbera-2

In the present study, 7-8 weeks old culture containing multiple shoots raised from flower bud explants were used for gamma irradiation (Fig. 39a). MS media supplemented with 6.0 mg/l BAP and 1.0 mg/l NAA was used for shoot induction and development, both before and after irradiation. Subsequent subculture was then carried out at a three weeks interval to develop M_1V_1 to M_1V_3 shoots. Several shoots died in each subculture and M_1V_4 shoots were developed during the 3rd subculture on ½ MS+2.0 mg/l BAP medium. It was observed that the growth of the shoots reduced with the increase of the radiation doses (Fig. 39b-e). During this period LD 50 were determined for both varieties (Fig. 40). Finally, the surviving M_1V_4 shoots were rooted on ½ MS+ 0.2 mg/l IAA medium and developed M_1V_5 plantlets. These M_1V_5 Plantlet (rooted shoots) were transplanted to soil and kept in the growth room for two weeks to acclimatize (procedure has been described in the method section). Using this method, the survival rate of the transplanted plantlets in the growth room was found to be about 99%. Variation in leaf structure, chlorophyll and height of the plant were observed in M_1V_5 plants (Fig. 41). Following proper acclimatization, the plantlets were transferred to greenhouse for their further growth (Fig. 41). The irradiated plants flowered during the flowering season and were used for evaluation of different morphological factor analysis to identify mutant lines.

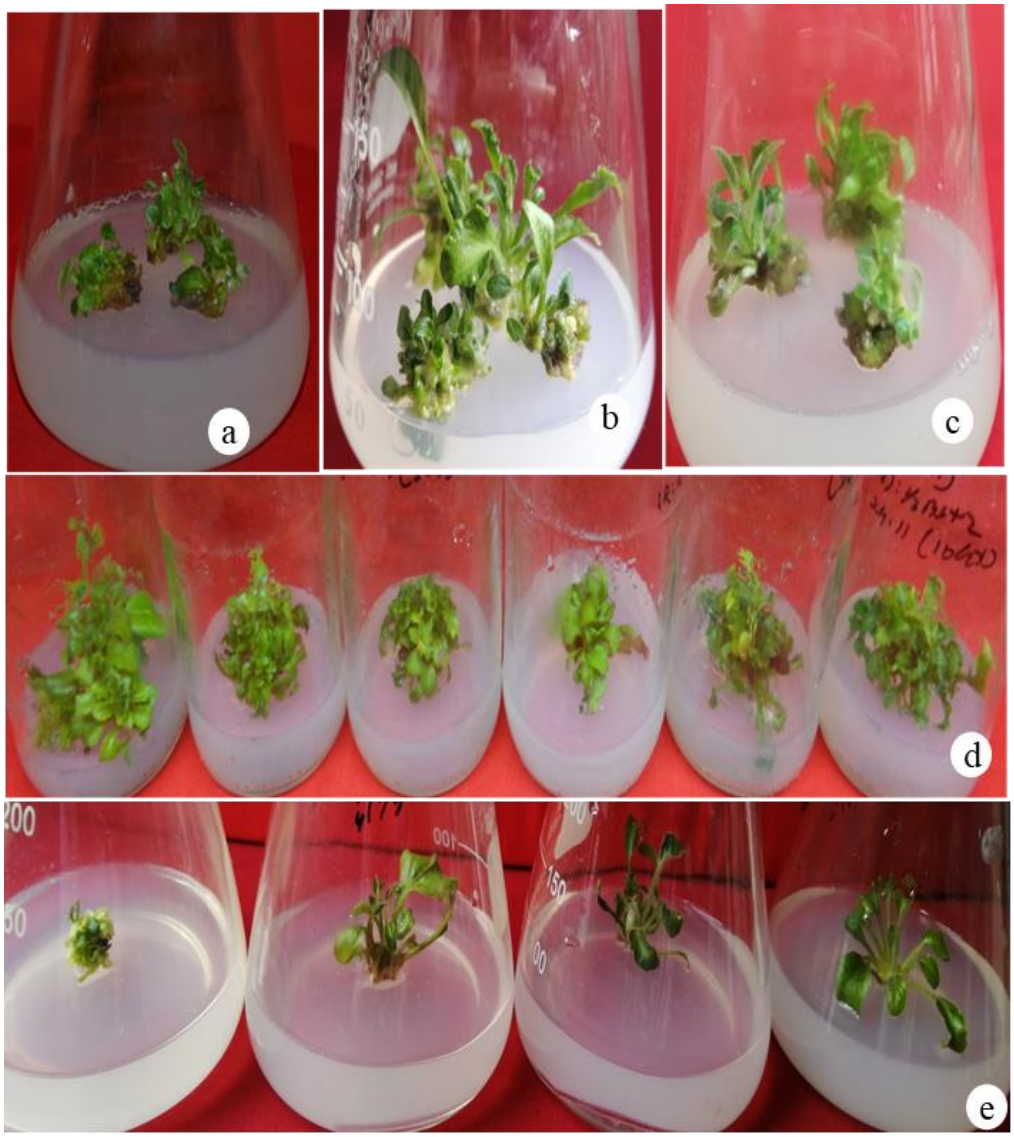

Fig. 39 (a-e): Explants used for mutation induction and their response of white variety. (a) 7-8 weeks old shoot primordial clump used in mutation induction; (b) Control clump (nonirradiated) after 4 weeks of irradiation date; (c) 5Gy treated clump after 4 weeks of irradiation date; (d) 0, 5, 15, 20, 25 and 10 Gy (left to right) treated clamps after 7-8 weeks of irradiation date; (e) 8 weeks old shoots of white variety inoculated on ½ MS + 2 mg/l BAP medium and the effect of different radation doses (from left to right, 15Gy, 10Gy, 5 Gy and control).

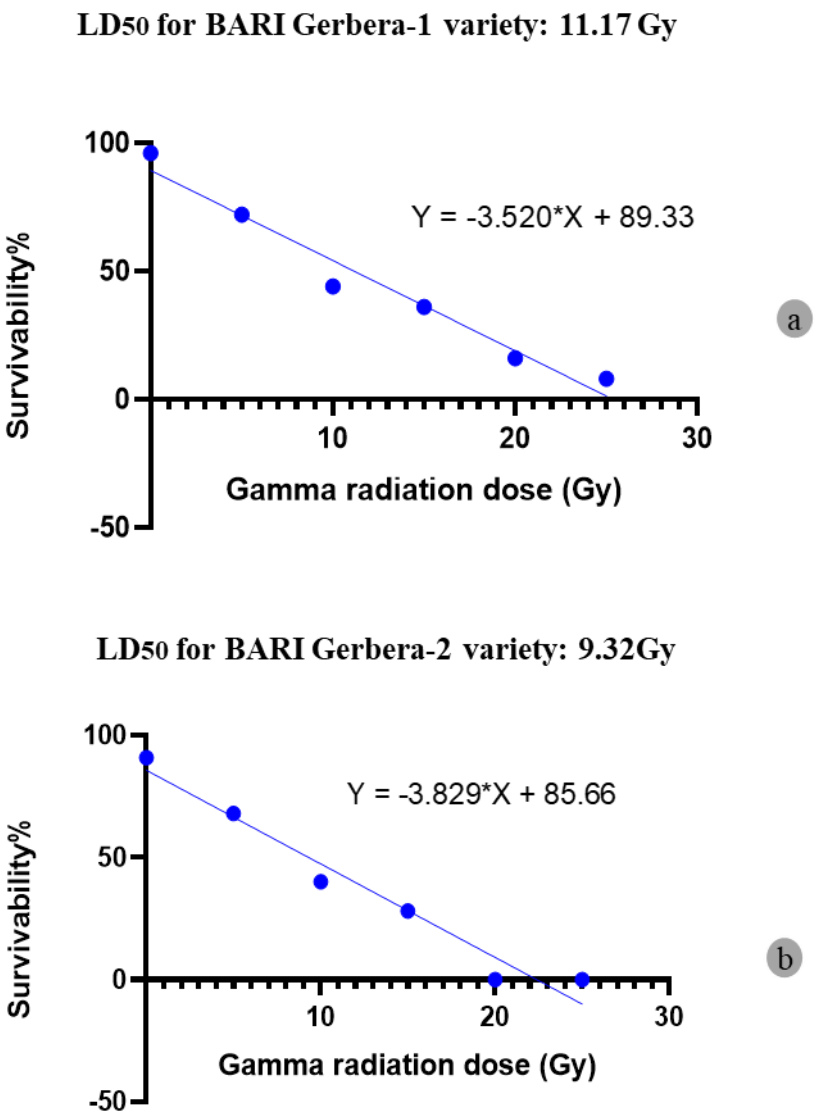

Fig. 40 (a-b): Determination of LD_{50} dose for both varieties of Gerbera. (a) LD_{50} for BARI Gerbera-1; (b) LD_{50} for BARI Gerbera-2.

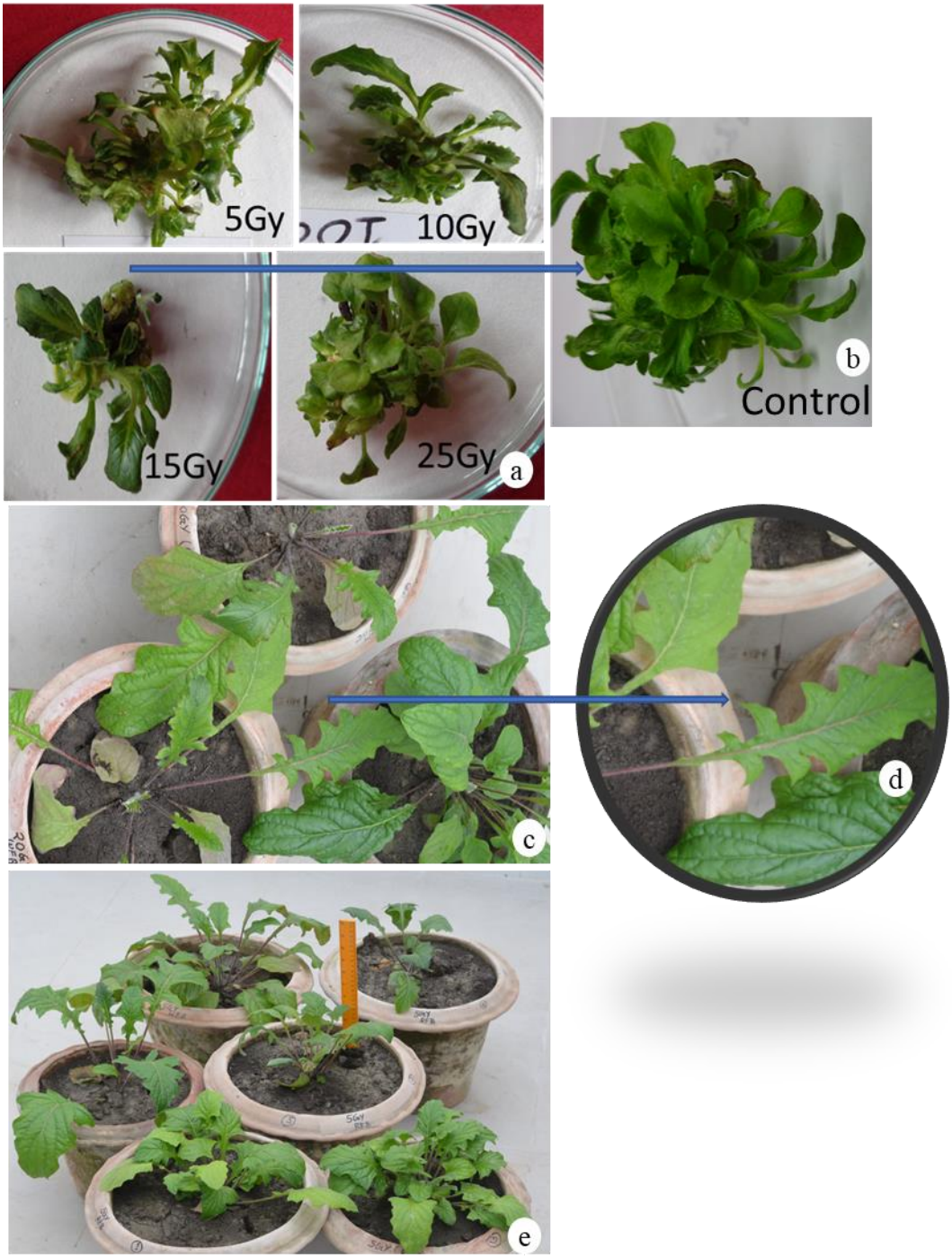

Fig. 41 (a-e): Variations in leaf size, shape and chlorophyl content observed in *in-vivo* condition M1V5 of BARI Gerbera-1. (a) Plants showing variation in leaf size, shape and chlorophyl content towards different doses response; (b) Leaf of control plants; (c) Plants showing variation in leaf size, shape and chlorophyl content in different dose response before anthesis in the greenhouse; (d) Magnified view of (c); (e) Changes noted regarding height and growth towards different doses before anthesis in the greenhouse.

4.2.2.1.1 Effect of gamma irradiation on *in vitro* grown shoots survival (60 days after irradiation) in two Gerbera varieties

During optimization of regulatory factors for induction of mutation, it was found that the increase in radiation dose has direct effect on the *in vitro* shoot regeneration percentage. In this experiment it was tried to find out the lethality percentage following five doses of radiation on 7-8 weeks old shoots derived from leaf explants of both gerbera varieties. Data for *in vitro* plant survival were recorded after 7 - 8 weeks following different irradiation doses (Table 27). A comparative study on the effects of five radiation doses (5 Gy,10 Gy, 15 Gy, 20 Gy and 25 Gy) on the shoot survival percentage of two varieties of Gerbera indicated that highest dose (25 Gy) showed highest lethality as the survival percentage for both varieties was low (5.80% and 0% for white and red varieties). However, the highest survival percentage was observed for 5 Gy irradiated shoots for both varieties as 72.77% for white and 68.09% for red varieties respectively (Table 27).

4.2.2.1.2 Determination of LD50 for two Gerbera varieties

In this study, the effects of gamma radiation doses on the micro shoots survival and plant regeneration from irradiated (shoots) were determined as percentages. The LD50 were calculated and recorded after evaluation of 60 days of irradiation of the micro shoots. After regression analysis of the percentage of plant survival data (Table 27), LD50 (50% lethal dose) was found at 11.17 Gy for BARI gerbera -1 and 9.32 Gy for BARI gerbera-2 variety (Fig. 40). LD_{50} is an important factor for successful mutation induction.

Table 27. Comparison of the effect of gamma irradiation on *in vitro* grown shoot clump survival (8 weeks after irradiation) in BARI Gerbera-1 (white color) and BARI Gerbera–2 (red color)

Dose of gamma rays (Gy)	Plant survival (%)	
	BARI Gerbera–2 (Red color)	BARI Gerbera-1 (White color)
0	90.8	96.0
5	68.09	72.77
10	40.0	44.0
15	28.0	36.0
20	0	10.8
25	0	5.80

Note # Number of plants used for each experiment = 30

4.2.2.1.3 Response of the *in-vitro* grown irradiated shoots of two Gerbera varieties towards variation induction

In this experiment, regeneration response as well as morphological changes of surviving M_1V_4 to M_1V_5 shoots were recorded while growing on ½ MS+2 mg/l BAP medium. These M_1V_5 shoots were rooted on the rooting medium (TR5). They were transplanted to soil and kept in the growth room for two weeks to acclimatize and variation in leaf structure, chlorophyll and height of the plant were observed and listed in Table 28 & Fig. 41). The frequency of morphological variation at different radiation doses were measured as the index reflecting the effect of radiation on plant morphological changes. According to the formula (Walther, 1969), the frequency of morphological variation was calculated as: Variation frequency (%) = No. of variations/No. of treated plants × 100 (Table 28). Plants derived from 15 Gy irradiated explants of BARI Gerbera- 1 variety showed highest variation frequency percentage, regarding leaf shape change percentage (50%), chlorophyll change percentage (30%) and plant height percentage (55%). Minimum changes occurred on plantlets developed from 5 Gy treated explants. In case of BARI Gerbera- 2, plants developed from 10 Gy produced the highest variation frequency percentage, regarding leaf shape change percentage (45%), chlorophyll change percentage (15%) and plant height percentage (5%). None of the 15-25 Gy treated shoots could survive during the elongation or rooting phage So variation frequency regarding all four measured aspects were 0 % for BARI Gerbera- 2. It was also noticed that variation frequency was not directly increased with the increased radiation doses.

