Mutation Induction by Gamma Irradiation in Chrysanthemum

[*Chrysanthemum x grandiflorum* (Ramat.) Kitam.]

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

Prince of Songkla University

2012

Thesis Title	Mutation Induction by Gamma Irradiation in Chrysanthemum [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.]
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ชื่อวิทยานิพนธ์ การชักนำการกลายพันธุ์ในเบญจมาศ [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.] โดยการฉายรังสีแกมมา

ผู้เขียน นางสาวชาลินี ถังมณี

สาขา ชีววิทยา

ปีการศึกษา 2554

บทคัดย่อ

ได้เพาะเลี้ยงกลีบดอกของเบญจมาศ [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.] บนอาหาร สูตร Murashige and Skoog (MS) ที่เติม BA และ 2, 4-D ที่ความเข้มข้นต่าง ๆ ผลการทดลองพบว่า บนอาหารสูตร MS ที่มี BA ความเข้มข้น 13.3 ไมโครโมลาร์ ร่วมกับ 2, 4-D ที่ความเข้มข้น 0.5 ไมโครโมลาร์ ให้ประสิทธิภาพการเกิดแคลลัสสูงสุด หลังจากการเพาะเลี้ยง 1 เดือน แคลลัสพัฒนายอดจำนวนมาก ยอดเกิดสูงสุด (45%) บนอาหารที่มี Kinetin ความเข้มข้น 9.3 ไมโครโมลาร์ ร่วมกับ IBA ที่ความเข้มข้น 4.9ไมโครโมลาร์ การเกิดรากจากยอดเป็น 100 % ในทุกการทดลอง แต่อย่างไรก็ตาม อาหารสูตร MS ที่มี IBA ความเข้มข้น 12.3 ไมโครโมลาร์ ให้จำนวนรากต่อยอดสูงสุด (40.0 ราก) การศึกษาทางเนื้อเยื่อวิทยาแสดงให้เห็นว่ายอดมีจุดกำเนิดมาจากเซลล์เนื้อเยื่อเจริญเพียงเซลล์เดียวที่มีไซโทพลาสซึมเข้มข้น และนิวเคลียสย้อมติดสีได้ดี

แคลลัสอายุ 1 เดือนของเบญจมาศ ที่เพาะเลี้ยงบนอาหาร สูตร MS ที่มี BA ที่ความเข้มข้น 13.3 ไมโครโมลาร์ ร่วมกับ 2, 4-D ที่ความเข้มข้น 0.5 ไมโครโมลาร์ ฉายด้วยรังสีแกมมา แบบเฉียบพลันที่ความเข้มของรังสี 0, 5, 10, 15, 20, 25 และ 30 เกรย์ ผลการทดลองพบว่า เปอร์เซ็นต์การรอดชีวิตของแคลลัสลดลงเมื่อเพิ่มปริมาณความเข้มของรังสี แคลลัสที่ไม่ผ่านการฉายรังสีให้การรอดชีวิต 100% แคลลัสที่ฉายรังสีที่ความเข้ม 30 เกรย์ มีอัตรารอดชีวิตต่ำสุด 38.7% ความเข้มของรังสีแกมมาที่ฉายแบบเฉียบพลัน

แล้วทำให้เกิดการตายของแคลลัสครึ่งหนึ่ง (LD_{50}) คือ ที่ความเข้ม 26 เกรย์ ปริมาณของรังสีเพิ่มขึ้นส่งผลให้เปอร์เซ็นต์ของการเกิดยอดจากแคลลัสลดลง เปอร์เซ็นต์การเกิดยอดสูงสุดเป็น 42.3 จากแคลลัสที่ผ่านการฉายรังสี 5 เกรย์ แต่อย่างไรก็ตามจำนวนของยอดเฉลี่ยต่อแคลลัสสูงสุดที่ 15 เกรย์ โดยมีนัยสำคัญทางสถิติ นอกจากนี้ แคลลัสที่ผ่านการฉายรังสีในทุกการทดลองให้จำนวนของยอดต่อแคลลัสสูงกว่าในการทดลองชุดควบคุมที่ไม่ฉายรังสี

ต้นเบญจมาศที่เจริญเติบโตมาจากแคลลัสที่ผ่านการฉายรังสี และไม่ฉายรังสี ร่วมถึงเบญจมาศที่เจริญมาจากสภาพธรรมชาติ ถูกนำมาวิเคราะห์ปริมาณ DNA โดยใช้วิธีโฟลไซโทเมทรี พบว่าปริมาณ DNAในนิวเคลียสของเบญจมาศที่เจริญมาจากแคลลัสที่ไม่ผ่านการฉายรังสี และเบญจมาศในสภาวะธรรมชาติเป็น 24.28 และ 24.98 พิโคกรัมตามลำดับ ส่วนเบญจมาศที่เจริญมาจากแคลลัสที่ผ่านการฉายรังสี มีปริมาณ DNA ต่ำกว่าในทุกชุดการทดลอง พืชที่ได้มาจากแคลลัสที่ผ่านการฉายรังสีที่ 15, 20, และ 25 เกรย์ มีปริมาณ DNA ในนิวเคลียสลดลงเป็น 10.99, 18.80, และ 16.91 พิโคกรัม ตามลำดับ จำนวนโครโมโซมของเซลล์ปลายรากของต้นที่พัฒนาจากแคลลัสที่ได้รับรังสีความเข้มข้นข้างต้นมีจำนวน 27, 37, และ 34 แท่ง ตามลำดับ ส่วนโครโมโซมของเบญจมาศที่เจริญในสภาพธรรมชาติมีจำนวน 54 แท่ง

การเปลี่ยนแปลงรูปร่างลักษณะภายนอกของยอด และใบถูกพบในพืชที่ได้จากแคลลัสที่ผ่านการฉายรังสี ลักษณะไคเมอราของใบปรากฏในพืชที่เจริญมาจากแคลลัสที่ผ่านการฉายรังสีที่ความเข้ม 30 เกรย์ ในขณะที่ลักษณะยอดสั้นเป็นกระจุก การเชื่อมต่อกันเป็นมัดของท่อลำเลียงของลำต้น ใบมีลักษณะคล้ายกับดอกกุหลาบ และลักษณะใบเป็นแก้ว ปรากฏในพืชที่ผ่านการฉายรังสีที่ 15 เกรย์

ความแปรปรวนทางพันธุกรรมของต้นเบญจมาศที่เจริญมาจากแคลลัสทั้งที่ไม่ได้รับ และได้รับรังสี ถูกตรวจสอบโดยวิธีอาร์เอพีดี จากการใช้ไพรเมอร์แบบสุ่มจำนวน 13 ไพรเมอร์ ซึ่งแต่ละไพรเมอร์มีขนาด 10 นิวคลีโอไทด์ พบว่าต้นเบญจมาศที่เจริญมาจากแคลลัสของกลีบดอกที่ผ่านการฉายรังสีแกมมาที่ความเข้มต่างๆ มี 5 ไพรเมอร์ คือ

OPAB-09, OPA-03, OPAB-14, OPB-08, OPB-18 ให้แถบดีเอ็นเอ ลักษณะ polymorphic สามารถใช้แยก mutant ได้ โดยให้แถบดีเอ็นเอที่มีขนาดโมเลกุลแตกต่างกันทั้งหมด 97 แถบ ซึ่งเป็นแถบดีเอ็นเอลักษณะ polymorphic จำนวน 31 แถบ จำนวนแถบดีเอ็นเอที่ได้ต่อไพรเมอร์มีจำนวน 6 ถึง 28 แถบ ขึ้นอยู่กับไพรเมอร์แต่ละชนิด โดยมีค่าเฉลี่ยของแถบดีเอ็นเออยู่ที่ 19.4 แถบต่อไพรเมอร์ และมีขนาดโมเลกุลอยู่ในช่วง 300-3000 คู่เบส ผลจากการใช้ไพรเมอร์ OPB-18 ตรวจสอบความแปรปรวนทางพันธุกรรมของต้นเบญจมาศที่เจริญมาจากแคลลัสของกลีบดอกที่ผ่านการฉายรังสีแกมมาที่ความเข้มต่างๆพบว่า ต้นเบญจมาศที่เจริญมาจากแคลลัสที่ผ่านการฉายรังสี 20, 25 และ 30 เกรย์ ไม่ปรากฏแถบดีเอ็นเอขนาด 500 และ 700 คู่เบส เมื่อเปรียบเทียบกับพืชในชุดการทดลองอื่นๆ แสดงให้เห็นว่าพืชที่ได้จากการฉายรังสีที่ความเข้มดังกล่าวมีความแปรปรวนทางพันธุกรรมเกิดขึ้นเมื่อเปรียบเทียบกับพืชในชุดการทดลองอื่นๆ นอกจากนี้ยังพบแถบดีเอ็นเอขนาด 3,000 คู่เบส ปรากฏเฉพาะในต้นเบญจมาศที่เจริญมาจากแคลลัสที่ผ่านการฉายรังสีแกมมาที่ความเข้ม 30 เกรย์ เมื่อตรวจสอบจีโนมิกดีเอ็นเอด้วยไพรเมอร์ OPB-08 ชี้ให้เห็นว่าพืชที่เจริญมาจากแคลลัสที่ผ่านการฉายรังสีที่ความเข้มนี้ มีความแปรปรวนทางพันธุกรรมเกิดขึ้นเมื่อเปรียบเทียบกับพืชที่ได้จากสภาวะอื่นๆ และเช่นเดียวกันกับการไม่ปรากฏแถบดีเอ็นเอขนาด 900 คู่เบส ในต้นเบญจมาศที่เจริญในสภาพธรรมชาติ และที่เจริญมาจากแคลลัสที่ไม่ผ่านการฉายรังสี แต่กลับปรากฏแถบดีเอ็นเอดังกล่าวในต้นเบญจมาศทุกต้นที่ผ่านการฉายรังสี เมื่อตรวจสอบจีโนมิกดีเอ็นเอด้วยไพรเมอร์ OPB-08 เช่นเดียวกัน ผลการทดลองนี้ชี้ให้เห็นว่าต้นเบญจมาศที่เจริญในสภาพธรรมชาติและที่เจริญมาจากแคลลัสที่ไม่ผ่านการฉายรังสี มีพันธุกรรมที่แตกต่างจากต้นเบญจมาศที่ผ่านการฉายรังสี

จากผลการวิเคราะห์อาร์เอพีดี พบว่าความแปรปรวนทางพันธุกรรมของพืชเพิ่มขึ้นเป็นสัดส่วนโดยตรงกับปริมาณรังสีที่พืชได้รับ อย่างไรก็ตามไพรเมอร์ที่ใช้ในการศึกษาครั้งนี้ไม่สามารถแยกพืชสายพันธุ์กลายที่ผ่านการฉายรังสีแกมมาที่ 15 เกรย์ ออกจากสายพันธุ์กลายอื่นๆได้ แม้ว่าพืชสายพันธุ์กลายดังกล่าวจะมีลักษณะที่

แตกต่างจากพืชสายพันธุ์กลายอื่นๆอย่างชัดเจน (การเปลี่ยนแปลงรูปร่าง, การลดลงของจำนวนโครโมโซม และการลดลงของปริมาณของดีเอ็นเอ)

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Major Program	Biology
Academic Year	2011

ABSTRACT

Ray florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. were cultured *in vitro* on Murashige and Skoog (MS) medium containing various combinations and concentrations of BA and 2,4-D. The MS medium supplemented with 13.3 µM BA and 0.5 µM 2, 4-D yielded the highest efficiency of callus formation after 1 month of culture. The calluses differentiated into adventitious shoots. The highest number of regenerated shoots (45.0%) was obtained on MS medium supplemented with 9.3 µM kinetin and 4.9 µM IBA while the rooting of shoots reached 100% in all experiments. However, MS medium containing 12.3 µM IBA induced the highest number of roots per shoot (40.0). Histological observation showed that shoot formation originated from a single meristematic cell that had a dense cytoplasm and a well stained nucleus.

The one-month-old calluses of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. on MS medium supplemented with 13.3 µM BA and 0.5 µM 2, 4-D were acute irradiated with 0, 5, 10, 15, 20, 25, and 30 Gy gamma rays. The survival percentage of the calluses decreased with an increasing irradiation dose. The untreated calluses produced a 100% of the survival while the survival percentage of the calluses at 30 Gy was the lowest. The half lethal dose (LD_{50}) of acute gamma ray was found to be 26 Gy. The irradiation dose negatively affected regeneration rate of the calluses. With a gradual increase of irradiation doses there was a gradual decrease in the

percentage of shoot regeneration. The optimal percentage of adventitious shoot regenerated from the irradiated calluses was 42.3 at 5 Gy. However, the mean number of shoots per callus was significantly highest at 15 Gy ($p \leq 0.05$). In addition, the irradiated calluses in all experiments provided the higher numbers of shoots per callus than the untreated control.

The nuclear DNA content of treated, untreated calluses and natural plants was analyzed by flow cytometry. The nuclear DNA content in plants regenerated from untreated calluses and plants in natural condition was 24.98 pg while the nuclear DNA content in plants regenerated from the irradiated calluses was lower in all treatments. Plantlets derived from treated calluses with 15, 20 and 25 Gy have the nuclear DNA content of 10.99, 18.80 and 16.91 pg in concomitant with the somatic chromosome numbers of 27, 37, and 34, respectively while the natural plants was 54.

Variations in the morphological characteristic of shoots and leaves were found in the plants from the irradiated calluses. Leaf chimera was evidenced in plantlets regenerated from calluses treated with 30 Gy. While with 15 Gy treatment, short compact shoots, fasciations, rosette leaf and vitrification were observed.

Thirteen random primers were screened for their ability to amplify the genomic DNA of the *Chrysanthemum x grandiflorum* (Ramat.) Kitam. and its mutants, five informative RAPD primers (OPAB-09, OPA-03, OPAB-14, OPB-08, OPB-18) gave reproducible polymorphic bands to distinguish some of the mutants from their parents. These five primer combinations generated a total of 97 reproducible amplification fragments across all radiomutants and parent cultivars, among which 31 bands were polymorphic. The number of amplified RAPD bands varied from a minimum of 6 to a maximum of 28 depending on the primer and the DNA sample with a mean value of 19.4 bands per primer. The size of fragments ranged from 300 to 3,000 bp. PCR amplification with primer OPB-18 clearly revealed that in plant regenerated from irradiated calluses at 20, 25, and 30 Gy a highly specific band (500 and 700 bp) is absent in comparison to the plants in other doses

and the plants in natural conditions indicating that they are highly polymorphic when compared to other mutants. Moreover, a band of 3000 bp was noticed only in plants regenerated from irradiated calluses at 30 Gy when the genomic DNA was amplified with primer OPB-08, indicating that the plants regenerated from irradiated calluses at 30 Gy is polymorphic when compared to plants derived from other conditions. Similarly, a polymorphic band of 900 bp is absent in natural plants and plants regenerated from untreated calluses but present in all plants regenerated from irradiated calluses when RAPD marker OPB-08 was used. This result showed that their cultivars are highly polymorphic compared to plants regenerated from irradiated calluses. The analysis of RAPD result showed that genetic variation of regenerated plantlet was proportional to the dosage of gamma ray within a certain range. However, the primers used in the present study could not discriminate the radiomutants derived from 15 Gy gamma irradiation from the other radiomutants, although this radiomutants seemed to be the most characteristically different from other accessions of mutant (a change in morphology**,** a decreasing in chromosome number and also in the nuclear DNA content).

ACKNOWLEDGEMENTS

I wish to express my deepest appreciation and grateful thanks to my advisor, Associate Professor Dr. Kamnoon Kanchanapoom for his kindness, valuable advice, guidance, encouragement, and constructive criticism, which enable me to carry out this thesis successfully.

The sincerest thanks are expressed to my thesis co-advisor, Associate Professor Dr. Sompong Te-Chato who gave helpful suggestion on RAPD techniques.

Special thanks go to the examining committees: Associate Professor Dr. Sontichai Chanprame, Department of Agronomy, Faculty of Agriculture Kamphaeng Saen, Kasetsart University, and Associate Professor Ladda Eksomtramage, Prince of Songkla University for their helpful suggestions and for dedicating valuable time for the thesis examination.

I also wish to thank the staffs of Laboratory of Crop Biotechnology, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University especially Miss. Tassanee Niyakit, for their help while carrying out a part of RAPD research project there.

My very special appreciation also goes to my colleagues at the Plant Biotechnology Research Unit, Department of Biology, for their encouragement and help throughout my research work.

I am extremely thankful to the Prince of Songkla University Graduate Studies Grant for supporting my Ph.D. research.

Finally, I owe much love to all at home, my husband, my young brothers and my young sisters especially my parents who always give me love and care.

Chalinee Thangmanee

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LIST OF ABBREVIATIONS AND SYMBOLS

2, 4-D	=	2, 4-Dichlorophenoxyacetic acid
ABA	=	Abscisic acid
ACC	=	1-aminocyclopropane-1-carboxylic acid
BA	=	N^6-Benzyladenine
BAP	=	6-Benzylaminopurine
DAPI	=	4, 6-diamidino-2-phenylindole
FAA II	=	formalin–acetic acid–alcohol
Gy	=	gray
IAA	=	3-Indoleacetic acid
IBA	=	Indole-3- butyric acid
KN or Kinetin	=	6-furfurylaminopurine
kV/min	=	kilovolt per minute
kPa	=	kilopascals
LD_{50}	=	50% lethal dose
MS	=	Murashige and Skoog (1962)
NAA	=	1-Naphthaleneacetic acid
ng	=	nanogram
p	=	probability level
PCR	=	polymerase chain reaction
PDB	=	P-dichlorobenzene
pg	=	picogram
PGR	=	plant growth regulator
PI	=	propidium iodide
PVP	=	polyvinylpyrrolidone
RAPD	=	random amplified polymorphic DNA
rpm	=	revolution per minute
SE	=	standard error

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

TCLs	=	thin cell layers
UV	=	Ultra-violet light
λ DNA	=	lamda DNA

CHAPTER 1
INTRODUCTION

1.1 Introduction

Chrysanthemum [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.], also known as florist's chrysanthemum has become a major floriculture crop in the word. It has inflorescence in the form of calathidium. It is cultivated both as a cut-flower and as a potted plant (pot mums). The first cultivated species originated in China over 2000 years ago (Teixeira 2003 a). Conventional breeding techniques, such as hybridization breeding, play an important role in the development of chrysanthemum cultivars. However, with cross breeding, it takes long period of time to breed a satisfactory cultivar, on the other hand, there are limitations to this technique as follows:

Firstly, there is the limited gene pool of any species, e.g., no plant species possesses the genes required for producing varieties with the full spectrum of colors (Mol *et al.,* 1989).

Secondly, distant crosses may be limited by incompatibility or differences in ploidy level between putative parents (Lowe *et al.,* 1993).

Thirdly, characteristics such as uniform growth and synchronous flowering are polygenic. Hence, sexual crossing may alter the delicate balance of factors determining plant growth and shape (Mol *et al.,* 1989).

As the demand for novel, high quality cultivars is increasing, cross breeding alone can no longer meet this demand. Various alternative breeding techniques have been used to produce new chrysanthemum cultivars. Of all the alternative methods, mutation techniques, especially *in vitro* mutation technique, might be more suitable for chrysanthemum breeding (Broertjes *et al.,* 1976; Mandal *et*

al., 2000 a; Chen *et al.*, 2003). This is because most chrysanthemum cultivars are polyploids belonging to a hexaploid species with an average chromosome number of 54, possess complex genetic background, and are mainly propagated asexually (Dowrick, 1953; Li, 1993), resulting in a readily genetic variation when *in vitro* mutation is conducted.

In the mutation breeding program, breeders usually hope to get plantlets with greater genetic variation compared with the parents. Mutation breeding by radiation, an agricultural application of nuclear technology, has been widely utilized to upgrade the well-adapted plant varieties by one or two major traits and also develop new varieties with improved agricultural characteristics. Breeding programs have focused on important characteristics of ornamental value, including flower color, size and form, production quality and reaction to the environment (Broertjes *et al.*, 1980). Many techniques and sources of variability in chrysanthemum breeding are currently in use. Mutation induction is an important tool for breeding new cultivars, and many cultivars have been produced using spontaneous (sports) and induced mutation. Gamma rays and X-rays are widely used for mutation induction in chrysanthemum. However, radiation treatment induces damage, such as chromosomal aberrations, in plants. Dose of radiation is one important factor of radiation treatment and its effect has been investigated by evaluating various traits, including lethality (Broertijes, 1968; Killion and Constantin, 1971; Sripichit *et al.*, 1988), growth (Yamashita, 1964; Killion and Constantin, 1971; Killion *et al.*, 1971; Bottino *et al.*, 1975), and fertility (Yamashita, 1964; Killion and Constantin, 1971). Therefore, it is believed that the dose of radiation also influences damage in chrysanthemum.

1.2 Literature Review

1.2.1 Review of chrysanthemum *in vitro* culture

Chrysanthemum is usually cultivated by vegetative cuttings or suckers. This method has a low multiplication rate and often the plants are low quality. Since

cuttings are obtained repeatedly from mother plants, there may be subjected to any virus infection and degeneration, thereby increasing production costs (Hahn *et al.,* 1998; Kim *et al.,* 2003). These problems have been solved by applying micropropagation methods, which are routinely applied to the clonal propagation of a variety of horticultural plants including chrysanthemum (Ben-Jacov and Langhans, 1972). The large-scale propagation of disease-free plants by *in vitro* disease-free shoot proliferation would be costly. However, the production of mother stock plants by micropropagation (Previati *et al.,* 2008), starting from disease-free shoots could be effective and would guarantee a production of healthy cuttings. An alternative way to defend against pathogens, but also insect pests, could be breeding resistant genotypes.

A decade ago, the protocols for rapid true to type, disease-free propagation has been developed in chrysanthemum through bud/shoot proliferation (Grewal *et al*., 1996). Murashige (1990) stated that clonal plant propagation is the most extensive and visible application of tissue culture. Rapid clonal plant propagation *in vitro* can be obtained through bud or shoot proliferation (Pierik, 1990). Cell and tissue culture propagation has been reviewed in chrysanthemum (Horst, 1990). Widiastoety (1987) cultured three types of explant (shoot tips, axillary buds and leaf pieces) in a modified MS medium. Callus was formed by 81% of the shoot tips in 11-12 days, 86% of the axillary buds in 11-13 days, and 50% of the leaf pieces in 13-14 days.

Many workers in the past have reported micropropagation of *Chrysanthemum morifolium* Ramat. (now *Dendranthema grandiflora* Tzvel.) from shoot tips and axillary buds (Ben and Langhans, 1972; Roest and Bokelmann, 1973; Earle and Langhans, 1974 b; Bush *et al.,* 1976; Grewal and Sharma, 1978; Wang and Ma, 1978; Lee *et al.,* 1979; Lazar *et al.,* 1981; Lazar and Cachita, 1982; de Donato and Perucco, 1984; Gertsson and Andersson, 1985; Ahmed, 1986; Ahmed and Andrea, 1987; Sun and Li, 1987; Widiastoety, 1987; Kim and Kim, 1988; Prasad and Chaturvedi, 1988; Bhattacharya *et al.,* 1990; Endo *et al.,* 1991; Dikshit *et al.,* 1997; Deruiter, 1997; Radojevic *et al.,* 2000 and Wankhede *et al.,* 2000), callus derived from stem and leaf explants (Hill, 1968; Earle and Langhans 1974 b) and floral parts (Bush *et al.,* 1976; Roest and Bokelmann, 1975; Lee *et al.,* 1979; Sutter and

Langhans, 1981; Lazar *et al.*, 1981; Lazar and Cachita, 1982; Chen *et al.*, 1985; Prasad and Chaturvedi, 1988; Ohishi and Sakurai. 1988; Khalid *et al.*, 1989; Lu *et al.*, 1990; Kaul *et al.*, 1990; Bhattacharya *et al.*, 1990; Corneanu and Corneanu, 1992; Kumar and Kumar, 1995 and Kumari and Varghese, 2003), leaf explant (Jarzina *et al.*, 1982; Endo *et al.*, 1990; Ledger *et al.*, 1991 ; Dejong *et al.*,1993; Pavingerova *et al.*, 1994; Lee *et al.*, 1997; Sherman *et al.*, 1998; Oka *et al.*, 1999 and Park *et al.*, 2002).

