M. Imran Kozgar

Mutation Breeding in Chickpea: Perspectives and Prospects for Food Security

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Preface

The universal truth is that the agricultural practices and plant breeding strategies are essential tools to feed the inhabitants of the world. These practices become more important in the conditions where population grows rapidly especially the countries like India. In order to feed the people, the plant breeding strategies has to be developed as per need of an hour and mutation breeding has shown some light to curb the two jargon problems presently persisting in the world vis-à-vis food insecurity and malnutrition. Enhancing the genetic diversity is the prerequisite for utilizing the benefits of successful plant breeding in order to get the necessary yield plateau and induced mutagenesis is one of the techniques which have potential to increase the required genetic diversity which is necessary for crop improvement.

In the present monograph entitled "**Mutation Breeding in Chickpea: Perspectives and Prospects for Food Security"** an attempt has been made to cover various aspects of mutation breeding programmes of chickpea, based on the original compiled work of the thesis entitled "Studies on the Induction of Mutation for Quantitative Traits in Chickpea (*Cicer arietinum* L.)" awared for Doctor of Philosophy (Ph.D.) degree by the Faculty of Life Sciences, Aligarh Muslim University, Aligarh, India to the author.

Chickpea is the second most cultivated crop among pulses, after soybean, which is highly nutritious and protein rich. In addition, the chickpea is a seat of high iron and manganese contents.

The present monograph contains nine chapters covering various aspects of recent knowledge of mutation breeding on chickpea and other pulse crops. The case studies (experimented work of the thesis) for two selected varieties of chickpea have also been incorporated for chapters 3-8 in order to understand the genetic basis and scope to enhance the diversity in chickpea and their possible role for the development of cultivars superior to parental ones. Chapters 1 and 2 have been inducted to provide background information about chickpea, its position among crops and about induced mutagenesis. Chapter 9 on the other hand provides the advances and future strategies to be introduced in the chickpea cultivation *via* mutation breeding in coming years. The materials and methodologies adopted for the work of case studies have been integrated in a separate single heading as Appendix in order to avoid redundancy within the chapters involving the case studies.

I hope researchers working on mutation breeding, teachers and students interested in mutation breeding, agricultural policy and decisions makers will find this monograph a good source material. Every effort has been done to make this work error free. However there is a possibility of some errors creeping in the monograph, for which I seek reader's indulgence.

I wish to express my appreciation and gratitude to the **Mr. & Mrs. M Farooq Kozgar** (parents); **Er. M Irfan Kozgar** (brother); **Ms. Erum Jan** and **Dr. Neelofar Muneeb** (sisters); **Mr. & Mrs. Hakeem Mohammad Khalil** (uncle and aunt) for their

encouragement, prays and best wishes; **Dr. Samiullah Khan**, Associate Professor, Department of Botany, Aligarh Muslim University, India who exhorted and guided me with his exquisite observance and sapience; **Mr. Mukhtar Ahmad**, **Mr. Tariq Ahmad**, **Mr. Showkat Ahmad** and **Mr. Sufail Ahmad (**paternal cousins) for their moral support; **Mr. Mohammad Irshad** for his scared love, affection, noble ideas, good wishes and constant encouragement; **Board of Directors**, **KERB BIOTECH LLP, Bemina, Srinagar, Kashmir** for disseminating the novel ideologies for compiling this project; Online Journal Lab., Maulana Azad Library, Aligarh Muslim University, and the Seminar Library of the Department of Botany, Aligarh Muslim University for making available the important literature; University Grants Commission (UGC, New Delhi) for providing financial assistance in the form of Basic Scientific Research Fellowship (BSR-UGC) and to the Publication partner **De Gruyter Open, Poland**, especially **Dr. Katarzyna Michalczyk** and **Ms. Sara Suliman** for their undaunted help and cooperation in publishing **"Mutation Breeding in Chickpea: Perspectives and Prospects for Food Security".**

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M. Imran Kozgar

Contents

Author Dedicated the Present Monograph To
Mr. M. Farooq Kozgar (Father)
Mrs. Tahira Farooq (Mother)
Er. M. Irfan Kozgar (Brother)
Ms. Erum Jan (Sister)
Dr. Neelofar Muneeb (Sister)

1 Chickpea: In the Backdrop Note

Physical availability, economic accessibility and risks related to either availability or access of food are most important criteria to diminish the hunger and malnutrition, and avail food security for all into practical dimensions (von Braun *et al*., 1992). Food security has been defined as "access by all people at all time to enough food for an active and healthy life" (World Bank, 1986; Kharkwal and Shu, 2009). Although, several world food summits were organized during past decades, the number of hungry people keeps increasing and now exceeds one billion (Swaminathan, 2010). One of the ways to acheive food security is through focused and practical work by reviving and expanding research strategies and activities beyond traditional stars of food grains such as wheat, rice, barley etc. to the crops like pulses. The production rate of pulse crops will have and/or is to play an important role in curbing and declining the food insecurity and malnutrition problems in developing countries like India for better future.

1.1 General Account of Pulses and Chickpea

Pulse crops, commonly known as food grain legumes, are grown in wide range of agro-climatic zones and form an integral part of vegetarian diet of Indian sub-continent, thus, contributing around 25-28% of the total global production (Goyal *et al*., 2011). Certain unique characteristics of pulse crops like high protein content (2 to 3 times more than the cereals), nitrogen fixing ability, soil ameliorative properties and ability to thrive better under harsh conditions make pulses an essential component of sustainable agriculture particularly in dry land areas (Singh, 1987; Wani *et al*., 2011a). In addition, the Indian population is mainly dependent upon pulses for fulfilling their protein requirement because of high cost associated to animal based proteins and vegetarian food habits (Yadav, 1991).

Developing countries, such as India, can take a rightful pride in attaining self-sufficiency in the production of food grains but most of them have miserably failed in case of pulses because their production could not keep pace with the population growth. Indian population is growing at a rapid rate, and hence stagnation in pulse production has meant a severe decline in the per capita pulse availability. Per capita pulses consumption over the years has come down from 60.7 g/day in 1951 to 37.0 g/day in 2009 (DES, 2010). A solution to the problem of declining per capita availability has to, therefore, come from a rapid improvement in indigenous production levels. While efforts have been geared up to bring additional area under pulses, more important is to increase the production by exploiting yield potential of existing varieties through genetic manipulations. The estimates at the end of first decade of 21st century (for 2009-2010) indicate that the pulses occupy an area of 23.35 million hectares in India and produce 14.60 million tonnes with an average yield of 625 kg/ha

(Fig. 1.1; for latest data information's log onto http://agricoop.nic.in/Agristatistics.htm). The average growth rate of pulses production during this period was 3.64 percent, which is far below the required growth rate to meet domestic requirements during the last one and half decade, 1995-2010 (DES, 2010). The non-availability of high yielding varieties is a major constraint in achieving higher pulse productivity. Non-synchronous maturity, long duration, flower drop and presence of different kinds of natural (like valconic eruptions, tsunami, forest fire etc.), and man-created (waste products, pollution, sewage as outcome of various industries, thermal power plants, etc.) anthropogenic pressures are other problems associated which hinders the production of high yielding varieties of major pulses.

Chickpea (*Cicer arietinum* L.), also known as Bengal gram, Gram or Chana (Hindi), is an important ancient annual grain legume used in human diet. Chickpea is a winter (rabi) season crop and requires cool climate for its growth and high temperature for maturity. Chickpea performs well when grown on sandy/loam soils associated to good drainage system.

Based upon seed size and colour, chickpea is classified as "kabuli type", or "macrosperma" and "desi type" or "microsperma". Kabuli type chickpea seed is bold and has thin seed coat ranging from white to pale cream in colour. On the other hand, desi type chickpea seed is small compared to the kabuli type chickpea and has thicker seed coat ranging in colour from brown to yellow. The kabuli type chickpea is believed to have developed from desi type chickpea through natural mutation and selection (Moreno and Cubero, 1978; Hawtin and Singh, 1980; Salimath *et al.*, 1984; Gil and Cubero, 1993). Toker (2009) reported that kabuli chickpea could have originated from spontaneous mutants of *C. reticulatum*.

Chickpea is the second largest grown food legume. It ranks third among pulses and 119th among the grain crops of the world (Gaur *et al.*, 2008; Kulthe and Kothekar, 2011). India leads the list of chickpea producing countries in area, as well as production. In India, chickpea is grown in the drier areas as they are best suited for its production. Chickpea producing states in India are Madhya Pradesh, Maharashtra, Rajasthan, Uttar Pradesh and Karnataka. Data on production, area harvested, and yield quantity of chickpea in India for the first decade of 21st century (since 2001-2010) are given in Fig. 1.2. The area of chickpea under cultivation in India is 8.21 million hectares with an annual production of 7.35 million tonnes in 2009-2010. The average yield is 895 kg/ha and average growth rate production was 4.58 percent (DES, 2010), which is low for this crop because it engages the large area under its cultivation as compared to other pulse crops and is not sufficient to meet the growing demand. For latest data information's log onto http://agricoop.nic.in/Agristatistics.htm)

In order to break the yield plateau in chickpea, efforts are needed to develop high yielding varieties with appropriate growth habit (ICAR-ICARDA, 2010). Genetic enhancement for yield, synchronization in maturity, tolerance to major biotic and abiotic stresses and increasing the nutrient composition of the crop to large extent is a major concern due to less genetic variability in chickpea. The possibility offered

by mutagenic agents to induce new genetic variation is, therefore, of extreme interest and importance. Since chickpea is a self-pollinating crop, mutation breeding could be rewarding for broadening the genetic base of total plant yield, yield contributing traits and other important traits like nutritive composition.

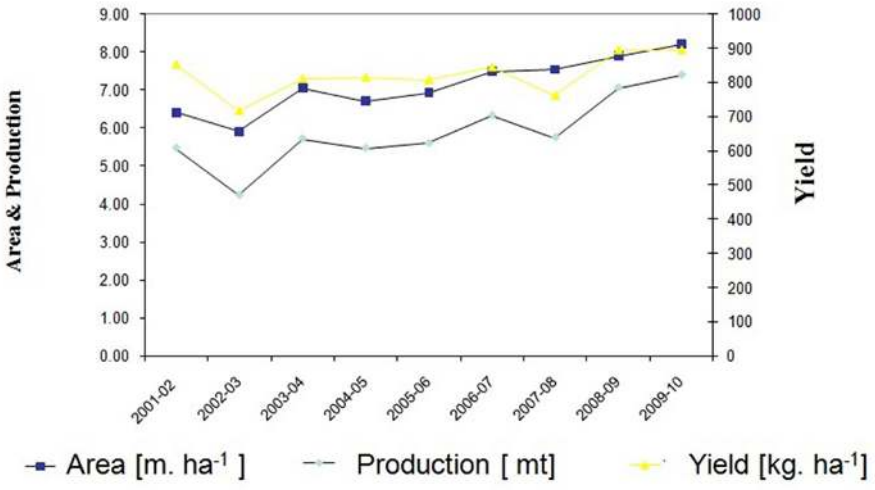

Fig. 1.1: National trends in area, production and yield of pulses. (Source: Directorate of Economics and Statistics (DES), Department of Agriculture and Cooperation, Ministry of Agriculture, Government of India)

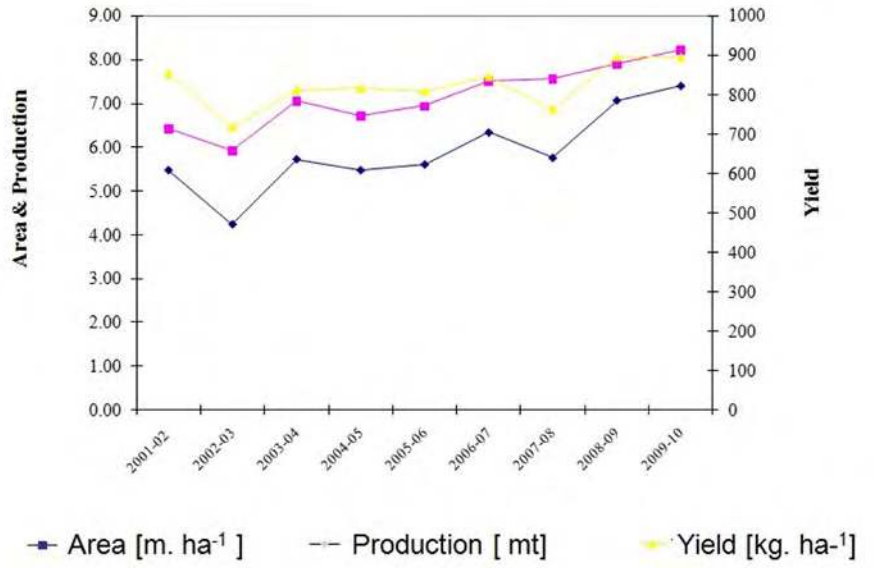

Fig. 1.2: National trends in area, production and yield of chickpea. (Source: Directorate of Economics and Statistics (DES), Department of Agriculture and Cooperation, Ministry of Agriculture, Government of India)

1.2 Botanical Description of Chickpea

The genus *Cicer* belongs to the family Fabaceae. The genus *Cicer* consists of 44 species, including 35 perennial and eight annual wild species and one the domesticated chickpea, *Cicer arietinum* L. (van der Maesen *et al*., 2007; Toker, 2009). The plant has a deep tap-root with well defined root-nodules. Stem is mostly erect and green. Leaves are stipulate and imparipinnately compound, usually small leaflets in each leaf, which are arranged on a rachis with a small petiole. Glandular hairs cover all external surfaces of the plant, with the exception of corolla. Flowers are pedicellate bisexual with papilionaceous corolla, borne singly in axillary racemes. The staminal column is diadelphous (10 stamens with an arrangement of 9+1). Ovary is monocarpellary, unilocular, 1-2 ovules and superior with a terminal slightly bent style and blunt stigma. Pistil and anthers usually remain inside the keel. Pollination takes place before the opening of the bud. Thus self-pollination is the rule (Auckland and van der Maesen, 1980; Singh and Ibrahim, 1990; Toker *et al.*, 2006). The fruit of chickpea is the inflated pod with 1-2 seeds. The surfaces of the seeds are wrinkled or smooth and the germination is hypogeal in chickpea.

1.3 Economic Importance of Chickpea

Chickpea seeds are very rich source of carbohydrates, proteins, fats and many other essential nutritive components, which are consumed by humans. In fact, chickpea has one of the highest nutritional compositions of any dry edible legume and does not contain any specific major antinutritional factors (CGKB, 2010; Malikarjuna *et al.*, 2011) and could be a useful source of dietary nutrients, especially in malnourished populations (Ibrikci *et al.*, 2003). The nutritional composition of chickpea is given in Table 1.1. Chickpea seeds are eaten fresh as green vegetables, parched, fried, roasted, broiled as snack food, sweets and condiments and their flour is used in soup/salads and to make bread.

Among the food legumes, chickpea is the most hypocholesteremic agent as the germinated chickpea is effective in controlling cholesterol level (Geervani, 1991). Glandular secretion of the leaves, stems and pods consists of malic and oxalic acids, which are collected and used for medical purposes, particularly in India. Medicinal applications include use for aphrodisiac, bronchitis, catarrh, cutamenia, cholera, constipation, diarrhoea, dyspepsia, flatulence, snakebite, sunstroke, and warts (Muehlbauer and Tullu, 1997). Seeds of chickpea are considered antibilious and also yield 21% starch, which is suitable for textile sizing, giving a light finish to silk, wool and cotton cloth (Duke, 1981).

Table 1.1: Nutritional composition of mature chickpea seeds (value per 100 g edible portion).