Table 28. Percentage of variations observed in growth room during hardening of *in-vitro* gamma irradiated two Gerbera varieties (M1V5)

Dose Gy	BARI Gerbera- 1(White)			BARI Gerbera- 2 (Red)		
	Leaf shape change %	Chlorophyl change %	Plant height %	Leaf shape change %	Chlorophyl change %	Plant height %
Control (0)	0	0	0	0	0	0
5	0	0	5	25	0	5
10	25	15	5	45	15	5
15	50	30	55	0	0	0
20	0	0	50	0	0	0
25	10	0	10	0	0	0

[According to Walther, 1963, Formula for physiological variation frequency % = No. of plants showing variation / No. of treated plants * 100%] Here n=20]

4.2.2.1.4 Establishment of plantlets from selected variants M_1V_5 produced in growth room

After sufficient development of roots the selected variant plantlets (M_1V_5) of both Gerbera varieties were successfully transplanted into small plastic pots (Fig. 42 a). The transplantation procedure has been described in the method section. Using this method, the survival rate of the transplanted plantlets was found to be about 99%. Following proper acclimatization, the plantlets were transferred to green house for their further growth and flowered during flowering season (Fig. 42 a-d).

4.2.2.2 Establishment of M_1V_5 mutant lines in greenhouse and morphological evaluation for selection of variant lines

During this series of experiments, plants (M_1V_5) showing vegetative variations were grown in the greenhouse till flowering and their performance was recorded along with the control plants (Fig. 43 a-b). Observation and selection were made for desirable variants at flowering time. Changes in flower color, form and shape were observed in plants treated with gamma rays of 5, 10, 15, 20 and 25 Gy for both Gerbera varieties. Irradiation dose 5Gy and 20 Gy induced 20 and 30 percent of mutation frequency of flower form and color in case of BARI Gerbera-1. In the case of BARI Gerbera-2 irradiation dose 5Gy and 10 Gy induced 45 and 20 percent of mutation frequency of flower form and color. Due to irradiation stress 10 Gy,15 Gy and 25 Gy treated shoots of BARI Gerbera-1 and 15 Gy, 20 Gy and 25 Gy treated shoots of BARI Gerbera-2 could not survive during the elongation or rooting phage, so mutation frequency was 0% in those cases (Table 29).

During this screening several variant lines from both varieties were identified. Screening was made for the 1st vegetative generation (greenhouse) and identified three mutant lines from BARI Gerbera -1 (white) variety and three mutant lines from BARI Gerbera -2 (red) variety. It may be mentioned that only the data regarding flowers has been listed in Tables 30 - 31 as the flowers are the focal object in the present study. Finally, in the 2nd vegetative generation (greenhouse) all these six mutant lines regarding flower shape and color were evaluated and considered as true mutants. Phenotypic evaluation of these promising mutants for two vegetative generations along with the detailed flower morphology has been discussed in following heads.

Table 29. Flower form and color mutation frequency in M_1V_5 of both Gerbera varieties in greenhouse during flowering

Radiation dose (Gy)	BARI Gerbera- 1		BARI Gerbera- 2	
	No. of plant Investigated	Flower form and color mutation frequency (%)	No. of plant Investigated	Flower form and color mutation frequency (%)
5	20	20	20	45
10	20	0	20	20
15	20	0	0	0
20	20	30	0	0
25	20	0	0	0

4.2.2.2.1 Phenotypic evaluation of promising mutants (M_1V_6 and M_1V_7) developed from M_1V_5 variant lines (1st and 2nd vegetative generation)

In this experiment, plants (M_1V_6) showing flower variations in the greenhouse were multiplied through conventional cutting. Observation and selection of desirable variants at flowering time for the second vegetative generation (M_1V_7) were made to see the status of isolated variants. Data were recorded on days required for bud initiation; days required for full bloom, number of flowers per plant and diameter of flower (cm) in the greenhouse condition. These data were recorded on an individual plant basis from five randomly selected plants per mutant type (genotype). Their performance stability and uniformity were evaluated through comparison with their mother plants where significant differences among them were observed (Tables 30-31 and Figs.44a-d).

Fig. 42 (a-d): Putative mutants derived from various doses and their growth inside green house before anthesis. (a) Growth of the plants derived from different doses just after hardening in growth room; (b) Growth of the control (non-irradiated BARI Gerbera -1 in green house before anthesis; (c) Same for 5 Gy irradiated plant (BARI Gerbera -1); (d) Same for 5 Gy irradiated BARI Gerbera-2.

Fig. 43 (a-b): Putative mutants derived from various doses and their growth inside green house before and after full bloom (a) Growth of the plants derived from different doses just before anthesis inside greenhouse; (b) full bloom of the plants derived from different doses inside greenhouse.

Table 30. Evaluation of the performance of the mutants developed from BARI- 1 (White) gerbera plants M_1V_6-M_1V_7 (1st - 2nd vegetative generation)

Selected Mutant	Plant height during anthesis (cm)	Days required for bud initiation after plantation in greenhouse	Days required to full bloom (day)	No. of flower/Plant	Flower Diameter (cm)
WV1	33±0.72	100±0.22	20±0.55	30±0.25	11.33±0.15
WV2	35±1.3	120±0.32	22±0.45	32±0.35	12.0 ±0.34
WV3	31±0.93	110±0.43	25±0.93	32±0.13	9.8±0.85
Control	30±0.22	120±0.55	30±0.48	20±0.5	9.53±0.90

Note # Number of plants evaluated n=5

Increased plant height, no. of flower/plant and flower diameter was observed in all the mutants derived from BARI Gerbera-1 and BARI Gerbera-2. Only mutant line RV2 derived from BARI Gerbera -2 was found shorter (22±0.13 cm) than the mother (25±0.86 cm) plants (Tables 30-31). Days required for bud initiation after plantation in the greenhouse were found to be the lowest in case of WV1 and RV2 (100±0.22 and 110±0.12 days) mutant lines. These two mutants can be considered as early varieties. Considerable large flowers were produced by WV2 (12.0 ±0.34 cm) and RV1 (10.5±0.41 cm) mutant lines (Tables 30-31).

Table 31. Evaluation of the performance of the mutants developed from BARI-Gerbera-2 (Red) plants M_1V_6-M_1V_7 (1st - 2nd vegetative generation)

Selected Mutant	Plant height during anthesis (cm)	Days required for bud initiation (cm)	Days required to full bloom (day)	No. of flower/Plant	Flower Diameter (cm)
RV1	25±0.82	110±0.12	23±0.15	25±0.25	10.5±0.41
RV2	22±0.13	100±0.22	22±0.35	30±0.17	8.5±0.4
RV3	30±0.58	110±0.44	20±0.99	25±0.76	9.3±0.14
Control	25±0.86	100±0.22	20±0.31	30±0.13	9.0±0.82

Note # Number of plants evaluated n=5

Fig. 44 (a-d): Putative mutants derived from various doses and their growth inside green house after full bloom (BARI Gerbera -2). (a) Change in plant height of the mutants during anthesis in greenhouse; (b) Change in plant height and length of flower stalk of the mutants during anthesis in greenhouse; (c-d) Changes noted in floret of the mutants.

Fig. 45 (a-h) Six promising mutants isolated from irradiated BARI Gerbera-1 and BARI Gerbera-2. (a) Control BARI Gerbera -1; (b) WV1 mutant of BARI Gerbera-1 developed from 20 GY; (c) WV2 mutant of BARI Gerbera-1 developed from 20 GY; (d) WV3 mutant of BARI Gerbera-1 developed from 20 GY; (e) Control BARI Gerbera -2; (f) RV1 mutant of BARI Gerbera-2 developed from 5 GY; (g) RV2 mutant of BARI Gerbera-2 developed from 10 GY; (h) RV3 mutant of BARI Gerbera-2 developed from 5 GY.

4.2.2.3 Description of the mutant lines

The control BARI Gerbera-1(white) and BARI Gerbera -2 (red) flowers were labeled as CW and CR respectively. Finally, six mutants were developed from both Gerbera varieties after gamma irradiation. BARI Gerbera-1 produced three mutants (WV1, WV2 and WV3) and BARI Gerbera-2 also produced three mutants (RV1, RV2 and RV3) (Figs. 45-49). No variation was seen on the *in-vitro* cultured both Gerbera varieties. These new cultivars consist of different flower forms, shapes, and colors (Figs. 45a-h). Following visual observation, color of the flowers of M1V7 generation of both varieties were confirmed through (RHSC) Colours of the Royal Horticulture Society Colour Charts Edition V (Royal Horticultural Society Colour Charts Edition V (orgfree.com) & RHS Fan 3 - Azalea Society of America) (Table 32).

The description of the mutant flowers are as follows:

4.2.2.3.1 WV1: These daisy-like flowers are very attractive yellowish colored and have a few extra layers of trans florets (7-8 layers). It also has more ray florets than the control and, in the inflorescence, they are very tightly attached and nicely oriented. The ray florets are changed (flat) regarding their size and shape Figs.45b & 46b. This mutant was derived from BARI Gerbera-1 (White) using 20 Gy treatment.

4.2.2.3.2 WV2: These sunflower-like flowers are very fascinating yellowish or off-white colored and showed a completely different shape (oval). They have a smaller number of ray and trans florets and they are loosely attached in 2-3 layers. All the ray florets are not equal in size some of them are considerably smaller. This mutant was derived from BARI Gerbera-1 (White) using 20Gy treatment. Completely developed disk floret is full of open banana shaped mature anther (Figs. 45c and 46c). This mutant was derived from BARI Gerbera-1(White) using 20Gy treatment.

4.2.2.3.3 WV3: These daisy- like flowers are very attractive and almost the same as WV1. The color of these flowers is cream white and flat ray florets are high in volume. They also have a high volume of trans florets. In the trans floret area, they have a faint

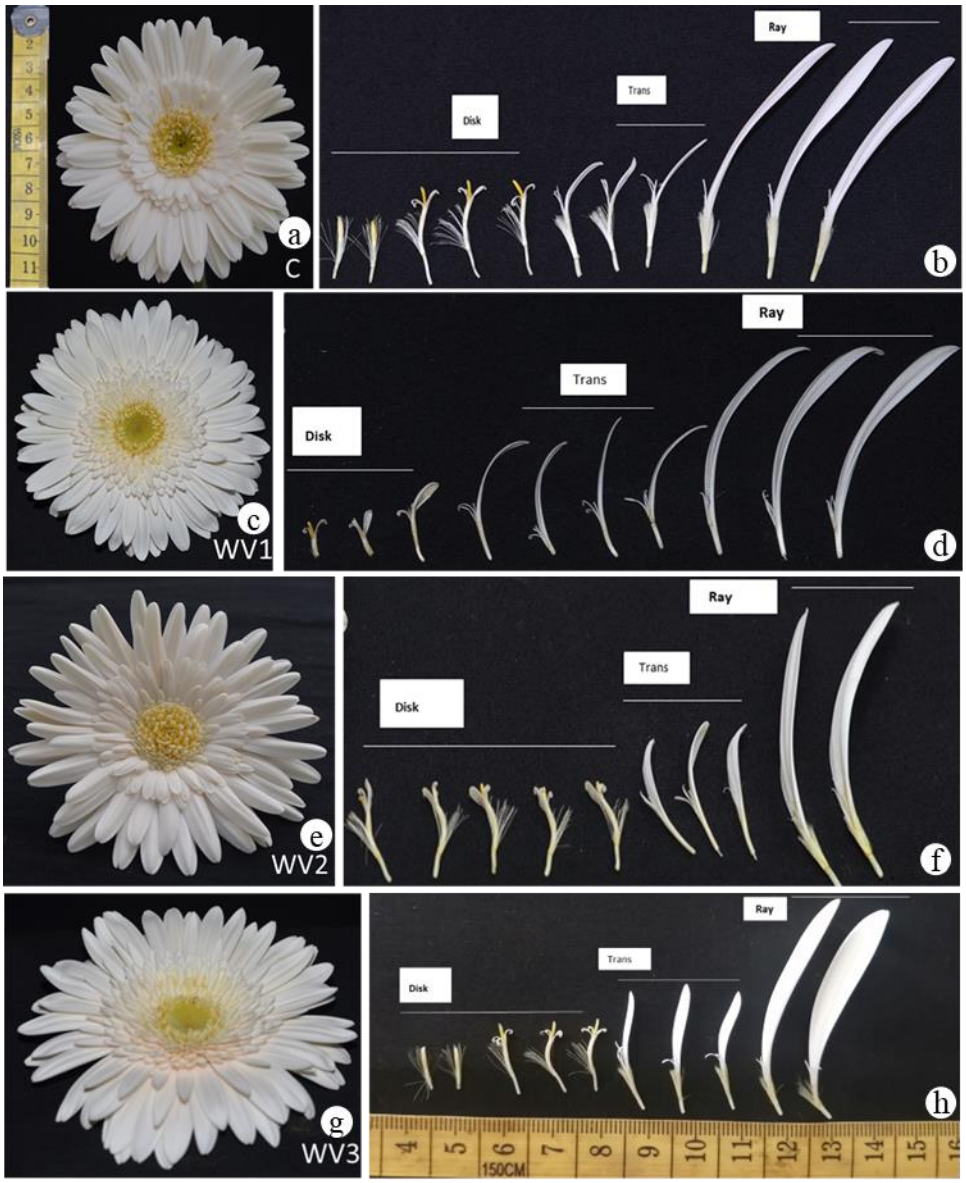

Fig. 46 (a-h) Comparison of the floral morphology of the mutants (WV1, WV2 and WV3) isolated from BARI Gerbera -1 plants irradiated with 20 Gy along with the control/mother flower (C). (a) Control (BARI Gerbera -1) flower; (b) Different types of ray and disk florets of BARI Gerbera -1 control flower; (c-d) WV1 flower and its different types of ray and disk florets; (e-f) WV2 flower and its different types of ray and disk florets; (g-h) WV3 flower and its different types of ray and disk florets.