Liu *et al.* (1994) achieved the highest production frequency of adventitious buds from shoot tip explants of *Dendranthema grandiflora*. Dejong *et al.* (1993) reported that explants from leaves of *in vitro* grown chrysanthemum (*Dendranthema grandiflora* Tzvel.) cultivars regenerated adventitious shoots without an intermediate callus phase. Chen *et al.* (1985) cultured young leaves from 8 chrysanthemum cultivars on Murashige and Skoog (MS) medium supplemented with BAP at 3-5 mg/l + NAA at 2 mg/l and found it best for bud induction. Urban *et al.* (1994) reported the regeneration of shoots from the leaf segments of Iridon and Helka cultivars of chrysanthemum. Mityushkina *et al.* (1995) regenerated adventitious shoots from leaf explants of 32 of 40 *Chrysanthemum morifolium* cultivars. Oka *et al.* (1999) reported that adventitious buds were mainly formed at the cut ends of primary leaves of garland chrysanthemum after 6-9 days culture on MS medium supplemented with 0.1 mg/l BAP and 0.1 mg/l NAA. Lazar and Cosma (1983) used buds of the cv. ‘Super Yellow’ and reported that good bud and shoot differentiation was obtained with IAA at 0.1 mg/l and BA at 10 mg/l.

Kaul *et al.* (1990) regenerated adventitious shoots from leaf and stem explants of 11 chrysanthemum cultivars on MS basal medium supplemented with BA and NAA 5 μM each. Regeneration of chrysanthemum plants has also been reported from florets (Lizuka *et al.*, 1973; Roest and Bokelmann, 1975; Bush *et al.*, 1976; Singh *et al.*, 1996; Enjoo *et al.*, 1998; Annadana *et al.*, 2000). Kim and Kim (1988) regenerated shoots directly from internodes and leaf segments of *Dendranthema indicum* and *Dendranthema zawadskii* on basal media supplemented with 3.0 mg/l BAP and 0.2 mg/l IAA. Datta *et al.* (2002) stated that leaf explants of chrysanthemum

differentiated shoot buds in the presence of BA and IAA, while the flower head and receptacle differentiated in the presence of BA and NAA.

Khalid *et al.* (1989) reported that florets of chrysanthemum are ideal explants for inducing somaclonal variations. Plants raised through florets produced more variation than those rose through leaf and stem explants. Mizutani and Tanaka (1994) cultured florets and corollas of 15 cultivars of chrysanthemum (*Dendranthema morifolium*) *in vitro* and reported that for floret and corollas the survival rate ranged 32-100 and 0-100%, respectively, whilst regeneration rate ranged 0-52 and 0-64%, respectively.

Lu *et al.* (1990) achieved direct plant regeneration using fresh *Chrysanthemum morifolium* Ramat. cv. 'Royal Purple' stem segments cultured on MS media supplemented with BA at 0.5-2.0 mg/l and NAA at 0.2-2.0 mg/l.

Yang *et al.* (1995) reported that the regeneration of shoots had occurred directly from internode explants of *in vitro* grown plants of *Dendranthema grandiflora*.

Hoque and Fatema (1995) reported that shoot tips showed comparatively better response to multiple shoot regeneration than the nodal segments, whereas in contrast, Hoque *et al.* (1998) obtained best response towards multiple shoot regeneration from the nodal explants on MS medium containing 1.0 mg/l BAP and 0.5 mg/l NAA. The response of node and young leaf explants was better compared with inter-nodal explant in terms of callus induction and growth. Regeneration was best in nodal explant followed by young leaves and internodes (Kumari *et al.,* 2001).

Gao *et al.* (2001) confirmed that stem explants were superior to leaf explants. It appears from the literature that there is still a need to study the regeneration of chrysanthemum *in vitro* culture using different explants sources.

The potential for micropropagation and rapid commercial multiplication of chrysanthemum has been significantly increased by several researcher workers using different sources of explants including petals segments (Jesmin *et al.,* 2007; Nahid *et al.,* 2007; Barakat *et al.,* 2010).

1.2.2 Callus formation and its culture

In nature, callus develops by infection of microorganisms from wounds due to stimulation by endogenous growth hormones, the auxins and cytokinins. However, it has been artificially developed by adopting tissue culture techniques. This technique has a great potential particularly in creating genetic variability. Callus has been successfully induced from numerous explants. However, the success of callus initiation is dependent on the explants source and the composition of the culture medium. Especially, culture medium which supplemented with auxins induces quickly cell division. The size of the explants and in a few cases the mode of culture or polarity of the explants in the medium affect callus development. Smaller explants are more likely to form callus while the larger ones maintain greater morphogenetic potential (David *et al.*, 1981). A callus is an amorphous mass of loosely arranged thin walled parenchyma cells developing from proliferating cells of the parent tissue (Dodds and Roberts, 1985). The unique feature of callus is that the abnormal growth has biological potential to develop normal root, shoots and embryoids ultimately forming a plant. Callus is formed through 3 developmental stages: induction, cell division and differentiation. During induction metabolic rate of cells is stimulated, duration of which depends on physiological status, and nutritional and environmental factors. Owing to increased metabolic rate, cells accumulate high contents and finally divide to form many number of cells. Cellular differentiation and expression of certain metabolic pathways start in the third phase leading to secondary products. Sometimes callus appears of different colors, for example, yellow, white, green or red. Within the cell population of callus, the genetic instability results in variations in phenotypes which may be attributed to developmental (epigenetic) or genetic basis. Epigenetic changes involve selecting gene expression. They are stable and heritable at cellular level. When callus has grown on nutrient medium after a long time it becomes essential to subculture it within 28 days on a fresh medium. Otherwise there develops nutrient depletion in original medium which results in paucity of water and accumulation of toxic metabolites. http://www.eplantscience.com/index_ (files/biotechnology/Plant%20biotechnology/In%20Vitro%20Culture%20Techniques/biotech_organogenesis.php)

1.2.3 Regeneration

The ability to regenerate whole plants from tissue culture is a prerequisite for most transformation systems especially plant regenerated from callus that is important for an integral part of genetic transformation and selection via somaclonal variations. In chrysanthemum, petals are another candidate as a source for organogenesis and plant regeneration (Bush *et al.,* 1976). A plant regeneration protocol mediated from petals callus is not only a prerequisite for the exploitation of improvements to this plant but also important for selection of somaclonal variations. In addition, the ability to regenerate plants from a single cell of florets is a useful approach to establish a mutant in pure form and facilitate the production of a wide range of new flower cultivars as stated by Mandal *et al.,* (2000). Previously, the isolation of a new flower color mutant through direct floret regeneration and isolation of a flower-shape mutant for the first time through management of a chimera has been reported by Datta *et al.,* 2001b; Misra *et al.,* 2003 ; Datta and Chakrabarty, 2009. Plant regeneration has been achieved in chrysanthemum by a number of groups using various species and cultivars, basal media, different plant growth regulator (PGR) and media additive combinations and concentrations, derived organogenesis from a number of explants sources including: stems (node and internode), axillary buds, leaves, shoot tips or apical meristem, protoplasts, roots, pedicels and florets. On stem explants, shoots originate from cortical cells, but from individual epidermal cells in flower pedicels. Indirect regeneration via callus was reported from stem, petal and shoot tips, but adventitious shoot regeneration derived from an initial callus phase may result in somaclonal variation and in chimerism while direct shoot regeneration from leaf or stem explants may eliminate such undesirables. Generally stem material results in a greater shoot regeneration capacity than leaf (Gao *et al.,* 2001).

Chrysanthemum is traditionally micropropagated on agar solid-based, sugar-supplemented media, but has also been propagated photoautotrophically or photomixotrophically under CO_2 enrichment, with reduced contamination levels and production costs (Mitra *et al.,* 1998), and tissue-cultured plants perform just as well as hydroponic systems (Hahn *et al.,* 1998). A mixed organogenic response (i.e. shoots, roots, callus and embryos forming together) or multiple-organ formation (Rout and

Das, 1997) is as a result of the cellular heterogeneity present in the initial explants source (Tanaka *et al.,* 2000) but this can be overcome by the use of thin cell layers (TCLs) in conjunction with a single PGR application (Nhut *et al.,* 2003a,b; Teixeira da Silva, 2003a,c) In most studies on chrysanthemum, the shoot regeneration capacity is reported as the number of surviving explants or as the number of shoots formed per explant, and in transformation experiments, this is normally on a selective (antibiotic containing) medium.

Propagation and plant regeneration through callus has already been reported in chrysanthemum. Hill (1968) established callus from shoot tip explant of chrysanthemum on a medium containing 2 mg/l NAA and 0.8 mg/l Kinetin. Roest and Bokelmann (1975) investigated the role of growth regulators, explant length, sugar, vitamins and minerals on adventitious shoot formation via callus from pedicle explant. They also confirmed that a combination of BAP and IAA was most favorable for shoot bud regeneration. In the absence of vitamins and minerals, or in the presence of the macro and micro elements (devoid of KNO_3 and NH_4NO_3), adventitious shoot formation was completely suppressed.

Lee *et al*. (1979) cultured shoot apices of chrysanthemum cv. 'Shin Dong Ah' on a Murashige and Skoog medium supplemented with 0.5 mg/l Kinetin and 1.0 mg/l NAA which produced the greatest fresh weight of callus. Bhattacharya *et al.* (1990) reported the influence of auxins on callus production and obtained good green calluses from both leaf and stem segments on MS basal salts supplemented with 2 mg/l 2, 4-D within 2 weeks of culture. They also reported that a combination of 0.1 mg/l IAA and 0.2 mg/l BAP was most appropriate for callus formation from nodal segments, shoot apices and leaf, for the regeneration of shoots from callus. An average shoots height of 3-4 cm after 4 weeks of regeneration of shoots from each explant was achieved. Kaul *et al.* (1990) regenerated adventitious shoots from leaf and stem explants of 11 chrysanthemum cultivars. The optimum medium for both explant types contained Murashige and Skoog basal medium supplemented with 5 μM BA and 5 μM NAA. Generally, stem explants were superior to leaf explants. The morphogenic development was noted 4-6 days after the initiation of cultures depending upon the explant type.

The highest percentage of shoot formation (100%) and greatest average number of shoots per explant (14.6) were observed on stem segments taken from the apical portion of the stem. Organogenetic capacity decreased in the more mature stem (Lu *et al.,* 1990). A high concentration (5.0 mg/l) of BAP and NAA promoted callus induction and formation of shoots from achenes and petals of *Chrysanthemum coccineum* but a high concentration (5.0 mg/l) of 2, 4-D inhibited shoot formation (Fuji and Shimizu, 1990). Callus clumps (1 mm diameter) were transferred to shoot regeneration medium 21 days after isolation. After a further 33 days leaves became visible (Lindsay and Ledger, 1993). Callus age was an important factor for shoot bud regeneration. The regeneration ability decreased when calluses were stored at low temperatures for long periods. Eight cultivars were tested for their regeneration ability using different types of explants on the same media. Cultivar and explant type had a greater effect on regeneration than the type of medium (Rademaker and de Jong, 1990). Sutter and Langhans (1981) compared 9 years old leaf callus of *Chrysanthemum morifolium* with those of 1 month old callus derived from leaf explants to assess the regeneration ability of long term cultures. Aberrant forms, variable leaf shapes and stunted growth were observed in 15% of them while the remaining 85% were characterized by excessive growth of lateral shoots.

Aribaud *et al.* (1994) reported that BA plus 2, 4-D caused callus formation and proliferation in leaf explant of chrysanthemum. Kumari and Varghese (2003) determined the effects of 2, 4-D (1 or 2 mg/l), NAA (1 or 2 mg/l) and Kinetin (0.5 and 1 mg/l) on callus of node, young leaves and inter-nodal explant of chrysanthemum. Amongst the various explants used, nodal explants showed the highest fresh weight in two combinations and the inter-node and young leaf in one combination. Dry matter accumulation was higher in nodal explant followed by young leaves and inter-node in both cultivars. Out of different media combinations, MS supplemented with 2.0 mg/l 2, 4-D and 1.0 mg/l Kinetin was superior in terms of fresh weight and dry matter accumulation in all explants.

Sherman *et al.* (1998) reported that the leaf explants when cultured on an embryogenesis-type medium containing a high concentration of 2, 4-D promoted callus formation. Chakrabarty *et al.* (2000) cultured yellow florets on MS medium

supplemented with 0.2 mg/l NAA, 1 mg/l BAP and 3% sucrose. Callus formed on 82% of the explants. Wankhede *et al.* (2000) studied callus induction in chrysanthemum cv. 'Zipri' and 'Shyamal Dark Pink'. Cultivar 'Zipri' exhibited greater callus induction than cv. 'Shyamal Dark Pink'. Callus induction and growth were most pronounced in cv. 'Zipri' grown in the MS medium containing either 1 mg/l 2, 4-D, 0.2 mg/l BAP plus 0.2 mg/l NAA, or 0.5 mg/l BAP, and in cv. 'Shyamal Dark Pink' grown in the MS medium containing either 2 mg/l 2, 4-D, 0.1 mg/l BAP plus 0.1 mg/l NAA, or 0.25 mg/l BAP. In both cultivars, leaf disc explants produced more calluses than stem disc explants. Gul (2001) induced callus on the medium supplemented with BAP plus 2, 4-D. Callus was obtained from stem nodal segments as well as shoot tips. Up to 60 plantlets were obtained from a single flask containing callus. The plantlet grew rapidly on plain ½ MS basal medium and up to 20 cm long shoots and 50 cm long roots were recorded. Sarker and Shaheen (2001) observed the best response for callus induction and consequent development of shoots when 1.5-2.0 cm long leaf explants were cultured on MS medium containing 5.0 mg/l BAP and 0.5 mg/l Kinetin, in chrysanthemum. The maximum number of shoots from the callus developed along the entire surface of the leaf. Shoots also regenerated from midrib callus on MS medium fortified with the similar hormonal supplements but comparatively with a less frequency.

1.2.4. *In vitro* root induction

Chrysanthemums are usually propagated as vegetative cuttings by dipping cut stems into formulated auxin-containing powder for adventitious root induction, the rooted plantlets being resilient to desiccation, resulting in ca. 100% acclimatization (Nishio and Fukuda, 1998). Cellulose plug-saturated liquid medium is one way of increasing survival and rooting of *in vitro* developed plantlets once transferred *ex vitro* (Roberts and Smith, 1990). *In vitro* adventitious roots can be established easily by placing *in vitro*-formed adventitious shoots onto a PGR-free medium, albeit with a lower than ideal acclimatization percentage. The *in vitro* induction of shoots from both stem and leaf explants has been well documented.

Despite adventitious root formation (or rhizogenesis) of shoots being well documented in chrysanthemum, there is only one report on the *de novo* formation of roots (Teixeira da Silva, 2003a), which may allow this procedure to be used for cryopreservation, artificial seed production (synseeds), secondary metabolite production (root-specific) and improvement of the genus through genetic engineering.

The effect of different plant hormones is a major topic in the study of root formation. Several hormones are known to influence root architecture, usually through the inhibition or induction of lateral roots. Auxin is a major player in shaping root systems by regulating growth of primary and lateral roots. Specifically, normal auxin transport and signaling are indispensable for the initiation and development of lateral root (Reed *et al.,* 1998; Fukaki *et al.,* 2002). Interplay between auxin and ethylene in root growth was described in pea in the 1970s (Chadwick and Burg, 1970). Recently, this interaction has been studied in more detail. It includes low concentrations of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), which induce auxin biosynthesis, while high concentrations were thought to increase auxin concentrations up to levels inhibitory for root growth and lateral root development (Ivanchenko, 2008). Furthermore, high ACC concentrations also increased the capacity of auxin transport by regulating the transcription of auxin transport components. Cytokinin and auxin have antagonistic effects on root formation. By influencing auxin transport and homeostasis, cytokinin inhibits lateral root formation (Laplaze *et al*., 2007). Auxin can, in turn, directly down regulate cytokinin biosynthesis (Nordstrom *et al.,* 2006). In rice, cytokinin was also shown to inhibit lateral root initiation, but stimulate lateral root elongation (Rani Debi *et al.,* 2005). Recently, abscisic acid (ABA) was also identified to play a role during root development. Although its exact role during lateral root initiation is not clear, it has been suggested that auxin and ABA act antagonistically during lateral root initiation (De Smet *et al.,* 2006). In later stages during lateral root formation, ABA can inhibit the growth of primordial prior to the activation of the lateral root primordial or shortly after emergence (De Smet *et al.,* 2003). Jasmonic acid has been shown to induce lateral root formation in rice, but knowledge has been limited on the mechanisms. As there is no correlation between the number and distribution of lateral roots induced by

auxin and jasmonic acid, both hormones are thought to act independently (Wang *et al.*, 2002). Nevertheless, results of these studies indicate the pivotal role of hormonal cross-talk in controlling the final architecture of the root systems. Future research in this area will be essential to disentangle the underlying mechanisms.

Root development (Yi *et al.,* 2003; Panfilova and Andrianov, 1996) or callus induction (Yiyao *et al.,* 2002) was enhanced by sound wave treatment while electro stimulation at low field intensity (10 kV/min) resulted in an increase in the number of roots, while higher field intensity (40 kV/min) resulted in a higher average number and total length of roots. The use of nitrate or urea was sufficient to ensure the induction and formation of roots of *in vitro Chrysanthemum morifolium* axillary shoots (de Argollo *et al.,* 1998).

Moreover, the induction of roots in regenerated shoots depends on the composition of mineral nutrients and growth regulators in the medium. Hill (1968) reported rooting of shoots on filter-paper bridges in liquid medium without sugar and growth substances. Ben-Jaacov and Langhans (1972) noted that the development of roots only occurred after some elongation of the shoots on MS medium containing BAP and IAA. They also reported that roots were easily induced when the cultures were still in the rotating flask by increasing the concentration of IAA to 1.0 ppm in the medium. Rooting, however, was not desirable at this stage since it was easier to transfer without roots. Roest and Bokelmann (1975) successfully induced roots in the adventitious shoots of *Chrysanthemum morifolium* Ramt cv. ‘Super Yellow’ and 'Bravo' on liquid MS nutrient media supplemented with 10^{-7} g/ml IAA. Similarly, Sangwan *et al.* (1987) transferred well-developed shoots (4-5 cm) to a root-inducing medium (MS medium with 1% sucrose and 0.1 mg/l IAA). Chrysanthemum shoots readily rooted when transferred to a half strength MS medium containing IAA (Bhattacharya *et al.,* 1990; Aribaud *et al.,* 1994; Gul, 2001). Guan *et al.* (1981) reported that rooting was induced in MS medium plus 1 mg/l IAA, or MS medium plus 2 mg/l 2, 4-D in chrysanthemum. Chrysanthemum shoots rose from tissue culture, developed roots within 4-5 days on ½ MS plus 0.25 mg/l IBA (Khan *et al.,* 1994; Karim *et al.,* 2002). Singh and Arora (1995) reported rooting *in vitro* raised from shoots of chrysanthemum cv. ‘Riot’ on half-strength MS semi-solid and liquid

media (on filter paper bridges) containing 1 mg/l IBA as well as under *in vivo* conditions. A total of 505 plants were raised and transferred under field conditions. These plants exhibited uniform growth and flowered true-to-type.

Various concentrations of IBA used for root induction showed maximum response (100%) on MS medium containing 0.2 mg/l IBA (Hoque and Fatima, 1995; Hoque *et al.,* 1998; Sarker and Shaheen, 2001). The regenerated shoots with well-developed roots were successfully transferred into small pots; and after proper hardening they were established into clay pots. Similarly, Sood *et al.* (1999) recorded in chrysanthemum, the longest root in plants treated with 2 ppm IBA (12.76 cm), whereas, paclobutrazol reduced the root length with increased concentration.

Ahmed (1986) reported that half of the chrysanthemum shoots rooted on a medium containing MS salts, with or without the addition of NAA at 0.1 mg/l. Root growth was best on half strength MS medium supplemented with 0.1 mg/l NAA in *Dendranthema grandiflora* (Ohishi and Sakurai, 1988; Liu *et al.,* 1994; Chakrabarty *et al.,* 2000). Transplantation from test tube to soil was most successful when plantlets were 3-4 cm high with 5 to 7 rootlets of 0.5 to 1.0 cm (Liu *et al.,* 1994).

Faisal and Amin (2000) reported that in chrysanthemum, *in vitro* regenerated shoots developed roots on all media combinations; the maximum number being found in both cv. ‘Super Yellow’ and ‘Light Violet’ in half strength of MS medium supplemented with 0.2 mg/1 IBA and 0.2 mg/1 IAA, respectively. Good rooting of cv. ‘Deep Pink’ was achieved on half strength basal MS salts supplemented with 0.25 mg/l IBA or IAA (Rout *et al.,* 1996). The maximum percentage of rooting was obtained on ½ strength basal MS salts supplemented with 0.25 mg/I IBA and 2% (w/v) sucrose. The performance of rooting was better in the liquid medium than on the solid medium after 7-8 days of culture. Rooting was delayed in MS media supplemented with IAA (0.1 - 0.5 mg/I) with the formation of callus at the basal end. Chlorosis of the leaves occurred when the shoots were maintained for a longer period on the rooting medium (Rout *et al.,* 1996).

Rooting depended on the age of the explant, better rooting was achieved when the shoots (1.5 - 2.0 cm) were transferred to sterile pots containing ½ MS medium without growth regulators (Bush *et al.,* 1976).

The effects of different levels of salt and sucrose on root development in chrysanthemum were reported (Earle and Langhans, 1974a, b; Roest and Bokelmann. 1975; Sun and Li, 1987; Tian *et al.,* 1993). There have been very few reports with regard to the role of the culture environment, such as temperature and light, on rooting. Rooting was achieved in 90% of the cultures of *Chrysanthemum morifolium* Ramat. cv. 'Deep Pink' rooted with about 2.0 Klux of light, whereas higher light intensities (3.0 Klux) gave a lower rooting percentage (Rout *et al.,* 1996). Kumari and Varghese (2003) achieved rooting of shoots by culturing on MS medium supplemented with 1.0 mg/l NAA.

1.2.5 Organogenesis

In plant tissue culture, organogenesis is a process of differentiation by which plant organs viz. roots, shoots, bud flowers, stem, etc. are formed while adventitious refers to the development of organs (roots, buds, shoots, flowers, etc.) or embryos (embryo-like structures) from unusual points of origin of an organized explant where a preformed meristem is lacking. Adventitious shoots or roots are induced on tissues that normally do not produce these organs. Adventitious shoots are stem and leaf structures that arise naturally on plant tissues located in sites other than at the normal leaf axil regions. Plant development through organogenesis is the formation of organs either *de novo* or adventitious in origin. Whole plant regeneration via organogenesis is a monopolar structure. It develops procambial strands which establish a connection with the pre-existing vascular tissue dispersed within the callus or cultured explant. This organogenesis process is much more common than somatic embryogenesis and has far more potential for mass clonal propagation of plants.