Energy (kcal)	**364**				
Proximates (g)					
Carbohydrate	60.65	Protein	19.30	Fiber	17.4
Water	11.53	Fat	6.04	Ash	2.48
Amino acids (g)					
Glutamic acid	3.375	Aspartic acid	2.270	Arginine	1.819
Leucine	1.374	Lysine	1.291	Phenylalanine	1.034
Serine	0.973	Alanine	0.828	Isoleucine	0.828
Valine	0.809	Glycine	0.803	Proline	0.797
Threonine	0.716	Histidine	0.531	Tyrosine	0.479
Cystine	0.259	Methionine	0.253	Tryptophan	0.185
Vitamins (mg)					
Choline	95.2	Ascorbic acid	4.0	Pantothenic acid	1.588
Niacin	1.541	Vitamin E	0.82	Vitamin B_6	0.535
Thiamine	0.477	Riboflavin	0.212		
Minerals (mg)					
Potassium	875	Phosphorus	366	Magnesium	115
Calcium	105	Sodium	24	Iron	6.24
Zinc	3.43	Manganese	2.204	Copper	0.847

Source: USDA National Nutrient Database for Standard Reference, Release 23 (2010)

1.4 Origin, Biosystematics and Cytogenetics

The domesticated chickpea (*Cicer arietinum* L.) is one of the seven Neolithic founder crops (other crops being einkorn wheat, emmer wheat, barley, lentil, pea and bitter vetch) and the wild progenitors of all of them are found together in the core area of the Fertile Crescent, a place near the upper reaches of the Tigris and Euphrates rivers in present-day southeastern Turkey/northern Syria (Lev-Yadun *et al.*, 2000). It is also believed that cultivated chickpea have originated at least 7000 years ago in south eastern Turkey and adjoining areas of Iraq, Iran and former Soviet Union (Ramanujam, 1976) with *Cicer reticulatum* L. being the wild progenitor (Ladizinsky and Alder, 1976). Some believe that *C. reticulatum* is sub-species of *C. arietinum* L. (Singh and Singh, 1997). Earlier botanists had postulated several different origins. De Candolle (1883) traced the origin of chickpea to an area south of Caucasus and in the north of Persia. Vavilov (1926) recognized the Mediterranean, Central Asia and Indian regions as the probable centers of origin of chickpea. There is linguistic evidence that the large seeded chickpea reached India *via* Afghan capital, Kabul, about two

centuries ago and acquired a name in Hindi as "Kabuli Chana" (van der Maesen, 1972). The small seeded chickpea is called "desi" (local) and these denominations are commonly used to distinguish the two main groups of the cultivars. The earliest record of chickpea in India is from Atranji Khera in Uttar Pradesh and this dates back to 2000 B.C. (Chowdhury *et al.*, 1970). It was introduced in Peninsular India probably between 500 and 300 B.C. (Vishnu-Mitre, 1974).

The genus *Cicer* belongs to the family Fabaceae and the tribe *Cicereae* (Iruela *et al.*, 2002). However, some argue that it belongs to tribe *Vicieae* (Singh *et al.*, 1997). The genus consists of 44 species of which 35 are perennial and nine are annual (van der Maesen *et al.*, 2007; Toker, 2009), including the cultivated one.

Chickpea (*C. arietinum* L.) is a diploid and self-pollinating annual pulse crop known to have somatic chromosome number to be 2n = 16 (Singh and Singh, 1997; Bharadwaj *et al.*, 2006), with genome size 1C = 740 Mbp (million base pair). There are reports of 2n = 14 chromosome number, but presumably such plants of *Cicer* are rare and may not be able to maintain themselves in nature (Singh *et al.*, 1997).

1.5 Case Studies

Keeping in view the socio-economic and nutritional importance of chickpea, a self-fertilized crop, and its role in food security, various studies were encased for the present monographic work in upcoming chapters (chapter numbers 3, 4, 5, 6, 7 and 8) in order to understand the genetic basis of quantitative traits in chickpea (*Cicer arietinum* L.). The detailed background information and their comparative discussion to the estimated case study results has been well explained. Overall, an attempt has been made through specialized chapters of case studies to evaluate quantitative traits in M_1 to M_3 generations following mutagenesis with gamma rays, ethylmethane sulphonate (EMS) and their combination treatments in two varieties of chickpea viz., Pusa- 256 and BG-1053.

2 Induced Mutagenesis

Hugo de Vries advocated the concept of utilizing induced mutations in breeding new forms for the first time (1901). Later, various experimental and philosophical works in the field added exhaustive information that has been generated on the role and application of induced mutations in several crop plants and were taken up by many breeders all around the world (Muller, 1927; Stadler, 1928; Goodspeed, 1929; Gaul, 1958, 1964; Brock, 1965; Micke, 1995; Kharakwal, 1996; Khan and Rehman, 1999; Chhun *et al.*, 2003; Ilbas *et al.*, 2005; Talame *et al.*, 2008; Toker, 2009; Goyal and Khan, 2010a, Nakagawa *et al.*, 2011)

In the beginning, mutation breeding was based primarily upon X-rays, gamma rays, thermal neutrons and radioisotopes of certain heavy elements. However, the discovery of chemical mutagens was an important event in the history of plant breeding. It was in 1930's when two chemicals, iodide and copper sulphate, where already known to act as a weak mutagens on *Drosophila* and during the World War II the mutagenic activity of urethane was demonstrated (Donini and Sonnino, 1998). However, the first elaborate report was presented by Auerbach and Robson (1942) who showed that mustard gas could induce mutations as well as chromosomal breaks in *Drosophila*. Now it is well known fact that, in addition to several ionizing radiations, a number of chemical mutagens induce mutations in plants, when applied singly or combined with other chemicals, and successively or simultaneously with physical mutagens (Ehrenberg *et al.*, 1956, 1961; Wallace, 1965; Konzak *et al.*, 1965; Ahloowalia and Maluszynski, 2001; Saleem *et al.*, 2005; Encheva, 2009). Although there are several unanswered questions regarding the classification and mechanism of action of mutagens, a more comprehensive account of them was given earlier by Sharma (1985) and later by van Harten (1998), Micke (1999) and Kodym and Afza (2003). The nature, essential properties and mode of action of physical and chemical mutagens have also been reviewed by Gottschalk (1978 a,b), Gottschalk and Wolff (1983a), Kaul (1989), Crueger (1993), Khan (1997), Kaul and Nirmala (1999), Siddiqui (1999), Ahloowalia *et al.* (2004), Hegde (2006), Al-Qurainy and Khan (2009), Goyal and Khan (2010a) & Nakagawa *et al.* (2011), which has fairly widened and enriched our knowledge on fundamental aspects of the mutational process and the possible mechanism of action of various physical and chemical mutagens.

Experiments on higher plants have shown that chemical mutagens, apart from easy handling and better efficiency, have much greater advantage and specificity than ionizing radiations due to a milder effect on the genetic material of a cell as opposed to the physical mutagens which break chromosomes (Auerbach, 1965; Handro, 1981). Rapoport (1966) discovered overwhelming majority of strong chemical mutagens, which are being used widely in genetic and breeding research. The chemical mutagens can be divided into three groups, viz. A) alkylating agents like ethyl methanesulphonate (EMS), methylmethane sulphonate (MMS), ethylethane sulphonate (EES), ethylene imines, diethylsulphate (dES), nitroso compounds; B) base analogues like 5-bromo

uracil, 5-bromo deoxyuridine; and C) others like azides (sodium azide), antibiotics, acridines, nitrous acid and hydroxylamine. The speed of hydrolysis of the chemical mutagens is usually measured by the half-life of the chemicals. Half-life is the time required for disappearance of half of the initial amount of active reaction agent. The preferred mutagens belong to the class of alkylating agents. Alkylating agents (AA) are potent mutagens and can be classified broadly into monofunctional, bi- or polyfunctional ones, depending upon the number of alkyl groups present in the compound (Natarajan, 2005). Alkylation refers to the substitution of an alkyl group (e.g. C_2H_5 of EMS) for hydrogen in the nitrogenous bases (Sharma and Chopra, 1994). As per the works of Haughn and Somerville (1987), Ashburner (1990), Sharma and Chopra (1994), Siddiqui (1999), and Natarajan (2005) the alkylation of DNA leads to the following effects:

1. Alkylation of the phosphate groups of DNA: Alkylation leads to the formation of phosphate triesters, which are unstable and release the alkyl group. However, if enough alkyl groups remain unreleased, then the attached alkyl groups interfare with DNA duplication. Sometimes the phosphate triester is hydrolyzed between the sugar and the phosphate and results in the breakage of the DNA backbone.
2. Alkylation of bases: The seventh position in the guanine is a preferred site for alkylation but it has been established that the major mutagenic effects arise from O^6 alkylation of guanine. O^6 alkyl-guanine can pair with thymine and leads to base pair transition.
3. Depurination: The alkylated guanine can separate from the deoxyribose leaving it depurinated. Any of the bases can fill the gap during DNA replication leading to transversion or transition type of mutation.

The mutagenic action of ethylmethane sulphonate (EMS) on various morpho-physiological and biochemical parameters was studied earlier in *Drosophila* (Fahmy and Fahmy, 1957), bacteriophage (Loveless, 1959), *Escherichia coli* (Strauss, 1964), barley and wheat (Gustafsson, 1960; Ehrenberg, 1960; Swaminathan *et al.*, 1962), *Arabidopsis* (Greene *et al.*, 2003), *Lycopersicon esculentum* (Saba and Mirza, 2002), soybean (Karthika and Subbalakshmi, 2006), *Jatropha curcas* (Dhakshanamoorthy *et al.*, 2010), *Vigna* sp. (Kozgar *et al.*, 2011) and other cultivated crops of human importance. EMS is now being widely accepted as a powerful mutagen and is used commonly in the induction of mutations in various crop plants because of its ability to induce a high frequency and wide spectrum of mutations (Swaminathan *et al.*, 1962; Hussein *et al.*, 1974; Khan *et al.*, 1998; van Harten, 1998; Barro *et al.*, 2001; Perry *et al.*, 2003; Hohmann *et al.*, 2005; Tabata *et al.*, 2013; Girja *et al.*, 2013).

Since chemical mutagens have been proved to be more potent and efficient in inducing mutations than physical ones (Sharma, 1965; Blixt and Mossberg, 1967; Kharkwal, 1998a; Kharkwal, 1998b), henceforth they have become the method of choice for genetic studies and remain popular even with the advent of new technologies (Jain, 2002; Greene *et al.*, 2003; Perry *et al.*, 2003).

With the discoveries of mutagenic effects of radiations and chemical mutagens, combined treatments of them were used by the breeders in order to increase the mutation frequencies. Mehandjiev (2005) reported that combined treatments of physical and chemical mutagens induced a wider mutation spectrum, which is of great significance to the experimental mutagenesis. He further suggested that the combined treatments enhance quantitative and qualitative changes in the spectrum of mutations, thus increasing or decreasing the frequency of the particular mutation types, which do not occur in the spectra of the individual treatments. van Harten (1998) suggested that combined treatments would be even more attractive if synergistic effects would occur, either in that way that mutation frequencies should reach levels beyond the sum of both individual treatments, or if "unique' mutations should arise in this way. Various reports have been put forward which confirm the usefulness of combination treatments of physical and chemical mutagens for the improvement of crop varieties due to the results of superior mutation frequencies as compared to those of single treatments like in *Gylcine max* (Patil *et al.*, 2004; Khan and Tyagi, 2010b), *Lens culinaris* (Reddy and Annadurai, 1992), *Lycopersicon esculentum* (Zeerak, 1992a), *Papaver somniferum* (Chauhan and Patra, 1993), *Vigna unguiculata* (Girija and Dhanavel, 2009), *Vigna mungo* (Goyal and Khan, 2010b), *Vigna radiata* (Grover and Tejpaul, 1982; Singh, 2007a), *Vicia faba* (Bhat *et al.*, 2007a) and *Brasicca napus* (Siddiqui *et al.*, 2009). There are also many reports of the formation of different types of mutants with different attributes produced through combination treatments of different types. Some of the noteworthy ones are an early maturing mutant variety "Boriana" of soybean, produced in M_2 by the combined treatments of gamma rays and EMS, which has a vegetation period 30 days shorter than the parental variety (Gecheva, 1983); mutant variety of sea buckthorn (*Hippophea rhamnoides*) with high yield and an increased content of the medicinal oil when combination treatment of 150 Gy of gamma rays and 0.01% nitrosomethyl urea were given (Privalov, 1986); mutant variety "Biser" of soybean, a highly productive and resistant to lodging was produced by combined "Beeson" variety seeds treatment with gamma rays and EMS (Mehandjiev, 1991); semi dwarf mutants of oats (*Avena sativa*) obtained from seeds that were treated with 0.1 and 0.2 % EMS for 2 h, followed by treatment with SA for 1 h (Konzak, 1993); androgenic double-haploid mutants in barley after SA treatment to anther and microspore culture (Castillo *et al.*, 2001) and rice mutants with different genetic lesions, after treated with different chemical and physical mutagens (Wu *et al.*, 2005).

2.1 Dose Effect and Genotypic Sensitivity

Plant materials differ in sensitivity to mutagenic treatments. According to Acquaah (2007), it is difficult to find the precise dose but careful experimentation can identify an optimum dose rate. Overdose kills too many cells and often produces crippled

plants, whereas underdose tends to produce few mutants. Many workers feel that a dose close to lethal dose 50 (LD_{50}) is optimal. It is that dose of the mutagen that would kill 50% of the treated individuals. Solanki and Waldia (1997) are of the opinion that an optimum dose is the one that produces maximum frequency of mutations and causes minimum killings. van Harten (1998) also reported that it is better to perform a "seedling growth test" with a range of doses to determine the optimal treatment conditions for a specific cultivar. Since, the genetic architecture of an organism is an important factor in determining the genotypic difference towards mutagens, henceforth, LD_{50} of a particular mutagen for a particular genotype varies greatly (Khan, 1990). Inter-varietal differences with regard to LD_{50} in pulses were reported by several workers like Khan (1988) in *Vigna mungo*, Singh (1983) in *Lens culinaris*, and Kharakwal (1981a; 1981b) in *Cicer arietinum*. Bykovets and Vasykiv (1971) also conducted mutation studies in pulse crops like *Glycine max*, *Pisum sativum* and *Lathyrus* using chemical mutagens and found that all crops are not mutable to the same extent and that the maximum mutagenic effect appeared in peas followed by *Glycine max* and *Lathyrus*. Khan *et al.* (1998) studied the mutagenic effect of maleic hydrazide (MH) in two varieties of *Vigna radiata* and found the variety PS-16 to be more sensitive than the variety K-851. Dose-linked effectiveness of the mutagens like EMS and gamma rays, in terms of germination, reduction in pollen fertility, chlorophyll mutations and seedling height, were noted in peas (Salim *et al.*, 1974), *Pennisetum glaucum* (Singh *et al.*, 1978), *Vigna radiata* (Singh and Chaturvedi, 1980; Khan and Wani, 2004), *Lens culinaris* (Khan, 2002; Wani, 2003), *Lablab purpureus* (Kamau *et al.*, 2011). Khamankar (1984) while working on tomato plant, showed that the rate of mutation was different with different physical and chemical mutagens at certain loci, since some of the gene loci were affected by one mutagen but not by the other. This type of differential sensitivity of genes to different mutagens is of considerable interest and pointed out that the mutation process with the chemical mutagens may be different from radiations. The effective dose of any mutagen in an individual crop is also varying. It has been found that polyploid species are slightly resistant to the action of mutagens than their diploid ones (Reddy *et al.*, 1991). Although, it is difficult to pinpoint the precise and exact cause for differential sensitivity of genotypes to different mutagenic doses, several possible explanations have been put forward by several workers. Akbar *et al.* (1976) concluded from their studies in rice that the differences in radio sensitivity among rice varieties may be due to the difference in their recovery process involving enzyme activity.