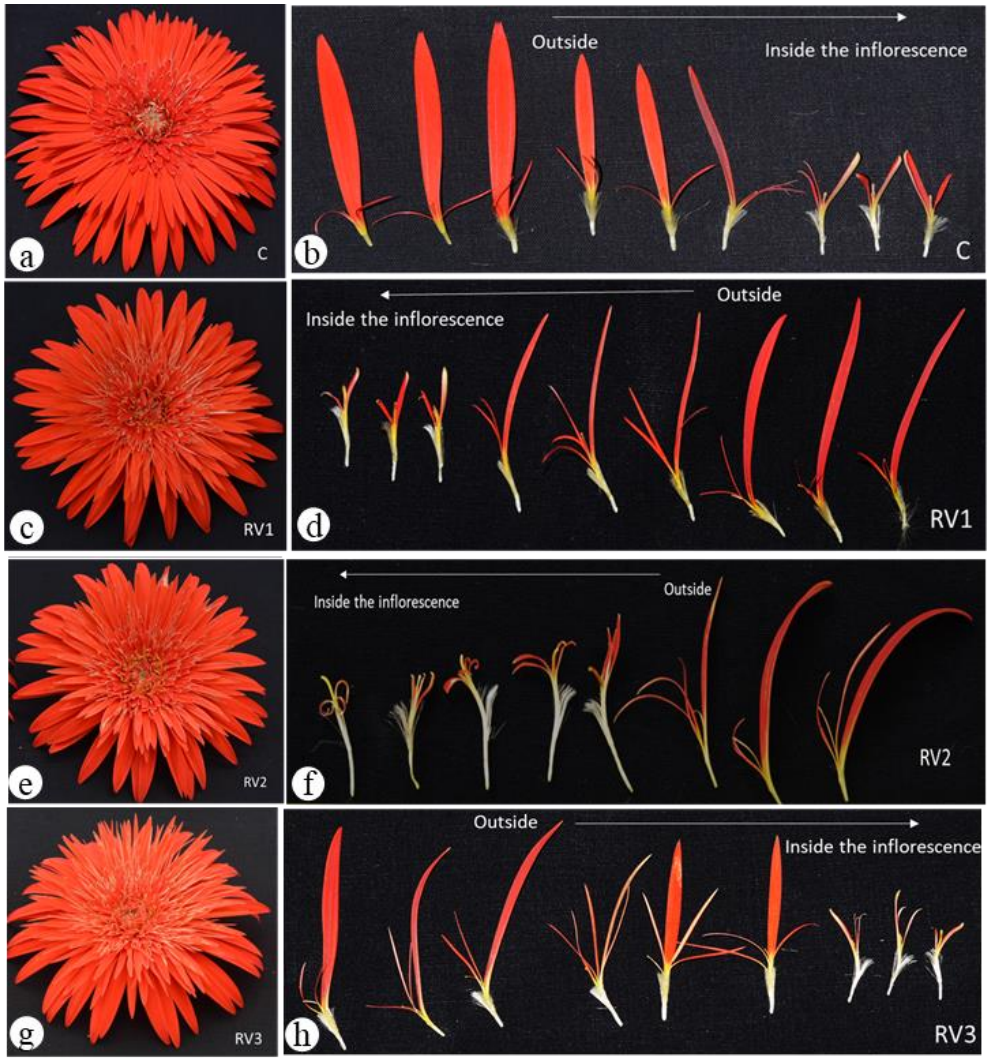

Fig. 47 (a-h) Comparison of the floral morphology of the mutants (RV1, RV2 and RV3) isolated from BARI Gerbera -2 plants irradiated with 5 Gy, 10 Gy and 5 Gy doses of radiation along with the control /mother flower (C). (a) Control (BARI Gerbera -2) flower; (b) Different types of ray and disk florets of BARI Gerbera -2 control flower; (c-d) RV1 flower and its different types of ray and disk florets; (e-f) RV2 flower and its different types of ray and disk florets; (g-h) RV3 flower and its different types of ray and disk florets.

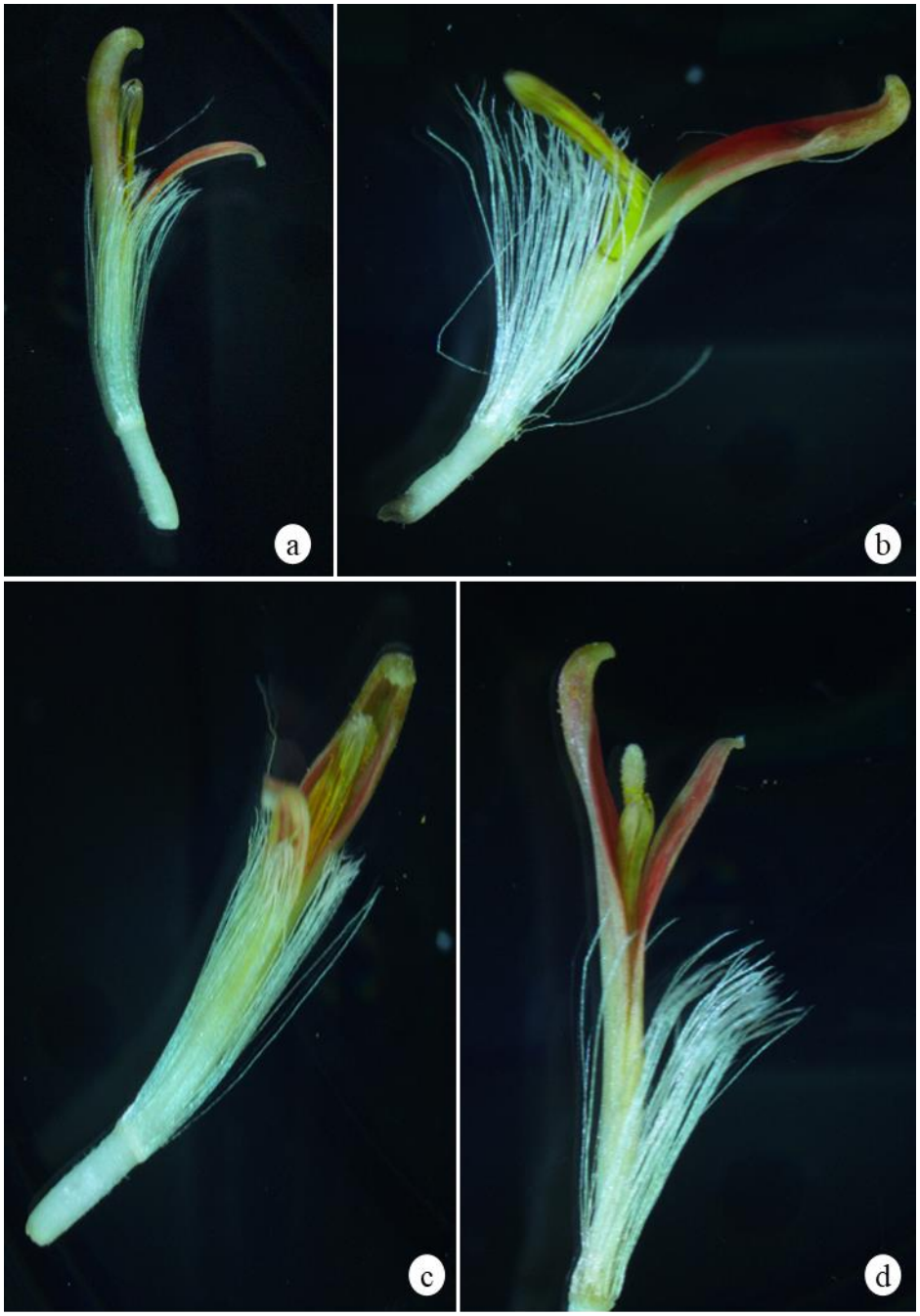

Fig. 48 (a-d): Comparison of the floral morphology of the mutants (RV1, RV2 and RV3) isolated from BARI Gerbera -2 plants irradiated with 5 Gy, 10 Gy and 5 Gy radiation along with the control or mother flower. (a) Control disk floret; (b) Disk floret of RV1; (c) Disk floret of RV2; (d) disk floret of RV3 at early stage.

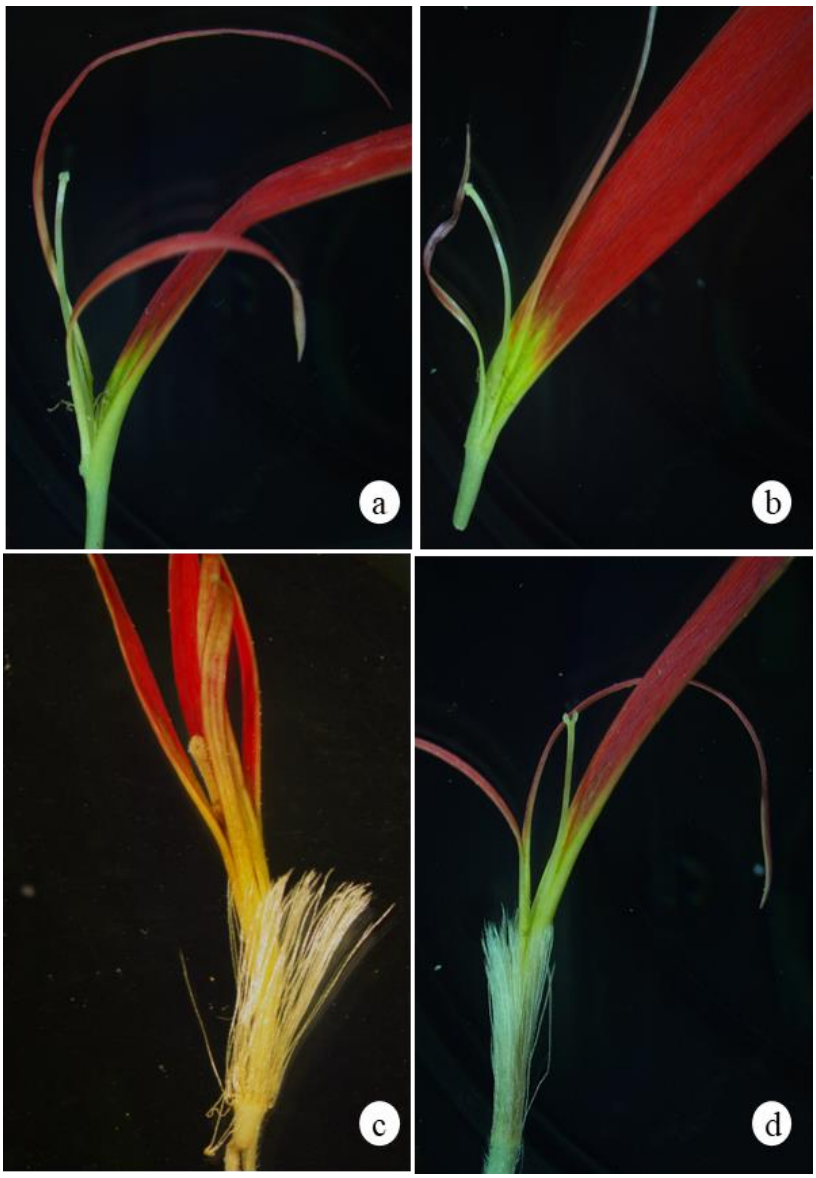

Fig. 49 (a-d): Comparison of the floral morphology of the mutants (RV1, RV2 and RV3) isolated from BARI Gerbera -2 plants irradiated with 5 Gy, 10 Gy and 5 Gy radiation along with the control/mother flower (C). (a) Control Ray floret; (b) Ray floret of RV1; (c) Ray floret of RV2; (d) Ray floret of RV3 at early stage.

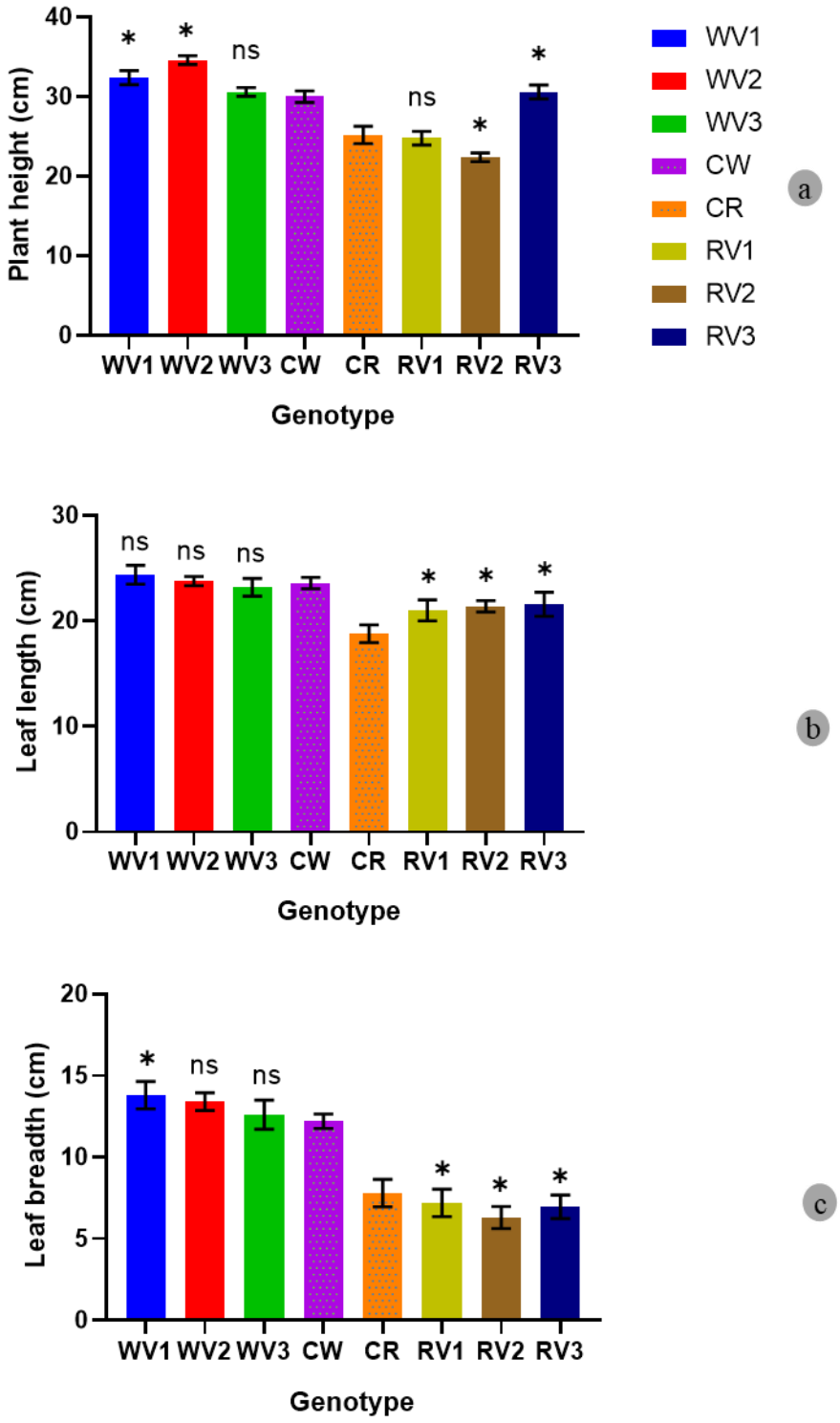

Fig. 50 (a-c): Changes in morphological characteristics of mutant lines. (a) Changes in plant height (cm); (b) change in leaf length (cm); (c) change in leaf breadth (cm). Each bar represents mean ± SME (n=5). * Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one-way ANOVA.