Plant production through organogenesis can be achieved by two modes: (i) organogenesis through callus formation with *de novo* origin and (ii) emergence of adventitious organs directly from the explant

Organogenesis via callus formation

Plant regeneration from cultured explant involves the initiation of basal callus and then shoots bud differentiation. Establishment of callus growth with subsequent organogenesis has been obtained from many species of plants and from numerous explants viz. cotyledon, hypocotyl, stem, leaf, shoot apex, root, young inflorescence**,** flower petal, petiole, embryo, etc. cultured *in vitro* (Yancheva *et al.,* 2003; Gahan and George, 2008). For any given species or variety, a particular explant may be necessary for successful plant regeneration. Explants from both mature and immature organs can be induced to form callus and then plant regeneration. However**,** explants with mitotically active cells are generally good for callus initiation. Immature tissues and organs are invariably more morphogenetically plastic *in vitro* than mature tissues and organs. The size and shape of the explant is also crucial. The increased cell number present in bigger explants increases the probability of obtaining a viable culture. It has been seen that only a small percentage of cells in a given explant contribute to the formation of callus. The site of initiation of callus is generally at the peripheral surfaces of the inoculum or at the excised surface. Callus is produced on explants *in vitro* as a result of wounding and in response to hormones, either endogenous or exogenously supplied in the medium. Meristematic tissues or organs like shoot tip, lateral bud, inflorescence, rachis, leaf, petiole, root should be selected in preference to other tissues because of their clonal properties, culture survival, growth rates and totipotency *in vitro.* Meristems, shoot tips, axillary buds, immature leaf, and immature embryos in cereals are particularly suited as good explants. The explants like mature leaves, roots, stems, petioles, and flower parts from herbaceous species can often be successfully cultured to initiate plantlets through organogenesis (David *et al.,* 1981).

The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures. Various culture media are used for organogenesis which include MS, B_5, White's medium (White, 1963) and SH (Schenk and Hildebrandit, 1972). MS medium contains a high concentration of nitrogen as ammonium unlike other media. It may be difficult to transfer cells from White's, B_5 or SH medium to MS medium.

Two modes of cell culture are generally used for organogenic path: (i) the cultivation of cell clusters on a solid medium and (ii) the cultivation of cell suspensions in liquid medium. A suspension cell culture is usually initiated by placing friable callus into liquid culture medium. The suspension usually consists of free cells and aggregates of 2-100 cells. Suspension cultures should be subcultured at least once a week while callus culture should be subcultured after 3-4 weeks.

Growth regulator concentration in the culture medium is critical for morphogenesis. Skoog and Miller (1957) first indicated its role in morphogenesis. Auxin at a moderate to high concentration is the primary hormone used to produce callus. Often 2, 4-D a very potent auxin is used alone to initiate callus. In some species a high concentration of auxin and a low concentration of cytokinin in the medium promote abundant cell proliferation with the formation of callus. Cytokinins, if supplied are Kinetin or benzyl adenine. Callus tissue comprises a wide range of cell type and characteristically consists of irregularly differentiated, vacuolated cells interspersed with smaller more meristematic cells. The nature of any callus will depend on the explant tissue or tissues from which it has arisen and also on the composition of medium used to induce and maintain it. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favored by the medium and come to dominate the culture. Under conditions favouring unorganized growth, the meristems in a callus are random and scattered. Transfer of callus pieces to conditions supporting organized growth leads to the formation of meristemoids, also termed as growth centers. The meristemoids are localized clusters of cambium-like cells which may become vascularized due to the appearance of tracheidal cells in the center. These are the sites of organ formation in the callus and can form shoots or roots (David *et al.,* 1981).

By varying the growth regulator levels and types, one can determine the route of morphogenesis *in vitro*. As mentioned earlier, medium containing high auxin levels will induce callus formation. Lowering the auxin and increasing the cytokinin concentration is traditionally performed to induce shoot organogenesis from callus. In tobacco, the presence of adenine or Kinetin in the medium leads to the promotion of bud differentiation and development. Auxins inhibit bud formation. The

next phase involves the induction of roots from the shoots developed. IAA or IBA alone or in combination with a low concentration of cytokinin are important in the induction of root primordia. Organogenesis can be induced from either cell suspension or callus cultures. One needs to transfer only cells from callus medium to regeneration medium and then continued subculturing on regeneration medium. Thus organ formation is determined by quantitative interaction, i.e. ratios rather than absolute concentrations of substances participating in growth and development.

Direct adventitious organ formation

The somatic tissues of higher plants are capable under certain conditions, of regenerating adventitious plants. Adventitious buds are those which arise directly from a plant organ or a piece thereof without an intervening callus phase. Induction of adventitious shoots directly on roots, leaves, bulb scales and other organs of intact plants is a common method of propagation. In culture, this method is particularly suitable to herbaceous species. The literature records numerous examples throughout the plant kingdom of shoots arising adventitiously on many different organs. In *Begonia*, for example, buds normally originate along the leaf. But in a medium containing BAP, the bud formation from the cut end of a leaf is so profuse that the entire surface of the cutting becomes covered with shoot buds. Promotion of bud formation by cytokinin occurs in several plant species. However, the requirement for exogenous auxin and cytokinin in the process varies with the tissue system, apparently depending on the endogenous levels of the hormones in the tissue. These observations led to the concept of totipotency, i.e. the capacity of all cells to regenerate a complete new plant even after differentiation within the somatic tissues of the plant. Every cell in the plant is derived from the original zygote through mitotic divisions and should contain the complete genome. Thus, the formation of adventitious organs will depend on the reactivation of genes concerned with the embryonic phase of development (Chawla, 2009).

In conventional propagation, the main stimulus for adventitious shoot formation arises from the physical separation of cutting from the parent plant, causing

changes in the production and distribution of endogenous hormones. The same applies to explants used for *in vitro* procedures, and in some species shoot formation may occur spontaneously on a medium lacking any growth regulators. But in most species the addition of growth regulators to the medium is required to initiate shoot formation. Two main types of growth regulating substances, auxins and cytokinins, are employed at different concentrations with respect to one another and depending upon the explant taken, age of the plant and growing conditions. Adventitious *in vitro* regeneration may give a much higher rate of shoot production than is possible by proliferating axillary shoots. Adventitious shoot proliferation is the most frequently used multiplication technique in micropropagation system (Chawla, 2009).

Organogenesis *in vitro* consists of several factors, such as PGR perception and transduction, redifferentiation after dedifferentiation of differentiated cells, organization for specific organ primordial and meristems, etc. The process depends on external and internal factors, such as exogenously applied PGRs, and the ability of plant tissue to perceive these PGRs. According to the requirement for a specific PGR balance, three phases of organogenesis are distinguishable (Sugiyama, 1999):

1. Competence (dedifferentiation): cells become able to respond to hormonal signals of organ induction (Howell *et al.* 2003). Dedifferentiation means the acquisition of organogenic competence. Wounding usually triggers dedifferentiation in tissue culture (Sugiyama, 1999).

2. Determination: the competent cells in explants are determined for specific organ formation and this process is influenced by a specific PGR balance. From competent cells, adventitious shoot formation could be induced by cytokinins (Gahan and George, 2008).

3. Morphogenesis: this proceeds independently of exogenously added PGRs (Sugiyama, 1999). However, Yancheva *et al.* (2003) found that the type of auxin and the length and timing of its application are critical for both activation and progression of the plant-cell developmental program.

In apple regeneration systems, both regeneration pathways can be detected depending on the genotype (Korban *et al.*, 1992; Gercheva *et al.,* 2000).

Direct organogenesis was reported by Pawlicki and Welander (1994) in regeneration experiments of apple rootstock cv. ‘Jork9’. Indirect organogenesis was detected by Dufour (1990) on leaves of apple cv. ‘Gala’. In a separate study, both regeneration pathways were observed in cv. ‘Greensleaves’: rapid regeneration occurred without a callus phase in 2 weeks of culture and slow regeneration via a callus phase occurred within 12 weeks (James *et al.*, 1988). The origin of regenerants depends on species and explants type. Welander and Maheswaran (1992) proposed that, in apple leaf explants, the mesophyll parenchyma cells around vascular tissues are the main origin of adventitious shoots. Yancheva *et al.* (2003) distinguished two types of regenerants: early shoots which appeared on the explant surface within 14 days and originated from epidermal and subepidermal cell layers, and shoots that regenerated later, which originated from internal de novo formed meristematic centers.

In Malus sp., the most frequently used explant for regeneration is the *in vitro* developed and fully expanded young leaf. Welander (1988), using different explants for regeneration, found shoot development only on leaf explants and no organogenesis was observed on stem segments. Shoot regeneration was higher on the leaf lamina (22%) than on petioles (5%) (Rugini and Muganu, 1998), and more shoots developed on basal and middle segments closer to the leaf petiole than on segments toward the leaf tip (Sriskandarajah *et al.*, 1990; Yepes and Aldwinckle, 1994; Caboni *et al.*, 1996).

In general plants, larger leaves were more productive than smaller ones (Sriskandarajah *et al.,* 1990). In addition, Leaves were cut into three segments and the middle segments were the most regenerative, possibly because the middle segments had two cut edges (the others had one) and the majority of adventitious shoots originated from cut areas (Sriskandarajah *et al.,* 1990). Cut edges may provide a way for nutrients and PGRs to be absorbed efficiently from the medium (Sarwar and Skirvin, 1997). Moreover, wounding in general causes a stress-related response, which results in the production of a whole series of compounds that could also induce callusing and may be even differentiation. The age of leaves can also affect regeneration ability. Young expanding leaves were the most suitable for regeneration (Welander, 1988; Fasolo *et al.,* 1989; Famiani *et al.,* 1994). Leaves collected from

rooted shoots were found to be more suitable for regeneration (James *et al.,* 1988; James and Dandekar, 1991). This might be because of inter-shoot competition for light and nutrients of micropropagated cultures. As a result, the excised tissues from rooted versus micropropagated plantlets could differ in their responses (James and Dandekar, 1991). Another reason for using rooted shoots as a source of explant material is a practical kind, in that the tissue is more uniform and reproducible between experiments (Bulley and James, 2004). The orientation of the leaf explant significantly influenced the outcome of regeneration: when the abaxial surface was uppermost, many more shoots regenerated than if the adaxial surface was oriented upwards (Welander, 1988; Gamage and Nakanishi, 2000).

Although leaf explants are used the most frequently for shoot regeneration, several attempts have been made with other types of explants. Regeneration was successful on cotyledons and embryonic axes of 'Gloster' (Keulemans and de Witte, 1994), on etiolated internodal explants of 'Royal Gala' (Liu *et al.,* 1998), on cotyledons of 'Golden Delicious' (Barbieri and Morini, 1987), and on the roots of intact micropropagated plantlets (Malus prunifolia Borkh. var. ringo Asami) (Masuda *et al,.* 1988). Pawlicki-Jullian *et al.* (2002) examined the regeneration ability of 'Jork9' rootstock and found that regeneration rate was much lower on transformed roots (16.7%) than on non-transformed ones (77.8%).

Caboni *et al.* (2000) used vegetative shoot apices as explants, and many shoots could be recovered for all tested genotypes ('Jork9', 'M26', 'Gala', and 'McIntosh'). Within genotype differences could be observed in regeneration capacity of culture lines, which were maintained for different subculture lengths (Standardi and Houshmand, 1992).

Summarizing the results, as a general rule, young fully expanded leaves of *in vitro* apple shoots are the best explants for regeneration. Young leaf explants excised from rooted shoots generally provide material that is more uniform and reproducible in response (Bulley and James, 2004). Explants should be oriented on media with the abaxial surface uppermost, and additional wounding or fragmentation of leaves can increase the regeneration capacity. Alternative explants such as vegetative shoot apices could be also be considered for especially recalcitrant

varieties, but these require larger amounts of starting tissue and handling time, and so are less convenient than leaves.

1.2.6 Mutation breeding

Plant breeding requires genetic variation of useful traits for crop improvement. Often, however, desired variation is lacking. Mutagenic agents, such as radiation and certain chemicals, then can be used to induce mutations and generate genetic variations from which desired mutants may be selected.

Mutation induction has become a proven way of creating variation within a crop variety. It offers the possibility of inducing desired attributes that either cannot be found in nature or have been lost during evolution. When no gene, or genes, for resistance to a particular disease, or for tolerance to stress, can be found in the available gene pool, plant breeders have no obvious alternative but to attempt mutation induction.

Gene mutations occur naturally as errors in deoxyribonucleic acid (DNA) replication. Most of these errors are repaired, but some may pass the next cell division to become established in the plant offspring as spontaneous mutations.

Although mutations observed in a particular gene are rare, there are probably 100,000 genes in a cell of a higher plant. This means that every plant may carry one or more spontaneous mutations into the next generation. Gene mutations without phenotypic (visible) expressions are usually not recognized. Consequently, genetic variation appears rather limited, and scientists have to resort to mutation induction. There are no other economic ways of altering genes, except to wait a long time for spontaneous mutations to occur.

Artificial induction of mutations by ionizing radiation dates back to the beginning of the 20^{th} century. But it took about 30 years to prove that such changes could be used in plant breeding. Initial attempts to induce mutations in plants
mostly used X-rays: later, at the dawn of the "Atomic Age", gamma and neutron radiation were employed as these types of ionizing radiations became readily available from newly established nuclear research centres.

Major efforts were devoted during this initial phase of mutation induction to define optimal treatment conditions for reproducibility. Research focused on changing "random" mutation induction into a more directed mutagenesis to obtain more desirable and economically useful mutations. However, it did not lead to the desired alterations in the mutant spectrum. Limitations were the concomitant increase of plant injury with increasing radiation dose and the low frequency of economically useful mutations. This led scientists to search for potentially better mutagens. As a result, new methods of radiation treatment, as well as chemical agents with mutagenic properties, were found (Novak and Brunner, 1992)**.**

1.2.6.1 Mutation induction by gamma irradiation

Induced mutation has been reported to be an efficient technique to achieve the desirable characters in flowers and ornamental plants (Maluszynski, 1995). The successful improvement of chrysanthemum through induced mutation and *in vitro* culture have been demonstrated (Nagatomi, *et al.*, 1996 ; Ahloowalia, 1992 ; Datta and Banerji, 1993 ; Matsumoto and Onozawa, 1990 ; Neto and Latado,1996). In the mutation breeding program, breeders usually hope to get plantlets with greater genetic variation compared with the parents, for the chance of developing novel cultivars might be greater if the mutants with higher genetic variation are selected during early growth stages (Broertjes *et al.*, 1976; Li, 1993; Banerji *et al.*, 1996; Mandal *et al.*, 2000).

Mutation induction by treatment with gamma or UV radiation is a common practice in plant breeding programs. The biological effect of gamma-rays is based on the interaction with atoms or molecules in the cell, particularly water, to produce free radicals, which can damage different important compounds of plant cell. The biological effect is due to these processes. Gamma-rays irradiation at high levels can cause different effects on plant growth and development such as: prevents seed growth, disrupts stomata resistance, damages plant cells, increases cell mutations, and

reduces plant fertility.

Recently, using of gamma irradiation technique for inducing mutation has been reported in chrysanthemum by several researchers.

Lamseejan *et al.*, (2000) studied the effect of gamma radiation on *in vitro* culture of chrysanthemum (*Chrysanthemum morifolium*). They reported that multiple shoots produced from ray-florets were irradiated with gamma rays at 0, 10, 30, 50, 70, 90 and 110 Gy. Shoots irradiated at 50 Gy and over died within 25-30 days. LD50 for this purple clone of chrysanthemum was 14 Gy. Only the controls and treated plants at 10 Gy were able to survive and gave rise to the full grown plants. After transplanting into the greenhouse for 60 days, control plants and treated ones were found to be different in four traits which were average height, average number of leaves, average number of nodes and % flowering. Plants were trimmed twice at three month intervals and allowed to produce flowers. Changes in flower characters were found in both controls and treated plants. However, the treated plants had much more variation than the controls and new flower color (yellow tinge) was only obtained from the treated ones.

Form their experiment on the effect of gamma irradiation on *in vitro* culture of chrysanthemum, they found that irradiated shoots at 50 Gy and over were unable to produce shoots and soon die. This finding is consistent with the precious detailed study of Thinh *et al.* (2000). They reported that by irradiating nodal explants of two cultivars of chrysanthemum with gamma rays at 50 Gy, none of nodal explants were able to produce shoots. Then the useful doses for mutation induction in chrysanthemum have been suggested as 10–20 Gy for *in vitro* cultures and 10–25 Gy for rooted cuttings (Thinh *et al*. 2000; Broerties and Van Harten, 1988). There are numerous reports on alteration of flower colour 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 colour and 15% in flower morphology. To create flower colour variability by mutation in chrysanthemum, selection of an appropriate genotype would be helpful. This would be seen from many reports on induced mutations in chrysanthemum. For example, Nagatomi (2000) demonstrated that pink genotype ‘taihei’ has given rise to many

flower colour mutants ranging from white, light pink, dark pink, orange, yellow, bronze and striped. Moreover, pink genotypes gave rise to several sports that included most of the colours seen in chrysanthemum (Schum and Preil, 1998).

However, the emergence of new cultivars is closely connected with the problem of identifying them and distinguishing between them. Thus, it is very necessary to quickly evaluate the degree of genetic variation of plantlets in the early stage of breeding. Traditionally, the identification of ornamental plant cultivars has been based on morphological characters and, in particular, on the colour of the inflorescence, but this method needs the plants to be seen in flower and it is very subjective. A better method involves DNA analysis e.g. flow cytometry analysis and random amplified polymorphic DNA (RAPD), utilizing polymerase chain reactions (PCR). The fact that only small samples derived from the leaves are sufficient for analysis also confirms greater applicability of the method for identifying cultivars as early as the cutting stage.

1.2.7 Flow cytometric investigation

Flow cytometry is a general method for rapidly analyzing large numbers of cells individually using light-scattering, fluorescence and absorbance measurement. The power of this method lays both in the wide range of cellular parameters that can be determined and in the ability to obtain information on how these parameters are distributed in the cell population. Flow cytometric assays have been developed to determine both cellular characteristics such as size, membrane potential, and intracellular pH, and the levels of cellular components such as DNA, protein, surface receptors, and calcium. Measurements that reveal the distribution of these parameters in cell populations are important for plant biotechnology, because they better describe the population than the average values obtained from traditional techniques (Rieseberg *et al.,* 2001).

1.2.7.1 Principles of flow cytometry

A flow cytometry system consists of five main operating units: a light source (mercury lamp or laser), flow cell, optical filter units for specific wavelength detection over a broad spectral range, photodiodes or photomultiplier tubes for sensitive detection of the signals of interest, and a data processing and operating unit. A cell suspension is injected into the flow cell where the cells pass one after another across a laser beam (or mercury lamp light) that is orthogonal to the flow. This is achieved by hydrodynamic focusing of the sample stream, wherein the sample stream is injected into the sheath stream inside the flow cell. Using this technology, it is possible to detect up to 10,000 cells/second. In some cytometers, the laser beam is split to give both exact cell size determination via transmission measurement and velocity determination (Eisert, 1981). When it impacts a cell, the excitation light is scattered in both forward and sideways directions. The forward-scattered light provides information on the size of the cells and can be detected without further manipulation. The sideways-scattered light is affected by several parameters, including granularity, cell size and cell morphology. The resulting fluorescent light is processed through the photomultiplier to the data processing system, and the resulting data are analyzed by the cytometer software.

1.2.7.2 DNA content analysis

Genome size or DNA content (C-value) is receiving more attention during recent years. Diversification in genome size depends on accumulation of many steps and can, therefore, be an important taxonomic characterized (Ohri, 1998). Major interspecific variation in DNA amount often correlates with cell size and generation time (Bennett, 1972). Nuclear DNA content, which can conveniently be measured by flow cytometry using propidium iodide, a stoichiometric DNA stain that intercalates in the double helix, is more and more used for taxonomic purposes and ploidy variation. This method has been used successfully to investigate ploidy in diploid, triploid, tetraploid and chimera in many plant species (Palomino *et al.,* 1999; Roux *et al.,* 2001; Tuna *et al.,* 2001; Roy *et al.,* 2001).

Actually, nuclear DNA content can be estimated through several methods, such as chemical analysis, feulgen microdensitometry and flow cytometry. The latest offers a quick and sensitive method for quantifying DNA amounts (Arumuganathan and Earle, 1991; Michaelson *et al.*, 1991), but is still subject to debate (Dolezel *et al.*, 1992; Bennett and Leitch, 1995). This might be attributed to technical differences and differences in the staining properties of the fluorochromes. Propidium iodide (PI) and ethidium bromide are considered to be useful fluorochromes for flow cytometric estimation of DNA content. These compounds intercalate with double-stranded nucleic acids and are independent of base composition (Le Pecq and Paoletti, 1967). On the other hand, the staining with DNA intercalators may be influenced by the spatial organization of the DNA molecule (Darzynkiewitz *et al.*, 1975).

Plants show considerable variation in nuclear DNA content (Bennett and Smith, 1976; Bennett and Leitch, 1995; 1997). Variation in DNA content is usually much smaller within species than between them (Bennett and Leitch, 1995). Dolezel *et al.* (1998) revealed that flow cytometry with DNA intercalators is a reliable method for estimation of nuclear genome size in plants. However, the study confirmed an urgent need for an agreement on standards. Given the small but systematic differences between different types of flow cytometers, analysis of very small differences in genome size should be made in the same laboratory and using the same instrument.

DNA content analysis has already been reported in chrysanthemum.

Yamaguchi *et al.* (2008) used flow cytometer to analyze nuclear DNA content in irradiated chrysanthemum. They confirmed that the nuclear DNA content was affected by gamma ray irradiation. The relative DNA content in plants regenerated from non-irradiated leaf ranged from 0.97 to 1.03 pg while in the plants regenerated from the irradiated leaf, there were plants which had less than 97% of the nuclear DNA content as compared to the control plant. The greatest decline was 10% in some of the plants that received 60 Gy at the dose rate of 2 Gy/h. Variance in the nuclear DNA content increased significantly ($p<0.05$ Hartley's test) among the doses at dose rates of 0.5 and 2 Gy/h.

Yamaguchi *et al.* (2010) reported that the relative nuclear DNA content in chrysanthemum that were regenerated from non-irradiated leaf segment ranged from 0.98 to 1.30 pg, with an average of 1.00 pg. In contrast, some plants that were regenerated from irradiated leaf segments had low relative nuclear DNA content. The lowest relative nuclear DNA content was 0.92 pg, that is, an 8% reduction compared to the control plant, equivalent to a reduction of approximately four of the 54 chromosomes of *Chrysanthemum morifolium.* Chrysanthemum has the basic chromosome numbers throughout (x=9) (Dowrick, 1952). In all irradiation treatment in their study, the number of plants with a relative nuclear DNA content below 0.97 pg, which is below the range for plants regenerated from non-irradiated leaf segments, increased significantly (chi-square test for independence, $P<0.01$), and the average nuclear DNA content was significantly lower (Kruskal-Wallis test, $P<0.01$) than that of plants regenerated from non-irradiated leaf segments. Thus, irradiation treatment caused a reduction in the nuclear DNA content in chrysanthemum.

1.2.7.3 Ploidy analysis

It has been known for long that the most accurate way to determine chromosome number or ploidy level is the classic method of counting chromosomes but it is very laborious to use in large numbers of samples. Recently, flow cytometry has been used as an efficient tool to estimate DNA content and ploidy in several plant species such as banana (Dolezel *et al.*, 1994; Lysak *et al.*, 1999), oil palm (Rival *et al.*, 1997), hop (Sesek *et al.*, 2000), and water yam (Egesi *et al.*, 2002). It is utilized routinely for ploidy analyses and is now regarded as the most accurate tool for ploidy determination (Dolezel *et al.*, 1994; Duran *et al.*, 1996). The main advantages of flow cytometry include its simplicity, speed, accuracy, convenience and ability to screen large numbers of samples per day. In addition, the method requires only a small amount of tissue and is therefore non-destructive, and has the ability to analyze large populations of cells where the possibility of mixoploidy or aneuploidy exists (Roux *et al.*, 2001; 2003).