2.2 Induced Mutagenesis and Chickpea

Conventional methods of plant breeding had a limited scope in the improvement of chickpea (Bharadwaj *et al.*, 2010). Mutation breeding has become a proven way of creating variation within a crop variety and offers the possibility of inducing desired

attributes that either cannot be found in nature or have been lost during evolution (Novak and Brunner, 1992). Micke (1999) advocated that the mutation approach is superior to other methods of crop improvement especially in cases where the required amount of variation could be produced rapidly. Mutation breeding combines several advantages in plant improvement by upgrading a specific character without altering the original genetic make-up of the cultivar, and is a well functioning branch of plant breeding supplementing to conventional methods in a favourable manner (Gottschalk, 1986; Toker *et al.,* 2007) as can be viwed by the release of various types of mutant varities released for different crops and well documented in IAEA Mutant Varieties and Genetic Stocks Database (MVGS), with their peculiar characteristic features for human welfare (see http://mvgs.iaea.org/Search.aspx). In that sense, it provides a rapid method to improve local crop varieties, without going through extensive hybridization and backcrossing, which is used in conventional breeding. In contrast, transposon or T-DNA insertional mutagenesis generally leads to complete disruption of gene function rather than in generating allelic series of mutants with partial loss of function and thus not producing the range of mutation strengths necessary for crop improvement (Chopra, 2005; Parry *et al.*, 2009). In addition, the insertion sites within the genome may not be distributed randomly (Zhang *et al.*, 2007) and hence, increasing the number of insertion lines required for full genome coverage to unrealistic level (Parry *et al.*, 2009). However, chemical and physical mutagenesis have a number of advantages over such approaches, since mutagens introduce random changes throughout the genomes, generating a wide range of mutations in all target genes, and a single plant can contain a large number of different mutations resulting a manageable population sizes (Parry *et al.*, 2009)

In recent years, a lot of work has been undertaken on induced mutagenesis through physical and chemical mutagens with keen interest to know its impact on food security and malnutrition conditions. It has been clearly shown in a number of plant species that the effect induced, varies with the varying mutagens and with the variation in mutagen doses. Thus selecting a mutagen and its optimum dose for a genotype in any plant species is an important step in mutation breeding programme. By June 2012, more than 3200 varieties in more than 175 plant species derived from mutagenesis programmes have been officially released worldwide as listed in the FAO/IAEA Mutant Varieties and Genetic Stocks Database (MVGS), including 410 mutant varieties of pulses. In chickpea, 21 such mutant varieties have been released for cultivation. Among which, 6 varieties have been released from India, which in reality is not augmenting the efforts of Indian plant breeders in achieving the target of food self-sufficiency and strong economic growth. Mutation breeding technique may have a greater role in crops like chickpea especially in India, where a large part of the natural variability has been eliminated in the process of adaptation to the stress of the environment. Mutations are grouped into two major categories on the basis of their phenotypic manifestations:

1. Micromutations: These involve changes in quantitative traits and can be measured at the level of population using various statistical parameters, such as character mean, variance, heritability etc. and,
2. Macromutations: These involve large changes in the characters which can be detected even without instrumental help at the level of individual plant.

The interest in micromutations for generating polygenic variability increased after Brock (1965) proposed the hypothesis of induction of quantitative variability through mutagenic treatment. Micromutations produce genetic variability in quantitative traits of the crop plants. Hence, they deserve full attention of plant breeders. Such mutations should be useful for improving quantitatively inherited traits (such as yield) without disturbing the major part of the genotype and the phenotypic architecture of the crop. In recent years, a number of attempts to assess mutagen-induced genetic variability in quantitative traits of pulses were elucidated out. However, breeding programmes to improve chickpea, a highly nutritious pulse crop, have not been commensurate with its full potential and role in the Indian diet.

3 Induced Mutagenesis: Biophysiological Damages and Cytological Abberrations

3.1 Bio-Physiological Damages

The variations in terms of bio-physiological damages, gene mutations and chromosome mutations induced by mutagens in any mutation breeding programme have been used as criteria in determining the mechanism of action of the mutagen in question and also the sensitivity of the biological material towards the mutagenic treatments. Among the variations caused, gene and chromosomal mutations may be transferred from M_1 to the subsequent generations, whereas, biological and physiological damages are generally restricted to the M_1 generation.

It is possible to identify plants, which suffer maximum damage due to mutagenic treatment using different types of parameters and in different experimental layout, either individually or in combination, of M_1 generation plants. Different parameters like seed germination, seedling height, plant survival at maturity, pollen and seed fertility, cytological abnormalities, aberrations on leaf surface, estimation of chlorophyll and biochemical contents, the activities of certain enzyme assays etc. have been studied by workers to analyse the biological and physiological changes in M_1 generation due to mutagenic treatments, both in laboratory and in field conditions. The effects of single and combination treatments of physical and chemical mutagens on different biological and physiological parameters in M_1 have been reported in *Oryza sativa* (Fujimoto and Yamagata, 1982; Sarawgi and Soni, 1994; Cheema and Atta, 2003), *Vigna radiata* (Khan, 1990; Sharma *et al*., 1995; Khan *et al*., 1994; Khan *et al*., 1998; Rehman *et al*., 2000; Khan and Wani, 2005a), *Vigna mungo* (Gautam *et al*., 1992), *Triticale* (Edwin and Reddy, 1993a), *Brassica juncea* (Singh *et al*., 1993), *Triticum* spp. (Xiuzher, 1994), *Eleusine coracana* (Kumar *et al*., 1996), *Plantago ovate* (Sareen and Kaul, 1999), *Lens culinaris* (Reddy *et al*., 1992; Verma *et al*., 1999), *Capsicum annum* (Siddiqui and Azad, 1998; Dhamayanthi and Reddy, 2000), *Gossypium hirsutum* (Muthusamy and Jayabalan, 2002), *Stipa capillata* (Zaka, *et al*., 2002), *Cicer arietinum* (Barshile *et al*., 2006; Hameed, *et al*., 2008), *Solanum melongena* (Alka *et al*., 2007), *Euryale ferox* (Verma *et al*., 2010), *Lathyrus sativus* (Kumar and Dubey, 1998b), *Nicotiana tabacum* (Amarnath and Prasad, 1998) and many such examples are being cited by scientific community.

Brunner (1995) in *Vicia faba* reported that M_1 parameters as seedling height, survival and fertility decrease with increasing doses of gamma and fast neutron radiation while chlorophyll and morphological mutant frequencies in segregating M_2 population, increase up to a maximum, and decrease thereafter due to M_1 injury.

Different workers have proposed different parameters as important indicators to determine the mutagenic actions for specific plants by studying the biological and physiological damages. Konzak *et al*. (1972) in wheat, Joshua and Bhatia

(1983) in legumes and Kumar and Mani (1997) in rice proposed that seedling height is a quick and simple method used as an index in determining the biological effects of various mutagens in M_1 generation. Kodym and Afza (2003) observed that germination is not a good indicator for an effective mutagen dose. Blixt (1972) in *Pisum* and Solanki and Sharma (1999) in *Lens culinaris* found that seedling damage (leaf aberrations) to be the most effective index among all M_1 parameters, whereas Gautam *et al.* (1992) in *Vigna mungo* reported that there is a direct relationship of pollen and ovule sterility with higher doses of gamma rays and EMS. Mutagenic efficiency based on injury and lethality was found higher in combined treatments of gamma rays and *N*-nitroso-*N*-methylurea (NMU) than their respective individual treatments (Dixit and Dubey, 1986a). Combined treatments also showed greater reduction in seedling survival than the individual treatments. Lal *et al.* (2009) studied mutagenic effect of gamma rays and sodium azide and their different combinations in blackgram in M_1 and observed that an increase in azide concentrations resulted in decrease in M_1 germination. The plant survival was also affected with different doses of gamma rays and SA and was decreased with increasing doses. The combination treatments of gamma rays and sodium azide had more depressive effect on seedling growth. Singh and Chaturvedi (1980) reported mutagen induced damage such as plant injury and lethality in M_1 generation arises due to physiological and chromosomal mutations. Verma *et al.* (2010) suggested that the alteration in chlorophyll contents in M_1 generation of makhana (*Euryale ferox*) due to gamma irradiation is a vital index in determining the mutagenic action. Gamma irradiation induces various physiological and biochemical alterations in the plants, like carbohydrate metabolism (Joshi *et al.*, 1990; El-Fiki *et al.*, 2003), photosynthetic activities (Kim *at. al.*, 2005; Seung *et al.*, 2007; Moussa and Jaleel, 2011) and interference in the nucleic acid metabolism (Kovalchuk *et al.*, 2001). The effect of the mutagens on the protein contents and the nitrate reductase activity (NRA) has also been reported by Barshile *et al.* (2009) in *Cicer arietinum* and Kozgar *et al.* (2011) in *Vigna* spp.

3.2 Cytological Aberrations

Induction of mutation in plant species is often associated with cytological abnormalities (Reddy, 1990). Cytogenetic information and the degree of cytological aberrations, either in mitosis or meiosis, is regarded as one of the dependable criteria used by plant mutation breeders for estimating the effect of mutagens on the crop plants and to manipulate chromosome segments or whole individual chromosome or sets of chromosomes to solve particular problem. Swaminathan *et al.* (1962) reported that the chromosomal mutations leading to the formation of non-functional gametes are the most common effect of mutagen-induced sterility with reduced reproductive capacity. They further reported that the spectrum of meiotic chromosomal

abnormalities is broad during diakinesis-metapahse and includes high proportion of univalents, moderate frequencies of multivalent, stickiness of chromosomes and non-orientation of bivalents. Studies on different plant species have shown that the decline in seed production is correlated with meiotic irregularities (La Fleur and Jalal, 1972; Smith and Murphy, 1986; Consolaro *et al*., 1996; Kumar and Rai, 2007; Kumar and Gupta, 2007).

In higher plants cytological abnormalities due to single and combination treatments of mutagens have been extensively reported by several workers (Swaminathan *et al*. (1962) and Kumar and Singh (2003) in *H. vulgare*; Kalloo (1972) in *Pisum sativum*; Chowdhury and Nirmla (1976), Reddy (1990) and Edwin and Reddy (1993b) in *Triticale*; Datta and Biswas (1985) and Kumar and Gupta (2007) in *Nigella sativa*; Bandyopadhyay and Bose (1983), Grover and Virk (1986), Ignacimuthu and Sakthivel (1989) and Goyal and Khan (2009) in *Vigna* spp.; Gupta and Roy (1985) in *Physalis*; Jayabalan and Rao (1987) in *Lycopersicon esculentum*; Siddiqui (1987) and Zeerak (1991) in *Solanum melongena*; Reddy and Annadurai (1992) in *Lens culinaris*; Ahmad (1993) and Saeed (1993) in *Cicer arietinum*; Ansari and Ali (2009), Fatma and Khan (2009) and Husain *et al*. (2013) in *Vicia* sp.; Kumar and Dubey (1998b) in *Lathyrus sativus*; Raghuvanshi (1984) and Dhamayanthi and Reddy (2000) in *Capsicum annuum*; Pagliarini *et al*. (2000) in *Chlorophylum comosum*; Venkateswarlu *et al*. (1988) in *Catharanthus roseus*; Mitra and Bhowmik (1996) in *Nigella sativa* and Suganthi and Reddy (1992) in some cereals).

Different types of meiotic abnormalities like chromatin bridges, laggards, fragments, cytomixis, inversion, micronuclei and unequal separation of chromosomes were reported in pearl millet following treatments with gamma rays and EMS (Laxmi *et al*. 1975). They further reported that gamma rays were more effective than EMS or combination treatments in inducing chromosomal abnormalities. Chromosomal abnormalities, and disturbed meiotic behaviour at different stages like sticky chromosomes, fragments and ring chromosomes at metaphase and the laggards and bridges at anaphase were reported by Grover and Tejpal (1982) in *Vigna radiata* using gamma rays, MH and their combinations. Misorientations at metaphase, bridges at anaphase, fragmentation and multinucleolate conditions were observed by Shah *et al*. (1992) in *Vigna mungo* using gamma rays. The chromosomal aberrations were found to be significantly correlated with dose and the combined treatments enhanced chromosomal aberrations. Kumar and Gupta (1978) observed an induced asynaptic mutant of blackgram, which had shown a large number of univalents and an irregular anaphase division.

A relative account of cytological effects of gamma rays, EMS and MMS on meiotic features and pollen fertility in *Vicia faba* was provided by Bhat *et al*. (2007b). There is a close relationship between pollen sterility and meiotic abnormalities as has been reported by Khan (1990), Bandyopadhyay and Bose (1980), Goyal and Khan (2009). Kleinhofs *et al*. (1974) reported that sodium azide induces mostly gene mutation with negligible frequency of chromosomal aberrations.

3.3 Case Study 1

In order to reveal the affects of defined doses/concentrations of the potent physical mutagen - gamma rays and potent chemical mutagen - ethylmethane sulphonate (EMS), alone and in combination in terms of the biophysiological damages and cytological aberrations two varieties of chickpea, Pusa-256 and BG-1053, were tried. The detailed methodology and description are encased in Appendix. However, the main results related to this chapter 3.3 and thereafter discussed with available literature is given herewith.

3.3.1 Results

The effects of gamma rays and ethylmethane sulphonate (EMS) alone and their combination treatments on two varieties of chickpea (Pusa-256 and BG-1053) were studied on seedling height, nitrate reductase activity (NRA), chlorophyll and carotenoid contents, seed germination, pollen fertility, meiotic abnormalities and plant survival at maturity in M_1 generation.

3.3.1.1 Seed Germination

Data recorded on seed germination in petri dishes (in laboratory) and in field conditions are given in Figs. 3.1-3.2. A gradual decrease was observed in seed germination in both the experimental conditions, viz. in laboratory as well as in field with increasing doses of gamma rays and EMS employed alone or in combination in both the varieties of chickpea (Pusa-256 and BG-1053). In variety Pusa-256, the percentage of seed germination was higher in laboratory experiment than in the field experiment, whereas it was higher in the field in comparison to the laboratory experiment in the variety BG-1053. Both varieties differed in the extent of reduction in seed germination. Seed germination was affected more adversely in the variety BG-1053 than in the variety Pusa-256. Combined gamma rays + EMS treatments were found to cause maximum reduction in seed germination followed by EMS and gamma rays treatments in both varieties.

Germination started the third day (in laboratory) and seventh day (in field) after sowing in controls of both varieties. However, it was delayed by two days (in laboratory) and more than four days (in field) in the lots treated with higher doses of mutagens. The delay in seed germination was found to be more pronounced in combined gamma rays + EMS treatments in both varieties.

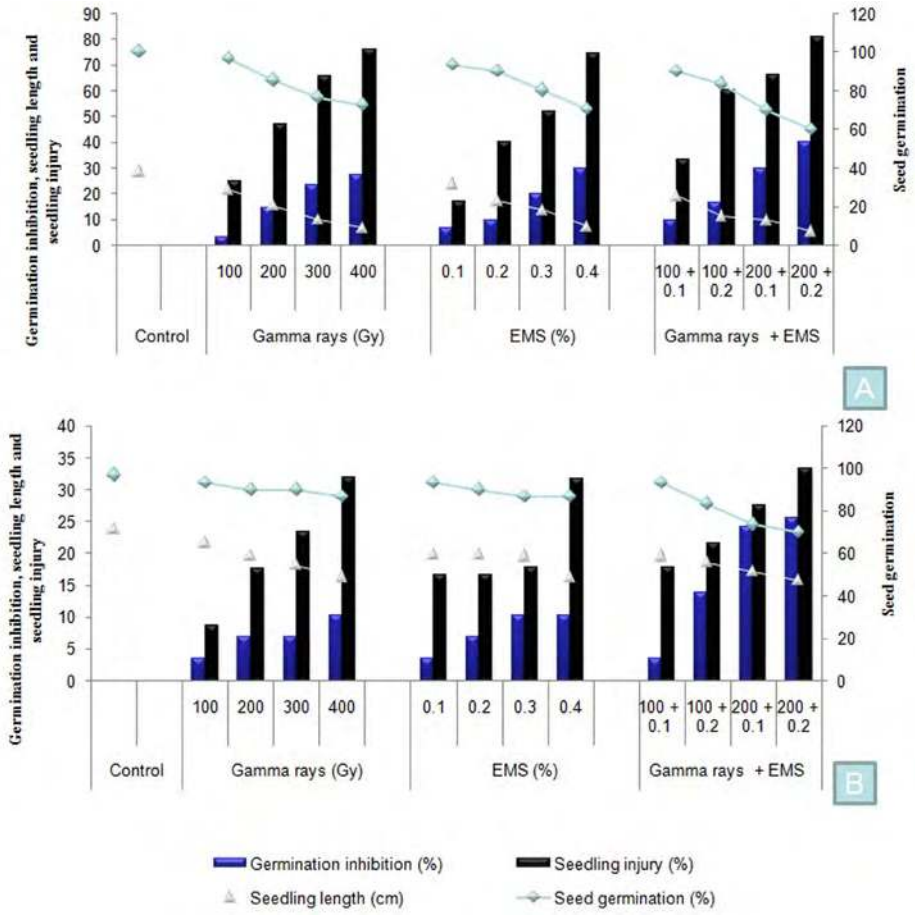

Fig. 3.1: Effects of mutagenic treatments on seed germination (%), germination inhibition (%), seedling length (cm) and seedling injury (%) in chickpea variety BG-1053 (A) and variety Pusa-256 (B) in M_1 generation (in laboratory conditions).