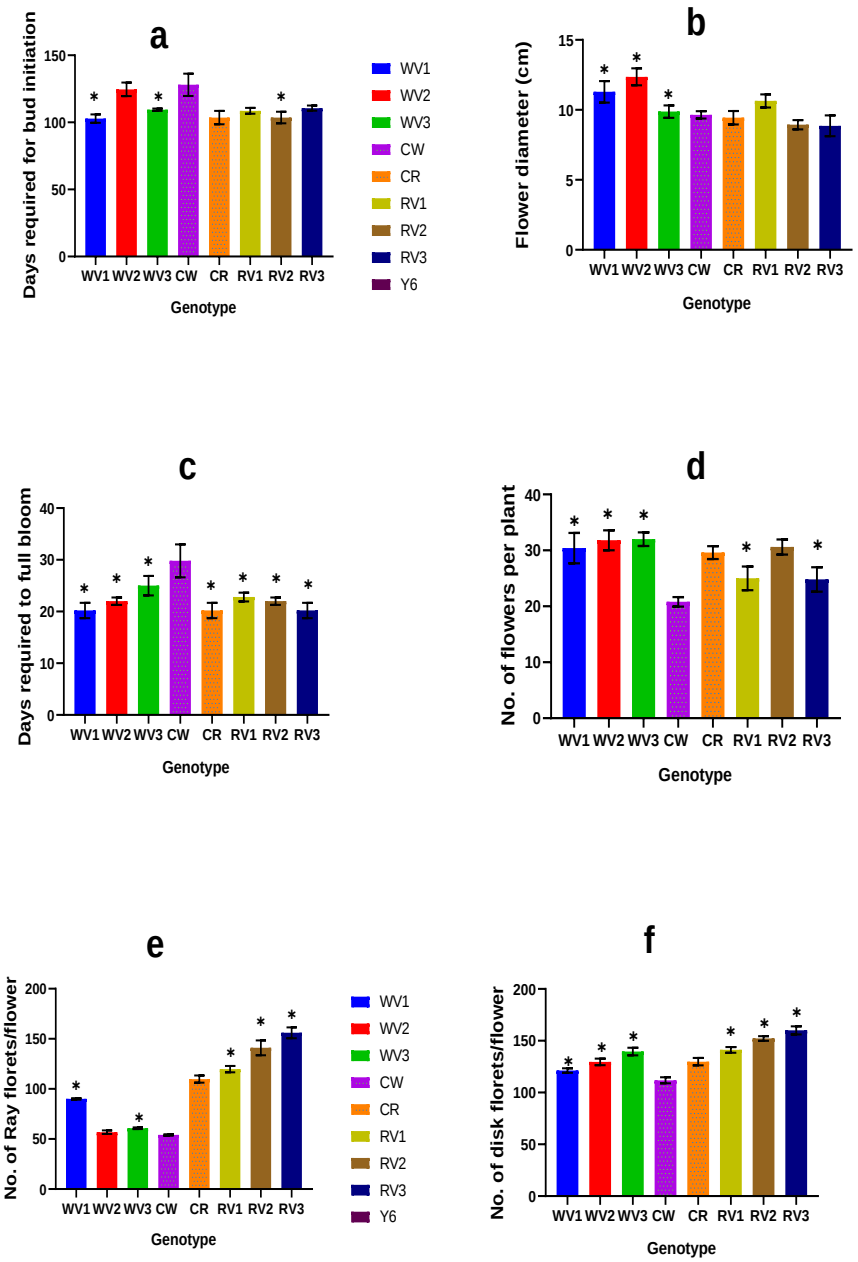

Fig. 51(a-f): Changes floral morphology of the mutant lines in M1V7 plants of control and mutants of two tested varieties. (a) Changes in day required for bud initiation; (b) Changes in flower diameter (cm); (c) Changes in days required for full bloom; (d) Changes in flower no per plant; (e) Changes in the no. of ray floret in the flowers; (f) Changes in the no. of disk floret in the flowers. Each bar represents mean ± SME (n=5). *Values are significantly different at P≤0.05 during Tukey's multiple comparisons test following one way ANOVA.

pink tinge, and the flower stalks length were very long (Figs.45d and 46d). This mutant was derived from BARI Gerbera-1 (White) using 20 Gy treatment.

4.2.2.3.4 RV1: These daisy- like flowers are very attractive dark red colored nice looking and have lesser number of ray and trans floret. The trans florets are completely changed in shape by forming spike like appearance. This narrowing of the petal may occur due to the curling of the disk or trans florets.

Table 32. Color and flower size of selected M1V7 mutants derived from both BARI Gerbera-1 and BARI Gerbera -2

Selected Variants	Radiation dose (Gy)	No. of ray florets	No. of disk florets	Length of ray florets (cm)	Length of disk florets (cm)	Length of flower stalk (cm)	Flower color code	Group/Type
GCW	0	54±0.81	110±0.66	3.33±0.16	1.5 ±0.37	77±0.31	Cream white	Double
WV1	20	90±0.83	120±0.66	4.1±0.21	0.6±0.34	64±0.91	Cream white	Double
WV2	20	57±0.65	130±0.66	3.8±0.82	1.2±0.22	81±0.71	Yellowish white/ off white	Double
WV3	20	60±0.87	140±0.66	4.5±0.66	2.2±0.32	67±0.31	Cream white	Double
CR	0	110±0.38	130±0.66	3.1±0.64	0.9±0.12	45±0.52	Dark red/46 B	Semi- double
RV1	5	120±0.58	140±0.64	3.6±1.3	1.5±0.58	52±0.77	Dark red/46B	Semi- double
RV2	10	140±0.24	150±0.44	3.2±0.75	0.5±0.66	46±0.92	Orangish red/44A	Semi- double
RV3	5	150±0.18	160±0.16	3.1±0.39	0.8±0.85	48±0.14	Orangish red/44A	Semi- double

A few, flowers showed discoloration in trans floret and disk area. Disk florets are also changed as they have an open banana shaped anther, and the tip of the floret is sharper than the control one (Figs.45f and 47c-d). This mutant was derived from BARI Gerbera-2 (red) using 5Gy treatment.

4.2.2.3.5 RV2: These daisy-like flowers are orangish red colored and have a spiky center. They are like RV1 regarding the shape of flower and the orientation of the florets as well. But they have differences in the structure of ray, trans, disk florets (Fig. 45c and 47e-f). This mutant was derived from BARI Gerbera-2 (red) using 10Gy treatment.

4.2.2.3.6 RV3: These daisy- like flowers are orangish red colored and have a spiky trans and disk florets containing center. They are like RV1 regarding the shape of flower and the orientation of the florets as well. But they have differences in the structure of ray, trans, disk florets (Fig.45d and 47g-h). This mutant was derived from BARI Gerbera-2 (red) using 5Gy treatment.

4.2.2.3.7 Analysis of morphological features of the new Gerbera mutant lines

Six different mutant lines were isolated from both BARI Gerbera-1 and BARI Gerbera -2 after *in-vitro* culture and irradiation of those cultures with gamma ray. *In-vitro* grown non-irradiated and irradiated plants brought up in the greenhouse condition in the earthen pot after hardening. During the analysis of vegetative and floral data of the mutants, those that were consistent in the subsequent generations (M_1V_6 and M_1V_7) were included. Different morphological characteristics such as, plant height, days required for bud initiation after plantation, days required for full bloom, diameter of the flower, flower stalk, flower number per plant, no. of ray floret and no. of disk floret were observed for M_1V_7. Data based on these characters was recorded in the greenhouse condition during the pick anthesis period for all those selected mutants. The recorded data are presented in Table 32.

It was observed that all the mutant lines are different from their mother plants regarding flower structure and shape. But they have not changed their type or group, BARI Gerbera-1 (Double) and all the mutants derived from this variety are Double. Whereas, BARI Gerbera-2 (Semi double) and all the mutants derived from this variety are also Semi-double (Table 32). In the case of BARI Gerbera-1 a very long flower stalk was observed in its mutant WV2 (81±0.71 cm). All the mutants developed from BARI Gerbera -2 also have longer flower stalks than the mother plant (Table 32). WV2, RV2 and RV3 are the color mutants. Again, the trans florets of the BARI Gerbera-1 showed significant changes in the structure, shape, and size (Fig. 46). In BARI Gerbera-2 also trans and ray florets are significantly changed in all the mutants (Fig. 48- 49). One way ANOVA following Tukey's multiple comparisons test result has been presented in Figs. 50 and 51. It was found that the mutants developed were significantly different than the control plants regarding all the parameters discussed in Table 32.

Finally, these promising six mutant lines derived from both Gerbera varieties have been included in the result for further molecular study.

4.2.2.3.8 Some anomaly (fasciation) observed after irradiation in both types of flowers varieties

During this study several abnormal morphological changes were observed regarding vegetative as well as floral aspects. As the flower is the focal point of present investigation, data with floral morphology has been included and presented in Figs. 52-54.

4.2.2.3.8.1 Some abnormalities observed after irradiation in both types of flower varieties

In the case of BARI Gerbera-2, flowers from M1V6-M1V7 in the greenhouse showed abnormal morphology with the changed color and structure of florets. Figs. 52 a-d showed the variation with control one.

4.2.2.3.8.2 Some abnormalities observed after irradiation in both types of flower varieties

In the case of BARI Gerbera-1, flowers from M1V6-M1V7 in the greenhouse showed abnormal flower head morphology, known as fasciation. Single, double, and triple headed fasciation was noticed more frequently in the mutants. Different stages of fasciation have been presented in Figs. 53 and 54.

4.2.3 Molecular analysis of the Gerbera mutants

4.2.3.1 Analysis of genetic diversity among the six promising mutants and two mother varieties of Gerbera plants using ISSR markers

Molecular marker technology provides information that can help to define the differences of regenerated mutants and their status according to the number of close relatives and their phylogenetic position. DNA marker technology is a new approach based on DNA polymorphism among tested genotypes and thus applicable to biological research. Among several molecular markers ISSR (Inter-simple sequence repeats) is the widely used most accountable molecular marker. In this experiment, 13 primers were used to find out the variation among 6 mutants generated from two varieties of Gerbera and mother plant of both varieties. 6 primers produced clearly scorable and reproducible bands in all these tested 9 groups (6 mutants and both BARI Gerbera-1 as well as BARI Gerbera -2 mother plants). Results of these experiments are presented under the following heads:

4.2.3.2 Optimization of PCR amplification conditions and selection of primers for ISSR analysis

PCR amplification conditions play a crucial role in amplification of DNA. In this part of the study, PCR amplification conditions were optimized by analyzing several factors. For this reason, various concentrations of the components of the reaction mix as well as variable amplification conditions were tested in obtaining most reproducible results. In most cases template DNA concentration over the range of 20-60 ng gave a constant banding pattern but higher concentrations resulted in smear in gel. At lower template concentrations, there was a tendency for the appearance of non-reproducible low molecular weight bands. Template DNA concentrations of 30 ng/µl were found to be the most suitable for getting reproducible banding patterns. Primers were selected carefully as some primers produced non-reproducible bands and some others could not amplify the DNA in all the investigating plants. These primers used in the present study are 10-18 nucleotides long and have the possibility of annealing at a random number of locations in the genome. However, all the primers do not produce reproducible and polymorphic banding patterns in all plant species. Initially, 13 primers were used. Among them five primers gave reproducible and scorable bands across the investigated samples. Therefore, these six primers were used for further analysis and mostly bright, prominent bands were scored. The bands amplified with ISSR primers were in the range of 110-4000 bp (Table 33).

Fig. 52 (a-d): Some abnormality found in 5 Gy and 10 Gy treated BARI Gerbera-2. (a) BARI Gerbera -2 control (C) flower and its different types of florets; (b) RV4 a colour and flower shape variant; (c) RV5 a colour and flower shape variant; (d) RV6 a colour and flower shape variant.

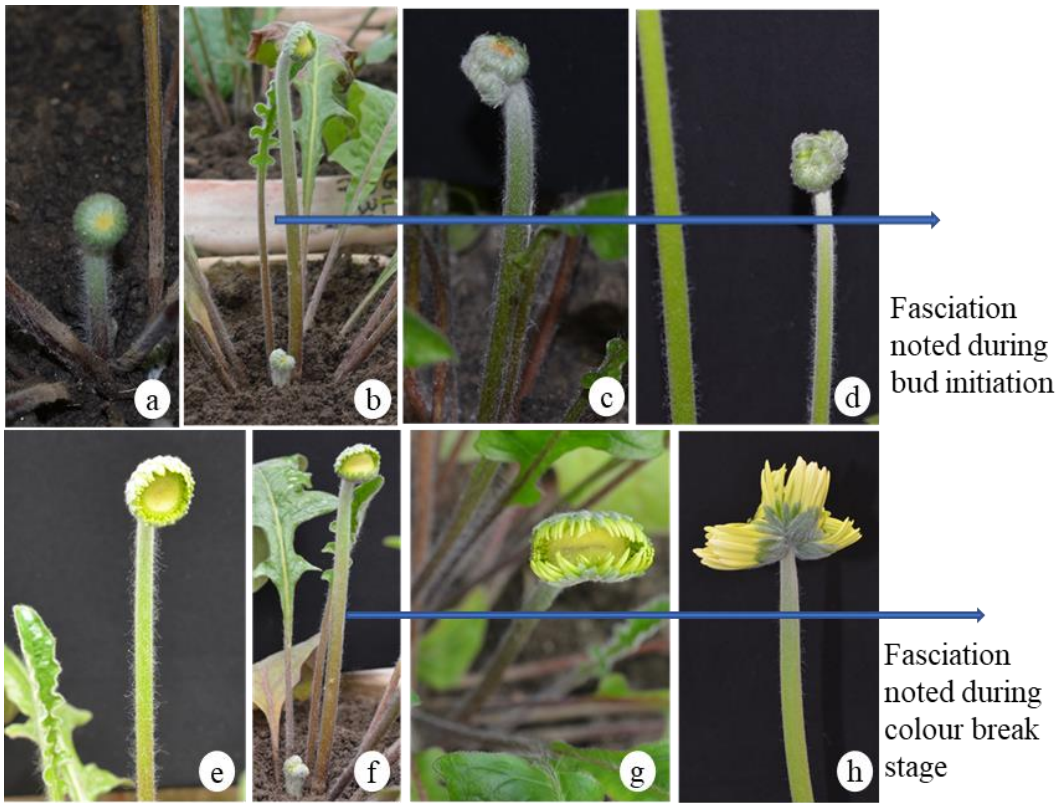

Fig 53 (a-h): Three types of fasciation during bud formation in BARI Gerbera-1. (a) Normal flower bud initiation; (b-d) singles of fasciation during flower bud initiation; (e) Colour break stage of normal bud; (f-h) Singal, double and triple fasciation noted during colour break stage of flower bud (left to right).

Fig. 54 Three types of fasciation during full bloom in BARI Gerbera -1. (a) Control; (b) Single fasciation; (c) Double fasciation; (d) Triple headed fasciation in flower.

4.2.3.3 Analysis of ISSR profiles

The selected 6 primers produced highly reproducible banding patterns in all the developed mutants as well as the two mother plant varieties investigated. However, the primers varied considerably in case of number of bands produced, intra-plants polymorphism and discriminating capacity. A particular band has been described by the primer name by which it was amplified suffixed with the molecular weight of the band (for example, the band UBC 835_{1265} denotes that it was amplified with the primer UBC 835 and its molecular weight was 1265 bp). Compilation of six ISSR primer profiles during the analysis of the six Gerbera mutants along with the two mother plants has been presented in Table 17. During this study, 89.06% polymorphism was recorded for the two cultivated Gerbera varieties and for the mutants developed from them. This broad range of polymorphism revealed wide diversity among the mutants developed through gamma radiation. The diversifications would be useful for different breeding programs of floriculture and can contribute to the global floral market. The primer wise ISSR profiles and banding patterns of 8 tested groups (6 mutants and both BARI Gerbera -1 and BARI Gerbera -2 mother plants) of plants are given below:

4.2.3.3.1 ISSR profile using primer UBC 812:

The primer UBC 812 produced a total of six bands in both mother varieties of Gerbera and mutants. This primer produced 2nd lowest bands among all the primers used in the present investigation. The lowest number of bands (1 band) were produced by red mutants RV1, RV2 and white mutants WV1 and WV2. WV3 produced three unique bands (UBC $812_{550,}$ UBC 812_{800}, UBC 812_{1300}). The primer produced only one monomorphic band (UBC 812_{1120}) and showed 83.33 % polymorphism. The investigated mutants belonging to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 55a).