Some fluorochromes, such as DAPI (4, 6-diamidino-2-phenylindole) and mithramycin bind preferentially to adenine-thymine (A-T) or guanine-cytosine (G-C) rich regions (Manzini *et al.,* 1983), leading to over or underestimation of the nuclear DNA content. Although DAPI and mithramycin fluorescence alone cannot be used to estimate nuclear DNA content, it may give additional information on the arrangement of base pairs and chromatin structure, when compared with data obtained by using DNA intercalators (Dolezel *et al.,* 1992).

At present, flow cytometry has been applied to estimate ploidy level in several plant species (Bennett and Leitch, 1995; Dolezel *et al.,* 1998). For chrysanthemum, discrepancies are quite large and might be attributable not only to the difference among the techniques, but also to the type of standard used for estimation. In Arecaceae, *Petunia hybrid*a (2C=2.85) was mostly used as a reference in flow cytometric analysis such as that of date palm (Siljak-Yakovlev *et al.,* 1996), oil palm (Rival *et al.,* 1997) and coconut. In banana, *Glycine max* cv. Polanka was used as internal reference marker (Lysak *et al.,* 1999). The majority of previous works used reference plants as an internal marker. However, when the references were used as an internal marker, interference between the staining solution and the genome of the two species resulted in poor reading in peak quality (Amsellem *et al.,* 2001). It is therefore necessary to clearly define that the reference plants should be used as internal references in each plant.

1.2.8 Molecular marker

A molecular marker is a DNA sequence which is readily detected and whose inheritance can easily be monitored. The use of molecular markers is based on naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes. A marker must be polymorphic, that is, it must exist in different from so that chromosome carrying the mutant gene can be distinguished from the chromosome with the normal gene by a marker it also carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two or more discontinuous variants or genotypes.

One of molecular markers is the PCR based technique RAPD (Random Amplified Polymorphic DNA). RAPD has been a powerful technique for genetic analysis of plants (Williams *et al.,* 1990; Kiss *et al.,* 1993). This technique enables one to show differences at the DNA sequence level in an easy and fast way, even if only small amounts of tissue are available. Williams *et al.* (1990) introduced the use of general purpose ten-mer primers in the RAPD technique. With these single primers arbitrary fragments were amplified and generally high levels of polymorphism were obtained, without any prior knowledge of the DNA sequences amplified. The first study on the identification of chrysanthemums with the application of RAPD markers was carried out by Wolff and Peters-Van Rijn (1993). Wolff *et al.* (1993, 1995) showed that with the RAPD technique reproducible patterns were obtained in chrysanthemum and that with these patterns cultivars could be distinguished. It was, however, also demonstrated that cultivars from one family, differing for flower colour, could not be distinguished with several DNA techniques. Furthermore, sporting and chimerism of chrysanthemum also revealed different DNA patterns among cultivars in two families and among the layers of one cultivar by RAPD analysis (Wolff, 1996). Moreover, it has also been applied for identifying cultivar characteristics and detecting genetic diversity of chrysanthemum (Wilde *et al*., 1992; Martin *et al*., 2002; Sehrawat *et al*., 2003; Lema-Ruminska *et al.*, 2004; Barakat *et al*., 2010), roses (Matsumoto and Fukui 1996) and *Alstroemeria L.* (Dubouzet et al., 1997, 1998).

1.2.8.1 Principles of RAPD

RAPD (Random Amplified Polymorphic DNA) analysis is a PCR based molecular marker technique. Here, single short oligonucleotide primer is arbitrarily selected to amplify a set of DNA segments distributed randomly throughout the genome. A number of closely related techniques based on this principle were developed almost at the same time and are collectively referred to as Multiple Arbitrary Amplicon Profiling (MAAP). These techniques are:

Randomly Amplified Polymorphic DNA (RAPD)

DNA Amplifying Fingerprinting (DAF)

Arbitrarily Primed Polymerase Chain Reaction (AP-PCR)

All these techniques refer to DNA amplification using single random primers and share the same principle with some differences in the experimental details (Chawla, 2009). Of these, RAPD was the first to become available and is most commonly and frequently used. Williams *et al.*, (1990) showed that the differences as polymorphisms in the pattern of bands amplified from genetically distinct individuals behaved as Mendelian genetic markers. Welsh and McClelland (1990) showed that the pattern of amplified bands so obtained could be used for genomic fingerprinting.

RAPD amplification is performed in conditions resembling those of polymerase chain reaction using genomic DNA from the species of interest and a single short oligonucleotide (usually a 10-base primer). The DNA amplification product is generated from a region which is flanked by a part of 10-bp priming sites in the appropriate orientation. Genomic DNA from two different individuals often produces different amplification patterns [randomly amplified polymorphic DNAs (RAPDs)]. A particular fragment generated for one individual but not for other represents DNA polymorphism and can be used as a genetic marker. Using different combinations of nucleotide, many random oligonucleotide primers have been designed and are commercially available. Such primers can be synthesized in an oligonucleotide synthesizing facility based on sequences chosen at random. No separate information is required from the plant to be studied. The choice of single primer (or RAPD primer) to use is done operationally. Since each random primer will anneal to a different region of the DNA, theoretically many different loci can be analyzed (Chawla, 2009).

The PCR reaction typically requires cycling among three temperatures, the first to denature the template DNA strands, the second to anneal the primer and the third for extension at temperatures optimal for *Taq* polymerase. This cycle is usually repeated 35 to 45 times (Fig. 1).

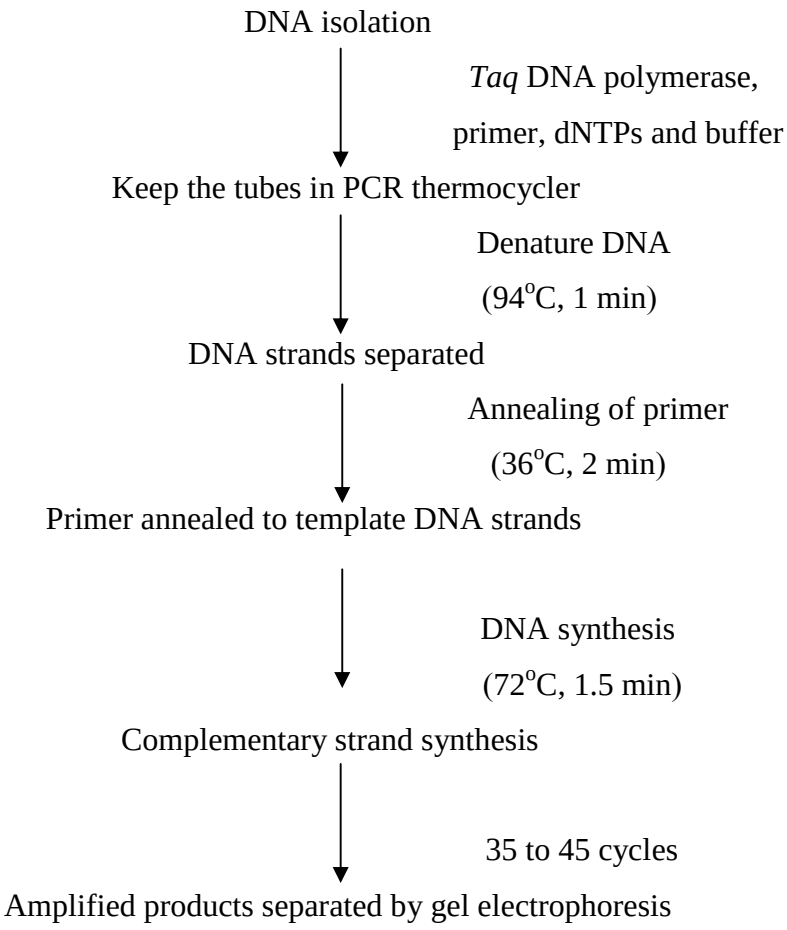


Figure 1 An outline of RAPD analysis

It is usually sufficient to heat the reaction mixture at 94^{o}C for 0.5 to 1min. However the initial denaturation step for the plant DNA should be for at least 3 min. Insufficient denaturation is a common problem leading to the failure of the PCR reaction.

The temperature for the annealing step will depend to some extent on base composition and the length of primers. Generally, an annealing temperature of 35-40 ^{o}C is used in PCR reactions for RAPD analysis. Annealing may require only a few minutes, but this will depend on a variety of factor such as interference from

secondary structure and primary concentration. A time in the range of 0.5 to 1 min is usually sufficient.

The extension temperature depends on length and concentration of the target sequence, and in general 72 ^{o}C temperature is used. As a thumb rule 1 min per kilo base is probably ample. After cycling, an extended extension period of 5-10 min is included to ensure that all annealed templates are fully polymerized.

Most genomes from bacteria to higher plants to human beings contain enough perfect or imperfect binding sites for a short primer of arbitrary sequence. These markers cover the entire genome, i.e. single to repeated sequences. Binding of primer to the complementary sequences allows the complementary sequences allow the amplification of several bands. A DNA sequence difference between individuals in a primer binding site may result in the failure of the primer to bind, and hence in the absence of a particular band among amplification products. The reaction products are conveniently analyzed on agarose gels, stained with ethidium bromide and seen under UV light (Chawla, 2009).

The advantages of RAPD technique (Chawla, 2009)

1. Need for a small amount of DNA (15-25 ng) makes it possible to work with populations which are inaccessible for RFLP analysis.
2. It involves nonradioactive assays.
3. It needs a simple experimental set up requiring only a thermo cycler and an agarose assembly.
4. It does not require species- specific probe libraries; thus work can be conducted on a large variety of species where such probe libraries are not available.
5. It provides a quick and efficient screening for DNA sequence based polymorphism at many loci.
6. It does not involve blotting or hybridization steps.

The limitations of RAPD technique

1. RAPD polymorphisms are inherited as dominant-recessive characters. This causes a loss of information relative to markers which show codominance.
2. RAPD primers are relatively short, a mismatch of even a single nucleotide can often prevent the primer from annealing, hence there is loss of band.
3. The production of nonparental bands in the offspring of known pedigrees warrants its use with caution and extreme care.
4. RAPD is sensitive to changes in PCR conditions, resulting in changes to some of the amplified fragments.

1.2.8.2 Review of using RAPD technique for identifying cultivar characteristics and detecting genetic diversity in chrysanthemum

Previously, RAPD technique has been applied for identifying cultivar characteristics and detecting genetic diversity of chrysanthemum (Wild *et al.,* 1992) Huang *et al*. (2000) reported that forty-five random primers were screened of which twenty-two primers were selected to detect the molecular marker in three hybrid combinations of *Chrysanthemum* by using random amplified polymorphic DNA (RAPD). From this study, the patterns of DNA could be classified into seven type: Type I markers shared bands in both parents, and offspring; Type II markers shared bands in male and female parents; Type III markers shared bands in male parent and offspring; Type IV marker shared bands in female parent and offspring; Type V markers were presented in the male parent only; Type VI markers were present in the female parent only; Type VII markers were present in offspring only. Of these, only Type III markers were suitable for identifying the true male parent. Different unique markers of Type VII in offspring are quite suitable as identifying markers of new hybrids to protect the rights of plant breeders. In this study, 34.4% to 48.9% of the RAPD markers were found to reveal additively among parents and offspring in three hybrid combinations of *chrysanthemum*. However, 38% to 52.6% of markers (Type II, V, and VI) were absent in offspring, but 11.6% to 113.1% of unique markers (Type VII) were present in offspring. Moreover, there were no definite rules as to whether

markers in offspring were more similar to female or to male parents by similarity analysis. In two hybrid combinations, the parents were more similar to each other than either was to the offspring. The above results illustrate that the genetics of chrysanthemum are very complex. RAPD, however, is a powerful tool to detect different DNA patterns in hybrid populations of chrysanthemum cultivars.

Martin *et al.* (2002) also reported that characterization of fifteen commercial varieties of chrysanthemum was carried out through RAPD analysis. Varieties could be distinguished from each other and the level of similarity between varieties seemed to be not very high. *In vitro* cultures were establish from four varieties and were subjected to different proliferation conditions. Five individuals from each variety and treatment were analyzed using RAPD at the beginning of the treatment and after a month of culture. Variation was detected at both stages of the culture period. The rate of variation found showed differences between varieties, but no significant difference was found between culture conditions.

Lema-Ruminska *et al.* (2004) stated that RAPD markers were used to study the molecular characterization of 10 new radiomutants of chrysanthemum. The original cultivar “Richmond” differed in genetic distance from its Lady group mutants. The analysis of genetic similarity indices revealed low diversity within the radiomutants. The dendrogram obtained after cluster analysis separated the new cultivars as a group that differed from the original cultivar “Richmond”. The Lady group cultivars, derived from one original cultivar by radiomutation, could be distinguished from each other by using RAPD markers of only a single primer or sets of two or three primer. Polymerase chain reaction analysis proved the efficiency of the RAPD method for DNA fingerprinting of the original cv. “Richmond” and its new radiomutants.

Kumar *et al.* (2006) reported that eleven radiomutants from two chrysanthemum cultivars ‘Ajay’ and ‘Thai Chen Queen’ were characterized by RAPD to understand the extent of diversity and relatedness. Out of 40 random primers screened, 21 gave reproducible polymorphic bands. PCR product of radiomutant genome revealed a total of 156 bands, out of which 118 were found to be polymorphic. Cluster analysis of the radiomutants indicated that they were separated

into three major groups. Yellow and Bright Orange mutants derived from cv. Thai Chen Queen have been placed in a separate group, indicating their high genetic diversity from the rest of the mutants and parents. The study revealed that RAPD molecular markers can be used to assess polymorphism among the radiomutants and can be a useful tool to supplement the distinctness, uniformity and stability analysis for plant variety protection in future.

Bhattacharya *et al.* (2006) used RAPD analyses to understand the molecular systematic and genetic differences between 10 original chrysanthemum cultivars and 11 mutants. The similarity among the cultivars and mutants varied from 0.17 to 0.90, a simple but efficient method to distinguish cultivars and to assess parentage. Two distinct groups were found. Two cultivars were present as a separate group showing differences from all other cultivars. Mutants with different flower colour could be identified at the molecular level using RAPD technique holding promise to identify unique genes as SCAR markers. A high genetic distance among the different chrysanthemums showed that there exists a possibility of introgressing new and novel gene from the chrysanthemum gene pool.

Teng *et al.* (2008) showed genetic variation of regenerated plantlets following *in vitro* mutation was detected via RAPD in chrysanthemum. RAPD bands produced from eighteen 10-mer arbitrary primers were used to assess the genetic variation of plantlets regenerated from floret-derived calluses treated with 0, 10, 15, and 20 Gy gamma rays. These primers generated a total of 167 reproducible RAPD bands ranging in size from 0.3 to 2.0 kb, of which 61.7% were polymorphic and 38.3% monomorphic. The number of bands per primer ranged from 4 to 14, with an average of 9.3. The further analysis of RAPD result showed that genetic variation of generated plantlets was proportional to the dosage of gamma ray, while the 15 and 20 Gy treatments were not significantly different, which is consistent with the common conception that genetic variation of radiomutants is usually proportional to the dosage of mutagen within a certain range.

Barakat *et al.* (2010) used five RAPD primers to amplify DNA segments from the genomic DNA of chrysanthemum and its 13 somaclones. The

genetic similarity among the fourteen genotypes ranged from 0.43 to 0.95. The chrysanthemum cultivar and its 13 somaclones were classified in to five clusters.

Therefore, RAPD is a useful technique for the rapid and easy assessment of genetic variation of mutants and may become a potential tool for the quick selection of mutants with great genetic variation during early growth stages.

1.3 Objectives

1.3.1. To optimize the types and concentrations of growth regulators in culture media for callus induction, shoot and root regeneration.

1.3.2. To determine the origin, initiation site and development of adventitious shoots from the ray floret callus of chrysanthemum.

1.3.3. To investigate the suitable gamma radiation dose for induction of mutation in chrysanthemum [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.] callus.

1.3.4. To investigate the effect of gamma dose on nuclear DNA content, somatic chromosome number, genetic variation and morphological characteristics of regenerated plants derived from treated calluses of chrysanthemum.

CHAPTER 2
MATERIALS AND METHODS

Investigation of the effect of gamma radiation dose on mutation induction in regenerated plants derived from irradiated calluses, using a combined method of gamma ray irradiation and tissue culture is described. The research consisted of two parts as the following:

Firstly, *in vitro* propagation through ray florets culture of *Chrysanthemum x grandiflorum* (Ramat.) Kitam. This past was an attempt to optimize the suitable different plant growth regulators and their concentrations for callus induction, adventitious shoot formation and root induction. In addition, a histological study observing the ontogenetic stages through the process of adventitious shoots formation is studied from callus. Moreover, regenerated plants from calluses cultures and plants in natural condition were compared to confirm that no variation appearance in all regenerated plants from calluses.

Secondly, the effect of gamma radiation dose on morphological mutation and nuclear DNA content using flow cytometric and RAPD techniques were studied. In addition, Somatic chromosome number of regenerated plants derived from treated, untreated calluses and plants in natural condition (control) was determined.

2.1 *In vitro* propagation through ray florets culture

2.1.1 Plant materials and surface disinfection

One week old ray florets of *Chrysanthemum x grandiflorum* (Ramat.) Kitam. were collected from field grown plants. These florets were first washed in tap water with liquid detergent (Teepol™), and then rinsed with tap water 2-3 times. The

explants were surface sterilized with 70% ethanol for 1 min, followed by immersion for 15 min in a sterilization solution of 5% (v/v) Clorox™ containing 3-4 drops of Tween-20 emulsifier per 100 ml solution. Ray florets were then rinsed 3-4 times with sterile distilled water.

2.1.2 Callus induction

To investigate the suitable plant growth regulators for induction of callus, ray florets were cut transversely into 1 cm long segments and cultured on MS (Murashige and Skoog, 1962) medium containing 3% sucrose. N^6- benzyladenine (BA) at concentrations of 0, 4.4, 8.9, 13.3 μM and 2, 4-dichlorophenoxyacetic acid (2, 4-D) at concentrations of 0, 0.5, 2.2, 4.5 μM were employed in various combinations. The obtained light green compact calluses were subcultured at 2-week-interval for 2 times to the same media.

2.1.3 Plant regeneration and root induction

The calluses were transferred to the adventitious shoot regeneration MS medium supplemented with different combinations of Kinetin (0, 4.6, 9.3, 13.9 μM) and indole-3-butyric acid (IBA; 0, 2.5, 4.9, 7.4, 9.8 μM). All experiments consisted of 30 calluses and 2 replications per treatment. The effects of Kinetin and IBA for plant regeneration from calluses were determined as percentage, and the total number of shoots were evaluated and recorded after 3 months of culture.

Regenerated shoots obtained in the best condition were transferred to MS medium containing IBA at concentrations of 0, 7.4, 12.3, 17.2 μM and chitosan at concentrations of 0, 43.5, 93.2 μM in various combinations for 1 month to optimize the type and concentration of growth regulators required for rooting. All experiments consisted of 2 replications and 30 shoots per replication were used in each treatment for the rooting experiments.

2.1.4 Culture conditions

All kinds of the medium were solidified by 0.17% Gelrite™ and the pH was adjusted to 5.7 prior to autoclaving at 121°C, 1.1 kPa pressure for 20 min. The cultures were maintained at 25 ± 1°C with 16-h photoperiod cool white fluorescent light at photon flux density of 36 μmol s^{-1} m^{-2}, daily.

2.1.5 Histological studies of callus

The calluses cultured on MS medium containing 13.3 μM BA and 0.5 μM 2, 4-D were selected for histological analysis after 5, 6, 7, 8, 9, 10, 11, 12, or 15 days of culture. The calluses were fixed in FAA II (ethanol: acetic acid : formaldehyde = 90 : 5 : 5), (Johansen, 1940) for 24 h. They were dehydrated through an ethyl-butyl alcohol series, after which the calluses were infiltrated and embedded in Paraplast-plus (melting point 56°C) using Histo-embedder (model Jung, Leica, Germany). Then serial sections of 10-12 μm thickness were cut on a rotary microtome and stained with haematoxylin and safranin (Ruzin, 1999). Serial sections were observed under a light microscope.

2.1.6 Statistical analysis

Each experiment consisted of 2 replications and 30 explants per replication were used in each treatment. The percentage of callus induction and efficiency of callus formation were monitored as growth parameters. Shoot and root regeneration were examined by analysis of variance. The differences among the mean values of treatments were compared using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

2.2 Gamma irradiation in chrysanthemum

2.2.1 Irradiation treatment

The calluses from ray florets of chrysanthemum were initiated on MS medium supplemented with 13.3 µM BA and 0.5 µM 2, 4-D. The one-month-old calluses were irradiated with 0, 5, 10, 15, 20, 25, or 30 Gy gamma rays with Mark 1 irradiator at the Gamma Irradiation Service and Nuclear Technology Research Center (GISC), Kasetsart University, Bangkok, Thailand. The irradiated calluses were transferred to the MS medium containing 13.9 µM Kinetin for shoot regeneration. All experiments consisted of 80 calluses and 2 repetitions per treatment. The irradiated calluses were subcultured at 2-week-intervals. The effects of gamma radiation doses on the survival and plant regeneration from irradiated calluses were determined as percentages, and the LD_{50} were evaluated and recorded after 3 months of culture.

2.2.2 Flow cytometric analysis

Nuclear DNA content as an index of irradiation damage in chrysanthemum was measured by flow cytometric analysis. Two plants from each treatment were measured using two leave from each plant on two different days. The plantlets regenerated from untreated calluses and plants in natural condition were used as control. For each sample, about 2 mg of leaf was chopped with a razor blade in 1 ml of cystain UV ploidy extraction buffer containing DAPI dye. After filtration through a 30-µm nylon mesh, the filtrate was analyzed using flow cytometer (Partec PAII, Partec, Munster, Germany). Leaves of the *Zea mays* cv. 'CE-777' were used as an internal reference standard, and the nuclear DNA content of the sample was determined by comparing the peak position of *Zea mays* nuclei with that of the sample.

The ratio of G_1 peak means was taken as the basis for the calculation of the 2C nuclear DNA content of the each treatment, according to the formula

$$\text{2C DNA content (pg)} = \frac{\text{Sample } G_1 \text{ Peak Mean} \times \text{Standard 2C DNA content (pg)}}{\text{Standard } G_1 \text{ Peak Mean}}$$

2.2.3 Chromosome determination

Root tips approximately 0.5 cm in length of plantlets derived from irradiated calluses from each treatment were cut off at 9.00 a.m. and pretreated in saturated aqueous solution of paradichlorobenzene (PDB) for 4 h and then fixed in Carnoy's fluid (containing 3:1 95% ethanol and glacial acetic acid) for at least 24 h at room temperature. The root tips were rinsed 2-3 times in 95% ethanol before being kept in 70% ethanol at 10 ^{O}C. To continue the processes, the root tips were rinsed several times with distilled water and then hydrolyzed for 8 min with 1M HCl at 60^{0}C. After being soaked in distilled water for 30 min, the fixed root tips were stained with Carbol Fuchsin (Lanhua and Jianxian, 2000) for 4h to stain the chromosomes, and rinsed with de-ionized water several times to wash out the stain from the root tips. An Olympus BX40 microscope (Japan) was used for chromosome determination. The chromosome count of each treatment was repeated for at least 5 cells of each irradiated plantlet.