3.3.1.2 Seedling Height (cm)

Seedling height (length) decreased with increasing dose of physical and chemical mutagens and combination treatments in both the varieties (Figs. 3.1 and 3.2). Gamma rays treatments showed an average reduction in height of seedling than EMS treatments. Combination treatments showed an enhanced affect in reducing the height of seedlings when compared with single treatments. Variety BG-1053 was more sensitive as manifested by reduction in seedling height.

The percentage injury in seedling height, as measured by the reduction in length of 10 days old seedlings, increased with mutagen doses.

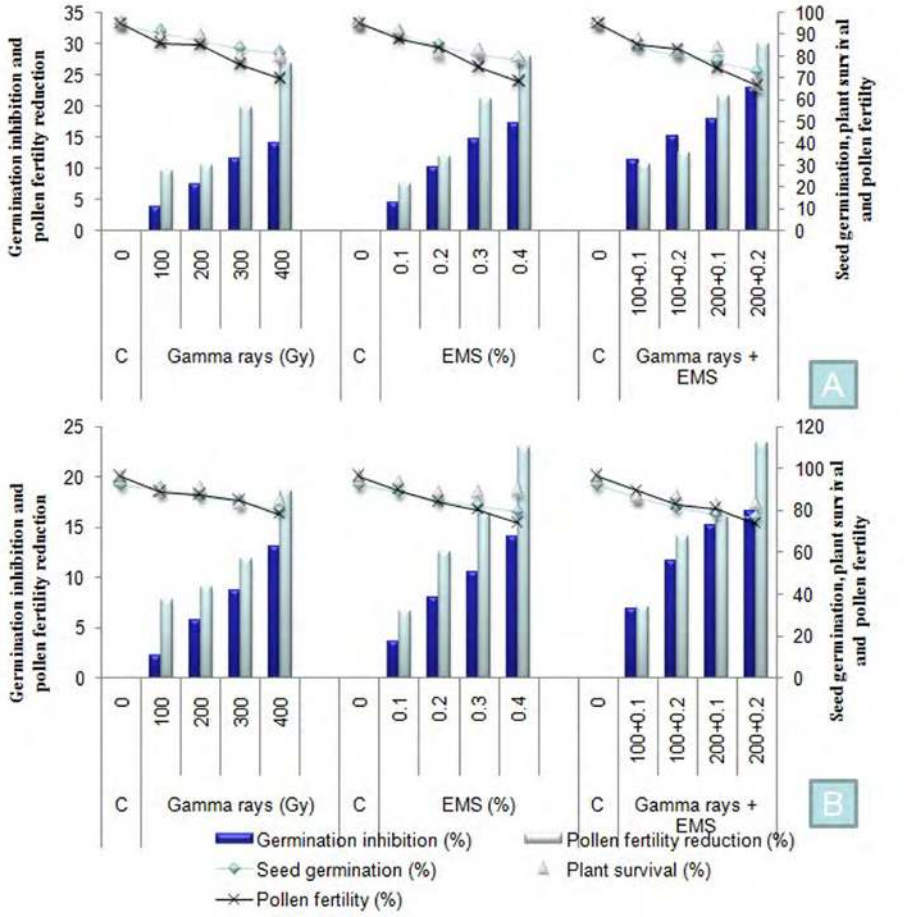

Fig. 3.2: Effects of mutagenic treatments on seed germination (%), germination inhibition (%), plant survival (%), pollen fertility (%) and pollen fertilty reduction (%) in chickpea variety variety Pusa-256 (A) and variety BG-1053 (B) in M_1 generation (in field conditions).

3.3.1.3 NRA, Chlorophyll and Carotenoid Contents

Estimated values for the assay of nitrate reductase activity (NRA), chlorophyll and carotenoid contents are presented in Fig. 3.3. The values of all the three parameters decreased in the leaves of seedlings raised from mutagen treated seeds than the control values. However, the reduction was dose independent. The combination treatments showed more reduction in NRA, chlorophyll and carotenoid contents than single treatments. Variety BG-1053 was found to be more sensitive to single and combination treatments.

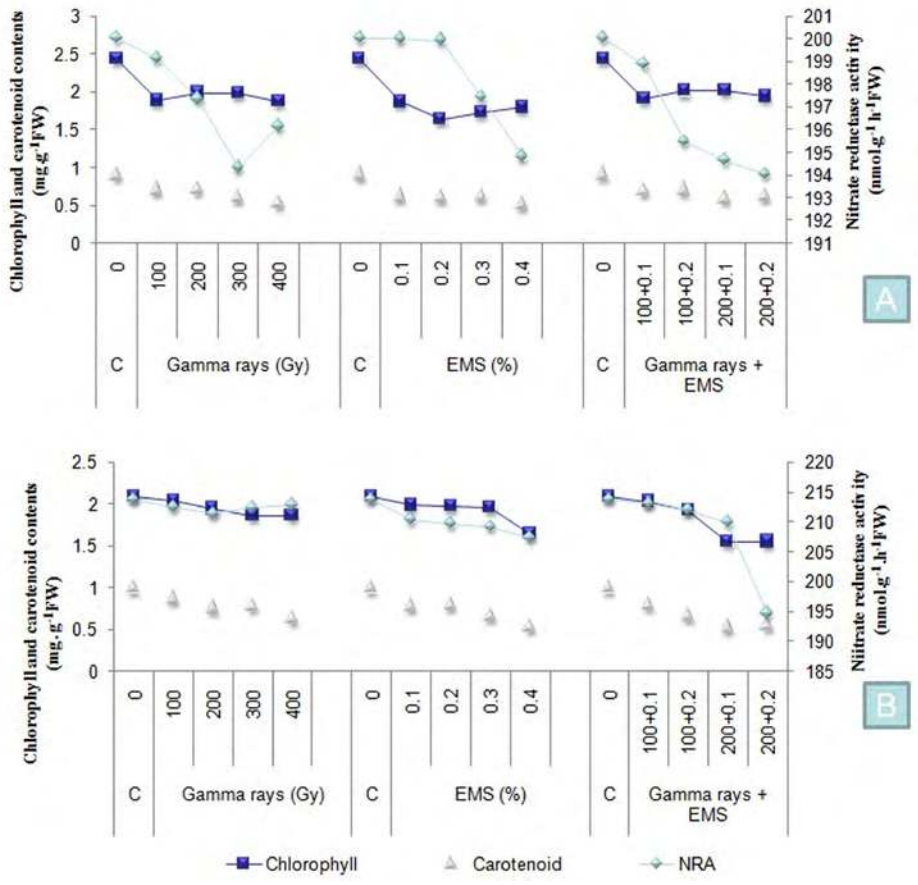

Fig. 3.3: Effects of mutagenic treatments on nitrate reductase activity (NRA), chlorophyll and carotenoid contents in the leaves of chickpea variety BG-1053 (A) and variety Pusa-256 (B) in M_1 generation (in laboratory conditions).

3.3.1.4 Cytological Observations

Chickpea contains 8 bivalents (2n = 16), which are small in size. Normal meiotic divisions with 8 bivalents were observed at metaphase-I in the floral buds from untreated (control) plants. However, various frequencies of meiotic abnormalities in the treated plants were observed in both of the varieties of chickpea (Pusa-256 and BG-1053). Both of the varieties had more or less similar meiotic abnormalities. The spectrum of abnormalities and the total number of pollen mother cells (PMCs) scored in single and combination treatments of gamma rays and EMS and in controls are presented in Fig. 3.4. The meiotic abnormalities observed in various mutagenic treatments included univalents, multivalents, stickiness, precocious separation, non-orientation of bivalents, unequal separation, bridges, laggards, non-disjunction,

disturbed polarity, micronuclei and cytomixis. The meiotic phase generally most affected by these abnormalities was metaphase in both of the varieties. A dose dependent increase in meiotic abnormalities was observed in EMS, gamma rays and combined treatments in both varieties of chickpea (Fig. 3.4). Meiotic abnormalities were comparatively more abundand in the variety BG-1053 than the variety Pusa-256 (Fig. 3.4). The combined gamma rays + EMS treatments were most effective in inducing the maximum total frequency of abnormalities in both the varieties of chickpea followed by gamma rays and EMS (Fig. 3.4).

Univalents and multivalents were observed at metaphase-I. Combination treatments produced a higher number of multivalents than gamma rays and EMS (Figs. 3.5-3.6). At metaphase-I varying number of stickiness (slight stickiness to

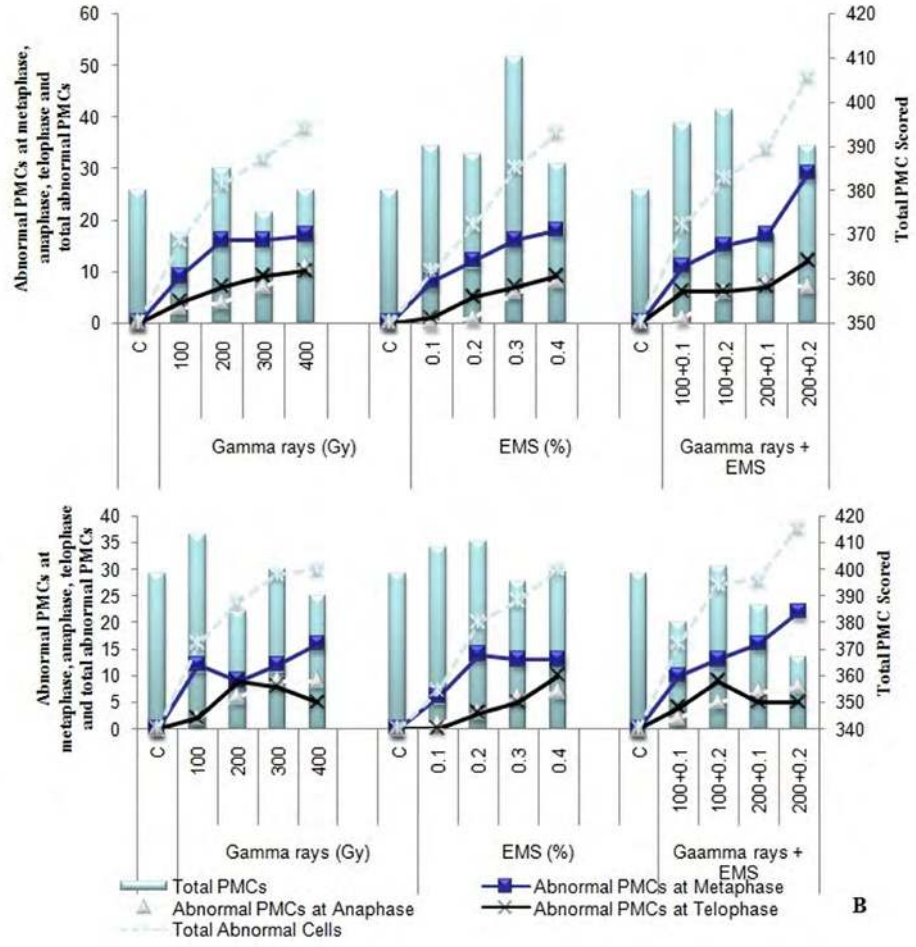

Fig. 3.4: Number of abnormal meiotic cells (pooled) at different stages of meiosis induced by mutagenic treatments in chickpea variety BG-1053 (A) and variety Pusa-256 (B) in M_1 generation.

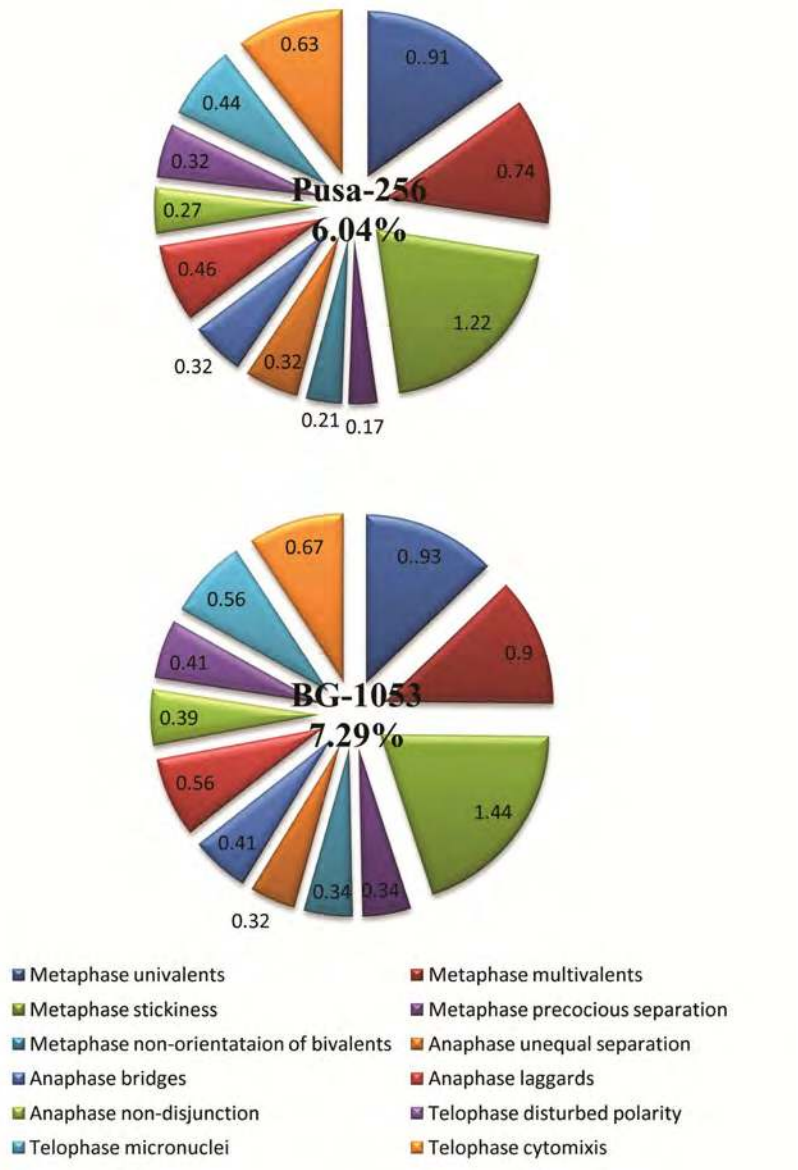

Fig. 3.5: Total (pooled) frequency (%) of meiotic abnormalities induced by gamma rays, EMS and their combinations at different stages of meiotic division in M_1 generation of chickpea (varieties pooled over mutagens).

compact chromatin mass) was noticed in almost all the treatments but their frequency was higher in the combined treatments. Precocious separation of chromosomes to the poles and non oriented bivalents at the equatorial plate were mostly present in higher doses of gamma rays, EMS and their combination treatments. Unequal separation,