4.2.3.3.2 ISSR profile using primer UBC 835:

The primer UBC 835 produced a total of 20 bands within both mother varieties of Gerbera and mutants. This primer produced the highest bands among all the primers used in the present investigation. The lowest number of bands was produced by RV2 red mutant (4 bands), and rest of the investigated materials produced bands that ranged from 4 to 12. The highest numbers of bands (12 bands) with one unique (UBC 835_{500}) band were produced by WV3 mutant developed from BARI Gerbera-1. It produced 1

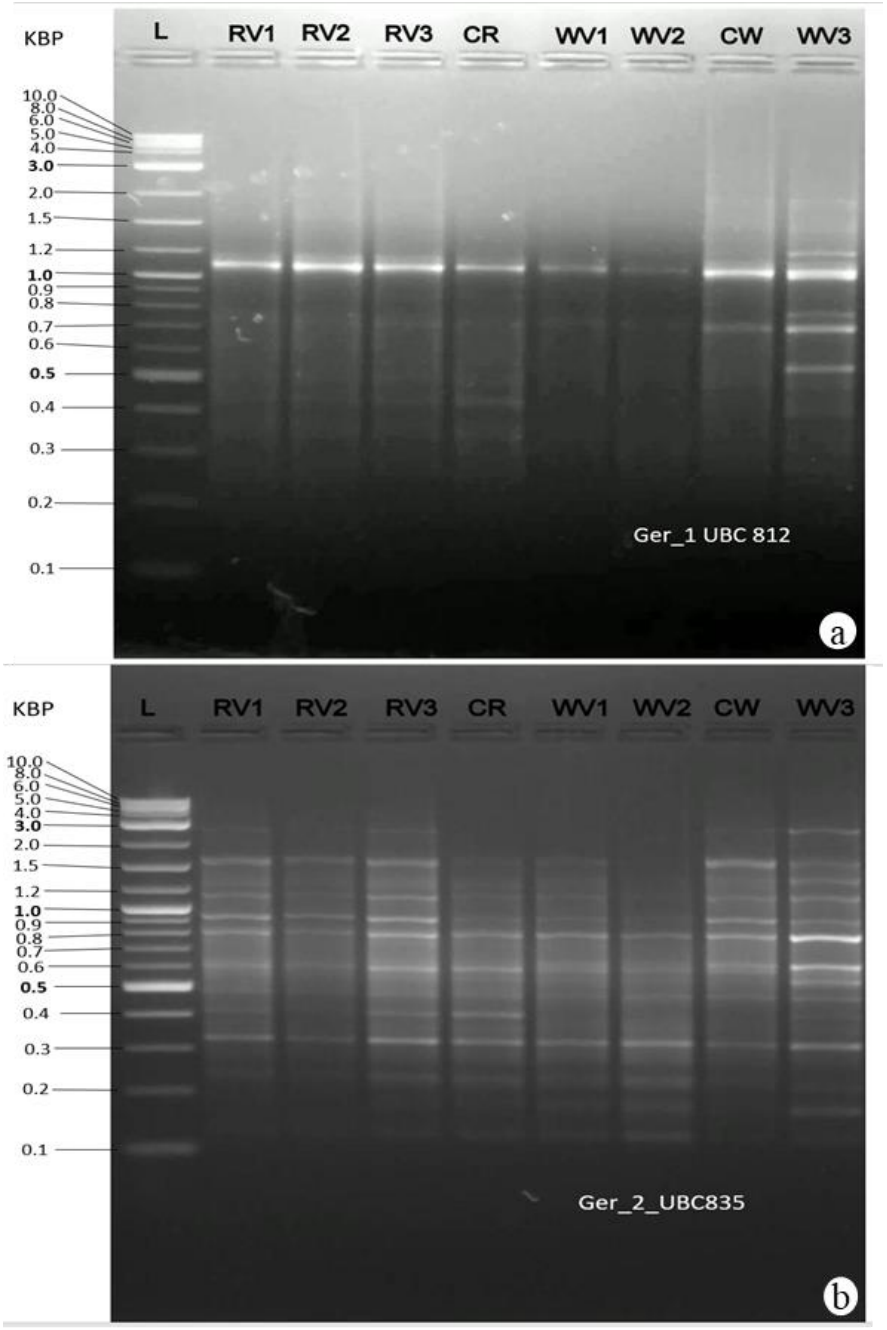

Fig. 55 (a-b): ISSR profiles of both varieties of Gerbera mothers and 6 mutants derived from gamma irradiation obtained with the primer UBC812 and UBC835. (a) Amplification of bands with UBC 812 primer. Lane L: Ladder (1 Kb plus); Lane 2,3,4&5: mutants RV1, RV2, RV3 and mother plant of BARI Gerbera-2 (CR); Lane (6-9): mutants WV1, WV2, CW(mother plant) and WV3; (b) Same as (a) but with UBC 835 primer and 1Kb + Ladder.

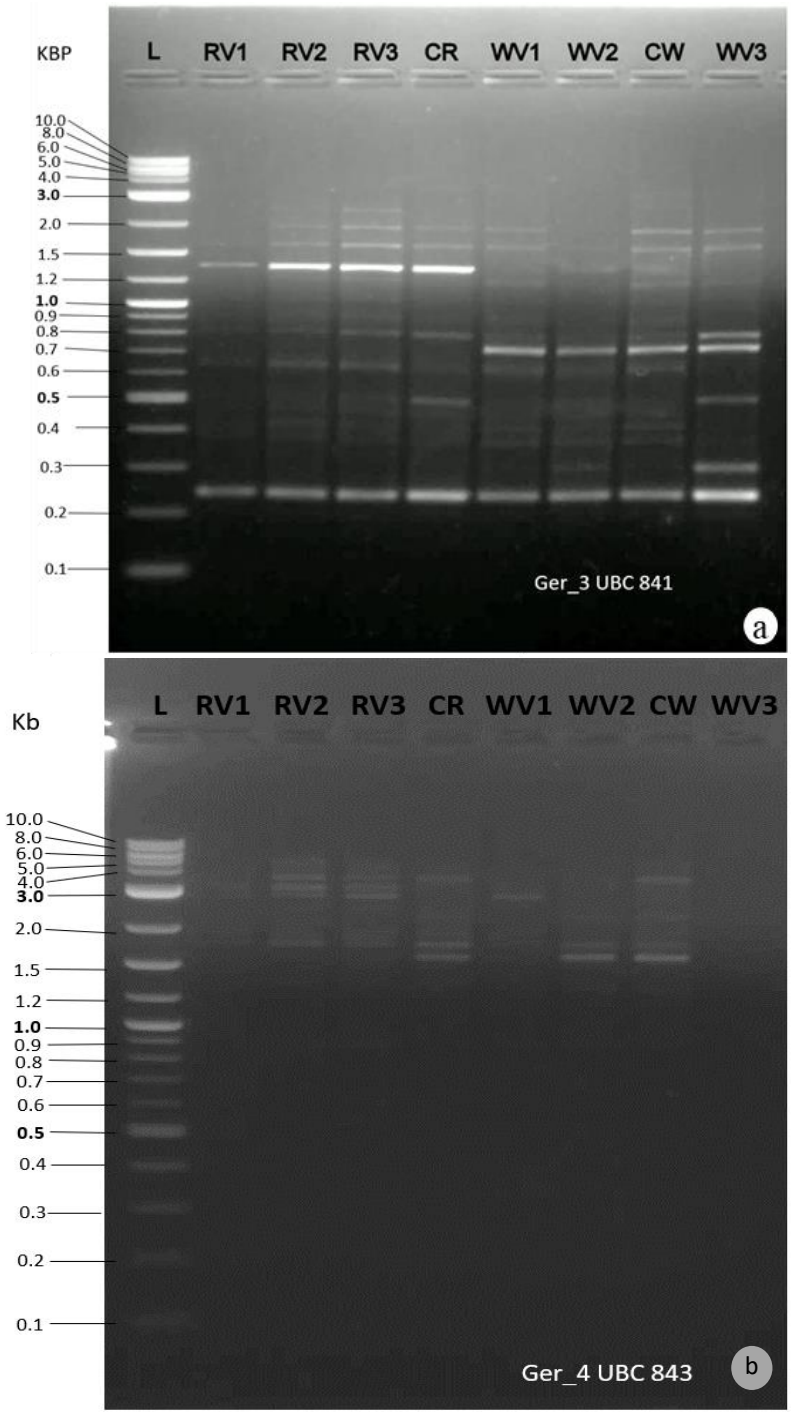

Fig. 56 (a-b): ISSR profiles of both varieties of Gerbera mothers and 6 mutants derived from gamma irradiation obtained with the primer UBC841 and UBC843 (a) Amplification of bands with UBC 841 primer. Lane L: Ladder (1 Kb plus); Lane 2,3,4&5: mutants RV1, RV2, RV3 and mother plant of BARI Gerbera-2 variety CR; Lane (6-9): mutants WV1, WV2, CW (mother plant) and WV3; (b) Same as (a) but with UBC 843 primer and 1Kb + Ladder.

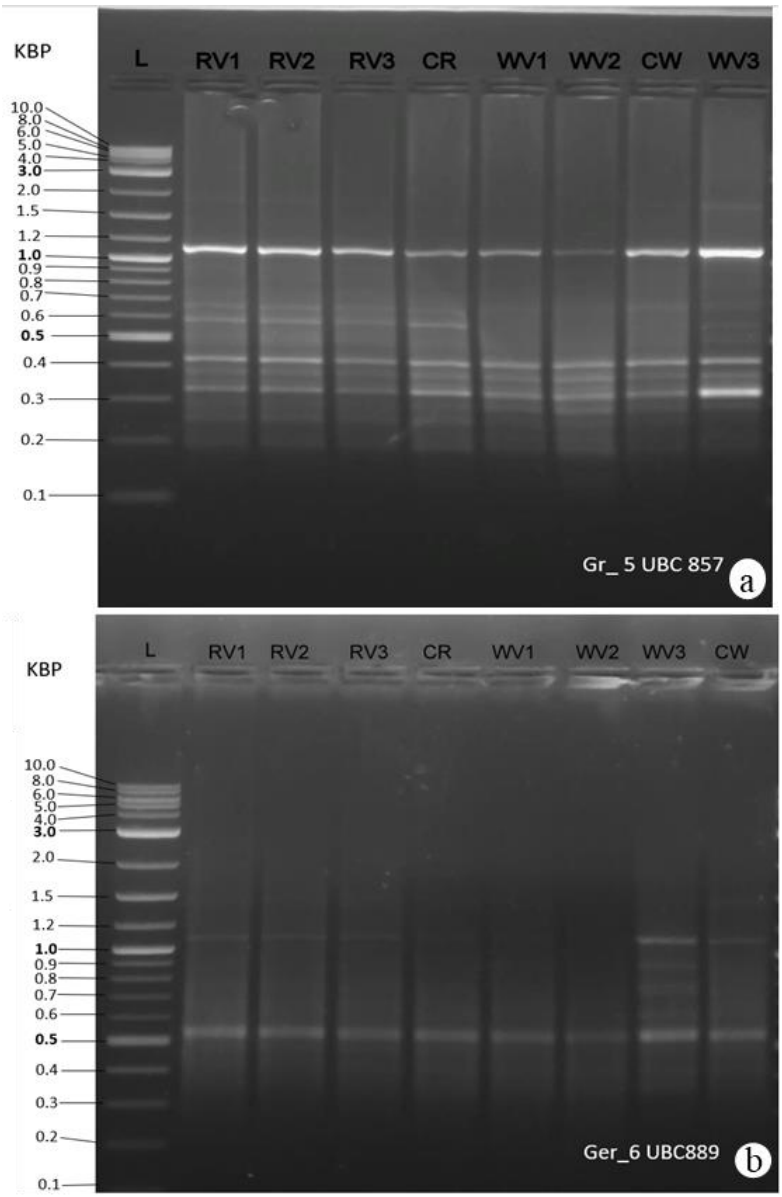

Fig. 57 (a-b): ISSR profiles of both varieties of Gerbera mothers and 6 mutants derived from gamma irradiation obtained with the primer UBC857 and UBC889 (a) Amplification of bands with UBC 857 primer. Lane L: Ladder (1 Kb plus); Lane 2,3,4&5: mutants RV1, RV2, RV3 and mother plant of BARI Gerbera-2 variety CR; Lane (6-9): mutants WV1, WV2, CW (mother plant) and WV3; (b) Same as (a) but with UBC 889 primer and 1Kb + Ladder.

monomorphic band and showed 95 % polymorphism (Table 33). The investigated mutants belonging to 8 groups were distinguishable by this primer as they produced different banding patterns (Fig. 55b).

4.2.3.3.3 ISSR profile using primer UBC841

The primer UBC 841 produced 13 bands within both mother varieties of Gerbera and mutants. It produced one unique band (UBC 841_{2700}) and one monomorphic bright band UBC 841_{250}. Only 2 bands were produced by RV1 and the rest of the investigated materials produced bands that ranged from 5 to 8. The highest number of bands (8) was produced by WV1 and it showed 92.31% polymorphism (Table 33). The investigated mutants belonging to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 56a).

4.2.3.3.4 ISSR profile using primer UBC843

The primer UBC 843 produced a total of 6 bands in both mother varieties of Gerbera and mutants. Both RV1 and WV3 produced no band and the rest of the material produced 1 to 4 bands. It showed 100% polymorphism with no monomorphic band (Table 33). The investigated mutants belonging to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 56b).

4.2.3.3.5 ISSR profile using primer UBC 857

The primer UBC 857 produced 14 bands within both mother varieties of Gerbera and mutants. This primer produced 2nd highest bands among all the primers used in the present investigation. White mutant WV3 produced 2 unique bands (UBC $857_{540,}$ UBC 857_{1350}) with this primer and other materials produced 4-10 bands. It produced 3 monomorphic band showing 78.57% polymorphism (Table 33). The investigated mutants belong to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 57a).

4.2.3.3.6 ISSR profile using primer UBC 889

The primer UBC 889 produced only 5 bands within both mother varieties of Gerbera and mutants. CR, WV1 and WV2 produced one band and the rest of the materials produced 2 to 3 bands. This primer produced three unique bands, two for GCW (UBC $889_{600,}$ UBC 889_{950}) and one for WV3 (UBC 889_{1600}). It produced one monomorphic

band showing 80% polymorphism (Table 33). The investigated mutants belonging to 9 groups were distinguishable by this primer as they produced different banding patterns (Fig. 57b).

Table 33. Compilation of five ISSR primer profile during the analysis of the six Gerbera mutants along with the two mother plants

Primer codes	Total loci	Size ranges (bp)	Number of Polymorphic loci	Number and size (bp) of unique bands	Polymorphism (%)
UBC 812	6	450-1300	5	WV3(550, 800, 1300)	83.33
UBC 835	20	110-2900	19	WV3 (500)	95
UBC 841	13	180-1500	12	RV3 (2700)	92.31
UBC 843	6	1600-4000	6	-	100
UBC 857	14	180-3000	11	WV3 (540,1350)	78.57
UBC 889	5	550-1600	4	GCW (600,950), WV3(1600)	80
Grand Total	64	110-4000	57	10	89.06

4.2.3.4 Analysis of genetic diversity

Genetic variation was high among all the materials investigated of Gerbera as revealed by the genetic distance presented in Table 34. In this study, the UPGMA analysis of Dendrograms and distance matrix coefficients revealed good relationships between some mutants (Table 34).

Primarily, genetic distance matrix was calculated according to Nei (1972) for all the 8 investigated Gerbera materials considering ISSR banding pattern (Table 34). Genetic variation among the germplasm is usually revealed by the genetic distance matrix. In this study, the lowest genetic distance was found between RV3 and CR (0.2469). On the other hand, the highest genetic distance was found 0.8630 for WV3.

The phylogenetic relationships among 8 tested Gerbera germplasm were analyzed by UPGMA method. The cluster result indicated that ISSR markers could distinguish all the mutants as well as the mother plants. Grouping of the investigated plants into each cluster or branch correlated with similarities in their ISSR DNA patterns. For example, the mutant that produced the same DNA banding patterns were also

recognized as being similar from phylogenetic analysis. That germplasm belonged to the same cluster has been presented in the resulting phylogenetic tree (Fig. 58).