2.2.4 Statistical analysis

Each experiment consisted of 2 replications and 80 explants per replication were used in each treatment. The percentage of the survival and plant regeneration from irradiated calluses were monitored as effect of radiated dose parameters. Shoot regeneration was examined by analysis of variance. The differences among the mean values of treatments were compared using DMRT at $p \leq 0.05$.

2.2.5 Molecular marker analysis

DNA extraction

Total genomic DNA from the leaves of chrysanthemum was prepared using an extraction technique modified from Doyle and Doyle (1990). Young fresh

leaves of plantlets regenerated from untreated calluses, irradiated calluses and plants in natural condition (200 mg) were ground to a powder with pestle and mortar in liquid nitrogen supplemented with 10 mg Polyvinylpyrrolidone 40 (PVP-40). The powder was poured into tubes, containing 750 µl of warm (60^0C) CTAB extracting buffer supplemented with 2% ß-mercaptoethanol. The tubes were incubated at 60^0C for 45-60 min. An additive of 800 µl chloroform was used and tubes were shaken in mixer for 10 min and centrifuged for 10 min at 13,000 rpm. The supernatants were transferred to a new tube and 750 µl isopropanol was added. The tube was inverted periodically to collect precipitated DNA and finally washed with 70% ethanol 2-3 times before pour off 70% ethanol and left overnight to dry the pellets DNA. Finally, the dried DNA was dissolved in TE Buffer (pH 8.0) (10 mM Tris-HCl, pH 8.0+1.0mM EDTA, pH 8.0) (Sagahi-Maroof *et al*., 1984). DNA was quantified by using agarose gel, and DNA samples were stored at -30 ^{0}C until use.

RAPD-PCR

Thirteen random 10-mer primers (Table 1), obtained from Operon Biotechnologies, Germany were tested in this experiment to amplify the template DNA. Amplification reaction volumes were 25 µl, each containing 1X PCR buffer with $MgCl_2$ [50 mM KCl, 10 mM tris = HCl (pH=9.0), 2 mM $MgCl_2$ and 1% Trition X-100], 200 µM of each of dATP, dGTP, dCTP and dTTP, 50 PM primer, 50 ng template DNA and 1.5 µl of Taq polymerase. Reaction mixtures were exposed to the following conditions: 3 min at 94^{o}C for the first cycle, followed by 45 cycles at a denaturation temperature of 94^{o}C for 1min, annealing temperature 36 ^{o}C for 1 min, and extension temperature 72^{o}C for 2 min. After the last cycle samples were incubated for 7 min at 72 ^{o}C.

Amplification products were visualized with DNA marker on 1.5 % agarose gel with 1X TBE buffer and were detected by staining with an ethidium bromide solution for 30 min. Gels were then destained in deionized water for 10 min. and photographed with Polaroid films under U.V. light.

Table 1. Primers used for the genetic analysis of irradiated chrysanthemum

Primers	**Sequence (5′ → 3′)**
OPAB-01	CCGTCGGTAG
OPAB-09	GGGCGACTAC
OPA-03	AGTCAGCCAC
OPAB-14	AAGTGCGACC
OPB-08	GTCCACACGG
OPR-11	GTAGCCGTCT
OPT-06	CAAGGGCAGA
OPN-15	CAGCGACTGT
OPB-04	GGACTGGAGT
OPB-18	GGGAATTCGG
OPA-05	AGGGGTCTTG
OPA-07	GAAACGGGTG
OPB-07	GGTGACGCAG

Data Analysis

Data were scored for computer analysis on the basic of the presence of the amplified products for each primer. If a product was present in a cultivar, it was designated as '1', if absent, it was designated as '0', Ambiguous bands that could not be easily distinguished were not scored (Williams *et al.*, 1990). Pair wise comparisons of cultivars, based on the presence or absence of unique and shared polymorphic products, were used to determine percentage polymorphism in parent and mutant cultivars of chrysanthemum.

CHAPTER 3
RESULTS

3.1 *In vitro* propagation through ray florets culture

3.1.1 Callus induction

After 2 weeks of initial culture on MS medium supplemented with various concentrations of BA and 2, 4-D, the ray florets became swollen and began to change from a yellowish to green color and subsequent callus development was observed (Figure 2a, b). Differences in the ability to form callus were observed. The medium containing a combination of BA and 2, 4-D produced 100% callus, while no callus initiation was observed on medium with BA alone (Table 2). In addition, the explants cultured on MS medium without growth regulators did not produce any callus. Moreover, with a gradual increasing concentrations of 2, 4-D alone there was a gradual increasing in the percentage of callus formation. By successive subculture, masses of proliferating callus cultures were established. The callus induction in all treatments resulted in developing in the beginning a light green compact callus starting from the adaxial surface and the cut end of the explants. Growth of the callus was rather slow but after 40 days of culture the speed of callus proliferation increased, resulting in a callus clump that covered the entire explants surface (Figure 2c).

Table 2 Effect of BA and 2, 4-D on callus induction (%) from the ray florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam.

BA (μM)	2, 4- D (μM)	Callus formation (%) (Mean ± SE)
0.0	0.0	0.0 ± 0.0a
	0.5	93.3 ± 4.6b
	2.2	96.0±0.0b,c
	4.5	100 ± 0.0c
4.4	0.0	0.0 ± 0.0a
	0.5	100 ± 0.0c
	2.2	100 ± 0.0c
	4.5	100 ± 0.0c
8.9	0.0	0.0 ± 0.0a
	0.5	100 ± 0.0c
	2.2	100 ± 0.0c
	4.5	100 ± 0.0c
13.3	0.0	0.0 ± 0.0a
	0.5	100 ± 0.0c
	2.2	100 ± 0.0c
	4.5	100 ± 0.0c

The different letters within column indicate significant differences analyzed by DMRT at $p \leq 0.05$.

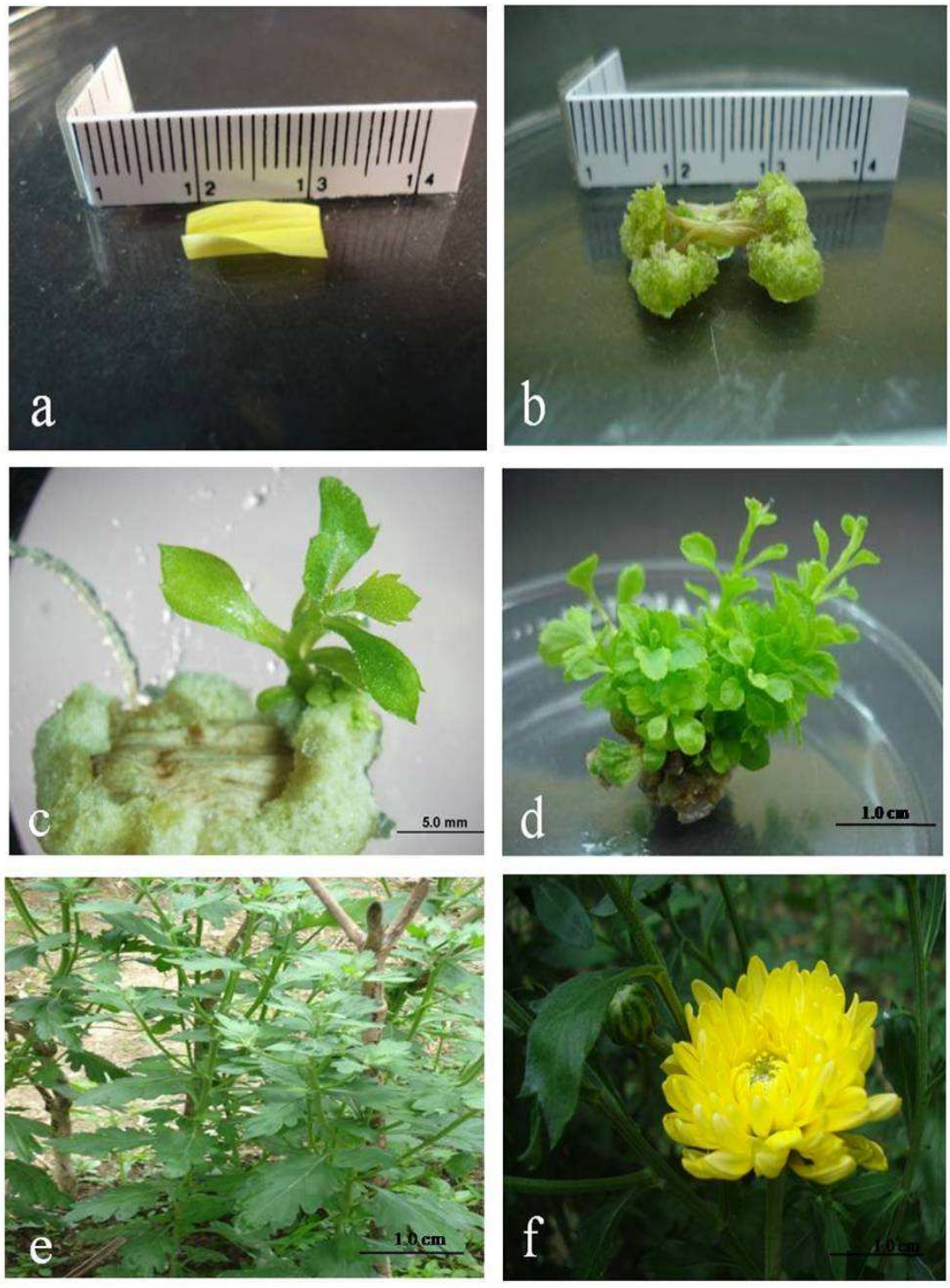


Figure 2 Plantlet regeneration from ray floret culture of chrysanthemum.

a) Ray floret, b) Callus formation on ray florets, c) Shoot regenerated from callus, d) Multiple shoots derived from callus, e) Hardened plantlets established in the green house, f) Fully developed flower.

3.1.2 Plant regeneration and root induction

When the calluses were transferred to MS medium supplemented with 9.3 μM Kinetin and 4.9 μM IBA the results revealed that calluses formed the maximum percentage of shoot regeneration (45.0%). However, the highest number of shoots per callus was found on MS medium supplemented with either 13.9 μM Kinetin or 9.8 μM IBA. The percentage of shoot regeneration and the number of shoots per callus showed significant differences among the treatments with $p \leq 0.05$ (Table 3). Shoots elongated on these media (Figure 2d) and plantlets regenerated from callus culture through organogenesis were allowed to grow for another 4 weeks. Kinetin and its concentrations were the main determinants of adventitious shoot production, since the addition of IBA into the culture media did not enhance shoot regeneration.

The rooting required presence of either IBA or chitosan in the medium. With a gradual increase concentration of IBA alone there was a gradual increase in the number of roots. Optimal number of roots per shoot was obtained on MS medium supplemented with 12.3 μM IBA, while higher concentrations of IBA reduced the number of roots per shoot (Table 4). It is interesting to note that when IBA was used in combination with chitosan, the number of roots per shoot declined compared to IBA alone. All propagated plantlets showed true-to-type leaf form, shape, growth pattern and the color of the ray florets of fully developed flowers (Figure 2e, f).

Table 3 Effect of different concentrations of Kinetin and IBA on shoot regeneration and number of shoots from ray floret calluses of *Chrysanthemum x grandiflorum* (Ramat.) Kitam. after 3 months of culture on MS medium

Kinetin (µM)	**IBA (µM)**	**Shoot regeneration % (Mean ± SE)**	**Number of shoots/callus (Mean ± SE)**
0	0.0	0.0 ± 0.0a	0.0 ± 0.0a
	2.5	17.6 ± 9.5bc	4.0 ± 1.9b
	4.9	0.0 ± 0.0a	0.0 ± 0.0a
	7.4	0.0 ± 0.0a	0.0 ± 0.0a
	9.8	4.3 ± 4.3ab	5.0 ± 2.1b
4.6	0.0	12.5 ± 12.5bc	3.0 ± 1.7ab
	2.5	5.8 ± 5.8ab	1.0 ± 1.0a
	4.9	7.1 ± 7.1ab	3.0 ± 1.7ab
	7.4	0.0 ± 0.0a	0.0 ± 0.0a
	9.8	7.1 ±7.1ab	1.0 ± 1.0a
9.3	0.0	37.0 ± 9.4de	3.0 ± 1.7ab
	2.5	28.5 ± 12.5cd	1.0 ± 1.0a
	4.9	45.0 ± 11.4e	3.0 ± 1.7ab
	7.4	30.4 ± 9.8cd	3.0 ± 1.7ab
	9.8	10.5 ± 7.2bc	1.0 ± 1.0a
13.9	0.0	44.4 ± 12.0e	5.0 ± 2.1b
	2.5	35.2 ± 11.9de	4.0 ± 1.9b
	4.9	39.1 ± 10.4de	3.0 ± 1.7ab
	7.4	23.0 ± 12.1cd	2.0 ± 1.4a
	9.8	43.7 ± 12.8e	1.0 ± 1.0a

The different letters within column show significant difference of shoot regeneration (Mean ± SE) analyzed by DMRT at $p \leq 0.05$.

Table 4 Effect of different concentrations of IBA and chitosan on root induction from shoots of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. after 1 month of culture on MS medium.

IBA (µM)	**Chitosan (µM)**	**Root induction %(Mean±SE)**	**Number of roots/shoot (Mean ± SE)**
0	0.0	100 ± 0.0a	15.0 ± 3.5a
	43.5	100 ± 0.0a	25.0 ± 4.3bcd
	93.2	100 ± 0.0a	35.0 ± 4.7de
7.4	0.0	100 ± 0.0a	28.0 ± 4.5cd
	43.5	100 ± 0.0a	19.0 ± 3.9ab
	93.2	100 ± 0.0a	21.0 ± 4.0bc
12.3	0.0	100 ± 0.0a	40.0 ± 4.9e
	43.5	100 ± 0.0a	12.0 ± 3.2a
	93.2	100 ± 0.0a	13.0 ± 3.3a
17.2	0.0	100 ± 0.0a	33.0 ± 4.7de
	43.5	100 ± 0.0a	13.0 ± 3.3a
	93.2	100 ± 0.0a	13.0 ± 3.3a

The different letters within column indicate significant differences analyzed by DMRT at $p \leq 0.05$.

3.1.3 Histological studies of callus

The present study indicates that shoots proliferating from ray floret were from a single origin. Microscopic examination showed scattered meristematic cells on the ray floret epidermis (Figure 3a). Serial sections of the 7-day-old light green compact calluses revealed well-organized small cells attached to each other. In this instance, these shoots had originated from a single meristematic cell that had a dense cytoplasm with a well stained nucleus (Figure 3b). Shortly after several divisions of this single cell culture several shoot apex-like structures or meristemoids

were observed (Figure 3c, d). Because of this observation it could be assumed that a shoot originated from a single cell since a shoot with a unicellular origin is widely encountered.

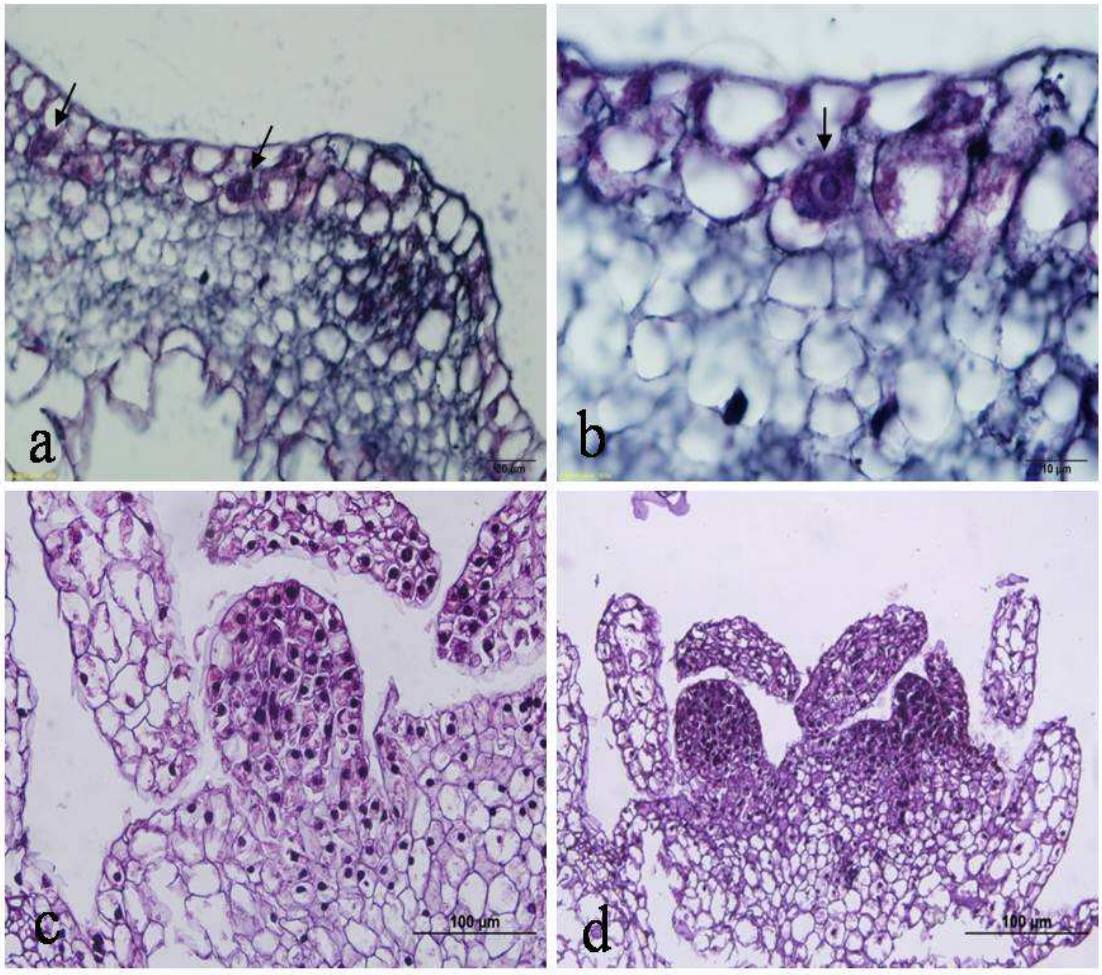


Figure 3 Histology of callus cultured on MS medium containing 13.3 µM BA and 0.5 µM 2, 4-D.

a) Scattered meristematic cells (arrows) on the ray floret epidermis,

b) A single meristematic cell with densely stained cytoplasm and nucleus (arrow), c) Spherical globules or meristemoids developed from meristematic cell, d) Formation of individual meristemoids.

3.2 Gamma irradiation in chrysanthemum

3.2.1 Effect of gamma ray irradiation on survival of irradiated calluses

The one-month-old calluses derived from ray florets initiated on MS medium supplemented with 13.3 µM BA and 0.5 µM 2, 4-D were irradiated with different doses of gamma rays and then transferred to the MS medium containing 13.9 µM Kinetin for shoot regeneration. The irradiated calluses were subcultured at 2-week-intervals. After 3 months of culture, the result clearly indicated that the survival percentage of the calluses was affected by the irradiation dose (Table 5). The survival percentage of the calluses decreased with an increasing irradiation dose. The untreated calluses produced a 100% of the survival while the survival percentage at 5 Gy was 75.0. At 10, 15, 20, 25 and 30 Gy of irradiation dose the survival percentage was significantly decreased to 72.5, 72.5, 65.0, 52.5, and 38.7 respectively. Moreover, the growth of treated calluses with 10, 15, 20, 25and 30 Gy of gamma rays was less than that of the controls. Treatments at 20 Gy and over caused yellow or green calluses to become browning wither and died within 30 days.

In order to obtain the LD_{50} (50% lethal dose) the data in Table 5 were plotted as shown in Figure 4. The LD_{50} obtained from the Figure 4 was found at 26 Gy.

Table 5 Survival rate of chrysanthemum calluses (as % of control) grown on MS medium containing 13.9 µM Kinetin after 3 months of irradiation with different doses of gamma rays.

Gamma ray dose (Gy)	Survival rate of calluses (% of control)
0.0	100.0
5.0	75.0
10.0	72.5
15.0	72.5
20.0	65.0
25.0	52.5
30.0	38.7

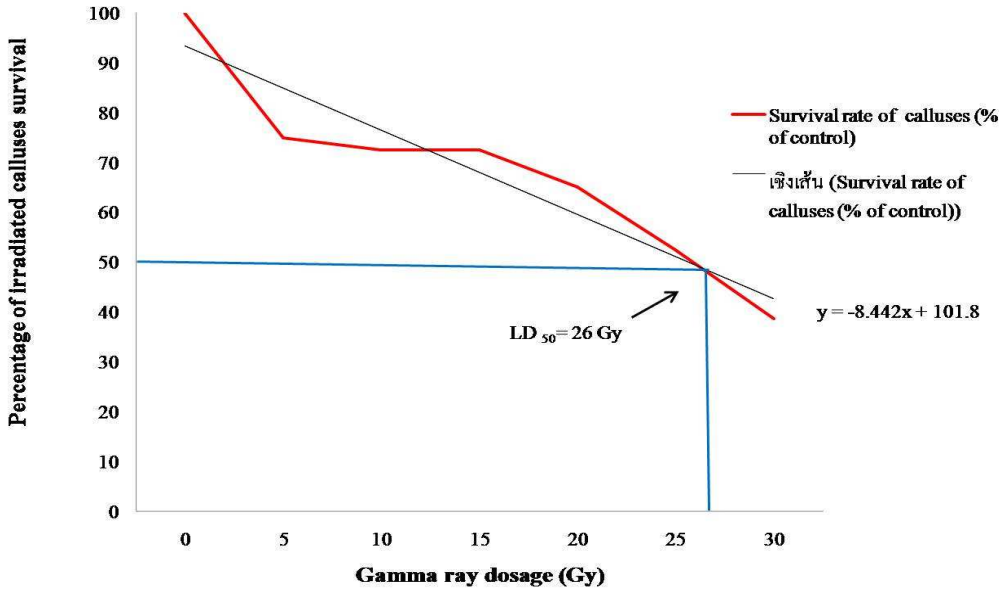


Figure 4 LD_{50} of 3 months old chrysanthemum calluses treated with gamma rays at different doses.

3.2.2 Effect of gamma ray doses on adventitious shoot regeneration from irradiated calluses

The results of gamma ray doses on percent of adventitious shoot regeneration and number of shoots per responding explant from irradiated calluses evaluated after 3 months on the MS medium containing 13.9 µM Kinetin are presented in Table 6. The irradiation dose negatively affected on regeneration rate of the calluses. That is with a gradual increase of irradiation doses there was a gradual decrease in the percentage of shoot regeneration. The highest percentage of adventitious shoot regenerated from the irradiated calluses was 42.3 at 5 Gy, which was lower than that in the control treatment (100%). When concentrations of gamma ray dose at higher than 5 Gy were applied, the percentage of shoot regeneration declined. The lowest percentage of adventitious shoot regeneration was 12.9 at 30 Gy. However, numbers of shoots per callus varied from 7.8 to 15.4 among the gamma ray doses. The mean number of shoots per callus was significantly highest at 15 Gy ($p \leq 0.05$). In addition, the irradiated calluses in all experiments provided the higher numbers of shoots per callus than the control treatment.

Table 6 The effect of gamma ray doses on adventitious shoot regeneration from irradiated calluses.

Gamma ray dosage (Gy)	Shoot regeneration % (Mean ± SE)	Number of shoots/callus (Mean ± SE)
0.0	100.0 ± 0.0d	5.0 ± 2.1a
5.0	42.3 ± 6.9c	9.8 ± 1.9b
10.0	39.6 ± 6.4c	11.2 ± 1.7c
15.0	23.8 ± 6.6b	15.4 ± 1.7d
20.0	20.0 ± 5.2b	12.8 ± 1.5c
25.0	18.9 ± 5.1a,b	10.8 ± 1.4b,c
30.0	12.9 ± 6.1a	7.8 ± 1.9b

The different letters within column show significant difference of shoot regeneration (Mean ± SE) analyzed by DMRT at $p \leq 0.05$.