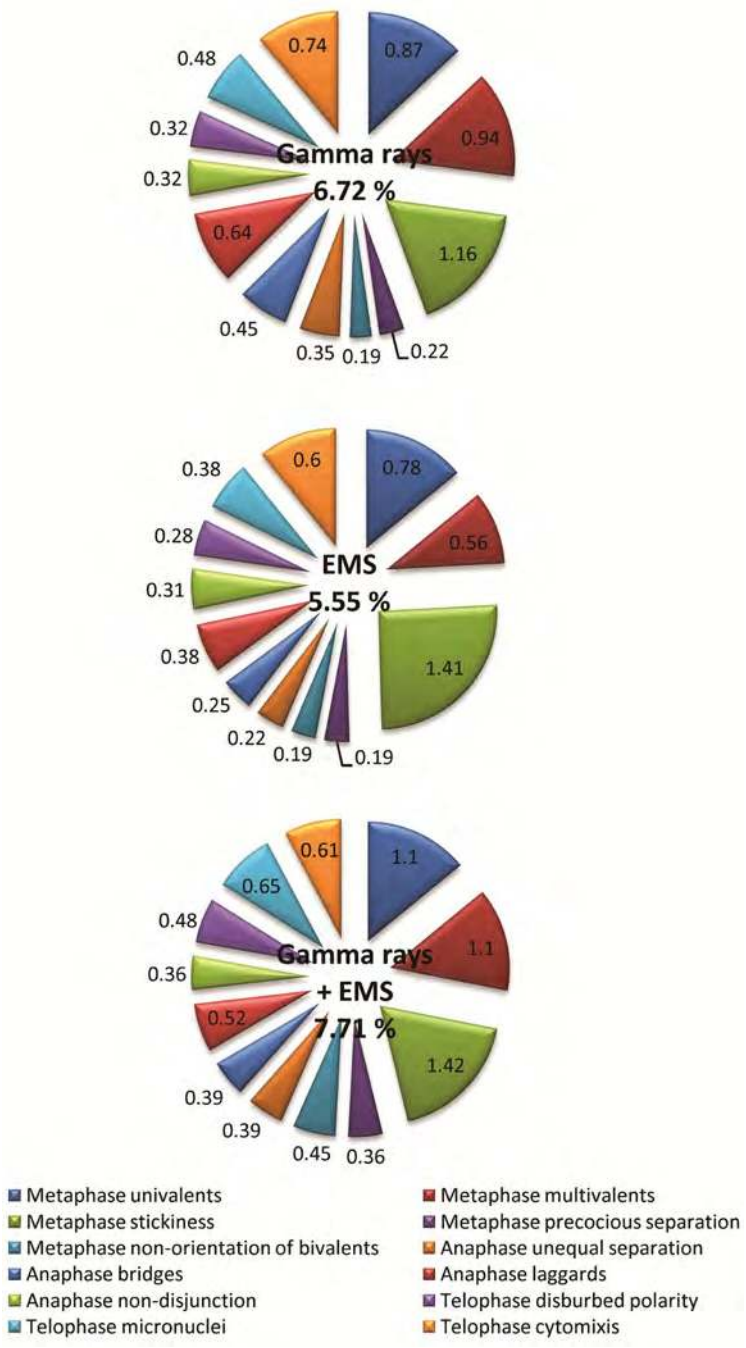

Fig. 3.6: Total (pooled) frequency (%) of meiotic abnormalities induced by gamma rays, EMS and their combinations at different stages of meiotic division in M_1 generation of chickpea (mutagen pooled over varieties).

bridges, laggards, non-disjunction of bivalents, disturbed polarity, micronuclei and cytomixis were mostly observed at first and second anaphase and telophase. Lagging chromosomes and bridges were frequently observed at anaphase-I. As a result of laggard chromosomes, some micronuclei were observed in telophase-I. Noticed bridges were usually of single type. Non-disjunction was mostly seen in all mutagen treated population of both the varieties, with the exception of 200 Gy, 300 Gy, 0.1% EMS and 100 Gy + 0.1% EMS treated population of the variety Pusa-256. Disturbed polarity was commonly observed at telophase-II. Cytomixis (cytoplasmic connections involving two PMCs) was another abnormality observed in the present study. In cytomixes, chromosome transfer between cells was not observed (Fig. 3.7).

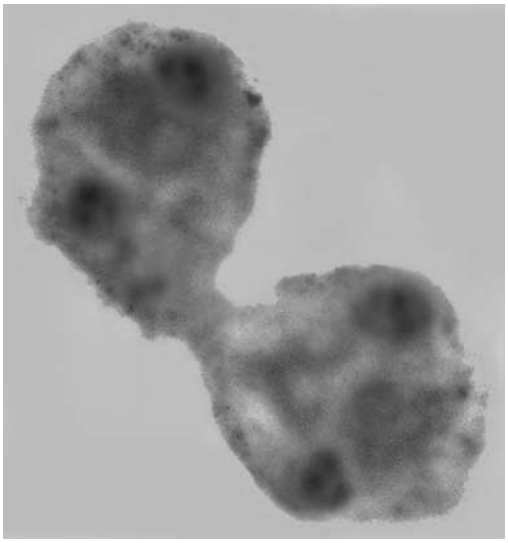

Fig. 3.7: Cytomixis without chromosomal transfer.

3.3.1.5 Pollen Fertility

In order to determine the efficiency of a mutagen and assessing any internal change in the plants, the study of pollen fertility in mutagen treated population forms a reliable index. Although around 4% pollen sterility was also observed in control plants, the reduction in pollen fertility in mutagen treated population was quite high and fertility was dose dependent as evident from a proportionate decrease with an increase in dose of mutagens in both the varieties (Fig. 3.8). The combined gamma rays + EMS treatments caused more drastic effects on fertility than gamma rays and EMS. EMS induced more sterility than the gamma rays. Variety BG-1053 showed the greater reduction in pollen fertility as compared with the variety Pusa-256.

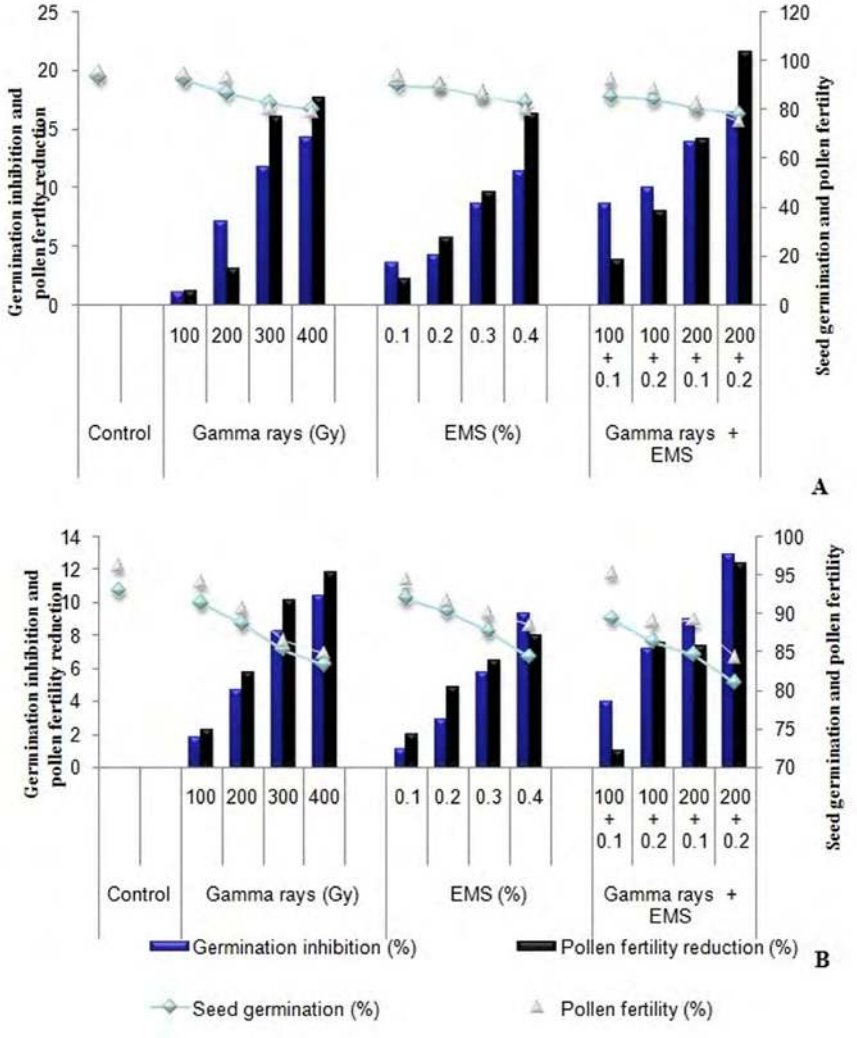

Fig. 3.8: Effects of mutagenic treatments on seed germination (%), germination inhibition (%), pollen fertility (%) and pollen fertility reduction (%) in chickpea variety BG-1053 (A) and variety Pusa-256 (B) in M_2 generation.

3.3.1.6 Plant Survival

Data on plant survival recorded at maturity in M_1 generation are presented in Fig. 3.2. Percentage of plant survival was noted to decrease gradually in single and combination treatments of gamma rays and EMS. However, it was dose independent. Around 95.65 percent in the variety Pusa-256 and 95.07 percent in the variety BG-1053 was observed in the controls.

Although the biophysiological damages and cytological aberrations are studied in terms of above highlighted parameters in M_1 generations, however, study of some parameters in M_2 generations are worthwhile to experiment viz. seed germination and pollen fertility which are closely related to the damages occurred in M_1 generations as they depict the recovery aspects of damages in M_2 generations.

In the present study, seed germination in M_2 generations decreased with increasing doses of gamma rays, EMS and their combination treatments in both the varieties (Fig. 3.8). However, the percentage of seed germination was higher in M_2 in comparison to M_1 generation. Compared to control, reduction in pollen fertility in the treated population was higher. Pollen sterility was lower in M_2 plants in comparison to M_1 plants. The combined gamma rays + EMS treatments showed more reduction in pollen fertility as compared to individual mutagen treatments of gamma rays and EMS.

3.3.2 Discussion

The estimation of bio-physiological damages caused by mutagens helps in determining the sensitivity of a biological material as well as the potency of a particular mutagen. In the present study, the effects of single and combination treatments of gamma rays and EMS measured in terms of seed germination, seedling height, nitrate reductase activity (NRA), chlorophyll and carotenoid contents at early stages and in terms of pollen sterility and plant survival at maturity in the population emerging from the treated seeds along with the controls of both the varieties were studied.

It was observed that gamma rays, EMS and their combination treatments bring about a reduction in seed germination, seedling height, NRA, chlorophyll and carotenoid contents, pollen fertility and plant survival. However, the extent of decrease differed among the mutagens and also between the two varieties. The activity of nitrate reductase (NR), chlorophyll and carotenoid contents and the survival of plants decreased appreciably in the mutagenic treatments in both the varieties, however, there was no direct relationship between the doses of mutagens and the parameters mentioned above. Earlier studies of Mahna *et al.* (1989), Toker and Cagirgan (2004a), Gaikwad and Kothekar (2004), Khan and Wani (2005a), Barshile *et al.* (2006), Štajner *et al.* (2009), Lal *et al.* (2009), Goyal and Khan (2010b) and Bhat *et al.* (2011) in different legumes have shown the adverse effects of mutagens on biological parameters. Promoting effects of low doses of mutagens on physiological parameters have also been reported earlier in several other crops (Mujeeb, 1974; Venkateshwarlu *et al.*, 1978; Vadivelu and Rathinam, 1980; Vandana and Dubey, 1988; Mahto *et al.*, 1989, Mensah *et al.*, 2003; Shereen *et al.*, 2009; Boureima *et al.*, 2009; Sarduie-Nasab *et al.*, 2010; Verma *et al.*, 2010 and Alikamanoglu *et al.*, 2011).

Seed germination decreased with the increasing doses of mutagens. Reduction in germination in mutagenic treatments has been explained due to delay or inhibition of physiological and biological processes necessary for seed germination, which include

enzyme activity (Kurobane *et al.*, 1979), hormonal imbalances (Chrispeels and Varner, 1967) and inhibition of mitotic process (Ananthaswamy *et al.*, 1971) and of course inhibition of DNA synthesis. The inhibition in seed germination was slightly higher in EMS than in gamma rays treatments. Similar results have also been reported earlier by Dhakshanamoorthy *et al.* (2010) in *Jatropha curcas*, Sagade and Apparao (2011) in *Vigna mungo* and Dube *et al.* (2011) in *Cyamopsis tetragonoloba*. The increase in sensitivity depends on the metabolic processes. Presoaking of seeds increases the sensitivity to chemical mutagens. The greater reduction in percent seed germination in the two varieties of chickpea caused by EMS might be due to the change in metabolic condition of the cells during presoaking. This may lead to decrease in the rate of respiration and lack of required enzymes for carrying out the normal metabolism, thus leading to decrease in the rate of seed germination. Gamma irradiation is known to cause disruption and disorganization of the tunica layer, thus inhibiting germination (Chauhan and Singh, 1975). Usuf and Nair (1974) inferred that gamma irradiation interfered with the synthesis of enzymes and at the same time, accelerated, the degradation of existing enzymes involved in the formation of auxins and thus reduces the germination of seeds. Reduced germination due to mutagenic treatments in the present study may be the result of the delay or the inhibition of metabolic activation necessary for seed germination or due to damage of cell constituents at molecular level. Seed germination was found to be affected more adversely in the combined treatments of gamma rays + EMS than individual mutagenic treatments, indicating synergistic effect by enhanced toxicity. The germination delayed by more than three days in the lots treated with high doses of mutagens may be attributed to inhibition of the mitotic proliferation in root and shoot meristems.

The seedlings raised from the treated seeds show a decrease trend, from lower to higher doses of mutagen, in root and shoot length. It could be due to the uneven damage to the meristematic cells as a consequence of genetic injury. The badly damaged cells would produce only a few cell progeny and growth will recur from those cells which are least damaged genetically. Variation in auxin level (Goud and Nayar, 1968), changes in the specific activity of several enzymes (Cherry *et al.*, 1962; Reddy and Vidyavathi, 1985), and physiological injury in the seeds and seedlings (Usuf and Nair, 1974) were correlated with reduction in seedling height after mutagenic treatments. Reddy *et al.* (1992) observed that the chromosomal damages and/or inhibition of cell divisions are the chief cause of a reduced growth. Combination treatments showed an enhanced affect in comparison to individual treatments of gamma rays and EMS. This is in support with the earlier report in urdbean (Ignacimuthu and Babu, 1988; Siddiqui and Singh, 2010).

Estimation of certain physiological parameters like NRA and photosynthetic characteristics (chlorophyll and carotenoid contents) in the M_1 seedlings raised from mutagen treated seeds help to understand the action of mutagens on plants. There was a decrease in the chlorophyll and carotenoid contents of the treated seedlings than compared to the controls, in both the varieties. Similar results were reported

earlier in different crop plants such as *Hordeum vulgare* (Kirk and Juniper, 1963), *Pisum sativum* (Feenstra and Jacobsen, 1980), *Eruca sativa* (Al-Qurainy, 2009) and *Satureja hortensis* (Rahimzadeh *et al.*, 2011). However, Borzouei *et al.* (2010) while working on the effects of gamma rays on wheat, reported that the chlorophyll contents in 100 Gy treated plant was more than the control in M_1 generation after gamma rays treatments. Shereen *et al.* (2009) also reported an increase in chlorophyll contents in M_1 generation in rice after gamma rays treatment. Alteration in the chlorophyll contents of mutagen-treated plants of *Euryale ferox* has also been reported by Verma *et al.* (2010). Decrease in the chlorophyll contents after mutagenic treatments in the present study might be due to the increased activity of chlorophylase, an enzyme, which is regarded as chlorophyll-degrading enzyme (Reddy and Vora, 1986). Hopkins (1995) considered the decline in the activity of NR, compared to the control, may be due to the inhibition and/or metabolic dysfunctions of the enzyme protein.

Physical and chemical mutagens are known to produce meiotic abnormalities leading to abnormal chromosomal behaviour. The cytological abnormalities during meiosis have been considered to be one of the reliable parameters for estimating mutagenic sensitivity of a species. In the present study, single and combined treatments of gamma rays and EMS induced a broad spectrum of meiotic abnormalities in the two varieties of chickpea. Different types of meiotic abnormalities observed in the present study have also been reported by different workers in various crop plants after mutagenic treatments (Ignacimuthu and Babu, 1989b; Zhou *et al.*, 2003; Goyal and Khan, 2009; Goyal and Khan, 2010c; Gulfishan *et al.*, 2011). These results support the view that physical mutagens produce more cytological abnormalities than chemical mutagens, but are contrary to the findings that EMS was more effective in inducing meiotic irregularities than gamma rays (Dhamayanthi and Reddy, 2000).

Among the chromosomal aberrations in PMCs, higher percentage of stickiness among the bivalents was recorded in the present study as compared to other types of meiotic abnormalities. The phenotypic manifestation of stickiness may vary from mild (few chromosomes of the genome are involved) to intense (invloving the entire genome). In both the varieties of chickpea, the stickiness was mostly of intense type where chromosome clumped into one group at metaphase, causing difficulty in normal disjunction. Mutagen induced chromosome stickiness has been described in *Lens culinaris* (Sinha and Godward, 1972), *Capsicum* spp. (Katiyar, 1978), *Turnera ulmifolia* (Tarar and Dnyansagar, 1980), *Lycopersicon esculentum* (Jayabalan and Rao, 1987), *Nigella sativa* (Mitra and Bhowmik, 1996) and *Secale montanum* (Akgün and Tosun, 2004). Rao and Laxmi (1980) reported that stickiness at meiosis was due to the disturbances in cytochemically balanced reactions by the effect of mutagens, while Klasterska *et al.* (1976) suggested that stickiness arises due to improper folding of chromosome fiber. Gaulden (1987) postulated that sticky chromosomes are likely to have resulted from the defective functioning of one or two types of specific non-histone proteins involved in chromosome organization, which are needed for chromatid separation and segregation. The altered functioning of these proteins leading to

stickiness is caused by mutation in the structural genes coding for them (hereditary stickiness) or by the action of the mutagens (induced stickiness). Chromosome stickiness has been reported to be one of the causes of decreasing pollen fertility in some species (Rao *et al.*, 1990; Pagliarini *et al.*, 2000).