Table 34. Summary of Nei's (1972) genetic distances of ISSR analysis of the six Gerbera mutants along with the two mother plants

pop ID	RV1	RV2	RV3	CR	WV1	WV2	GCW	WV3
RV1	****							
RV2	0.2671	****						
RV3	0.3087	0.2877	****					
CR	0.4453	0.3747	0.2469	****				
WV1	0.6035	0.4700	0.6325	0.4700	****			
WV2	0.6931	0.6624	0.8630	0.6035	0.2271	****		
GCW	0.6035	0.5754	0.6325	0.6325	0.3747	0.3977	****	
WV3	0.5754	0.8630	0.6624	0.6624	0.6624	0.7577	0.4953	****

Dendrogram produced by UPGMA method was distributed among six mutants along with the two mother varieties. There was a total of 7 clusters (1 - 7) present in the Dendrogram (Fig. 58). All the germplasm was grouped into two major branches (B1 and B2) representing BARI Gerbera-1 mutant WV3 (totally different than others) and the rest of the material tested. B2 further separated into two sub-branches (SB1 and SB2). SB1 has WV1 and WV2 mutants in one group and showed lowest genetic distance. CR showed similarity RV3 on the other hand. Thus, a clear difference was observed between the mutants and the mother varieties.

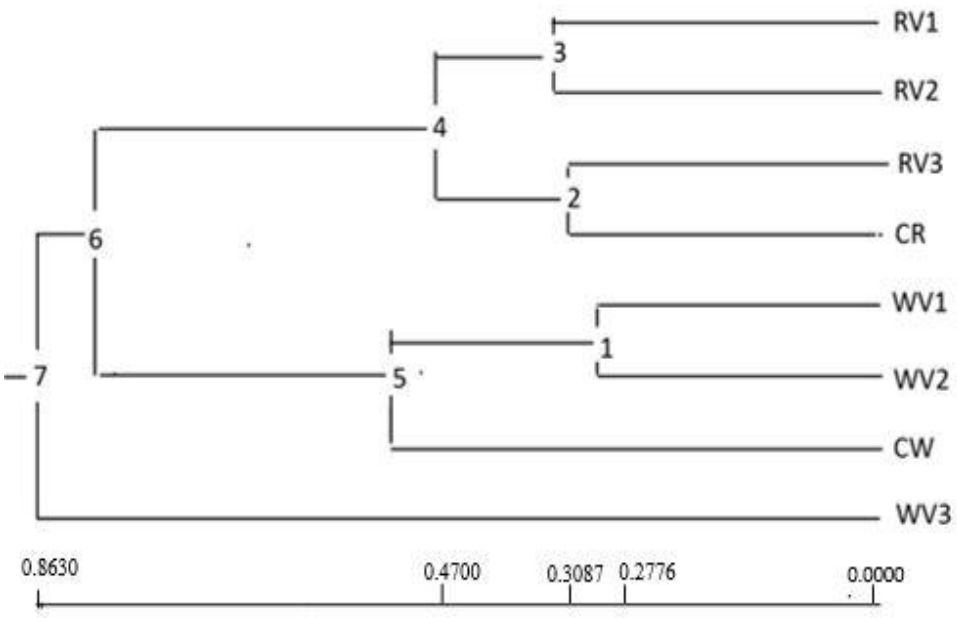

Fig. 58. UPGMA dendrogram constructed based on Nei's genetic distance summarizing the data on differentiation among the mother varieties of Gerbera and the developed mutants by ISSR analysis.

5. DISCUSSION

The main objectives of the present investigation were to establish a reliable protocol for *in vitro* plant regeneration and induction of mutation in two exotic flowering plants, Chrysanthemum (*Chrysanthemum morifolium* Ramat) and Gerbera (*Gerbera jamesonii* Bolus) through gamma irradiation. The ultimate goal of the present investigation was to induce mutation for producing new variant lines of the above two economically important flowering plants suitable for cultivation in Bangladesh. Two varieties of Chrysanthemum, namely, BARI Chrysanthemum-1 (yellow) and BARI Chrysanthemum-2 (white) and two varieties of Gerbera, viz. BARI Gerbera-1 (white) and BARI Gerbera- 2 (red), were used in the present study. These experiments were carried out in three phases for each of the two plant types. In the first phase, experiments were carried out for the development of *in vitro* plant regeneration protocols for the selected Chrysanthemum and Gerbera varieties. During this study the response of different explants of the experimental materials towards *in vitro* plant regeneration were studied. In the second phase, induction of mutation of selected Chrysanthemum and Gerbera varieties using gamma irradiation were carried out. Following irradiation, both *in vitro* and *in vivo* condition morphological changes regarding flowers were studied and finally seven mutants from Chrysanthemum and six mutants from Gerbera were developed. In the third phase, molecular characterization of the developed mutants was performed through ISSR marker analysis. In this chapter, aforementioned experimental results are discussed in the light of the findings of other researchers. Plant tissue culture is an important technique where numerous plants (clones) could be produced from a single explant. Therefore, the choice of explants is very important and makes an absolute difference between success or failure in inducing regeneration *in vitro*. In the present investigation, the regeneration experiments were mainly conducted using the shoot tip, leaf and internode explants for Chrysanthemum and flower bud, flower stalk and leaf explants for Gerbera.

The success of a regeneration protocol also depends on the selection of suitable explant, culture condition and regeneration medium with proper phytohormonal supplements.

During this study leaf explant of Chrysanthemum and flower bud explant of Gerbera showed best response towards multiple shoot regeneration among all other explants

used. Chrysanthemum leaf explants showed the highest 94.73% regeneration response for BARI Chrysanthemum -1 (yellow) and 90% for Chrysanthemum -2 (white) variety. Almost similar regeneration response was observed while using internode was used as explant. In this case 94.7% regeneration response was found for BARI Chrysanthemum-1and 88.2% for BARI Chrysanthemum-2. There are some previous reports on using leaf explant for shoot regeneration in chrysanthemum (Hodson de Jaramillo *et al.* 2008; Zalewska *et al*. 2011; Lim *et al*. 2012; Naing *et al*. 2014, Nasri *et al.* 2018; Miller and Kulus 2018).

Highest regeneration response, 73.3% for BARI Gerbera-2 (red) and 70% for BARI Gerbera-1 (white) of Gerbera was achieved when flower bud was used as explant. There are some previous reports on using flower bud as explant for shoot regeneration in Gerbera (Aswath and Choudhary 2002; Tyagi and Kothari 2004; Ray *et al*. 2005; Kumar and Kanwar 2007; Nhut *et al*. 2007; Akter *et al.* 2012; Talla *et al.* 2018).

In vitro regeneration of Chrysanthemum and Gerbera was found to be highly influenced by the interaction of plant growth regulators (PGRs), plant genotype and explant type (Orlikowska *et al.* 2000; Lim *et al.* 2012; Shabbir *et al.* 2012; Teixeira da silva *et al.* 2015; Nazari *et al.* 2016). Most of the studies revealed direct/indirect organogenesis/embryogenesis of *C. morifolium* and *G. jamesonii* in MS medium using different concentrations of auxins (NAA, IAA, 2, 4-D) along with cytokinins (BAP, BA and TDZ) (Akter *et al.* 2012; Lim *et al.* 2012; Huang *et al.* 2018; Kazeroonian *et al.* 2018; Talla *et al.* 2018; Rahman *et al.* 2018).

Present study revealed that the addition of auxin (0.5 mg/l) IAA in MS medium induced regeneration responses in leaf explants of Chrysanthemum. A significant increase in regeneration percentage was recorded (from 37.5 to 76% for BARI Chrysanthemum-1 variety and 35.7 to 70% for BARI Chrysanthemum-2 variety) using up to the 1.0 mg/l IAA. But higher concentrations of auxin, like 2.0 mg/l IAA in MS medium showed a negative effect on chrysanthemum regeneration percentages for both the varieties (60.3% for BARI Chrysanthemum-1 and 48.3% for BARI Chrysanthemum-2). These results in Chrysanthemum were found to be identical with previous studies using another auxin NAA (Xu *et al.* 2012; Naing *et al.* 2014; Nasri *et al.* 2018). Interesting results were found when a combination of auxin and cytokinin were used. Addition of 0.5 mg/l BAP along with 0.5 mg/l IAA in MS medium increased regeneration in two-fold. However, further increase of BAP levels (1.0 mg/l) did not show significant change towards regeneration response. Again, high

concentration of IAA (2.0 mg/l) along with low BAP (0.5 mg/l) in MS medium exhibited dramatic increase in Chrysanthemum regeneration response for both the varieties (94.73% for BARI Chrysanthemum-1 and 90% for BARI Chrysanthemum--2). While addition of more cytokinin (MS + 2.0 mg/l IAA+ 1.0 mg/l BAP) dropped the regeneration rate (74% for BARI Chrysanthemum-1and 70.33% for BARI Chrysanthemum-2). These findings indicated that lower concentrations of auxin (MS+ 1.0 mg/l IAA+ 1.0 mg/l BAP) showed an increase rate of regenerations responses (81.25% for BARI Chrysanthemum-1 and 87.5% for BARI Chrysanthemum-2 variety) for both the varieties. Similar findings were obtained in several previous studies with Chrysanthemum using the identical groups of hormones (Xu *et al*. 2012; Naing *et al*. 2014; Kazeroonian *et al*. 2018; Chowdhury *et al*. 2021).

During the present study, among the nine treatments tested MS+2.0 mg/l IAA + 0.5 mg/l BAP produced the highest regeneration response (94.73% for BARI Chrysanthemum-1 and 90% for BARI Chrysanthemum-2) as well as highest mean number of shoot/explant 8.67 and 7.67 for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties, respectively. Highest mean shoot length for both varieties were found to be approximately 3 cm after six weeks of culture initiation using the same treatment (MS+ 2.0 mg/l IAA+ 0.5 mg/l BAP). Similar regeneration response was observed (highest 94.7% and 88.2% for BARI Chrysanthemum-1 and BARI Chrysanthemum-2 variety respectively) using the same treatment for internode explant. Considering all investigating factors of regeneration, this treatment was found to be the best for both varieties and both explants tested. A similar treatment containing MS medium supplemented with 0.6 mg/l BAP and 2.0 mg/l IAA was also reported to be effective for *in vitro* regeneration using leaf, internode and single node explants by many researchers (Zalewska *et al.* 2011; Miller and Malgorzata 2014; Tymoszuk and Kulus 2020).

In the case of Gerbera regeneration, like Chrysanthemum high concentrations of auxin (1.0 mg/l NAA) were also found to show significant contribution for shoot induction in both tested varieties. Whereas low auxin (0.5 mg/l NAA) formed only callus and failed to form shoots in both varieties. Pierik *et al.* (1973) also reported that addition of NAA with BAP promoted better shoot formation compared to IAA.

In the present study, flower bud explants of both Gerbera varieties (BARI Gerbera-1 and BARI Gerbera-2) were cultured on MS medium supplemented with BAP (1.0 – 7.0 mg/l) and NAA (0.5 - 1.0 mg/l) to evaluate their combined effect to induce callus

and subsequent development of shoots. For white variety, highest response towards callus formation and shoot regeneration was observed on MS medium supplemented with 6.0 mg/l BAP+1.0 mg/l NAA. In this combination 70% of explants showed shoot initiation response where highest mean number of shoots per explant were found to be 10.33. This combination also found to be the best for red variety as it produced 73.3% regeneration within 40-45 days of inoculation having 12.33 shoot/explant.

Flower stalk explants of both Gerbera varieties showed early response (within 18-25 days) towards callus induction, but percentage of shoot formation was low in all the combinations of BAP and NAA compared to flower bud explants. Highest response towards shoot regeneration was 50% for white and 56.7% for red variety on MS+6.0 mg/l BAP+1.0 mg/l NAA medium. Same treatment produced the highest mean number of shoots per explant, 7.88 for white and 9.22 for red flower stalk explants for both the varieties. Hasbullah *et al.* (2008) also reported that a combination of auxin and cytokinin is essential to induce the formation of adventitious shoots in Gerbera.

Addition of cytokinin in the regeneration medium generally increases shoot number per explant. Several previous reports have confirmed that BAP accelerates the development of the shoot buds as well as enhancing the shoot primordial development in Chrysanthemum and in other plants which is in line with result of the present study (Lee *et al.* 1997; Yesmin *et al*. 2014; Kazeroonian *et al.* 2018; Chowdhury *et al*. 2021).

Current study also revealed that MS medium supplemented with low concentration of cytokinin (BAP) and high concentration of auxin (IAA/NAA) were optimum for organogenesis providing best regeneration responses of both Chrysanthemum and Gerbera tested. Xu *et al.* (2012) reported that low concentration of 6-benzyladenine (BA) and high concentration of NAA were most effective for the formation of calli and somatic embryogenesis. However, this result was different from many other studies showing importance on embryonic callus induction through 2,4-D along with BAP or other cytokinins (Hodson de Jaramillo *et al*. 2008; Huang *et al*. 2018). Aforementioned findings showed that lower concentration of NAA played a key role in the process of Chrysanthemum regeneration which was not similar to the results of the present study. This may be due to the use of different explant and genotypes of Chrysanthemum used in the present investigation (Xu *et al*. 2012).

Subculture is a very important step for the multiplication of shoots. It was observed that when regenerated shoots (both Chrysanthemum and Gerbera) were cultured for a longer period on MS medium supplemented with auxin and cytokinin combination for shoot multiplication then the shoots showed vitrification and abnormal morphology.

The present findings revealed that subsequent subculture of the Chrysanthemum leaf/internode explants after four weeks in a hormone free MS medium enhanced the production of multiple shoots. Therefore, MS medium (hormone free) were used for further shoot proliferation and shoot elongation for 5-6 weeks till the regenerated shoots achieved the optimum (3.0-3.5 cm) length. Several reports also suggested that the development of somatic embryo and plant regeneration can be achieved through the application of lower concentration of auxin or auxin free MS medium (Hodson de Jaramillo *et al*. 2008; Xu *et al*. 2012; Huang *et al*. 2018).

Regarding Gerbera subculture, full and ½ strength of MS medium was supplemented with 1.0 – 2.0 mg/l BAP and evaluated their response toward shoot multiplication and development. Best response (96.2%) towards multiple shoot formation was observed on ½ MS+2.0 mg/l BAP from flower bud explant while producing maximum number of shoots/explant (30-35 shoots/explant). Micro shoots isolated from a clamp developed on MS+6.0 mg/l BAP +1.0 mg/l NAA after the end of 9-10 weeks of culture, were transferred to ½ MS+2.0 mg/l BAP for minimizing time required for growth. After 20 days of culture new healthy shoot clumps were developed. In the case of Gerbera flower stalk explant, 18-20 shoots/explant were produced in the same medium. The regenerated shoots from both the explants increased 2-3 folds per subculture on the same medium and all the shoots were found healthy and normal in appearance. It may be mentioned that both the tested Gerbera varieties produced almost similar results during this study. Akter *et al.* (2012) reported that MS+2.0 mg/l BAP is the best for the same purpose.

Induction of the healthy root system from the regenerated shoots is an important step for a successful regeneration protocol to develop plantlets. During the present study, both Chrysanthemum and Gerbera produced spontaneous roots during shoot multiplication on the subculture medium.

In Chrysanthemum, during shoot elongation and multiplication spontaneous rooting was observed when consequent subcultures were done in hormone free MS medium.

In both the varieties more than 90-95% of shoots were found to produce healthy roots within 6 to 7 weeks of culture. These findings were identical to the other reports for different Chrysanthemum varieties (Hodson de Jaramillo *et al*. 2008; Naing *et al*. 2014; Kazeroonian *et al*. 2018). Better plant growth and rooting was observed on a hormone free MS medium solidified with Gelrite. Rooting occurred spontaneously in most cases without the application of any exogenous growth regulator in the culture medium (Naing *et al*. 2014). However, several studies showed healthy root induction using full, or half strength of MS medium supplemented with lower concentration of auxin (IBA/IAA/NAA) (Sarker and Ismat 2001; Waseem *et al.* 2011; Yesmin *et al.* 2014; Miler and Kulus 2018).