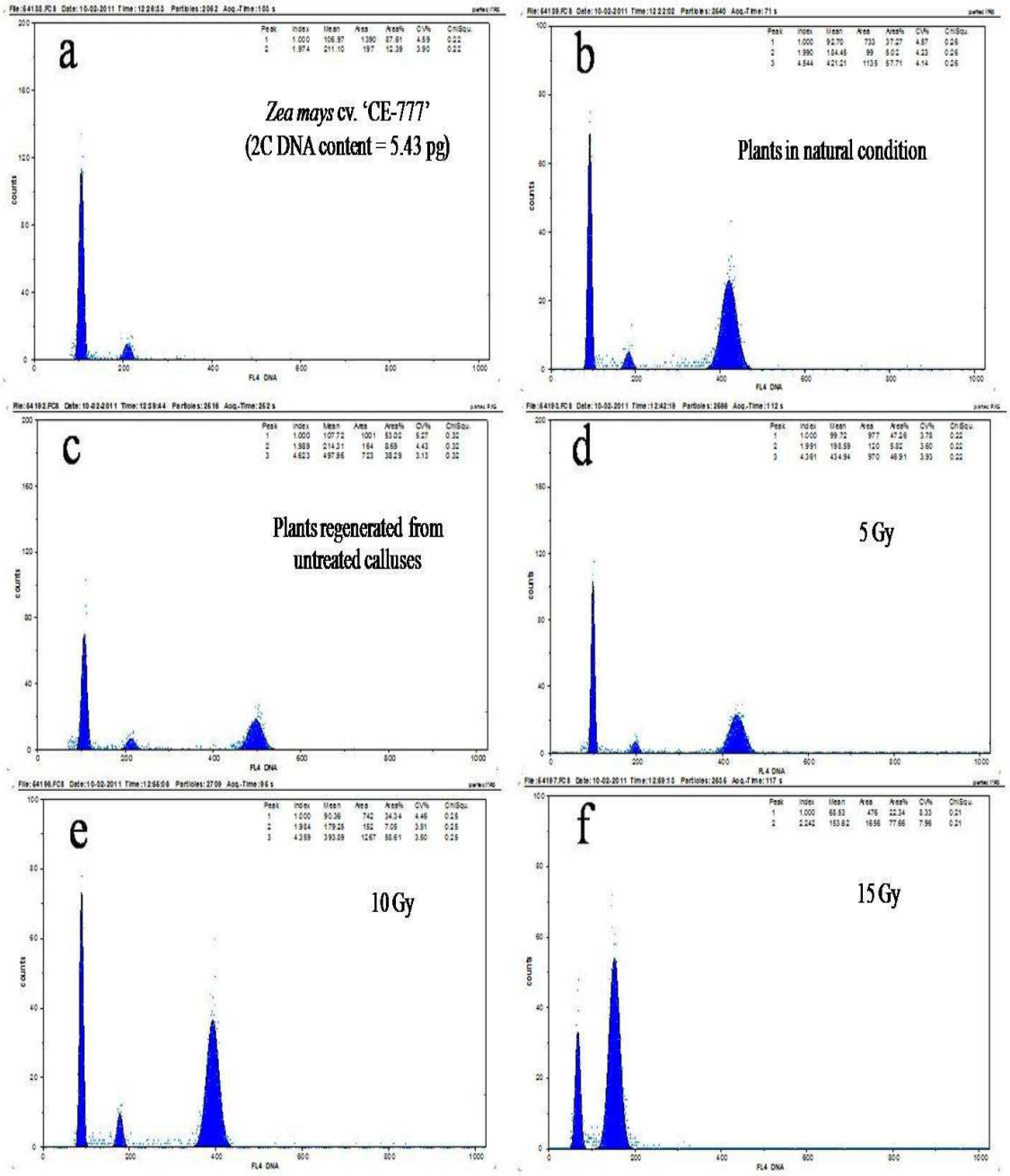
3.2.3 DNA content analysis in irradiated plantlets

Two leaves of regenerated plantlets from irradiated calluses in each treatment were subjected to flow cytometric analysis of nuclear DNA content on two different days. Chopping of young leaf tissues with a sharp razor blade lead to the release of high numbers of nuclei into the nuclei buffer solution. Representative histograms obtained during the analysis were shown in Figure 5. The histograms obtained from *Zea mays* cv. 'CE-777' (2C DNA content = 5.43 pg) used as an internal reference standard showed two peaks representing *Zea mays* nuclei in $G_1$and G_2 phase of cell cycle approximately on channel 100 and 200, respectively (Figure 5a, j). In addition, when chrysanthemum leaves in each experiment and *Zea mays* nuclei were isolated, stained and analyzed simultaneously, the histograms of fluorescence distribution contained three large peaks in experiments that plantlets derived from natural condition, regeneration from untreated calluses, and irradiated calluses (at 5, 10, and 30 Gy). Two peaks represent *Zea mays* nuclei in $G_1$and G_2 phase of cell cycle

approximately on channel 100 and 200, respectively and another peak represent chrysanthemum nuclei at about channel 400 (Figure 5b, c, d, e, i).

On the other hand, plantlets regenerated from irradiated calluses at 15, 20 and 25 Gy, the histograms of fluorescence distribution contained two large peaks corresponding to one peak of *Zea mays* nuclei in G_1 phase at about channel 100 and another peak of the irradiated plants nuclei in G_1 phase which was found to be the same position with the peak of *Zea mays* nuclei in G_2 phase at around channel 200 (Figure 5f, g, h).

The average values of nuclear DNA content of each treatment were listed in Table 7. Results obtained from flow cytometry analysis indicated that the average values of nuclear DNA content in plants regenerated from untreated calluses was 24.28 pg which closely with plants in natural condition was 24.98 pg while the nuclear DNA content in plants regenerated from the irradiated calluses in all treatments was significantly lower. The average values of nuclear DNA content in plants regenerated from the irradiated calluses at 5, 10, and 30 Gy was 23.68, 23.40, and 21.03pg, respectively. In contrast, the average values of nuclear DNA content in plants regenerated from the irradiated calluses at 15, 20, and 25 Gy was 10.99, 18.80, and 16.91pg, respectively. This result correspond with the reduction of peak position of the irradiated plantlets nuclei in G_1 phase from channel 400 to channel 200. The greatest decline was 10.99 pg at 15 Gy which was more than 50% reduction compared to the control plants. Variance in the nuclear DNA content increased significantly ($p \leq 0.05$) among the doses at 10 and 15 Gy. However, a flow cytometric measurement of DNA content always shows small changes resulting from variations in the preparation, staining and measurement procedures.


a
Zea mays cv. 'CE-777'
(2C DNA content = 5.43 pg)
b
Plants in natural condition
c
Plants regenerated from
untreated calluses
d
5 Gy
e
10 Gy
f
15 Gy
counts
FL4 DNA

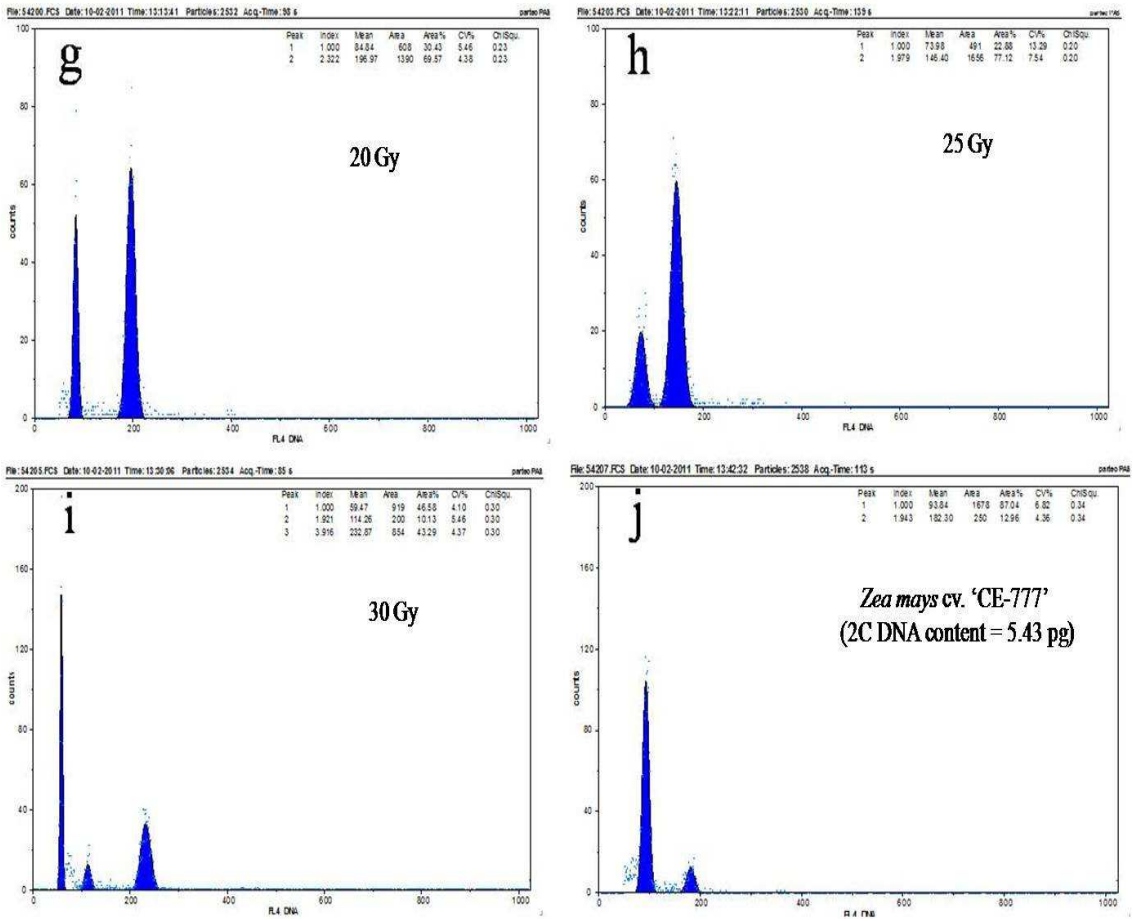


Figure 5 Nuclear DNA content histograms of irradiated calluses at various doses estimated by flow cytometry.

a and j) *Zea mays* cv. 'CE-777' (2C DNA content = 5.43 pg) used as an internal reference standard, b) Plants in natural condition ,c) Plantlets regenerated from untreated calluses, d, e, f, g, h and i) Plantlets regenerated from irradiated calluses at 5, 10, 15, 20, 25, and 30 Gy of gamma ray, respectively.

Table 7 Comparison of the relative DNA content in populations of the plants regenerated from irradiated calluses, untreated calluses and plants in natural condition.

Gamma ray dosage (Gy)	DNA content (pg) (Mean ± SE)	DNA content/DNA content of control
Plants in natural condition	24.98 ± 0.30 c	1.00
0	24.28 ± 0.80 c	0.97
5	23.68 ± 0.60 c	0.94
10	23.40 ± 0.26 c	0.93
15	10.99 ± 1.18 a	0.43
20	18.80 ± 6.19 b	0.75
25	16.91 ± 6.16 b	0.67
30	21.03 ± 0.22 c	0.84

The different letters within column show significant difference of the relative DNA content (Mean ± SE) analyzed by DMRT at $p \leq 0.05$.

3.2.4 Chromosome counting

Plantlets regenerated from the irradiated calluses exhibited highly significant decrease in number of chromosomes compared to plantlets regenerated from untreated calluses and plants in natural condition. Figure 6 showed chromosomes of root tip of the plants regenerated from irradiated calluses, untreated calluses and plants in natural condition. In root tip cells, plantlets regenerated from untreated calluses and natural plants contain 2n=54 chromosomes (Figure 6a) while root-tips squashed of plantlets regenerated from the irradiated calluses at 15, 20, and 25 Gy revealed 2n= 27, 37, and 34 chromosomes, respectively (Figure 6b, c, d).

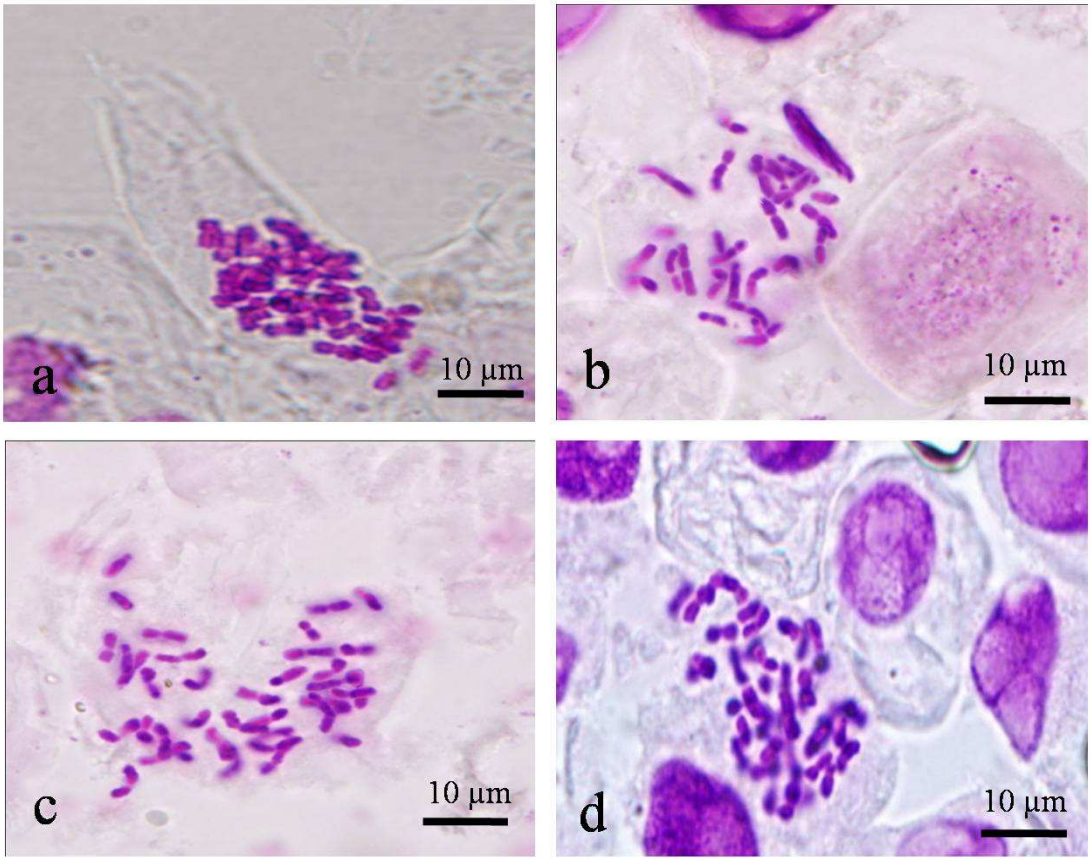


Figure 6 Mitosis metaphase chromosome of plantlets obtained from irradiated calluses.

(a) Control natural plants, 2n=54, (b) Treated plant with 15 Gy, 2n=27, (c) Treated plant with 20 Gy, 2n=37, (d) Treated plant with 25 Gy, 2n=34.

3.2.5 Morphological observation

Three months old plants regenerated from calluses were screened for the observation of any morphological abnormalities after treated with gamma ray at different doses. The results revealed that with 30 Gy treatment, leaf chimera was evidenced while with 15 Gy treatment, short compact shoots, fasciations, rosette leaf and vitrification were found (Figure 7).

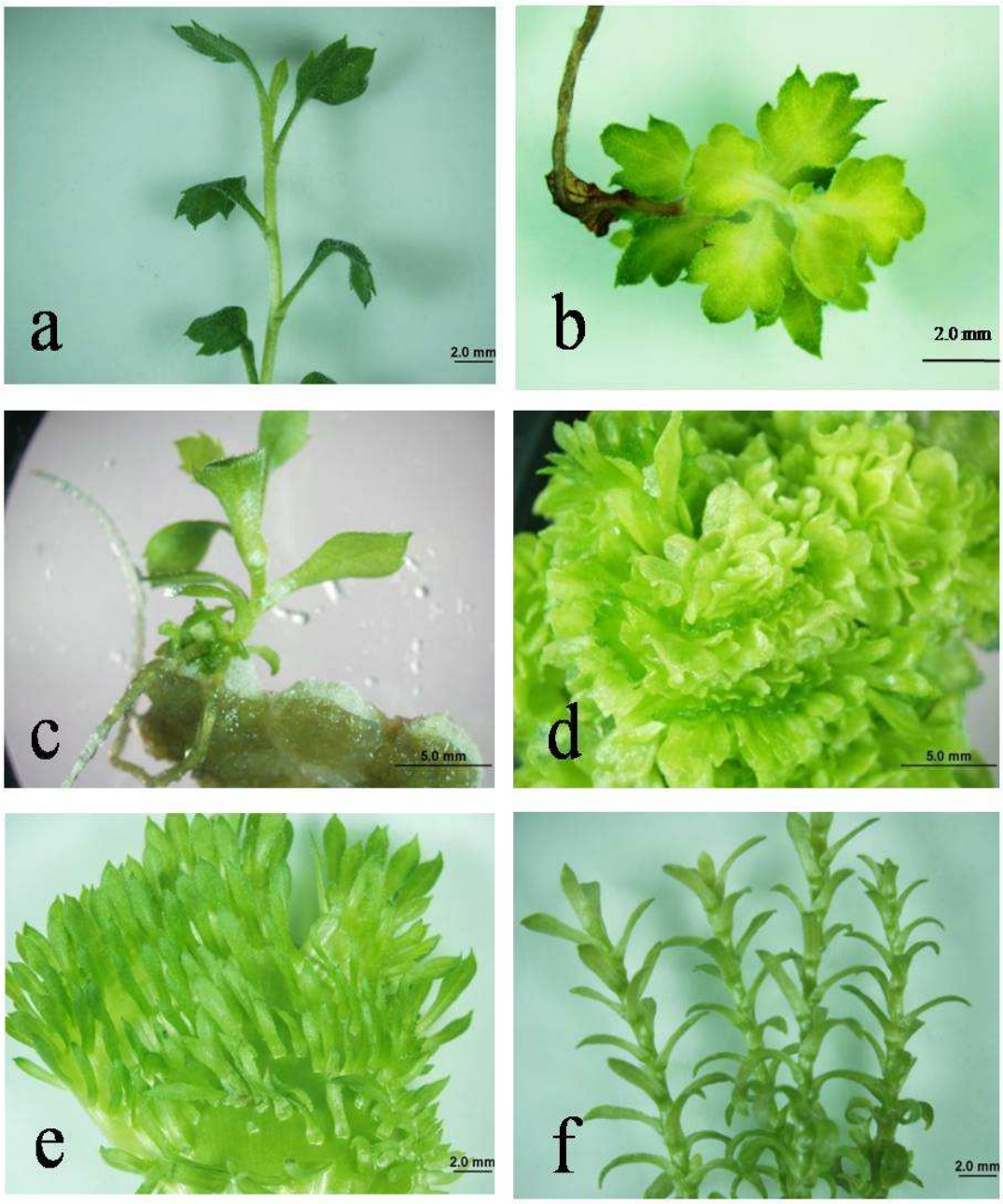


Figure 7 Variations in shoots and leaves of plants from the irradiated calluses.

(a) Untreated plant (control), (b) Chimera leaves after treated with 30 Gy, (c) Regenerated plant from callus, (d) Rosettes and vitrification after treated with 15 Gy, (e) Short compact shoots and fasciations after treated with 15 Gy, (f) Stem with short nodes after treated with 15 Gy.

3.2.6 Molecular marker analysis

In the present study, DNA extraction of the leaf tissue gave good yields (more than 80 ng DNA) for several RAPD reactions (Figure 8). The RAPD reaction generally gave good reproducible result; in all cases a polymorphic fragment pattern was repeated to confirm the results. Each accession of polymorphic band was scored for presence (1) and for absence (0). RAPD bands within accessions were scored as missing if they were poorly resolved on the gel or if the template DNA did not amplify well (Table 8).

Moreover, thirteen primers were screened for their ability to amplify the genomic DNA of the *Chrysanthemum x grandiflorum* (Ramat.) Kitam. and its mutants, five were selected on the basis of robustness of amplification, reproducibility, scorability of banding patterns and were employed for diversity analysis. The percentage of polymorphism was found varied from 14 (OPAB-09, OPA-03) to 66 (OPB-08) (Table 9). Five informative RAPD primer combinations generated a total of 97 reproducible amplification fragments across all radiomutants and parent cultivars, among which 31 bands were polymorphic (Table 9). The number of amplified RAPD bands varied from a minimum of 6 to a maximum of 28 depending on the primer and the DNA sample with a mean value of 19.4 bands per primer. The size of fragments ranged from 300 to 3,000 bp.

However, Figure 9 shows the amplification profiles, generated by primers OPB-18 across the chrysanthemum cultivar and its somaclones. PCR amplification with primer OPB-18 clearly revealed that in plantlets regenerated from irradiated calluses at 20, 25, and 30 Gy (lane 6, 7, 8), a highly specific band (500 and 700 bp) is absent in comparison to the plantlets in other doses and the plants in natural conditions indicating that they are highly polymorphic when compared to other mutants (indicated by arrow in Figure 9). Moreover, a band of 3000 bp was noticed only in plantlets regenerated from irradiated calluses at 30 Gy (lane 13, 14) when the genomic DNA was amplified with primer OPB-08, indicating that the plants regenerated from irradiated calluses at 30 Gy is polymorphic when compared to plants derived from other conditions (indicated by arrow in Figure 10). Similarly, a polymorphic band of 900 bp is absent in natural plants and plants regenerated from untreated calluses (lane 1, 2) but present in all plants regenerated from irradiated

calluses (indicated by arrow in the Figure 10) when RAPD marker OPB-08 was used. This result indicated that their cultivars are highly polymorphic compared to plants regenerated from irradiated calluses.

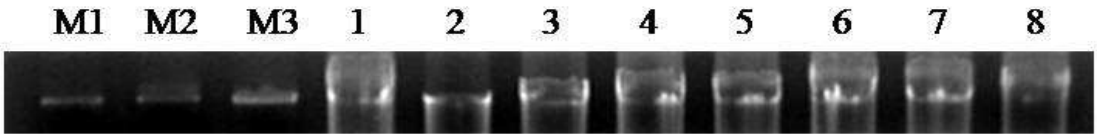


Figure 8 The quantity of DNA extracted from the leaf tissue of parent and mutant of chrysanthemum compared with lamda DNA (λ DNA) digested with *Eco*RI/*Hin*dIII.

Lane 1, plants in natural condition; lane 2, plants regenerated from non-irradiated calluses; lane 3-8, plants regenerated from irradiated calluses at 5, 10, 15, 20, 25 and 30 Gy, respectively; M1, M2, and M3, λ DNA at 40, 60, and 80 ng, respectively.

Table 8 DNA fingerprinting of the original *Chrysanthemum x grandiflorum* (Ramat.) Kitam. and its mutants using five primers: OPAB-09, OPA-03, OPAB-14, OPB-08, and OPB-18.

Primer	Size of fragment (bp)	Dose							
		plants in natural condition	0	5	10	15	20	25	30
OPAB-09	800	0	0	1	1	1	1	1	1
	600	1	1	1	1	1	1	1	1
	500	1	1	1	1	1	1	1	0
OPA-03	3000	1	1	1	1	1	1	1	1
	600	1	1	1	1	0	0	0	0
	500	1	1	1	1	1	1	1	1
	300	1	1	1	1	1	1	1	1
OPAB-14	400	1	1	0	1	1	1	1	0
OPB-08	3000	0	0	0	0	0	0	0	1
	900	0	0	1	1	1	1	1	1
	800	1	1	1	1	0	0	0	0
	700	1	1	1	1	1	0	0	0
	400	1	1	1	1	1	1	1	1
OPB-18	700	1	1	1	1	1	0	0	0
	500	1	1	1	1	1	0	0	0
	400	1	1	1	1	1	1	1	1

The band: present (1), absent (0) or without product (-).