The univalent is either a chromosome which failed to pair at zygotene or is one which paired to form a bivalent but whose two component chromosomes are separated at diplotene because of the absence of chiasma formation between them or it is due to precocious anaphase separation of bivalents (Sarbhoy, 1977). Precocious chromosome migration to the poles may have resulted from univalent chromosomes at the end of prophase-I or precocious chiasma terminalization at metaphase-I. Chiasmata are responsible for the maintenance of bivalents, which permit normal chromosome segregation. This process ensures pollen fertility (Pagliarini, 1990). The occurrence of univalents indicates non-homology between certain chromosomes in the complement and it seems more likely that mutagenic treatments induced structural changes in some of the chromosomes which restricted pairing and hence formation of univalents. While precocious migration of univalents to the poles is a very common abnormality in plants (Murata *et al.*, 2006; Khan et *al.*, 2009; Goyal and Khan, 2009), the occurrence of non-oriented bivalents observed in the chickpea varieties is rare. Non-orientation of bivalents appears to be manifestation of improper functioning or breakage of spindle fibres, which cause the chromosomes to lie scattered all over the cell space (Kumar and Singh, 2003). Stickiness of chromosomes at metaphase affects adversely the normal disjunction of chromosomes at anaphase, which resulted in the formation of laggards and unequal separation of chromosomes in daughter nuclei. The univalents and the laggard chromosomes, which fail to be included in the daughter nuclei during later stages of division generally lead to micronuclei formation (Koduru and Rao, 1981; Zeerak and Zargar, 1998; Maurya *et al.*, 2006; Azad, 2011). The laggards and micronuclei might be the major factor for causing pollen sterility in plants (Patil, 1992). The percentage of cells with univalents and laggards was higher than the percentage of cells with micronuclei, indicating that some chromosomes were included in the main nucleus. This seems to be normal behavior for many species (Khazanehdari and Jones, 1997; González-Sánchez *et al.*, 2004; Dewitte *et al.*, 2010; Koduru and Rao, 1981). Various reports (Schulz-Shaeffer, 1980; Zeerak, 1992a; Rebollo and Arana, 2001; Sharma and Kumar, 2004; Tel-Zur *et al.*, 2005; Miko, 2008) confirmed that the laggard chromosomes and their presence as univalents and unequal separation of chromosomes may result in the production of aneuploid gametes which may be utilized in breeding programmes. Multivalent formation have been reported in various plants like eggplant (Satyanarayana and Subhash, 1982), *Vigna* spp. (Ignacimuthu and Babu, 1989b), lentil (Gupta *et al.*, 1999), chilli (Dhamayanthi and Reddy, 2000), faba bean (Khan *et al.*, 2009; Gulfishan *et al.*, 2010) and *Aliuk* spp. (Sharma and Gohil, 2011). In most cases multivalent formation in mutagen treated plants has been attributed to reciprocal translocation, which results in segmental homology between non-homologous chromosomes. The occurrence of

chromosome bridges at anaphase-I could be due to stickiness of chromosomes ends. Ignacimuthu and Babu (1989b) reported that chromosome breakage and reunion of broken ends could lead to the formation of bridges. Disturbed polarity at telophase-II could be due to spindle disturbances. It may be because of alterations in the genes, controlling the biochemical pathways of the substance that determine the position of the spindle poles. Observations by Suarez and Bullrich (1990), and Suganthi and Reddy (1992) in *Triticum aestivum*, Zeerak (1992a) in *Lycopersicon esculentum*, Fatma and Khan (2009) and Ansari and Ali (2009) in *Vicia faba*, Kumar and Singh (2003) in *Hordeum vulgare*, Khan and Tyagi (2009b) in *Glycine max* and Gufishan *et al.* (2011) in *Capsicum annum* on anaphase bridges and disturbed polarity have been already reported. Chromosome bridges are often associated with segregating univalent, which forms due to failure of some chromosomes to attach at metaphase-I (Caryl *et al.*, 2003).

The migration of chromatin material from one cell to an adjoining cell through cytoplasmic connections is generally referred as cytomixis. Although cytoplasmic connections have been reported in several plant species, the movement of nuclear material through them is rare. Cytomixis may have serious genetic consequences by causing deviations in chromosome number and may represent an additional mechanism for the origin of aneuploidy and polyploidy (Sarvella, 1958; Thakur, 1978; Siddiqui *et al.*, 1979). In the present study, although no visual transfer of chromosome material could be detected, the formation of cytoplamsic channels between two cells suggests that screening of mutagen treated population for varied ploidy level and its utilization in plant breeding can yield interesting results (Fig. 3.8). Cytomixis was observed at telophase-I and PMCs were mostly connected with a single cytoplasmic channel. Mutiple cytoplasmic channels were also reported by Belluci *et al.* (2003) in *Medicago sativa* and Massoud *et al.* (2011) in *Astragalus cyclophyllos*. According to Kaul (1971), some chemical mutagens, which cause stickiness of chromosomes, may also be responsible for cytomixis. Cytomixis in the present study may be due to abnormal genetic behavior induced by mutagen treatments. Cytomixis has been detected at a higher frequency in genetically imbalanced species such as hybrids, as well as in apomictic and polyploidy species (Gottschalk, 1970; Yen *et al.*, 1993). Among the factors proposed to cause cytomixis are the changes in gene control, pathological conditions, fixation effects, herbicides, environmental stresses and temperature (Omara, 1976; Bobak and Herich, 1978; Haroun, 1995; Haroun *et al.*, 2004; Heslop-Harrison, 1996; Caetano-Pereira and Pagliarini, 1997; Heng-Chang *et al.*, 2007).

In the present study, varying degree of pollen sterility has been observed in the treatments with gamma rays and EMS alone or in combination. Studies on different plant species have shown that the decline in pollen fertility is correlated with the meiotic aberration (Gaul, 1970; Sinha and Godward, 1972; Ramanna, 1974; Larik, 1975; Patil, 1992; Dhamayanthi and Reddy, 2000; Kumar and Singh, 2003).

Khalatkar and Bhatia (1975) reported that among single and combination treatments of gamma rays and EMS, the pollen fertility was affected less in the

combined treatments in barley. Contrary to this finding, the pollen sterility was highest in combination treatments as compared to individual mutagenic treatments of gamma ray and EMS. Similar results were made earlier in *Phaseolus vulgaris* (Gautam *et al.*, 1998) and *Vigna mungo* (Gautam *et al.*, 1992; Makeen *et al.*, 2010). EMS treatments proved to be more efficient in inducing pollen sterility than gamma rays, although detectable chromosomal abnormalities were less frequent with it. Sato and Gaul (1967) attributed the high pollen sterility and low frequency of meiotic abnormalities in case of EMS treatments of barley seed to small undetectable deletions or gene mutations. Results indicate that present pollen sterility was higher than percent meiotic abnormalities in both single and combined treatments. It seems that in addition to chromosomal aberrations, some genic and physiological changes might have caused sterility. The percentage of pollen sterility was relatively lower in M_2 than in M_1 generation, indicating that some sort of recovery mechanism must be operating in the intervening period.

The survival of plants decreased in the mutagenic treatments in both the varieties of chickpea, however, there was no direct relationship between the dose of mutagens and the survival. A linear relationship between the dose of mutagen and survival of plants at maturity was observed in *Cajanus cajan* (Potdukhe and Narkhede, 2002), *Vigna mungo* (Sharma *et al.*, 2006) and *Glycine max* (Manjaya, 2009). The reduction in survival percentage of the treated population could be due to disturbed physiological processes or chromosome damage leading to mitotic arrest.

Differences in mutagenic sensitivity between the two presently used varieties have also been reported earlier in *Cajanas cajan* (Pandey *et al.*, 1996), *Vigna* sp. (Kozgar *et al.*, 2011; Khan and Siddiqui, 1995), *Oryza sativa* (Satoh and Omura, 1981, Rao and Rao, 1983), *Phaseolus vulgaris* (Svetleva and Crino, 2006, Al-Rubeai and Godward, 1981), *Jatropha curcus* (Dhakshanamoorthy *et al.*, 2010), *Lens culinaris* (Gaikwad and Kothekar, 2004), *Cicer arietinum* (Toker and Cagirgan, 2004a) and *Vigna mungo* (Goyal and Khan, 2010b). Such variation may be due to the fact that some of the gene loci affected by one mutagen were not necessarily affected by another (Khamankar, 1984) and even a single gene difference induced significant changes in mutagen sensitivity (Sparrow *et al.*, 1965). In the present study, the two chickpea varieties differ in their genome architecture not only due to the reason of being the kabuli (BG-1053) and desi (Pusa-256) types but also testified by the fact that the variety BG-1053 was developed by inter-varietal single cross hybridization, while the variety Pusa-256 developed by inter-varietal double cross hybridization involving three desi and one kabuli types which might have some impact and hence difference in sensitivety response outcomes.

4 Chlorophyll Mutations

There are two important goals of mutation research viz. the enhancement of mutation frequency and alteration of mutation spectrum in a predictable manner. In the past, different approaches have been tried to achieve these goals (Gustafsson, 1963; Nilan, 1967; Swaminathan and Sharma, 1968; Chakarbarti, 1975; Maluszynski, 2001; Mehandjiev *et al.,* 2001). The scoring of chlorophyll mutation frequency in M_2 generation is one of the most reliable measures for evaluating the mutagenic induced genetic alterations of the mutagen treatments used on the plant ideotype (Chaturvedi and Singh, 1990). Chlorophyll deficient chimers in M_1 generation and their segregation in M_2 generation are often observed in a mutagenized population. Several authors have reported the occurrence of different types of chlorophyll mutations such as albina, xantha, chlorina, viridis, virescent, tigrina etc., in M_2 generation following treatments with various mutagenic agents in different crop species. Some of the noteworthy examples are of Dahiya (1973) and Wani *et al.* (2011c) in mungbean; Venkateshwarlu *et al.* (1978) in pigeonpea; Venkateswarlu *et al.* (1988) in *Catharanthus roseus*; Prasad and Das (1980), Bawankar and Patil (2001) and Waghmare (2001) in grasspea; Dixit and Dubey (1986b), Reddy *et al.* (1993) and Paul and Singh (2002) in lentil; Arora and Kaul (1989) in *Pisum sativum*; Anwar and Reddy (1981), Reddi and Suneetha (1992) and Yamaguchi *et al.* (2006) in rice; Mohanasundaram *et al.* (1998), John (1999) and Girija and Dhanavel (2009) in cowpea; Singh *et al.* (1999) in urdbean; Prakash and Shambulingappa (1999) and Devi *et al.* (2002) in rice bean; Szarejko and Maluszynski (1999) in barley; Deepa and Devi (2000) in winged bean; Kumar *et al.*, (2003) in limabean; Toker and Cagirgan (2004b), Khan *et al.* (2005b), Barshile *et al.* (2006), Bhat *et al.* (2012) in chickpea and Khan and Tyagi (2009b) in soybean. The frequencies of chlorophyll mutations in different crops with different mutagens have been found to be markedly different. Chemical mutagens induce higher frequency of chlorophyll mutations than radiations (Blixt, 1972; Filippetti *et al.*, 1977; Sharma and Sharma, 1981b; Tripathi and Dubey, 1992; Vandana *et al.*, 1994; Kharkwal, 1998b; Singh *et al.*, 2000a; Waghmare and Mehra, 2001; Karthika and Subbalakshmi, 2006; Lal *et al.*, 2009; Arulbalachandran and Mullainathan, 2009a). Among the chemical mutagens, EMS was reported to induce a higher frequency of chlorophyll mutations. The superiority of EMS in inducing chlorophyll mutations at a higher frequency was also suggested by Swaminathan *et al.* (1962), Muntzing and Bose (1969), Hussein *et al.* (1974), Reddi and Suneetha (1992), Shah *et al.* (2006), Gupta (2008), and Wani *et al.* (2011b). Ionizing radiations generally produce a higher proportion of albina mutations than chemical mutagens (Swaminathan *et al.*, 1962; Ando, 1970; Gupta and Yashvir, 1975; Subramanian, 1980; Cheema and Atta, 2003; Karthika and Subbalakshmi, 2006). However, Hemavathy and Ravindran (2005) found that the occurrence of albina is less compared to the chlorina and xantha in gamma rays treated population of *Vigna mungo*.

The use of combination treatments of physical and chemical mutagens alters the mutation frequency and spectrum (Arnason *et al.*, 1963; Favert, 1963) and has gained a great momentum in its usage in the recent past in mutation breeding programmes. Chemical mutagens when combined with radiation are not only mutagenic themselves but also affect mutation in specific ways and are more effective in inducing chlorophyll mutations comparing to individual treatments (Sharma, 1969; Khalatkar and Bhatia, 1975 Reddy and Smith, 1981; Singh *et al.*, 1999; Khan and Tyagi, 2009b). Similar observations of synergistic effects were made on rice treated with thermal neutrons and diethyl sulphate, DES (Rao and Ayenger, 1964) and on black gram in the combination treatments of gamma rays and EMS (Gautam *et al.*, 1992). In some cases, the antagonistic effects have also been reported in the combined treatments of gamma rays and EMS (Arora and Kaul, 1989). However, in general, most of the mutagens given in combination exhibit synergism in legumes and cereals.

There is a strong indication that total mutations frequency and spectrum are associated with the dose of mutagen (Hussein *et al.*, 1974; Sarma *et al.*, 1979; Reddy and Gupta, 1989; Reddy and Revathi, 1991; Venkatachalam and Jayabalan, 1993; Singh *et al.*, 1999; Amarnath and Prasad, 2000; Das and Kundagrami, 2000). However, this claim was repudiated by some workers (Pipie, 1972; Khan, 1990; Yamaguchi *et al.*, 2009) who found no relationship between the dose of mutagen and the mutation spectrum. According to Gaul (1964), Vo Hung (1974), Khan and Siddiqui (1993), Kaul and Bhan (1977), Khan (1986), Reddi and Suneetha (1992), Raveendran and Jayabalan (1997), Mitra and Bhowmik (1999) and Ganapathy *et al.* (2008), the highest dose is not always the most effective treatment.

4.1 Case Study 2

Chlorophyll mutations are one of the reliable indices to assess the genetic effects of mutagenic treatments. The seeds of chickpea were harvested from the Pusa-256 and BG-1053 varieties in M_1 generations as per Case Study 1 and were sown in M_2 generations and the progenies developed were screened for the chlorophyll mutations as per methodologies explained in Appendix. The outcome results and discussion are given herewith.

4.1.1 Results

Six different types of chlorophyll mutants were recorded in the field in M_2 generation when seedlings were 10-20 days old. The spectrum of different M_2 chlorophyll mutants included: albina, chlorina, maculata, tigrina, viridis and xantha. All these chlorophyll deficient mutants were lethal except chlorine, maculata and viridis. A brief description of the isolated different chlorophyll mutants is given in Table 4.1 and Fig. 4.1.

Table 4.1: Characteristic features of chlorophyll mutants and percentage mutated plant progenies induced by gamma rays, EMS and their combinations in two varieties of chickpea in M_2 generation.