In the case of *in vitro* plant regeneration, root formation is an indispensable step to produce plantlets which is also true for Gerbera. Therefore, 3.5 – 4.5 cm long shoots were excised and cultured on half strengths of MS medium supplemented with different concentrations of IBA and IAA. Among the different auxins used 0.5 mg/l IAA showed the best response towards rooting considering all the factors tested. On this medium (½ MS + 0.5 mg/l IAA) 100% rooting was found to initiate within 15-20 days of culture. On the other hand, IBA supplemented media produces large callus masses and the percentage of root formation was found to be low. Similar results were found for both Gerbera varieties. But others reported IBA (0.2-0.5mg/l) supplemented ½ MS media is the best for rooting (Kanwar and Kumar 2008; Akter *et al.* 2012). Usage of different species in the present study may cause these differences.

Efforts were made to establish plantlets in pots with proper root systems. Plants with sufficient roots were transplanted to soil and their survival rate was 100% in the growth room and field/greenhouse condition. All the Chrysanthemum and Gerbera varieties that have been used in this study showed almost identical responses towards field performance following acclimatization.

Another objective of the study was to develop mutant lines through mutation induction using gamma radiation on *in vitro* cultures. Induced mutagenesis through radiation has been successfully used for the development of new flower color/shape mutants in Chrysanthemums (Misra *et al.* 2003; Dwimahyani and Widiarsih 2010) and various ornamental plants including Gerbera (Mandal *et al.* 2000; Yamaguchi *et al.* 2009). The selection of ornamentals is very easy when visible characters, such as flower color, shape and size, or leaf form and growth habit are concerned. According

to IAEA (2021) by 2005, 2,335 varieties were released through mutagenesis in the world, in which ornamental and decorative crops were 552. To date there have been only few reports of induction mutation through gamma radiation and the production of successful mutants in Chrysanthemum and Gerbera (Laneri *et al.* 1990; Jerzy and Lubomski 1992; Hasbullah *et al*. 2012; Kumari *et al*. 2013; Soliman *et al*. 2014).

The basic requirement for an effective use of mutation induction in plant breeding programmes is the analysis of radio sensitivity of the explants material (Walther and Sauer 1985). Several studies have been conducted on the radio sensitivity of *in vitro* cultures of several crops (Hell 1983; Walther and Sauer 1985; Wang *et al.* 1988; Cheng *et al.* 1990; Shen *et al.* 1990; Charbaji and Nabulsi 1999; Predieri and Gatti 2000). They studied the effect of gamma irradiation on *in vitro* cultures in crop breeding application, with an objective of developing a suitable *in vitro* mutagenic system for the induction and selection of desirable mutants.

Influence of various regulatory factors on the inducing mutation efficiency on various plantlets has been reported by several authors in a number of ornamental plants including Chrysanthemum (Mandal *et al.* 2000; Yamaguchi *et al.* 2009), rose (Ibrahim *et al.*1998), Gerbera (Hasbullah *et al.* 2012). The factors that influence successful mutation include radiation doses, genotype of the host (varieties), types of radiated plantlet stages, radiation period, period of stressing out the lethal effect of gamma doses, etc.

While considering all the regulatory factors for *in vitro* mutation induction through gamma radiation, the present study reveals that the increase in radiation dose affects the percentage of plant regeneration. A number of reports are available which reported irradiation with 5-25 Gy doses of gamma rays for successful mutation induction process (Jerzy and Lubomski 1992; Hasbullah *et al.* 2012; Soliman *et al.* 2014). So, considering all these studies the present experiments were conducted using five radiation doses, namely, 5Gy, 10Gy, 15Gy, 20Gy and 25Gy.

The growth stage of the *in vitro* grown culture is important for induction of mutation through gamma radiation. The present experiment was conducted using *in vitro* grown shoot primordia/microshoots for both Chrysanthemum and Gerbera. It was found that this stage of the explant (shoot primordia/microshoots) was very effective as it

produced a large number of shoots after overcoming the lethal effects of different radiation doses.

Lethal effects is common after irradiation during mutation induction. A comparative study on the effects of five radiation doses (5Gy,10Gy, 15Gy, 20Gy and 25 Gy) on the shoot survival percentage of two varieties of Chrysanthemum indicated that highest dose (25 Gy) showed highest lethality as the survival percentage for both varieties was found low (3.90% and 9.93% for BARI Chrysanthemum-1 and BARI Chrysanthemum-2). However, the highest survival percentage was observed for 5Gy irradiated shoots for both varieties (57.29% for BARI Chrysanthemum-1 and 64.08% for BARI Chrysanthemum-2). Similar results were reported by others working on Chrysanthemum mutation induction through gamma radiation (Wang *et al*. 2020; Nasri *et al*. 2021).

For Gerbera also, application of highest dose (25 Gy) showed highest lethality as the survival percentage for both varieties were found low (5.80% and 0% for BARI Gerbera-1 and BARI Gerbera-2 varieties). BARI Gerbera-2 also showed 0% survival in response to 20Gy. However, highest survival percentage was observed for 5 Gy irradiated shoots for both varieties (72.77% for BARI Gerbera-1 and 68.09% for BARI Gerbera-2 varieties). These results are in agreement with the report of Hasbullah *et al*. (2012)

The lethal dose (LD_{50}) for 50% of the regenerating explants in irradiated explants was estimated during the study. In the case of Chrysanthemum, LD_{50} was calculated as 9.25 Gy for BARI Chrysanthemum-1 and 11.19 for BARI Chrysanthemum-2 variety. Whereas, LD_{50} 11.17 Gy for BARI Gerbera-1 and 9.32 Gy for BARI Gerbera-2 variety was noted. Hasbullah *et al*. 2012 reported 25 Gy as LD_{50} for Chrysanthemum, but Lamseejan *et al*. 2000 observed 14Gy as LD_{50}. These differences regarding LD_{50} may be due to use of different genotype and culture conditions using different explants. It is also noted that the variation in LD_{50} values for different genotypes of the same species is a common observation in mutation studies depending upon the biological materials, their size, maturity, hardness, and moisture content at the time of treatment (Babaei *et al.* 2010; Tabasum *et al*. 2011). Lethal dose also depends on the age of the explant used for irradiation, rate of irradiation, genotype and culture condition. LD_{50} is important because the rate of mutation is high at this dose and in most of the mutation induction studies found successful mutation induction around this dose.

Mutation breeding studies using vegetatively propagated ornamentals including *C. morifolium* and *G. jamesonii,* the dose chosen should result in the highest survival of irradiated explants and a low inhibition of the rate of production of new shoots would give the highest efficiency in recovering useful mutants (Laneri *et al*. 1990; Hasbullah *et al*. 2012).

The growth of the regenerated shoots became slow and required more time for regeneration after irradiation during the present study. A considerable time was needed to recover multiple shoots (M_1V_3) after irradiation for both Chrysanthemum and Gerbera. Several shoots died in each subculture and M_1V_4 shoots were developed during the 3rd subculture on hormone free MS medium (Chrysanthemum) or on ½ MS+2 mg/l BAP medium (Gerbera). It was observed that the growth response reduced with the increase of the radiation doses. Similar responses were also reported by other researchers (Ibrahim 1969; Datta *et al.* 2005; Soliman *et al.* 2014).

Both Chrysanthemum and Gerbera irradiated shoots (M_1V_4) were rooted on respective rooting mediums and developed healthy rooted plantlets (M_1V_5). No inhibitory effect of radiation/ doses was observed during rooting in both Chrysanthemum and Gerbera varieties tested.

In the case of Chrysanthemum (M_1V_5), the frequency of morphological variation at different radiation doses was recorded. Highest variation frequency percentage (75-80%) regarding leaf shape change percentage, chlorophyll change percentage, internode size percentage and plant height percentage were found from dose 15 Gy for Chrysanthemum-1 variety. About 10 % variation regarding all concerned factors were observed in plants developed from 25Gy treated explants. Whereas dose 5Gy, 10 Gy and 15 Gy produced remarkable variation frequency percentage (90-35%) for BARI Chrysanthemum-2. None of the 20Gy and 25Gy treated shoots of BARI Chrysanthemum-2 could survive on the elongation or rooting phage, so no variation was observed regarding all four measured aspects.

In the case of Gerbera, highest variation frequency percentage (45-50%) regarding leaf shape change percentage, (15-30%) chlorophyll change percentage and (55-5%) plant height percentage were found for both varieties from dose 5-20 Gy. None of the 15, 20 and 25 Gy treated shoots could survive on the elongation or rooting phage, so variation frequency regarding all four measured aspects were 0% for BARI Gerbera-2.

For both Chrysanthemum and Gerbera, it was noticed that variation frequency is not directly increasing with the increased radiation doses. Some reports support this point for mutation induction in ornamentals as well as some crop plants (Hell 1983; Walther and Sauer 1985; Wang *et al.* 1988; Cheng *et al.* 1990; Shen *et al.* 1990; Charbaji and Nabuls 1999; Predieri and Gatti 2000; Datta *et al.* 2005; Soliman *et al.* 2014).

Plants (M_1V_5) showing vegetative variations were grown in the field/greenhouse till flowering and noted performance in greenhouse conditions along with the control plants. Observation and selection were made for desirable variants at flowering time for both Chrysanthemum and Gerbera. Changes in flower color, form and shape were observed in plants treated with gamma rays of different doses.

In Chrysanthemum (M_1V_5) mutation frequency of flower form and flower color was found to be different regarding radiation doses. Mutation frequency of flower form and flower color 45%, 15% and 20% was observed in 5Gy, 10Gy and 15Gy treated BARI Chrysanthemum-2 and 15%, 10% and 10% was observed in 10Gy, 15Gy and 25Gy treated BARI Chrysanthemum-1.

BARI Gerbera-1(M_1V_5) produced 20% and 30% mutation frequency of flower form and flower color at 5Gy and 20Gy doses. Whereas radiation doses 5Gy and 10Gy developed 45% and 20% mutation frequency of flower form and flower color for BARI Gerbera-2 (M_1V_5).

There are numerous reports on alteration of flower color of ornamental plants arising after mutagenic treatment. Schum and Preil (1998) reported that 55% of the records on induced mutation in ornamental plants concerned changes in flower color and 15% in flower morphology.

Another objective of this study was evaluation of morphological variation of the irradiated both Chrysanthemum and Gerbera plants of (M_1V_5) stage at field/greenhouse level. So, data on morphological characters such as leaf length, plant height, flower diameter, flower stalk length, flower stalk diameter, number of petals, etc. were collected from randomly selected mother and irradiated plants. In this step, both Chrysanthemum and Gerbera (M_1V_5) plants showing promising changes in flower form and color were vegetatively propagated for two successive generations to

isolate solid mutant and developed M_1V_6 - M_1V_7 generations. During analysis of the M_1V_6 generation only floral characters were evaluated and noted. Finally, for M_1V_7 generation both vegetative and floral characters were evaluated and compared with the mother plants used as control.

After proper evaluation of the M_1V_6 - M_1V_7 generations of Chrysanthemum, it has been possible to identify four mutants from BARI Chrysanthemum-1 variety (YM1, Y1, Y5 and Y6) and three mutants from white variety BARI Chrysanthemum-2 (M1, M2 and M6) which were developed through gamma radiation. These new cultivars consist of different flower forms, shapes, and colors. In BARI Chrysanthemum -2, 5Gy radiation dose developed mutant lines M1 and M2 while M6 were developed from 15Gy. All these mutants are different colors than the mother plants. They also differed from their morphological aspect of ray, trans and disk florets. In the mother plant only one type of tubular long florets was present. Whereas, all these three mutants have developed changed ray (flat), trans (tubular with star shaped opening) and disk (tubular) florets. Again, in Chrysanthemum-1 variety, 10Gy radiation dose developed mutant lines YM1, Y5 and Y6 while Y1 was derived from 25Gy treated plants. All these four mutants where color mutants as the color have changed compared to the mother plant. Outer most rows of ray florets of YM1 are flat and the innermost two to three rows of ray florets are inward curved or loosely incurved. Y1 has a large center disk with three to four or more rows of tubal but spoon shaped tip ray florets. Y5 has a large center disk with five to six or more rows of semi flat ray florets. Y6 has a very small center disk with six to seven or more rows of spoon shaped ray florets. The disk florets of all the mutants were tubular shaped.

Similar types of changes in flower form and color through gamma radiation were observed in Chrysanthemum by some previous researchers (Lamseejan *et al*. 2000; Misra *et al.* 2003; Datta *et al.* 2005; Barakat *et al*. 2010; Soliman *et al.* 2014; Wang *et al*. 2020).

During present investigation, gamma radiation dose 5Gy and 10Gy was found most efficient as they produced most of the mutants from the Chrysanthemum varieties studied. Many authors such as Broertjes and Van Harten (1988) and Tien *et al*. (2000) suggested that the useful doses for mutation induction in Chrysanthemum were 10–20 Gy for *in vitro* cultures and 10–25 Gy for rooted cuttings.

Again, plant height of the mutants was found to be shorter than the mother plants for both Chrysanthemum varieties. Similar results were obtained in other Chrysanthemum genotypes after treatment with gamma rays (Kim *et al*. 2016). In line with our findings, Datta *et al*. (2005) also observed that the height of the plantlet was decreased significantly because of the radiation treatment. Only the plant height of M6 (50-60 cm) was the same as the mother plant's height (50-60 cm). YM1 plants' height (60-65 cm) were too short compared to the mother plants (85-100cm). Several studies have reported that radiation reduces plant height (Wang *et al*. 2020). In the growth rate parameter, gamma rays show inhibitory effects (Cheng *et al*. 2010; Kim *et al*. 2016). Primary branch number, size of the internode, leaf length and leaf breadth remained almost unchanged in mutants compared to the mother plants regardless of the variety. It was observed that gamma radiation decreased the plant height of the mutants and had an obvious effect on this parameter. It was also noted that the dose of radiation is not directly affecting any concerned factor analyzed during the present study.

It was also observed that days required for bud initiation after plantation have been decreased in case of M1, M6, Y1 and Y5 mutant lines and these mutant lines can be considered as early varieties. Similar result was found by another researcher (Datta 2015). Similar result on delaying flower bud formation after radiation treatment was reported by Kumari *et al.* (2013).

Other mutant lines took almost similar time for bud initiation compared to their mother plants used as control. All three mutant lines derived from BARI Chrysanthemum-2 produced smaller flowers compared to the mother flowers. But all the mutant lines developed from BARI Chrysanthemum-1 produced larger flowers compared to the mother flowers. So, it can be assumed that gamma radiation has an influence on days required for bud initiation and flower diameter of the resultant mutants. However, the radiation dose did not show any direct effect related to the factors tested.

No variation was seen on the non-irradiated *in-vitro* cultured Chrysanthemum variety and field grown control BARI Chrysanthemum-1 (yellow) and BARI Chrysanthemum-2 (white) flowers.

Performance of both color and shape/form changes in flowers of Gerbera mutants M_1V_6 - M_1V_7 generations were evaluated and properly recorded. It may be mentioned here that three mutant lines (WV1, WV2 and WV3) of BARI Gerbera - 1 were derived through the application of 20Gy dose. On the other hand, one mutant line (RV2) of BARI Gerbera-2 was derived from 10Gy and two mutant lines (RV1 and RV3) of the same variety were derived from 5Gy. So, gamma radiation dose 5Gy-20Gy was found to be the most effective for mutation induction in both Gerbera varieties. Similar results on mutation induction in Gerbera and Chrysanthemum were also reported by some earlier workers (Mandal *et al*. 2000; Yamaguchi *et al.* 2009).

Plant height was increased in all mutant lines of Gerbera compared to the mother plants except RV2 mutant lines. This observed variable response on plant height in case of Chrysanthemum during the present study was also noticed by some other previous studies (Cheng *et al*. 2010; Kim *et al*. 2016; Wang *et al.* 2020). Most of the mutant lines took less time for flower bud initiation and attaining full bloom stage. They also produced larger flowers compared to the mother plants. Significantly, early flowering was induced in all mutated plants compared to non-mutated plants. It has been noticed that, in Gerbera mutant lines most of the changes were noted in trans and disk florets. A significant increase in the length of flower stalk was observed in almost all mutant lines. Similar result was also reported by Ghani *et al.* (2013). So, it can be concluded that all the changes observed in both Chrysanthemum and Gerbera are due to the application of appropriate doses of radiation.