Table 9 Percentage polymorphism in parent and mutant cultivars of chrysanthemum with reproducible random primer

Primer	Total no. of bands	No. of polymorphic bands	Percentage polymorphism
OPAB-09	21	3	14.29
OPA-03	28	4	14.29
OPAB-14	6	2	33.33
OPB-08	24	16	66.67
OPB-18	18	6	33.33
Total	97	31	-
Average	19.4	6.2	-

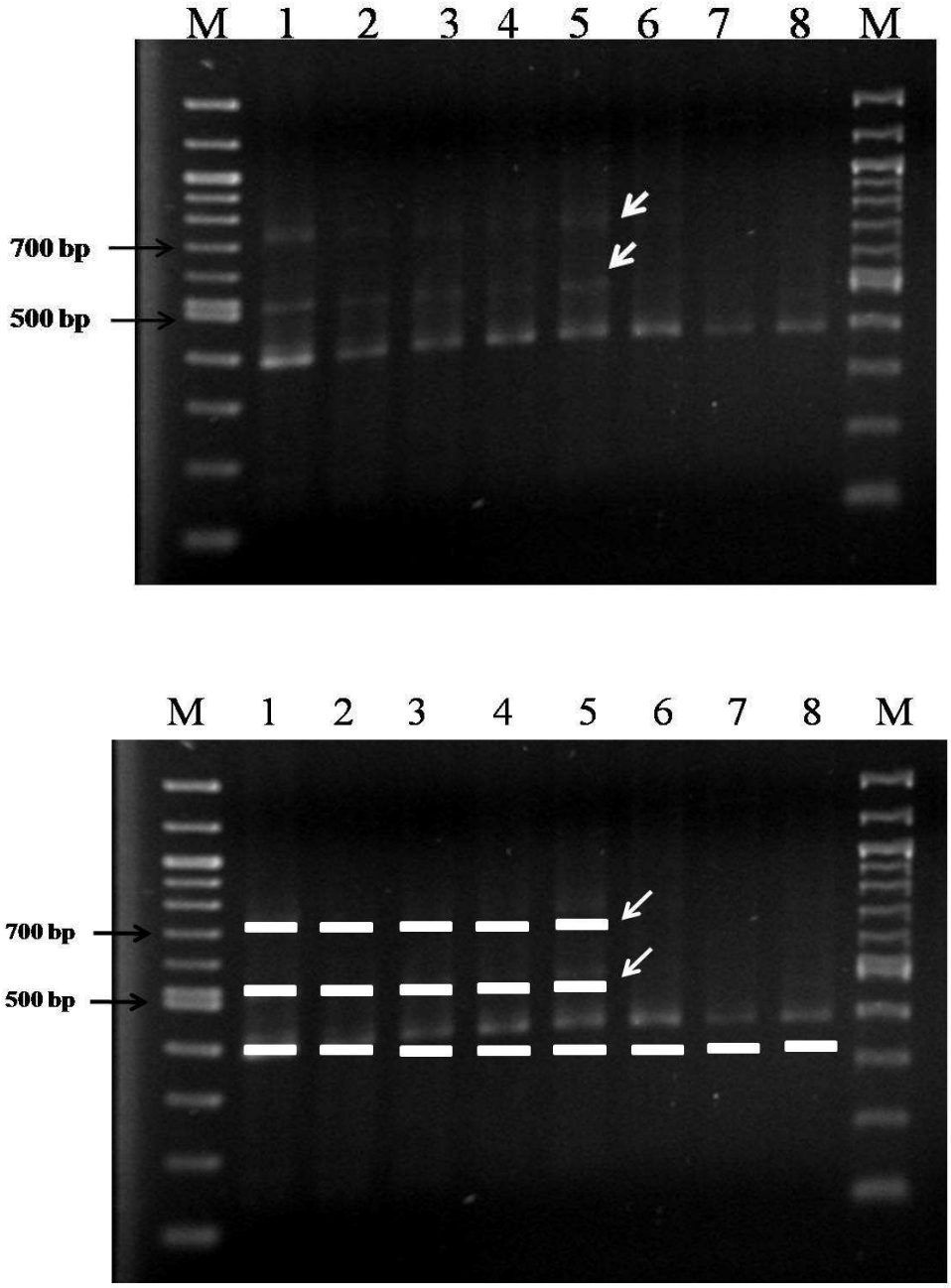


Figure 9 Amplification of parent and mutant cultivars of chrysanthemum using primer OPB-18.

Lane 1, plants in natural condition; lane 2, plants regenerated from non-irradiated calluses; lane 3-8, plants regenerated from irradiated calluses at 5, 10, 15, 20, 25 and 30 Gy, respectively. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

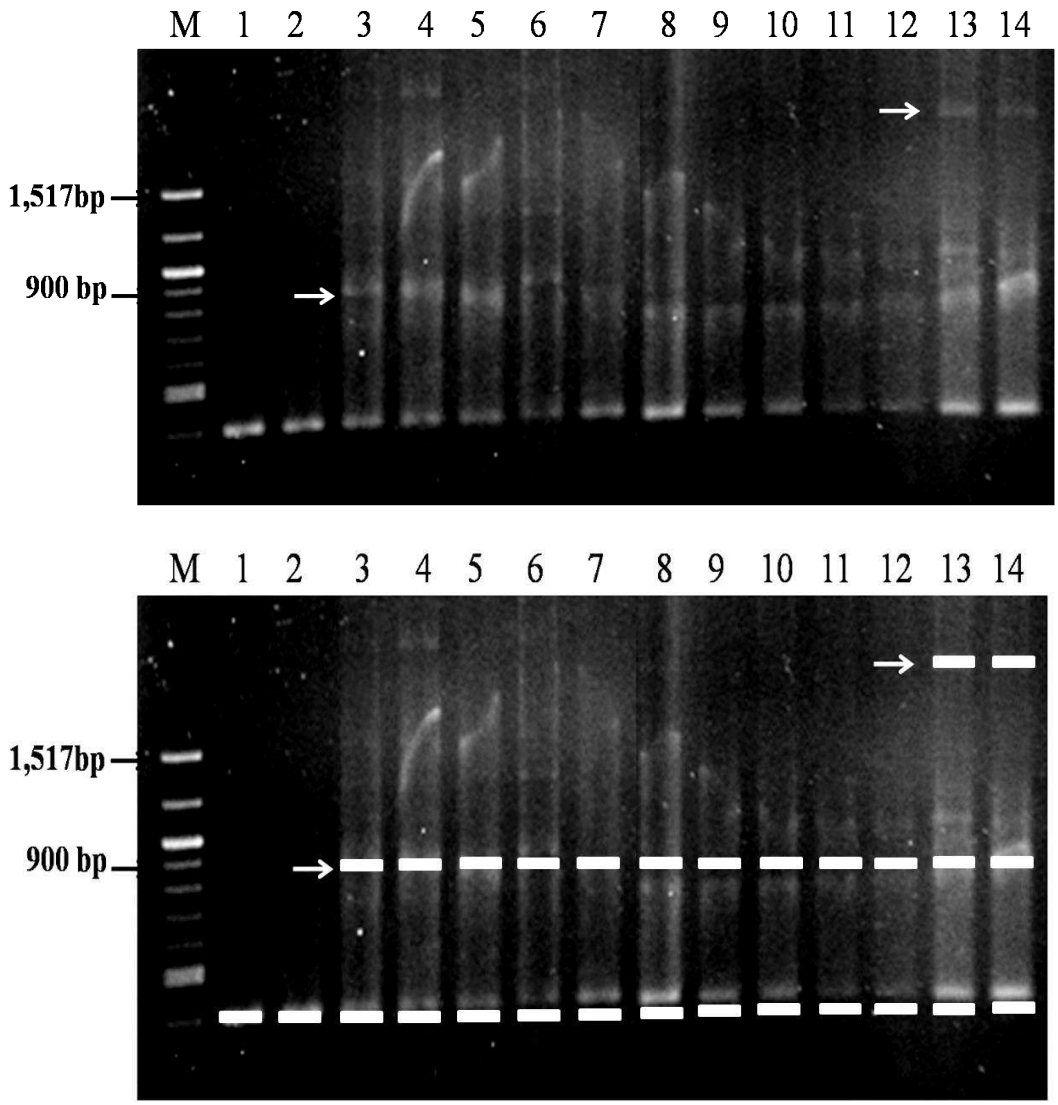


Figure 10 Amplification of parent and mutant cultivars of chrysanthemum using primer OPB-08.

Lane 1, plants in natural condition; lane 2, plants regenerated from non-irradiated calluses; lane 3-4, plants regenerated from irradiated calluses at 5 Gy; lane 5-6, plants regenerated from irradiated calluses at 10 Gy; lane 7-8, plants regenerated from irradiated calluses at 15 Gy; lane 9-10, plants regenerated from irradiated calluses at 20 Gy; lane 11-12, plants regenerated from irradiated calluses at 25 Gy; lane 13-14, plants regenerated from irradiated calluses at 30 Gy, respectively. M, DNA molecular weight marker λ DNA digested with *Eco*RI/*Hin*dIII.

CHAPTER 4
DISCUSSION

4.1 *In vitro* propagation through ray florets culture

4.1.1 Callus induction

Callus formation from ray floret segments of *Chrysanthemum x grandiflorum* (Ramat.) Kitam. was studied. MS medium was used throughout the experiments. The medium containing a combination of BA and 2, 4-D gave 100% callus production, while no callus initiation was observed on medium with BA alone. Moreover, with a gradual increasing concentrations of 2, 4-D alone there was a gradual increasing in the percentage of callus formation. From this and previous studies (Smith and Krikorian, 1990, 1991; Chang and Chang, 1998; Ignacimuthu *et al.*, 1999) it is evident that exogenous auxin is the most important growth regulator for the induction of embryogenic callus in the majority of angiosperms. Recently, Obukosia *et al.* (2005) also reported the influence of auxins on callus production from both leaf and stem segments of chrysanthemum cultivars. Among the growth regulators used, 2, 4-D was the most effective growth regulator for chrysanthemum callus induction either when used alone or in combinations with BA. Castillo *et al.* (1998) reported that 2, 4-D by itself or in combination with cytokinins has been widely used to enhance callus induction and maintenance. Moreover, many researchers observed 2, 4-D as the best auxin for callus induction in monocot and even in dicot (Evans *et al.*, 1981; Ho and Vasil, 1983; Jaiswal and Naryan, 1985; Chee, 1990; Mamun *et al.*, 1996). 2, 4-D acts as an auxin to induce cell division and enlargement at low concentration. Cell enlargement has been associated with an increase in activities of synthesis of new wall materials (Cleland, 1971). Similar results were found in achenes and petals of *Chrysanthemum coccineum* (Fuji and Shimizu, 1990).

However, as observed in the present study, only one species in this result requires supplementary cytokinin along with auxin for optimum response and in some cases to prevent necrosis of callus (Chang and Chang, 1998; Ishii *et al.,* 1998; Ignacimuthu *et al.,* 1999). This requirement for exogenous cytokinin could be related to the maintenance of a proper balance between auxin and cytokinin, which act synergistically to regulate cell division (Johri and Mitra, 2001), a process essential for callus formation. Moreover, Rout and Das (1997) reported that appropriate auxin and cytokinin levels are required for callus induction from each species or variety. Our results are also in agreement with those of Trewavas and Cleland (1983) who reported that combination of BA and 2, 4-D evoked the best response for induction of callus and was probably due to the difference in endogenous levels of growth regulators in this plant or to a difference in sensitivity.

Chai and Mariam (1998) reported that cytokinins, such as BA and Kinetin, at low concentrations, in combination with auxins were often used in plant species to promote callus initiation. Such effect of BA and 2, 4-D on callus induction agrees with the results of other experiments with chrysanthemum (May and Trigiano 1991, Mandal and Datta 2005). Some other studies produced different results using a combination of BA and NAA (Lu *et al.*, 1990; Mandal *et al.*, 2000 b; Nahid *et al.*, 2007) because callus formation takes place under the influence of exogenously supplied growth regulators present in the nutrient medium. The type of growth regulator requirement and its concentration in the medium depends strongly on the genotype and endogenous hormone content of an explant.

Moreover, using a combination of BAP and NAA to induce callus from chrysanthemum nodal explants have been reported by Ilahi *et al.,* (2007). They reported that the most suitable hormonal combination for callus induction was found to be 0.5 mg/l BAP, alongwith 0.1 mg/l NAA. These results are in agreement with those of Chakrabarty *et al.,* (2000). They also reported callus formation in chrysanthemum on MS medium supplemented with 1.0 mg/l BAP and 0.2 mg/l NAA. Their findings are also in conformation to the results obtained by Hussain *et al.,* (1994).

However, the explants cultured on MS medium without growth regulators did not produce any callus. This result is in support of the results obtained by Fiegert *et al.* (2000), Jayasree *et al.* (2001) and Yasmin *et al.* (2003).

So, many other factors are important for callus formation: genotype, composition of the nutrient medium, physical growth factors (light, temperature, ect.). The MS (1962) mineral medium or modifications of this are often used. Sucrose or glucose (2-4%) is usually employed as the sugar source. The effect of light on callus formation is dependent on the plant species; light may be required in some cases and darkness in other cases. A temperature of 22-28 ^{o}C is normally advantageous for callus formation.

4.1.2 Plant regeneration and root induction

Present results indicate that Kinetin and its concentrations were the main determinants of adventitious shoot production, since the addition of IBA into the culture media did not enhance shoot regeneration. Our results are in agreement with the findings of Jesmin *et al.* (2007) who reported that Kinetin was the most effective agent for regenerating shoots from callus of *Chrysanthemum morifolium*. They reported that 1 mg/l Kinetin combined with 0.1 mg/l NAA significantly promoted shoot induction compared to those grown at higher concentration of BA (5 mg/l), Kinetin (3 mg/l) or NAA (1 mg/l). Among the different treatments shoot regeneration frequency could be improved by manipulating the composition of Kinetin in culture medium. The number of regenerated shoot buds depends on the composition of culture medium, especially on level of plant growth regulators (PGRs) (Rout and Das, 1997).

Cytokinins especially Kinetin known as ethylene-inducing plant hormones, play an essential role in various aspects of plant growth and development. Plants that are wounded or exposed to environmental stresses or pathogen attacks usually exhibit an enhanced ethylene production. Moreover, ethylene also acts as an endogenous regulator of various morphogenic processes, including the determination of the size of organs (Bleecker *et al.,* 1998), and inhibition is often associated with the action of ethylene (Abeles *et al.,* 1992). Our results are consistent with this

observation, since shoot regeneration, and number of shoot per explants significantly increased on media containing Kinetin.

N^6- benzyladenine (BA), a synthetic cytokinin, synergistically enhanced ethylene production in the presence of IAA in mungbean hypocotyls (Yoshii and Imaseki, 1982). Kinetin alone slightly stimulated ethylene production by etiolated seedlings in several species (pea, mungbean) while the remarkable synergistic effect of Kinetin on IAA-induced ethylene production has been observed (Fuches and Lieberman, 1968).

Based on the foregoing discussion, it can be concluded that Kinetin plays a vital role in regeneration in relation to ethylene production. Therefore the effect of Kinetin was optimal on the regeneration from ray floret callus of chrysanthemum. In addition, low concentrations of IBA are good for shoot regeneration.

Root induction

Generally, adventitious root formation has many practical implications in horticulture and agronomy and there is a lot of commercial interest because of the many plant species that are difficult to root (Davies *et al.,* 1994; Kovar and Kuchenbuch, 1994). Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez *et al.,* 1989; Epstein and Ludwig- Muller, 1993; Blazkova *et al.,* 1997). It was shown, for example, that a difficulty-root cultivar of *Prunus avium* conjugated IBA more rapidly than an easy-to-root cultivar (Epstein *et al.,* 1993). Only in the easy-to-root cultivar was the appearance of free IBA observed after several days and the authors concluded that the difficult-to-root cultivar was not able to hydrolyse IBA conjugates during the appropriate time points of adventitious root development. Interestingly, it was possible to induce rooting of the difficult-to-root cultivar after application of an inhibitor of conjugation (Epstein *et al.,* 1993). It has been shown that having IBA is even more active than free IBA in the promotion of adventitious roots in mung bean, possibly due to its higher stability during the rooting process (Wiesman *et al.,* 1989).

However, other differences such as uptake and transport can also account for the differences in rooting behaviour (Epstein and Ludwig-Muller, 1993).

In this study, we confirmed with root initiation of the chrysanthemum cutting that chitosan can function as a plant growth stimulator for root initiation in chrysanthemum production or tissue culture. The optimal amount of 93.2 µM of chitosan supplementation in MS IBA free solid medium gave the highest root formation per explant. This information is similar to the results of Nge *et al.* (2006) who reported that the optimal concentration of chitosan for promoting growth of *Dendrobium* orchid tissue was also 15 mg/l. Barka *et al*. (2004) reported that 1.75% (v/v) chitosan solution in culture medium enhanced root and shoot biomass of grapevine plantlets *in vitro*. However, in this study we found that chitosan supplement to solid medium promoted root formation but this activity can be inhibited from the combination between chitosan and IBA. This indicates that chitosan may regulate the activity of IBA. Uthairatanakij *et al.* (2007) reported that chitosan may play a role in enhancing growth and development by some signaling pathway to auxin biosynthesis via a tryptophan-independent pathway.

Recently chitosan has attracted considerable interest due to its biological activities such as antimicrobial, antitumor and as a stimulant of plant growth (Chibu and Shibayama, 2001; Khin *et al.,* 2006). From this result MS medium supplemented with IBA and chitosan may bring about high level of auxin which can inhibit growth and kill the cutting. This information was supported by Sopalun *et al*. (2010) who reported that chitosan supplemented to agar medium promoted shoot formation but not rooting. However, supplementation at too high a level, such as 100 mg/l can inhibit growth and kill *Grammatophyllum speciosum* protocorm-like bodies.

In this study, the rooting required the presence of either IBA or chitosan in the medium. With a gradual increase of IBA alone there was a gradual increase in the number of roots. Optimal number of roots per shoot was obtained on MS medium supplemented with 12.3 µM IBA, while higher concentrations of IBA reduced the number of roots per shoot. This result indicates that IBA is an important factor for rooting. It is interesting to note that when IBA was used in combination with chitosan, the number of roots per shoot declined compared to when it was used alone.

However, No further report exists on the effect of combination between IBA and chitosan in root induction.

4.1.3 Histological studies of callus

The generation of genetic variation by induced mutations or genetic transformation occurs in a single cell within multicellular structures; therefore, knowledge on organ formation and location of the competent cells is important (Mendoza *et al*., 1993; Lee *et al*., 1997). Moreover, in order to understand the organogenic process, observations of cellular level changes and their correlation with biochemical modifications are necessary (Apezzato-Da-Gloria and Machado, 2004). The present study describes the organogenesis process from ray floret callus of *Chrysanthemum x grandiflorum* (Ramat.) Kitam. cultured on 13.3 µM BA and 0.5 µM 2, 4-D. In our experiments, a light microscope observations revealed two distinguishable groups of cells within the calluses: competent organogenic cells and non-organogenic cells. The former cells resemble meristematic or embryogenic cells. Studies using light microscopy in many plant species have provided detailed descriptions of the pluripotent cells within the shoot and root meristems. These cells are isodiametric and small with a dense cytoplasm, spherical shaped nucleus and prominent nucleolus and with a higher metabolic activity (Verdeil *et al*., 2007).

In contrast, the non-organogenic cells were large cells with small nucleus, agreeing with the observations of Popielarska *et al*. (2006) who described kiwi fruit non morphogenic cells as elongated and highly dissociated. Moreover, the TEM observations of Mendez *et al.* (2009) showed that organogenic competent cells have abundant endoplasmatic reticulum and mitochondria which suggest high rates of protein synthesis and cellular respiration in response to rapid cellular growth, as reported in *B. forficate* and *G. max* (Apezzato-Da-Gloria and Machado, 2004). All these events are essential for the cell division in the organogenic calluses for shoot and root formation. In general, these characteristics have also been described in embryogenic process of maize and are associated with high metabolic activity (Fransz and Schel, 1991a, b).

From histological analysis in this study, it indicates that shoots proliferating from ray florets were from a single origin. Microscopic examination showed scattered meristematic cells on the ray floret epidermis similar to that of the results obtained from Oka *et al.* (1999) who reported that their embryoids were shown to form directly on the cut edges of garland chrysanthemum leaf explants on medium containing 0.1 mg/l BA and 1 mg/l NAA, and originated from epidermal and sub-epidermal layers, but not from callus. Serial sections of our 7-day-old light green compact calluses revealed well-organized small cells attached to each other. In this instance, these shoots had originated from a single meristematic cell that had a dense cytoplasm with a well stained nucleus. Shortly after several divisions of this single cell culture several shoot apex-like structures or meristemoids were observed. Because of this observation we could assume that a shoot originated from a single cell since a shoot with a unicellular origin is widely encountered, for example, in Guinea grass (Lu and Vasil, 1985) and oil palm (Kanchanapoom and Domyoas, 1999).

Moreover, in other species there have been many reports about the origin of callus. To illustrate, in the rice calluses grew from vascular tissue of mesocotyl and epithelium tissue of scutellum. When they were reactivated, divisions led to derivatives which became arranged in a procambial strand zone which ensured the growth of the calluses (Yanvisadepakdee *et al.*, 2000). Proliferation of calluses from cells adjacent to the vascular bundles has already been observed in various monocotyledons (Schwendiman *et al.*, 1988). Previous reports on callus formation of rice also indicated that mesocotyl (Nishimura and Maeda, 1977) and scutellum (Nishimura and Maeda, 1977; Maeda, 1980) were the origin of callus formation. In other cereals such as rye and triticale calluses originated from the scutellum, scutellar node, and region of the radicle of cultured embryos (Eapen and Rao, 1982).

In orchid tissue culture from meristem, inflorescence and leaf, the calluses formed at first on explants and differentiated into plantlets. In the culture of buds of *Vanda* x Miss Joaquim reported by Kanchanapoom *et al.* (1991) showed that the single epidermal cells which retain meristematic potential develop indirectly in to somatic embryogenesis with an intervening callus stage. Such indirect embryogenesis has been described in *Ranunculus sceleratus* (Konar *et al.*, 1972) and *Daucus carota* (Kato and Takeuchi, 1966). In early stages of the regeneration of these plants, the

mode of epidermal cell division is quite similar to that of embryogenesis from Vanda leaf epidermis. In addition, Intuwong and Sagawa, (1973) reported that young inflorescence of Sarcanthine orchid showed clusters of meristematic activities in the hypodermal layers of rachis, from which protocorm-like bodies developed. Vajrabahya and Vajrabhaya (1970) noted that new tissues of *Dendrobium* inflorescence derived from epidermis.

4.2 Gamma irradiation in chrysanthemum

4.2.1 Effect of gamma ray irradiation on survival of irradiated calluses

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, 1986). Predieri (2001) reported that one of the first steps in mutagenic treatments is the estimation of the most appropriate dose to apply. Laneri *et al.* (1990) stated that in a mutation breeding experiment, the dose chosen for the main experiment should result in the highest survival of irradiated explants and that a low inhibition of the rate of production of new shoots gives the highest efficiency in recovering useful mutants. Several studies have been conducted on the radio sensitivity of *in vitro* cultures of several crops (Hell, 1983; Walther and Sauer, 1986; Wang *et al.*, 1988; Cheng *et al.*, 1990; Shen *et al.*, 1990; Charbaji and Nabuls, 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 suitable *in vitro* mutagenic system for the induction and selection of desirable mutants.