Isolated mutant types and their characteristics	Treatment	No. of M_1 plant progenies	No. of plant progenies segregating in M_2	% mutated plant progenies (M_p)
1. Albina		**Var. Pusa-256**		
Lethal mutation characterized by entirely white leaves of seedlings; seedlings survived for 10-12 days after germination.	Control	50	-	-
	100 Gy γ rays	50	1	2.0
	200 Gy γ rays	50	4	8.0
	300 Gy γ rays	50	5	10.0
2. Chlorina	400 Gy γ rays	50	5	10.0
Light green colour of leaves; most of the seedlings died within 20 days. However, few vigorous plants survived and were late in maturity.	0.1% EMS	50	1	2.0
	0.2% EMS	50	4	8.0
	0.3% EMS	50	4	8.0
	0.4% EMS	50	5	10.0
3. Maculata	100Gy γ rays + 0.1% EMS	50	4	8.0
Seedlings showed yellow or whitish dots on leaves; These mutants survived till maturity and produced few seeds.	100Gy γ rays + 0.2% EMS	50	6	12.0
	200Gy γ rays + 0.1% EMS	50	6	12.0
	200Gy γ rays + 0.2% EMS	50	7	14.0
		Var. BG-1053		
4. Tigrina	Control	50	-	-
Leaves yellow with green patches typical of the colour of the skin of Indian frog; survived for 15 days.	100 Gy γ rays	50	3	6.0
	200 Gy γ rays	50	7	14.0
	300 Gy γ rays	50	8	16.0
	400 Gy γ rays	50	8	16.0
5. Viridis				
Reduced plant height and viridine green colour of leaves; leaflets size reduced; plants were slow growing and had a low seed yield.	0.1% EMS	50	2	4.0
	0.2% EMS	50	6	12.0
	0.3% EMS	50	6	12.0
	0.4% EMS	50	7	14.0
6. Xantha	100 Gy γ rays + 0.1% EMS	50	6	12.0
Leaves were bright yellow in colour; seedlings survived for 10-20 days.	100 Gy γ rays + 0.2% EMS	50	7	14.0
	200 Gy γ rays + 0.1% EMS	50	7	14.0
	200 Gy γ rays + 0.2% EMS	50	8	16.0

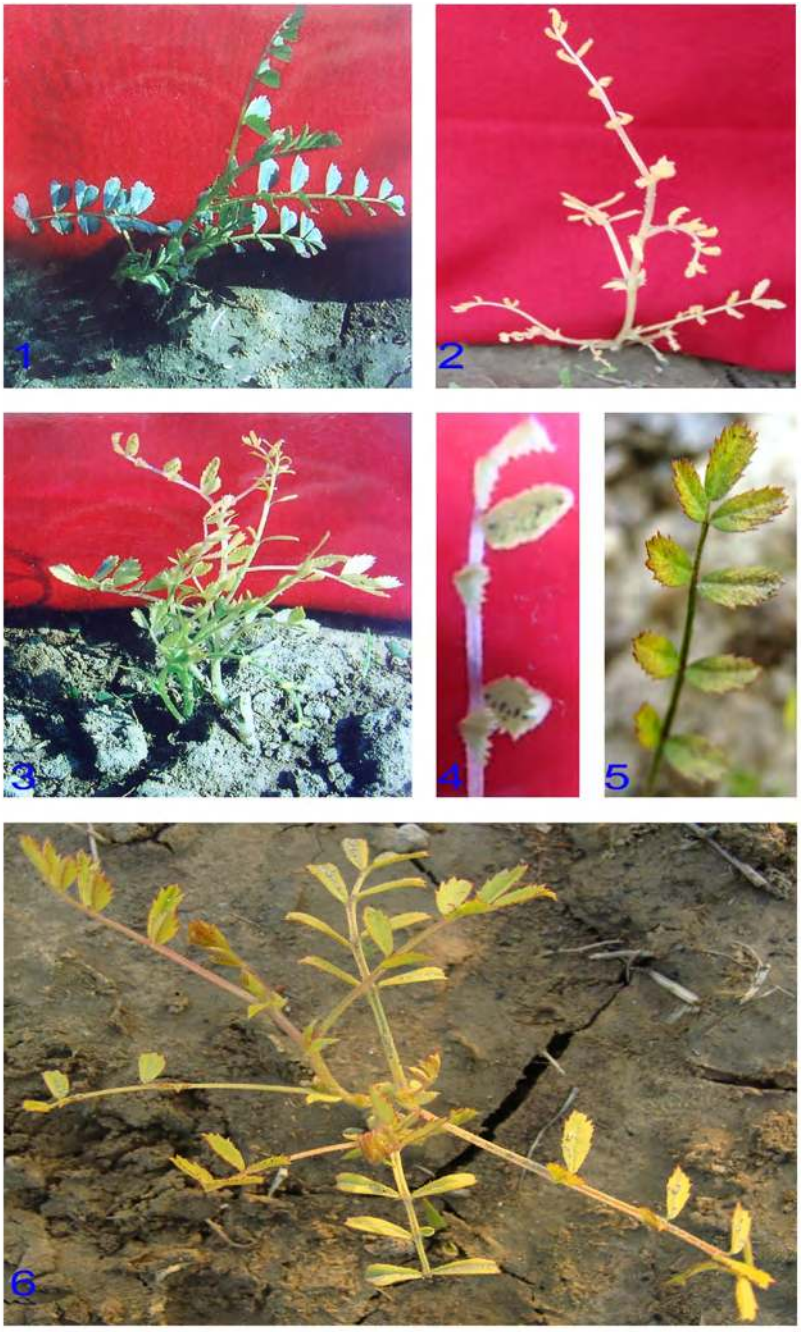

Fig. 4.1: Chlorophyll mutants: **1.** Control seedling; **2.** Albina mutant (white leaves of seedling); **3.** Chlorina mutant (light green colour); **4.** Tigrina mutant (patches of green dots on leaflets); **5.** Viridis mutant (viridine green colour); **6.** Xantha mutant (bright yellow colour). (Photographs by M I Kozgar)

The chlorophyll mutations frequency was calculated on progeny basis as well as on M_2 seedling basis. The trend of the mutation frequency was similar in both of the methods (progeny basis as well as on M_2 seedling basis). Therefore, results are presented on M_2 plant basis. A linear relationship between mutations frequency and the dose of gamma rays, EMS and their combinations was observed in both varieties of chickpea up to 300 Gy in gamma rays treatments, up to 0.3% in EMS treatments and up to 200 Gy gamma rays + 0.1% EMS in combination treatments. Among the mutagens used, the combined gamma rays + EMS treatments gave a higher mutation frequency in comparison to individualistic treatments of gamma rays and EMS. However, the EMS treatments were found to be more effective than gamma rays treatments. Variety BG-1053 responded with the greater mutation frequency as compared with the variety Pusa-256. In both varieties the frequency (1.06%) of albino mutants was the highest, followed by those of chlorina, xantha, maculata, viridis and tigrina. The ratio of albino/chlorine was found to be 1.51, 0.83 and 1.24 for gamma rays, EMS and gamma rays + EMS, respectively (Fig. 4.2).

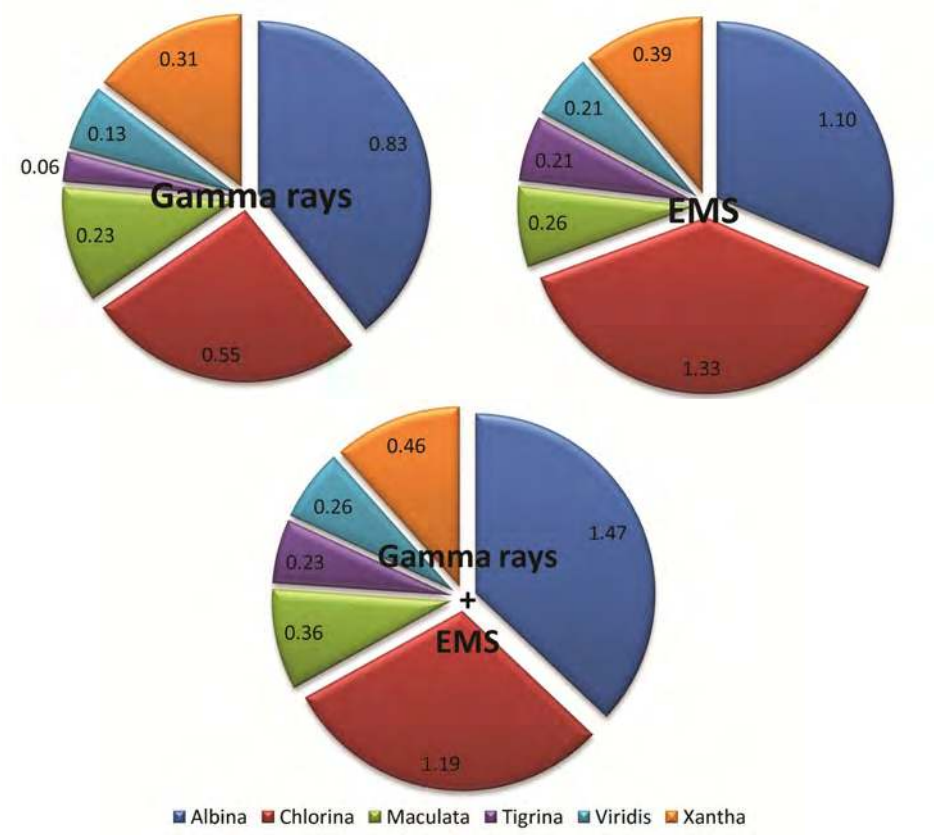

Fig. 4.2: Comparative frequency (%) and spectrum of chlorophyll mutations in chickpea based on pooled values of two varieties of chickpea (Pusa-256 and BG-1053).

The combined treatments did not show any additive effect in producing chlorophyll mutations. The results obtained in the present study showed negative synergism in both varieties of chickpea.

4.1.2 Discussion

The chlorophyll mutation frequency is useful in assessing the potency of a mutagen, genetic effects of mutagen and estimation of mutational events mainly because of ease of identification. From a breeder's point of view, the frequency of chlorophyll mutants expressed as per cent of M_2 population seems to be more realistic and helpful. Therefore, results were explained on M_2 plant basis. In the present study, a critical comparison of the chlorophyll mutations indicates that the mutation rate, in general, increased with an increase in dose up to a certain dose level beyond which it decreased. This trend was observed in both the varieties in treatments with gamma rays, EMS and their combination. Higher frequencies of chlorophyll mutations with medium or lower doses of mutagens were reported by Srivastava *et al.* (1973), Nadarajan *et al.* (1982), Toker and Cagirgan (2004a), Yamaguchi *et al.* (2009) and Pawar *et al.* (2010) in different crops including chickpea. The decrease in chlorophyll mutation frequency as observed at the highest doses of mutagens may be attributed to saturation in the mutational events, which may result in the elimination of the mutant cells during growth. Contrary to the results obtained, the works of Subramanian (1980), Grover and Virk (1984), Khan (1990), Khan and Siddiqui (1992), Reddy *et al.* (1993), Kharkwal (1998b), John (1999), Das and Kundagrami (2000), Barshile *et al.* (2006), Pavadai *et al.* (2009) and Kumar *et al.* (2009a) reported dose dependent increase in the chlorophyll mutations frequency with different mutagenic treatments. Occurrence of chlorophyll mutants in large number of crops have been attributed to different causes such as impaired chlorophyll biosynthesis, further degradation of chlorophyll and bleaching due to deficiency of carotenoids (Bevins *et al.*, 1992) and may be related to their preferential action on chlorophyll development genes (Reddy and Anndurai, 1991). Recovery of a higher number of albina from combination treatments of gamma rays + EMS is contrary to the contention of Arora and Kaul (1989) that gamma rays alone or in combination with EMS induced more chlorina mutants. Athwal *et al.* (1970) in chickpea and/or as well as Karthika and Subbalakshmi (2006) in soybean reported that albina constituted the largest single category of mutants in gamma rays treated population. In the present study, EMS produced more albino types than gamma rays. The frequency of chlorophyll mutations induced by gamma rays was less than that of EMS and combination treatments. These results are in contrary to the findings of Arora and Kaul (1989) who observed, in *Pisum sativum*, that gamma rays treatment was the most potent in inducing the highest chlorophyll mutations frequency. EMS is supposed to be specific to certain chromosomal regions (Goud, 1967; Khan and Siddiqui, 1993; Uchida *et al.*, 2011) containing genes for chlorophyll development and

has been reported to induce high frequency of chlorophyll mutations (Swaminathan *et al.*, 1962; Girija and Dhanavel, 2009; Pawar *et al.*, 2010; Bhat *et al.* 2012).

The combined treatments of gamma rays + EMS showed a considerable degree of synergism in increasing the frequency of chlorophyll mutations. Synergistic effects of physical and chemical mutagens have been reported when they are used in combination in various crops (Khalatkar and Bhatia, 1975 in barley; Agarkova and Yakovlev, 1977 in *Phaseolus vulgaris*; Ignacimuthu and Babu, 1988 and Singh, 2007a in *Vigna* spp.; Venkateswarlu *et al.*, 1988 in *Catharanthus roseus*; Reddy, 1992b in triticale). A probable reason for synergism is that the mutagen first applied may expose accessible the protected mutable sites to the second mutagen, and secondly, the repair enzymes may be rendered non-functional by the second mutagen, thereby promoting the fixation of already induced pre-mutational changes (Sharma, 1970; Makeen *et al.*, 2010). In the present study, negative synergism (less than additive) was observed in combination treatments of gamma rays + EMS (unpublished data). Less than additive effect may result from two mutagens competing for the same site (Aastveit, 1966). The negative synergism between hydrazine (HZ) and hydroxylamine (HA) for chlorophyll mutation frequency in M_2 may be explained by assuming that at least some of the mutational events produced by the first of applied mutagens might be getting reversed by the second mutagen (Reddy *et al.*, 1973).

Discrepancies regarding the mutation frequency for even similar treatments in the two varieties of chickpea suggested a possible influence of genome on mutagenic potency. Inter-varietal differences with regards to the mutation frequency as observed in the present study confirm the findings of Singh *et al.* (1998) in rice, Ahmed (1999) in cowpea and Kumar *et al.* (2003) in limabean. Genetic differences even of a single gene induce significant changes in mutagen sensitivity, which influences not only the rate but also the spectrum of recoverable mutations (Bhan and Kaul, 1976; Paul and Singh, 2002).

5 Mutagenic Effectiveness and Efficiency

Mutagenic effectiveness and efficiency are two different properties, which are important in mutation breeding progarmmes. Knowledge of relative biological effectiveness and efficiency of various mutagens and their selection is essential to recover high frequency of desirable mutations (Smith, 1972; Kumar and Mani, 1997). Mutagenic effectiveness is a measure of the mutations induced per unit dose of a mutagen (time × concentration/dose), while mutagenic efficiency gives an idea of genetic damage (mutation) in relation to the total biological damage caused in M_1 generation (Konzak *et al.*, 1965; Gautam *et al.*, 1992; Khan, 1997; Khan and Wani, 2006; Kamau *et al.,* 2011 and Singh, 2011). Although, both are two different properties but the usefulness of any mutagen in plant breeding programme depends on both of them. It is not necessary that an effective mutagen shall be an efficient one also (Koli and Ramkrishna, 2002; Gaikwad and Kothekar, 2004; Khan *et al.*, 2005b). Various factors like biological, environmental and chemical ones modify the effectiveness and efficiency of different mutagens and the mutation rate (Blixt, 1970; Fujimoto and Yamagata, 1982; Ogunbodede and Brunner, 1991; Kodym and Afza, 2003). Mutagenic effectiveness and efficiency were also found to depend upon mutagen type and the genotype. There have been a number of reports revealing that the effectiveness and efficiency of mutagens vary to a greater extent in various crop plants as in clusterbean (Velu *et al.*, 2007), cowpea (Dhanavel *et al.*, 2008; Girija and Dhanavel, 2009), garden pea (Sharma *et al.*, 2010), grasspea (Nerker, 1977; Kumar and Dubey, 1998a; Waghmare and Mehra, 2001), lentil (Dixit and Dubey, 1986a; Sharma, 1990; Gaikward and Kothekar, 2004; Solanki, 2005), limabean (Kumar *et al.*, 2003), mungbean (Mehraj-ud-din *et al.*, 1999; Singh, 2007a, Goyal *et al.*, 2009; Wani *et al.*, 2011b), soybean (Kavithamani *et al.*, 2008; Pavadai *et al.*, 2009; Khan and Tyagi, 2010b), rice (Rao and Rao, 1983; Reddi and Suneetha, 1992), wheat (Chowdhury, 1978), barley (Jagtap and Das, 1976), sorghum (Sree Ramulu, 1972; Reddy and Smith, 1984), fenugreek (Koli and Ramkrishna, 2002) and brinjal (Zeerak, 1992b). It has been noticed that among the monofunctional mutagens, while methylating agents are more toxic and thus have to be used only at lower concentrations (IAEA, 1970; Fujimoto and Yamagata, 1982; Khan and Siddiqui, 1992), ethylating agents, being less toxic, can be applied at relatively higher concentrations to yield more mutations. Khan and Wani (2005) found that the order of mutagens based on effectiveness was MMS > SA > EMS whereas on the basis of their efficiency, it was EMS > MMS > SA. It was also observed that moderate concentrations of mutagens were most effective and efficient in inducing mutations. Kaul and Bhan (1977) reported that EMS is more effective and efficient mutagen than dES and gamma rays in rice. According to Mahapatra (1983), sodium azide was more effective and efficient than gamma rays, EMS and NMU. Makeen and Babu (2010) in study on the mutagenic effectiveness and efficiency of gamma rays, SA and their combination treatments in urdbean, observed that the effectiveness of gamma rays was higher than SA and combination

treatments. HZ was found to be the most effective mutagen followed by SA and EMS in mugbean (Wani *et al.*, 2011b). According to Kaul (1989), the most desirable mutagen is the one that is least damaging and highly useful mutation yielder.