The final part of the present study was the confirmation of the genetic variations of the developed mutant lines through molecular characterization for using ISSR marker analysis. So, attempts were made to find out the genetic variation or polymorphism existed among mother and irradiated plants of *Chrysanthemum morifolium* Ramat and *Gerbera jamesonii* Bolus.

Molecular marker technology provides information that can help to define the differences of regenerated mutants and their status according to the number of close relatives and their phylogenetic position. DNA marker technology is a new approach based on DNA polymorphism among tested genotypes and thus applicable to identify mutant lines. Among several molecular markers ISSR (Inter-simple sequence repeats) has been considered as a widely used most accountable molecular marker. Several

researchers have used ISSR markers to study the polymorphism of Chrysanthemum and Gerbera (Bhatia *et al*. 2010; Shao *et al*. 2010; Gantait *et al*. 2011; Palai and Rout 2011; Baliyana *et al*. 2014; Wang *et al*. 2020; Gantait and Mahanta 2021; Nasri *et al.* 2021; Samarina *et al.* 2021). ISSR analysis showing genetic variation among untreated and radiated plants will be useful in distinguishing variants with differences in morphological characteristics (Ziarovska *et al.* 2013).

In ISSR marker study, PCR amplification conditions play a crucial role in amplification of DNA. During the present study, after analyzing several factors of PCR condition, template DNA concentration of 30 ng/µl was found to be the most suitable for obtaining reproducible banding patterns for both Chrysanthemum and Gerbera. Primers were selected carefully as some primers produced non-reproducible bands and some others could not amplify the DNA in all the investigating plants. Primers used in the present study were 10-18 nucleotides long and had the possibility of annealing at a random number of locations in the genome. The PCR cycle was also optimized during the experiment for both Chrysanthemum and Gerbera and found a total of 35 cycles was optimum for both cases. This result was in accordance with the studies on *Brassica* (Paul *et al.* 2020). However, in some other cases 28 to 45 cycles were found optimum for successful PCR amplification (Shao *et al*. 2010; Palai and Rout 2011; Ghani and Sharma 2019; Wang *et al*. 2020; Nasri *et al*. 2021).

During the present experiment, a total of 13 ISSR primers were used to find out the variation among the 9 populations (7 mutants generated from two varieties of Chrysanthemum and control mother plant of both varieties). Among all the primers tested, five primers produced clearly scorable and reproducible bands. A total of 33 reproducible and scorable bands were detected, of which 30 bands were found to be polymorphic. The number of bands produced by primers ranged from two to fourteen, with an average of 6.6 bands per primer obtained. The bands amplified with ISSR primers were in the range of 307 to 2665 bp. During this study, an average of 90.90% polymorphism was recorded for the two Chrysanthemum varieties and for the mutants developed from them. This broad range of polymorphism revealed wide diversity among the mutants developed through gamma radiation. Using ISSR marker Wang *et al*. (2020) reported 88.89% polymorphism in *Chrysanthemum morifolium* 'Donglinruixue' mutant lines produced from different gamma irradiation doses. Not

only this, Baliyan *et al*. (2014) observed 60.20% polymorphism while studying the genetic diversity of 24 genotypes of Chrysanthemum. Earlier, Kaul *et al.* (2011) also reported 100% polymorphism using RAPD marker in Chrysanthemum cv. 'Snow Ball' after gamma irradiation. These results indicate that the application of gamma irradiation may be an effective method of mutation induction, as proven previously (Pestanana *et al.* 2011, Lee and Han 2014).

The present study revealed genetic distances among different Chrysanthemum populations (mutant lines and mother plants) ranging from 0.0308 to 1.2993. It was an indication that DNA changes had occurred in these materials.

The dendrogram produced by the Unweighted Pair-Group Mean Average (UPGMA) method shows cluster analysis supported by distance linkage mapping along with the percentage of polymorphism present in each material investigated. The dendrogram formed two major clusters (7 and 6) of varieties representing BARI Chrysanthemum-1 and BARI Chrysanthemum-2 varieties along with the mutants developed from them respectively. The BARI Chrysanthemum-1 mother variety (CY) formed a separate subbranch (SB1), which was totally different from the other four mutant lines developed from BARI Chrysanthemum-1 varieties and BARI Chrysanthemum-2 as well. Among the four mutant lines developed from BARI Chrysanthemum-1 (yellow) variety Y6 and Y5 are mostly similar whereas YM1 is mostly different then Y1. Regarding BARI Chrysanthemum-2, M6 mutant lines were close to the mother plants (CW) whereas, M1 and M3 mutant lines were distinct from mother plants and included in a separate branch. Thus, there was a clear difference between the mutants and the mother varieties. This result was in accordance with the studies on *Sophora davidii* (Wang *et al*. 2017); lily (Xi *et al*. 2012) and banana (Khan, 2013).

In the case of Gerbera polymorphism study, 13 primers were used to find out the variation among six mutants generated from two varieties of Gerbera and mother plant of both varieties. Six primers produced clearly scorable and reproducible bands in all these tested 9 populations (6 mutants and both BARI Gerbera-1 as well as BARI Gerbera -2 mother plants). A total of 64 bands were produced, 57 of them were polymorphic and 10 of them were unique. The bands amplified with ISSR primers were in the range of 110-4000 bp. During this ISSR marker study, a high level of polymorphism (89.06%) was recorded for the Gerbera population studied. This broad

range of polymorphism revealed wide diversity among the mutants developed through gamma radiation. Ghani and Sharma (2019) reported 100% polymorphism when comparing gamma irradiated plants with control *G. jamesonii.* using RAPD markers. While Gong and Deng (2010) detected (67.7%) polymorphism among seven Gerbera cultivars representing three plant types (cut flower, pot, and garden) propagated by two methods (seed or tissue culture), using SSR markers. During the study, genetic distances among different Gerbera populations (mutant lines and mother plants) ranged from 0.2271 to 0.8630 and clearly indicated that in these tested materials DNA changes had occurred. It is clear evidence of successful mutation induction.

The dendrogram produced for Gerbera showed that all the germplasm was grouped into two major branches/clusters (B1, and B1, 6). The WV3 mutant line was found to be the most distinct mutant line produced during the study. The SB1, 5 separated BARI Gerbera-1 (GW) showing differences from WV1 and WV2 which are included in a sub-subbranch SSB1, 1. The SB1, 4 produced two sub subbranches where SSB1, 2 included BARI Gerbera-2 (GR) and RV3 as CR showed similarity with RV3 mutant lines. Another SSB2, 3 included RV1 and RV 2 as they have less genetic distance between them. Thus, the study indicated that there was a clear difference among the mutant lines developed through gamma radiation and the mother plant varieties.

From the foregoing discussion, it may be concluded that during the present investigation, it has been possible to develop high frequency regeneration system for the studied two varieties of Chrysanthemum and two varieties of Gerbera growing in Bangladesh. It has also been possible to develop seven mutants which were confirmed based on their morphological characters (color and shape) from two studied Chrysanthemum varieties using *in vitro* gamma radiation. Whereas six color and shape mutants has been developed from two Bangladeshi Gerbera varieties. Those mutant lines of Chrysanthemum and Gerbera have been confirmed through morphological study for two successive vegetative generations using vegetative and floral characters. Finally, PCR based molecular study ISSR was carried out to confirm stable mutation and isolate stable promising mutant lines.

There are some reports on the development of some mutant lines of Chrysanthemum and Gerbera plants through gamma radiation (Laneri *et al.* 1990; Jerzy and Lubomski 1992; Hasbullah *et al.* 2012; Kumari *et al.* 2013; Soliman *et al*. 2014) however, very

few follow up reports are available on the establishment of solid mutant lines followed by molecular marker ISSR study. To the best of our knowledge, this is the first report in Bangladesh, on the successful development of promising mutant lines through *in vitro* gamma radiation using Gerbera varieties as well as one Chrysanthemum variety and one local popular imported variety. The developed mutant lines of both Chrysanthemum and Gerbera were analyzed for the two vegetative generations (M1V6 and M1V7) in field/greenhouse. Based on this study, it can also be reported as first report in Bangladesh using Bangladeshi varieties on the successful development of stable promising Chrysanthemum and Gerbera mutants. During ISSR analysis, a high level of polymorphism 90.90% was recorded in Chrysanthemum. Whereas in Gerbera 89.06% polymorphism was observed. This high level of polymorphism is the confirmation of the change in DNA or mutation as well as diversity. Thus, newly developed seven Chrysanthemum and six Gerbera mutant lines can add more diversity in color and shape of these two economically important flowers of Bangladesh.

The promising mutant lines identified through the research of the present investigation need further advanced molecular testing to find out the genes responsible for flower color and shape changes. Moreover, before consideration for releasing these mutant lines (of Chrysanthemum and Gerbera) further confirmation using sequencing is needed.

Both Chrysanthemum and Gerbera are extreme heterozygous in nature and self-incompatible. Based on present experimental results, it may be concluded that the *in vitro* mass propagation protocol developed in the present investigation could be commercially profitable. Only a very limited number of varieties of Chrysanthemum and Gerbera are available in the local market. However, it is a natural demand from traders as well as from buyers to have newer and newer varieties of economically important ornamental plants. The present study clearly indicates that stable flower color and form/shape mutant lines can be developed through gamma radiation using *in vitro* techniques. The diversifications developed through gamma radiation would be useful for different breeding programs of floriculture and can contribute to the local and global floral market. So, our local varieties of Chrysanthemum and Gerbera with

new fascinating characters could be a major source of foreign exchange as non-traditional export items.

It may be mentioned that as a successful nation, Bangladesh is rising in many sectors including agriculture. But more attention is needed for floriculture, which is a new arena of agriculture. More tangible efforts are also needed for the promotion of investment from government and private companies in this sector.

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7. APPENDIX – A

ABBREVIATIONS

The following abbreviations have been used throughout the text:

%	:	Percentage
µ	:	Micron
µg	:	Microgram
µl	:	Micro liter
µM	:	Micromole
1 N	:	1 Normal
2, 4-D	:	2, 4-dichlorophenoxy acetic acid
AERE	:	Atomic Energy Research Establishment
ANOVA	:	Analysis of Variance
BAP	:	6-benzylaminopurine
BARI	:	Bangladesh Agriculture Research Institute
BBS	:	Bangladesh Bureau of Statistics
bp	:	base pair
C	:	Centigrade / Celsius
$CaCl_2$	:	Calcium chloride
Cm	:	Centimeter (s)
Co	:	Cobalt
Co_{60}	:	cobalt $_{60}$
CTAB	:	Cyle tetramethyl ammonium bromide
CIE	:	CIE Lab
cv.	:	cultivar
dNTP	:	Deoxy nucleoside tri-phosphate
EPB	:	Export Promotion Bureau
HRC	:	Ornamental and Floriculture Division of the Horticulture Research Center
e. g.	:	Example gratia, for example
et. al.	:	et alil and others
etc	:	et cetra, and the rest
FAO	:	Food and Agriculture Organization
Fig/s	:	Figure / Figures
FW	:	Fresh weight

G	:	gram (s)
Gy	:	Gray
Ha (s)	:	Hectare
$HCCL_3$	:	Chloroform
HCL	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
Hr (s)	:	Hour (s)
i. e.	:	id est = which to say in other words
IAA	:	Indole- 3 – acetic acid
IBA	:	Indole- 3 – butyric acid
IAEA	:	International Atomic Energy Agency
ISSR	:	Inter-simple sequence repeats
IFRB	:	Institute of Food and Radiation Biology
Kb	:	Kilo base pair
Kcal	:	Kilocalorie
Kn	:	Kinetin (6- furfurylaminopurine)
KNO_3	:	Potassium nitrate
L	:	Litre
LB	:	Liquid Broth
lb / sq. inch	:	Pound per square inch
LD_{50}	:	Lethal $Dose_{50}$
m	:	Meter (s)
MAP	:	Marker-assisted Plant
M	:	Molar
mg / l	:	Milligram per liter
mg	:	Milligram
min (s)	:	Minute (s)
ml (s)	:	Milliliter (s)
mm	:	Milimeter
mM	:	Millimolar
MS	:	Murashige and Skoog Medium 1962
MT	:	Metric tonne
MW	:	Molecular weight
AFLP	:	Amplified fragment length polymorphism
SSR	:	Short sequence repeat
Na_2 – EDTA	:	Sodium salt or ferric ethylene diamine tetra acetate

NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium hydroxide
NH_4NO_3	:	Ammonium nitrate
Nm	:	Nanometer
No.	:	Number
PCR	:	Polymerase Chain Reaction
pH	:	Negative logarithm of Hydrogen
RAPD	:	Random amplified polymorphic DNA
R.H.S	:	Royal Horticulture Society Color
RHSC	:	Royal Horticulture Society Color Charts
rpm	:	Rotation per minute.
sec.	:	Second
Sp. / Spp.	:	Species
Tk	:	Taka
TBq	:	Terabecquerel, 10^{12} Bq (Becquerel)
t	:	Ton
TAE	:	Tris-acetate-EDTA
US	:	United States
USD	:	United States dollar
$	:	United States dollar
USDA	:	United States Department of Agriculture.
UV	:	Ultraviolet Wavelength
UK	:	United Kingdom
UPGMA	:	Unweighted Pair Group Method of Arithmetic Means
v / v	:	Volume by volume
Viz	:	Namely
w / v	:	Weight by volume
Wt.	:	Weight
M1	:	Daisy- like bright purple BARI Chrysanthemum-1 mutant derived from 5Gy treatment
M2	:	Daisy- like light purple BARI Chrysanthemum-1 mutant derived from 5Gy treatment
M6	:	Daisy- like light purple BARI Chrysanthemum-1 mutant derived from 15Gy treatment
CW	:	BARI Chrysanthemum-1 mother plant
CY	:	BARI Chrysanthemum-2 mother plant

Y1	:	Daisy-like bright yellow BARI Chrysanthemum-1 mutant derived from 25Gy treatment
YM1	:	Daisy-like vivid yellowish orange mutant of BARI Chrysanthemum-1 derived using 10 Gy treatment.
Y5	:	Daisy- like vivid yellowish orange mutant of BARI Chrysanthemum-1 derived using 10 Gy treatment.
Y6	:	Daisy- like vivid yellowish orange mutant of BARI Chrysanthemum-1 derived using 10 Gy treatment.
GCW	:	BARI Gerbera-1 mother plant
WV1	:	Daisy-like yellowish white mutant of BARI Gerbera-1 derived using 20 Gy treatment.
WV2	:	Sunflower-like yellowish or off-white colored and oval shaped mutant of BARI Gerbera-1 derived using 20 Gy treatment.
WV3	:	Daisy-like yellowish white mutant (with a pink tinge) of BARI Gerbera-1 derived using 20 Gy treatment.
CR	:	BARI Gerbera-2 mother plant
RV1	:	Daisy- like dark red colored mutant of BARI Gerbera-2 derived using 5 Gy treatment.
RV2	:	Daisy-like orangish red colored and a spiky center containing mutant of BARI Gerbera-2 derived using 10 Gy treatment.
RV3	:	Daisy-like orangish red colored and a spiky center containing mutant of BARI Gerbera-2 derived using 5 Gy treatment.

APPENDIX - B

Murashige and Skoog (MS) Medium 1962

Components	**Concentration**
Macronutrients	**(mg/l)**
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.2H_2O$	440.00
$MgSO_4.7H_2O$	370.00
Micronutrients	
$FeSO_4.7H_2O$	27.80
Na_2-FeEDTA	37.30
$MgSO_4.4H_2O$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2MoO_4.\ 2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine-HCl	0.10
Inositol	100.00
Sucrose	30,000.00

pH adjusted to 5.8 before autoclaving.