In this investigation, the effect of gamma radiation on the survival percentage of calluses was observed that different doses of radiation had different effects on the survival percentage of calluses. After gamma ray irradiation, the calluses were grown for 3 months and the results showed that when concentration of gamma ray increased, the survival percentage of calluses decreased. Some calluses died as a result of high dosage of gamma ray. This result was the same as Jala (2005)

in the study of petunia that growth rate and rate of survival decreased when the plant was exposed to high dosage of gamma ray. The half lethal dose (LD_{50}) of acute gamma ray was 26 Gy. The effect of gamma radiation on calluses survival rate was gradual decreased depending on the exposure dose. A similar gradual reduction in the survival rate has been observed in many species, for example, X-ray irradiated wheat (Kikuchi *et al*., 2009). Since, acute irradiation applied to cultured materials at much higher doses led to physiological damage and the ultimate death of cells.

4.2.2 Effect of gamma ray doses on adventitious shoot regeneration from irradiated calluses

The irradiation dose negatively affected on regeneration rate of the calluses. The percentage of responded explants and the number of regenerated shoots per explant decreased with the increase in gamma radiation dose. In contrast to the findings of Chakravarty and Sen (2001), where regeneration was enhanced by increase in gamma radiation dose. However, low doses of gamma irradiation were used to enhance microtuber production in potato (Al-Safadi *et al.,* 2000). From this experiment, the mean number of shoots per callus was significantly highest at 15 Gy. When concentrations of gamma ray dose higher than 15 Gy were applied, the mean number of shoots per callus declined. Moreover, the irradiated calluses in all experiments provided the higher numbers of shoots per callus than the control treatment. These results agree on several studies that have been previously reported that low dosage of gamma radiation induced active cell proliferation as also noted in other species (Batra and Arya, 1974; Liu, 1982). The extent which the stimulatory effects are results of increase in cell division or cell-expansion has not been ascertained. High doses resulted in inhibition of cell division and depression of growth rate. This is due to depression of DNA, RNA and protein synthesis (Moustafa *et al.,* 1989).

Decrease in mitotic index and increase in mitotic abnormalities with increasing age in culture was observed in both control and irradiated callus cells. This may be due to accumulation of toxic metabolites in the system. This effect might be further enhanced by irradiation (Kar and Sen, 1985; Kharkwal, 1998).

Simultaneous occurrence of altered and normal cells suggests that cells may behave differently to the same dosage due to the effect at different stages of nuclear cell cycle with certain stages being more susceptible to damage by irradiation than others (Eapen, 1976). In addition, primary cultures with less polyploid are more radio-sensitive than long-term cultures with more polyploids since polyploidy has a protective effect on cell (Opatrny, 1974). An optimum dose was beneficial for improving regeneration from callus. This behaviour found parallel in low doses of gamma radiation which help in accelerating seed germination and also in improving the seed quality in different systems (Abo-Hegazi and Ragab, 1986; Prasad *et al.*, 1986). This study has thus shown that irradiation with gamma radiation can be used to increase regeneration capacity found *in vitro* in chrysanthemum. The genetic variants produced can be utilized for generating tolerant or resistant plants for commercial uses (Spiegel-Roy and Kochba, 1973). The extent to which variability in regenerants can be transmitted to the next generation is yet to be studied.

4.2.3 DNA content analysis in irradiated plantlets

Mutagenic treatment may cause histogenic disturbances. At the molecular level, mutagens cause random changes in the nuclear DNA or cytoplasmic organelles, resulting in gene, chromosomal or genomic mutations that create variability. The changes may be a result of alterations of the DNA sequence of gene such as gain, loss or substitution of one base pair by another. Therefore, monitoring of mutagen-induced DNA damage is potentially useful in mutation breeding studies.

The most reliable conventional method of ploidy level analysis is the counting of chromosomes of metaphase plates from root tips. However, the preparation and microscopic analysis is time consuming, and indistinguishable and frequently few cell divisions are visible in a single root tip. This has led to the development of flow cytometric techniques for rapid routine ploidy determination in chrysanthemum (Dolezel *et al.,* 1994). In addition, flow cytometry is recommended for the accurate estimation of nuclear DNA content (Novak, 1992; Dolezel *et al.* 1989). Flow cytometry involves the analysis of intact nuclei, hence dividing cells are not required, and the analysis is not limited to meristematic tissues. Moreover, a small

amount of fresh leaf tissue (5-50 mg) is sufficient, that means this method could be applied to plants at the early stage of development. The nuclear DNA content is easily measured by flow cytometry. The gain or loss of a single chromosome can be detected by this method, as shown in *Musa* (Roux *et al.,* 2003), ryegrass (Barker *et al.,* 2001), and wheat-rye addition lines (Bashir *et al.,* 1993; Pfosser *et al.,* 1995).

Analysis of the relative fluorescent intensity of DAPI stained nuclei yielded a histogram showing three dominant peaks corresponding to G_1 and G_2 nuclei of *Zea mays* cv. 'CE-777' and G_1 nuclei of chrysanthemum, respectively. The majority of cells in full-grown plants does not participate in cell division and reside in a so-called G_0 stage of the cell cycle. At this stage, the nuclear DNA content reflects the ploidy state of the plant. Cells, which are involved in divisions, start to from a comparable so-called G_1 state and subsequently pass through S (DNA-synthesis), G_2 (an interphase nuclear stage with a doubled DNA content preceding the actual nuclear division). Accordingly, nuclear DNA content in absolute units (genome size in pictogram (pg) DNA) was adopted for all samples used. Such estimation requires comparison with a reference standard having a known DNA content. In this study, *Zea mays* cv. 'CE-777' was used as an internal standard because its genome size is relatively constant (Dolezel *et al.,* 1994; Vinderlov *et al.,* 1983) and to avoid bias due to staining and instrumental changes when estimating nuclear DNA ploidy by flow cytometric analysis. The precision of the ratio between the standard and the sample (chrysanthemum) reflects the accuracy of DNA content measurements.

In this study, the nuclear DNA content in plantlets regenerated from untreated calluses and plants in natural condition was 24.28 and 24.98 pg, respectively while the nuclear DNA content in plantlets regenerated from the irradiated calluses was lower in all treatments. The greatest decline was 10.99 pg at 15 Gy which was more than 50% reduction compared to the control plants. One possible reason that the 15 Gy treatment showed the greatest decline in nuclear DNA content is that most of the cells composing in the tissues of calluses reside in dividing cells stage of cell cycle more than other treatments.

Generally, the cells composing in the tissues of callus have different stage of cell cycle and each stage has difference in the sensitivity to radiation. For

example, the dormant cells, less sensitivity to radiation, obtain little radiation damage on the nuclear DNA content, but the more sensitive dividing cells obtain more radiation damage resulting in more declines in nuclear DNA content.

The nuclear DNA content of irradiated chrysanthemums decreased with increasing dose of gamma ray. The results are in agreement with Yamaguchi *et al*. (2008) on their report that nuclear DNA content of chrysanthemums decreased with increasing dose and dose rate of gamma rays, indicating that the nuclear DNA content could be used as an index of radiation damage.

A decrease in the nuclear DNA content is undesirable in chrysanthemum, because it is correlated to reduction of inflorescence diameter as well as the chromosome number (Yamaguchi *et al*., 2008). Consequently, radiation that induces mutations at high frequency with little influence on the nuclear DNA content is desirable for chrysanthemum breeding.

The results indicate that gamma ray has a direct effect at the DNA level of chrysanthemum and this variation can be effectively measured using the flow cytometric technique. This technique has immediate application for selection of potentially useful mutants for chrysanthemum breeding programme. It may be expected that the number of practical applications will increase and plant breeders will even more extensively use flow cytometry to rejection of mutants with undesirable similar to Roux *et al.* (2003) who use flow cytometry to rejection of mutants with undesirable agro morphological traits in *Musa*. Moreover, flow cytometry can be used to estimate radiation damage in chrysanthemum and facilitate the rejection of mutants with severe radiation damage that results in smaller plants.

4.2.4 Chromosome counting

Although radiation treatment induces mutations, it causes radiation damage. Konzak *et al.* (1965) suggested that not only mutation induction effect but also the plant damage caused by irradiation treatment, such as chromosomal aberrations, should be considered for the use of any mutagen in plant breeding.

In this study, plantlets regenerated from the irradiated calluses exhibited highly significant decrease in number of chromosomes compared to

plantlets regenerated from untreated calluses and plants in natural condition. Plantlets derived from treated calluses with 15, 20 and 25 Gy have the somatic chromosome numbers of 27, 37, and 34, respectively while the natural plants was 54 in concomitant with the nuclear DNA content of 10.99, 18.80 and 16.91 pg.

Reduction in chromosome number was reported in chrysanthemums irradiated with gamma (Dowrick and El-Bayoumi, 1996) or X-rays (Ichikawa *et al.*, 1970). Reduction in chromosome number is generally undesirable in the development of commercial varieties of chrysanthemums because it is correlated with reduction in the diameter of inflorescence (Ichikawa *et al.,* 1970). Therefore, it is necessary to consider radiation damage to chromosomes in addition to mutation frequency in chrysanthemum breeding.

4.2.5 Morphological observation

The relationship between morphological variants and high doses of irradiation was apparent. The majority of leaf chimera, short compact shoots, fasciations, rosette leaf and vitrification were observed from 15 and 30 Gy treatments similar to those observed by Chutinthorn (1979) which reported in the study of many ornamental plants. When concentration of gamma ray increased, plant growth decreased and abnormal characters occurred. Some died as a result of high dosage of gamma ray. This result was the same as Jala (2005) in the study of petunia found that growth rate and rate of survival decreased when the plant was exposed to high dosage of gamma ray. Plant height increased in response to an increase of dosage of gamma rays. This was the same as *Curcuma alismatifolia* (Thohirah *et al.*, 2009).

Moreover, these variants that were found in this study also showed decrease in genomic DNA content ranging from 20% to 60%, suggesting that the variation in DNA content has phenotypic effects via its influence on cell size and mitotic cycle time (van Harten, 1998).

The morphological changes observed at 15 Gy and 30 Gy treatments might be due to broken segments lost or translocated or may originate from deletions in the nucleotide sequences in structural genes, changes in the promoter sequences, deletions of the introns, regulating gene and repressers, which may either cause frame

shift mutations or lead to the production of modified gene expression. It is estimated that at least 90% of the radiation-induced mutations refers to deletions (van Harten, 1998).

4.2.6 Molecular marker analysis

Molecular marker-assisted selection techniques have been widely used in plant breeding. Among those techniques, RAPD was the most commonly used (Williams *et al.,* 1990). RAPD has been successfully used for screening rice and fruit seedlings at the early stage since its invention (Kim *et al.,* 1999; Stefano, 2001). In chrysanthemum, it has been applied for identifying cultivar and detecting genetic diversity (Wilde *et al.,* 1992; Wolff *et al.,* 1995; Wolff, 1996; Shibata *et al.,* 1998; Trigiano *et al.,* 1998; Martin *et al.,* 2002; Sehrawat *et al.,* 2003; Lema-Ruminska *et al.,* 2004). For example, Martin *et al.* (2002) successfully identified fifteen commercial cultivars using RAPD and found that those cultivars could be distinguished from each other. Similarly, when conducting the analysis of genetic diversity of chrysanthemum radiomutants, Lema-Ruminska *et al.* (2004) also found that 10 new radiomutants could be distinguished from each other using RAPD marker and thus concluded that RAPD was an efficient method for characterization of the original cultivar and new radiomutants. Therefore, RAPD has become a popular useful tool in cultivar characterization of chrysanthemum.

When mutation breeding was performed, we always hope to get plantlets with greater genetic variation compared with the parent (Broertjes *et al.,* 1976; Li, 1993; Banerji *et al.,* 1996; Mandal *et al.,* 2000; Chen *et al.,* 2003). As a result, the chance of developing novel cultivars might be greater. If the plantlets with little genetic variation in comparison with the parent are selected at the early stage, then the chance to get new cultivars will be slim, thus the efficiency of this breeding program is low. Accordingly, a large amount of manual labor, time and money are consumed. Therefore, the selection of mutants possessed great genetic variation relative to the parents at early growth stage is crucial for developing new cultivars with excellent ornamental characteristics in chrysanthemum mutation breeding.

Given the link between genetic variation of mutants and possibilities of developing valuable cultivars (Li, 1993; Banerji *et al.*, 1996; Mandal *et al.*, 2000), it is necessary to develop a method that can quickly and readily detect genetic variation of regenerated plants in the early stage of breeding. In response to this, we have tentatively used RAPD to evaluate genetic variation of the mutants regenerated from the calluses treated with different dosage of gamma rays, with a hope that RAPD can detect the genetic variation. Interestingly, the analysis of RAPD result showed that genetic variation of regenerated plantlet was proportional to the dosage of gamma ray within a certain range, although the 5 and 10 Gy treatments were not significantly different. In fact, it is commonly accepted that genetic variation of radiomutants is well proportional to the dosage of mutagen within a certain range, which is in line with our RAPD results and confirms the applicability of the RAPD technique to assess genetic variation of regenerated plantlets. Therefore, we hold a belief that besides the characterization of cultivars and analysis of genetic diversity, RAPD can be also applied in the rapid selection of regenerated plants with great genetic variation during young stages in chrysanthemum mutation breeding.

In this present study out of 13 primers tested, five resulted in polymorphic bands which pointed to differences in the genotypes studied. Two of the primers, OPB-18 and OPB-08, made it possible to identify the genetic variation of radiomutants. However, the primers used in the present study could not discriminate the radiomutants derived from 15 Gy gamma irradiation from the other radiomutants, although these radiomutants seemed to be the most characteristically different from other accessions of mutant (a change in morphology**,** a decreasing in chromosome number and also in the nuclear DNA content). Possible causes of the lack of distinct differences of RAPD patterns between the radiomutants derived from 15 Gy gamma irradiation and other radiomutants could include that polymorphic RAPD markers resulted from the amplification of both coding and non-coding genome region, or the number of primers used was insufficient to test the whole genome in order to reveal changes in DNA related to the phenotypic traits observed for the radiomutants derived from 15 Gy irradiation.

According to Wolff and Peters-Van Rijn (1993), the *Dendranthema* genus has a high level of genetic variability. However, the same authors found only

one polymorphic band for a group of cultivars derived from a single original cultivar, but these cultivars were spontaneous mutants. Probably, in cultivars obtained as the result of mutagenic factors, namely X- and gamma radiation, the genome shows much greater changes which are more easily found with RAPD markers than spontaneous mutants. Wolff (1996) reported that out of nine cultivars obtained from a single original cultivar, two resulted from ionizing radiation and all the others were spontaneous. The two artificially induced mutants showed polymorphic bands which distinguished them from the others and suggested greater changes in the genome than those in the spontaneous mutants. The changes could have concerned point mutations in genes as well as greater chromosomal aberrations, e.g. a loss of a part or the entire chromosome (Wolff, 1996; Shibata *et al.*, 1998).

In summary, we applied RAPD technique to investigate the genetic variation of plantlets regenerated following *in vitro* mutation treatment in chrysanthemum. According to the results, we found that RAPD can satisfactorily detect the genetic variation. Thus, it is believed that RAPD-assisted mutant selection of plantlets during early growth stages might become a potentially useful technique in mutation breeding of chrysanthemum and other ornamental plants.

CHAPTER 5
CONCLUSIONS

5.1 Major findings of the thesis

The research was carried out to answer the question regarding the effect of gamma radiation dose on mutation induction in regenerated plants derived from irradiated calluses of chrysanthemum [*Chrysanthemum x grandiflorum* (Ramat.) Kitam.]. A combined method of gamma ray irradiation and tissue culture was used for the study. The main findings are briefly described as follows:

5.1.1 *In vitro* propagation

The combination of BA and 2, 4-D evoked the best response for induction of callus from ray florets of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. The MS medium supplemented with 13.3 μM BA and 0.5 μM 2, 4-D yielded the highest efficiency of callus formation. The explants cultured on MS medium without growth regulators did not produce any callus. Moreover, with a gradual increasing concentrations of 2, 4-D alone there was a gradual increasing in the percentage of callus formation. The callus started from the adaxial surface and the cut end of the explants.

5.1.2 Plant regeneration and root induction

Kinetin and its concentrations were the main determinants of adventitious shoot production from callus of *Chrysanthemum* × *grandiflorum* (Ramat.) Kitam. The highest number of regenerated shoots (45.0%) was obtained on MS medium supplemented with 9.3 μM Kinetin and 4.9 μM IBA. Present results also

indicate that low concentrations of IBA are good for shoot regeneration while the rooting required the presence of either IBA or chitosan in the medium. With a gradual increase of IBA alone there was a gradual increase in the number of roots. Optimal number of roots per shoot was obtained on MS medium supplemented with 12.3 μM IBA, while higher concentrations of IBA reduced the number of roots per shoot. This result indicates that IBA is an important factor for rooting. It is interesting to note that when IBA was used in combination with chitosan, the number of roots per shoot declined compared to IBA alone.

5.1.3 Histological studies of callus

Shoots proliferating from ray floret callus were from a single origin. Microscopic examination showed scattered meristematic cells on the ray floret epidermis. Serial sections of the 7-day-old light green compact calluses revealed well-organized small cells attached to each other. In this instance, these shoots had originated from a single meristematic cell that had a dense cytoplasm with a well stained nucleus. Shortly after several divisions of this single cell culture several shoot apex-like structures or meristemoids were observed.

5.1.4 Effect of gamma ray irradiation on survival of irradiated calluses

The effect of gamma irradiation on calluses survival rate depending on the exposure dose. The survival percentage of the calluses decreased with an increasing radiation dose. The untreated calluses produced a 100% of the survival rate while the survival percentage at 5 Gy was 75.0. At 10, 15, 20, 25 and 30 Gy of irradiation dose the survival percentage was significantly decreased to 72.5, 72.5, 65.0, 52.5, and 38.7, respectively. Moreover, the growth of treated calluses was slower than that of the controls. Treatments at 20 Gy and over caused yellow or green calluses to become browning wither and died within 30 days.. The half lethal dose (LD_{50}) of acute gamma ray was 26 Gy.

5.1.5 Effect of gamma ray doses on adventitious shoot regeneration from irradiated calluses

The radiation dose negatively affected on regeneration rate of the calluses. That is with a gradual increase of radiation doses there was a gradual decrease in the percentage of shoot regeneration. The optimal percentage of adventitious shoot regenerated from the irradiated calluses was 42.3 at 5 Gy, which was lower than that in the control experiment (100%). When concentrations of gamma ray dose higher than 5 Gy were added, the percentage of shoot regeneration declined. However, low dosage of gamma radiation induced active cell proliferation represent by the irradiated calluses in all experiments provided the higher numbers of shoots per callus than the control treatment.

5.1.6 DNA content analysis in irradiated plantlets

Gamma ray has a direct effect at the DNA level of chrysanthemum. The nuclear DNA content was affected by gamma ray irradiation. The nuclear DNA content of irradiated chrysanthemums decreased with increasing dose of gamma ray. The nuclear DNA content in plants regenerated from untreated calluses and plants in natural condition was 24.98 pg while the nuclear DNA content in plants regenerated from the irradiated calluses was lower in all treatments. Plants derived from treated calluses with 15, 20 and 25 Gy has the nuclear DNA content of 10.99, 18.80 and 16.91 pg in concomitant with the somatic chromosome number of 27, 37, and 34, respectively while the natural plants was 54. The greatest decline was 10.99 pg at 15 Gy which was a more than 50% reduction compared to the control plants.

5.1.7 Morphological observation

Variations in the morphological characteristic of shoots and leaves were found in the plants from the irradiated calluses. Plants regenerated from calluses revealed that with 30 Gy treatment, leaf chimera was evidenced while with 15 Gy treatment, short compact shoots, fasciations, rosette leaf and vitrification were observed. Furthermore, these variants also showed decrease in genomic DNA content

ranging from 20% to 60%, suggesting that the variation in DNA content has phenotypic effects via its influence on cell size and mitotic cycle time.

5.1.8 Molecular marker analysis

Thirteen primers were screened for their ability to amplify the genomic DNA of the *Chrysanthemum x grandiflorum* (Ramat.) Kitam. and its mutants. Five informative RAPD primer combinations generated a total of 97 reproducible amplification fragments across all radiomutants and parent cultivars. Among which 31 bands were polymorphic. The number of amplified RAPD bands varied from a minimum of 6 to a maximum of 28 depending on the primer and the DNA sample with a mean value of 19.4 bands per primer. The size of fragments ranged from 300 to 3,000 bp. PCR amplification with primer OPB-18 clearly revealed that regenerated plantlets from irradiated calluses at 20, 25, and 30 Gy a highly specific band (500 and 700 bp) is absent in comparison with the plants in other doses and the plants in natural conditions indicating that they are highly polymorphic when compared to other mutants. Moreover, a band of 3000 bp was noticed only in plants regenerated from irradiated calluses at 30 Gy when the genomic DNA was amplified with primer OPB-08, indicating that the plants regenerated from irradiated calluses at 30 Gy is polymorphic when compared to plants derived from other conditions. Similarly, a polymorphic band of 900 bp is absent in natural plants and plants regenerated from untreated calluses but present in all plants regenerated from irradiated calluses when RAPD marker OPB-08 was used. This result showed that their cultivated plants are highly polymorphic compared to plantlets regenerated from irradiated calluses. The analysis of RAPD result showed that genetic variation of regenerated plantlet was proportional to the dosage of gamma ray within a certain range. However, the primers used in the present study could not discriminate the radiomutants derived from 15 Gy gamma irradiation from the other radiomutants, although this radiomutants seemed to be the most characteristically different from other accessions of mutant (a change in morphology**,** a decreasing in chromosome number and also in the nuclear DNA content).

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APPENDICES

APPENDIX 1

Composition of Murashige and Skoog (MS) medium (1962)

	1 liter
Macroelements	
Ammonium nitrate, NH_4NO_3	1,650 mg
Potassium nitrate, KNO_3	1,900 mg
Calcium chloride, $CaCl_2.2H_2O$	440 mg
Potassium dihydrogen phosphate, KH_2PO_4	170 mg
Magnesium sulfate, $MgSO_4.7H_2O$	370 mg
Chelated iron	
Disodium ethylene diaminetetraacetate, Na_2-EDTA	37.3 mg
Ferrous sulfate, $FeSO_4.7H_2O$	27.8 mg
Microelements	
Boric acid, H_3BO_3	6.2 mg
Manganese sulfate, $MnSO_4.4H_2O$	16.9 mg
Zinc sulfate, $ZnSO_4.7H_2O$	6.14 mg
Potassium iodide, KI	0.83 mg
Sodium molybdate, $Na_2MoO_4.2H_2O$	0.25 mg
Copper sulfate, $CuSO_4.5H_2O$	0.025 mg
Cobalt chloride, $CoCl_2.6H_2O$	0.025 mg
Growth factor	
Myo-inositol	100 mg
Organic addenda	
Glycine	2 mg
Caseinhydrolysate (CH)	100 mg

Vitamins

Thiamine HCl	0.1 mg
Nicotinic acid	0.5 mg
Pyridoxin HCl	0.5 mg

APPENDIX 2

Hematoxylin staining solution

Composition

1.	Hematoxylin	8 g
2.	Ammonium alum	16 g
3.	Ethanol 95%	250 mL
4.	Potassium permanganate	0.4 g
5.	Glycerin	400 mL
6.	Distilled water	800 mL

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List of Publication and Proceedings

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