5.1 Case Study 3

A comparative study of the mutagenic effectiveness and efficiency in inducing mutation in M_2 generations in relation to the effects in M_1 generation in terms of biological and cytological aberrations (Case Studies 1 and 2) were studied and discussed in this section.

5.1.1 Results

In the present case study it was found that effectiveness and efficiency were higher at the moderate doses of gamma rays and EMS, while in case of combination treatments, 100 Gy gamma rays + 0.1% EMS and 100 Gy gamma rays + 0.2% EMS treatments were most effective and efficient (unpublished data).

Mutagenic efficiency, calculated on the basis of seedling injury (Mp/I), pollen sterility (Mp/S) and meiotic abnormalities (Mp/Me) at 200 Gy of gamma rays treatment was most efficient among gamma rays treatments and in EMS treatments, 0.2% was the most efficient in both varieties of chickpea. Among combination treatments, 100 Gy gamma rays + 0.1% EMS treatment was most efficient in both the varieties of chickpea.

On the basis of seedling injury and pollen sterility, the efficiency of mutagens in descending order was: Gamma rays + EMS > gamma rays > EMS in both varieties. On the basis of meiotic abnormalities, the combined gamma rays + EMS treatments were found to be more efficient and gamma rays treatments were less efficient in both the varieties (Fig. 5.1). The efficiency calculated on the basis of Mp/Me was generally higher as compared to Mp/I and Mp/S.

5.1.2 Discussion

The usefulness of a mutagen depends both on its effectiveness and efficiency and was well illustrated by (Konzak *et al.*, 1965). Therefore, mutagenic effectiveness is an index of the response of genotype to the increasing dose of the mutagen, while mutagenic efficiency shows the proportion of mutations in relation to the undesirable biological effects, such as pollen sterility, meiotic abnormalities and seedling injury induced by the mutagen in question. The order of mutagenic effectiveness as determined on the basis of chlorophyll mutated plant progenies was EMS > gamma rays + EMS > gamma

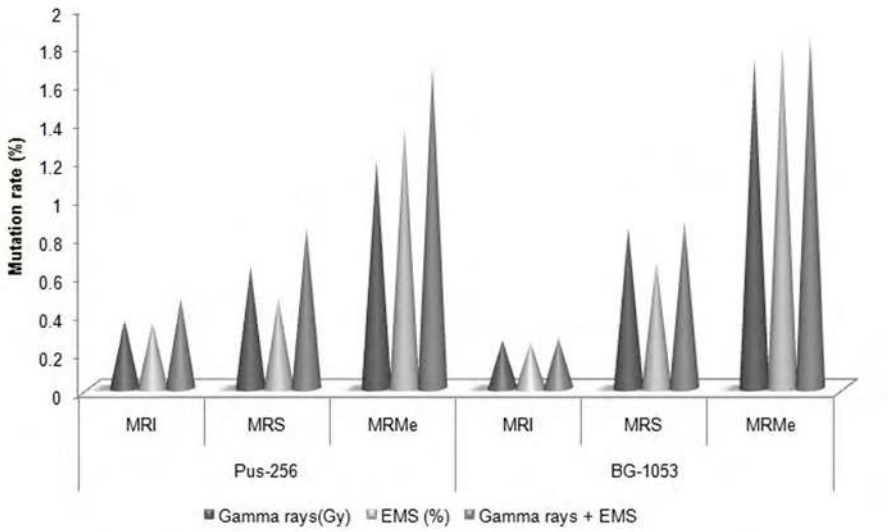

Fig. 5.1: Mutational rate of the mutagens in relation to biological damage in two varieties of chickpea. Abbreviations: **MRI** – Mutation rate based on seedling injury (seedling height reduction), **MRS** – Mutation rate based on pollen sterility, **MRMe** – Mutation rate based on meiotic abnormalities.

rays. EMS has been regarded as superior to gamma rays in inducing useful mutations in rice (Kaul and Bhan, 1977), lentil (Solanki and Sharma, 1994), mungbean (Singh, 2007a), chickpea (Shah *et al.*, 2008), urdbean (Thilagavathi and Mullainathan, 2009), cowpea (Girija and Dhanavel, 2009) and soybean (Khan and Tyagi, 2010a). Gamma rays and EMS were found to be effective at moderate doses, while the combined gamma rays + EMS treatments proved more effective at the lower doses, i.e. 100 Gy gamma rays + 0.1% EMS and 100 Gy gamma rays + 0.2% EMS. The decline in the mutagenic effectiveness recorded at higher doses shows that the increase in mutation rate was not proportional to the increase in the doses of mutagens.

Three criteria viz. seedling injury (Mp/I), pollen sterility (Mp/S) and meiotic abnormalities (Mp/Me) were taken into consideration to determine the efficiency of mutagens used. Zeerak (1992b) on the basis of his results on brinjal, rated the combined treatments of gamma rays and EMS to be less efficient than EMS and gamma rays. In the present study, the combined treatments proved to be the most efficient in comparison to individual mutagenic treatments of gamma rays and EMS.

Like effectiveness, moderate doses of gamma rays and EMS, in addition to the lower doses of gamma rays + EMS were more efficient, compared to the higher doses. Gautam *et al.* (1992) in black gram and Ganapathy *et al.* (2008) in millet have reported that mutagenic efficiency increased with the increase in the dose of the mutagens. However, Khan (1999) in blackgram reported higher mutagenic efficiency at the lower doses. The higher effectiveness and efficiency at lower and moderate doses of mutagens have also been reported in *Lathyrus sativus* (Nerkar, 1977), *Vigna mungo* (Sharma *et al.*, 2010), *Glycine max* (Khan and Tyagi, 2010a) and *Vigna radiata* (Wani *et al.*, 2011c).

6 Morphological Mutations

Alterations in the morphological pattern of any type, through mutagens, are regarded as morphological mutations. Morphological mutations affecting important plant attributes in many cases prove to be promising from breeder's point of view. Morphological mutants play a vital role in modifying the characteristics of cultivars for the construction of ideotype, and ultimately, lead to development of new variety of crops. For the development of improved varieties such mutants were found to be more productive, when used in cross breeding (Pawar *et al.*, 2000). Several morphological mutants based on different plant forms, leaf, maturity, pod and seed characters were reported with the application of single and combination treatments of physical and chemical mutagens in pulses (Blixt, 1972; Appa Rao and Reddy, 1975; Filippetti and De Pace, 1986; Jha, 1988; Vanniarajan *et al.*, 1993; Venkatachalam and Jayabalan, 1995; Ramesh and Dhananjay, 1996; Ravikesavan *et al.*, 2001; Henry, 2002; Khan *et al.*, 2005b; Solanki, 2005; Singh, 2007b; Selvam *et al.*, 2010, Khan and Tyagi, 2010a; Wani *et al.*, 2011a), cereals (Okuno and Kawai, 1978; Reddy and Gupta, 1988; Reddy, 1992; Singh *et al.*, 1998; Viswanatahan and Reddy, 1998; Kumar and Mani, 1977; Ali and Siddiq, 1999, Singh, 2006) and other plants of economic importance (Chaghtai and Hasan, 1980; Zeerak, 1990; Datta and Laxmi, 1992; Marry and Jayabalan, 1995; Kumar *et al.*, 1996; Amarnath and Prasad, 2000; Datta and Sengupta, 2002; Cagirgan, 2006). In chickpea, like other pulse crops, morphological mutations have been isolated for flower colour and structure, growth habit, size and colour of the seeds (Khosh-Khui and Niknejad, 1971; Muehlbauer and Singh, 1987; Davis *et al.*, 1990; Davis, 1991; Ahmed and Godward, 1993; Ghate, 1993; Knight, 1993; Pundir and Reddy, 1998; Gaur and Gour, 2001; Toker and Cagirgan, 2004b; Toker, 2009; Khan *et. al*, 2004 & 2011). The presence of more than one mutation in a single plant was termed as „multiple mutation" by Sharma (1969). According to him, agents with higher mutagenic efficiency induce more multiple mutations, and such mutations may accumulate several desirable mutations within one plant. Multiple mutations have been reported earlier by Kharkwal (1999) in *Cicer arietinum*, Appa Rao *et al.* (1975) in *Vigna mungo*, Odeigah *et al.* (1998) in *Vigna unguiculata*, Auti and Apparoa (2009) in *Vigna radiata*.

Tyagi and Gupta (1991) reported that each gene, which is of agronomic interest can mutate and, hence, a wide spectrum of viable mutants, morphological in nature, can be expected in mutation experiments. Based on segregation pattern of morphological mutants, Reddy and Gupta (1988) and also Thakur and Sethi (1993) observed that most of the true breeding mutants were conditioned by single recessive genes, however, Konzak *et al.* (1969) argued that the different morphological mutants which bred true in future generations like tall, dwarf, semi-dwarf, bushy, prostate and bold seeded mutant types were found to be under the influence of polygenes.

Frequency of morphological mutations has been found to increase with increases in the dose of mutagen (Thakur and Sethi, 1995). Datta and Sengupta (2002) reported that spectrum of viable mutations was wider at lower doses of mutagens. Vaniranjan *et al.* (1993) observed the higher frequency of viable mutations at medium doses of gamma rays and EMS treatments. Thus, the spectrum and frequency of morphological mutations vary with mutagen and duration of treatment (Kumar and Mani, 1997) and the genetic differences of the experimental organism, which have a key role in the recoverable frequency and spectrum of morphological mutations (Kharkwal, 1999; Sharma, 2001; Khan *et al.*, 2004b).

6.1 Case Study 4

In any mutation breeding programme, isolating and characterizing the morphological mutants in M_2 generation progenies plays an important role. The morphological mutants play an important role in selection of desired characteristics in any hybridization programmes. In the present study as revealed in past chapters in Case Studies 1-3 some of the morphological mutants with their characteristics features and some physiological studies associated are discussed.

6.1.1 Results

A wide range of morphological mutants was isolated in the population of two varieties of chickpea in M_2 generation (Figs. 6.1-6.5). These mutants involved traits affecting plant height, growth habit, leaf, flower and pod. Mutation frequency was calculated on M_2 plant basis. The highest frequency was noted in EMS treated population and the lowest with gamma rays treatments, while combination treatments of gamma rays + EMS were intermediate (Fig. 6.1). The frequency of morphological mutations was higher in the variety Pusa-256 than the variety BG-1053. The spectrum of mutations induced by EMS was comparatively wider than that of gamma rays and the combined gamma rays + EMS treatments in both varieties. Mutations affecting growth habit and leaf appeared more frequently followed by flower, plant height and pod in both the varieties (Fig. 6.1). In general, tall, broad/giant leaves, elongated rachis, double flower, open flower and bold pod mutants were predominant at the lower doses of gamma rays and EMS employed alone or in combination. In the two varieties of chickpea, the combined treatments of gamma rays and EMS did not produce additive effects on morphological mutations. A brief description of the morphological mutants identified is given below:

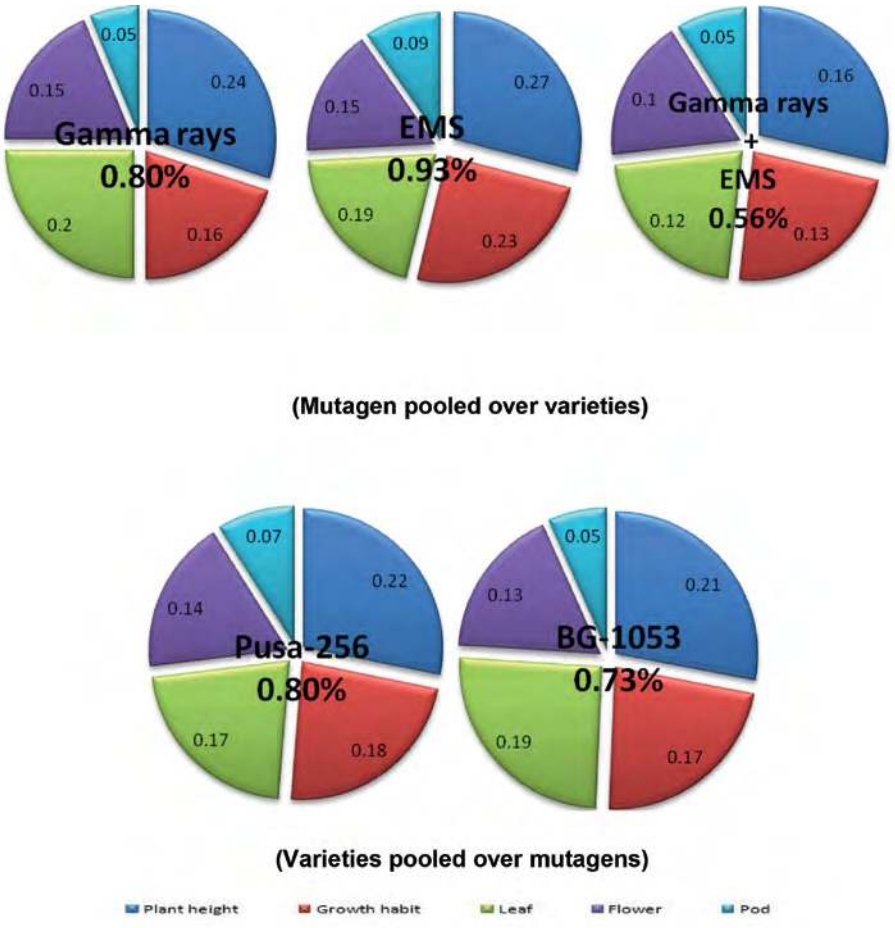

Fig. 6.1: Total (pooled) frequency and spectrum of morphological mutants induced in M_2 generation of chickpea.

I. Mutants with altered plant height

(i) Tall mutants: These mutants were observed at lower doses of single and combination treatments of gamma rays and EMS in both varieties. The mutants were considerably taller than the control plants, their height being 73-78 cm whereas it was 54-57 cm for the control plants. The pods and seed setting were low (Fig. 6.2-1).

(ii) Dwarf mutants: These mutants were isolated from the higher doses of the mutagens in both the varieties except in combination treatments of gamma rays and EMS in the Pusa-256. These mutants had short internodes and almost all yield components were reduced. Seeds were smaller in size. The plant height ranged from 20 to 25 cm (Fig. 6.2-2).

Fig. 6.2: Morphological mutants (mutants with altered plant height and growth habit): **1.** Tall mutant; **2.** Dwarf mutant; **3.** Bushy mutant; **4.** Prostrate mutant (Photographs by M I Kozgar)

II. Mutants with altered growth habit

(i) Compact/Bushy mutants: These mutants exhibited vigorous growth. Branches were short and the plant height was reduced giving it a bush like appearance. Yield per plant, more or less, was equal to the control plants. The mean height of the mutant was 35.40 cm. These mutants were isolated at higher doses of mutagens in both varieties (Fig. 6.2-3).

(ii) Prostrate mutants: These mutants had long internodes and failed to grow erect with trailing branches at the ground level. The mutants possessed small pods containing 1-2 shrivelled seeds with hard and rough seed coat. They were isolated from the combined gamma rays + EMS treatments in both varieties (Fig. 6.2-4).

(iii) Spreading mutants: These mutants were isolated at 300 Gy gamma rays in the variety Pusa-256 and 0.4% EMS in the variety BG-1053. These had long internodes and large canopy. The branches appeared to emerge erect separately at the ground. The leaves were mostly of brad type and the yield was reduced (Fig. 6.3-4).

(iv) One sided branching mutants: All branches were produced on one side of the stem. Plants were late in flowering, partially sterile with few pods only, seeds extremely shriveled, dark and non-viable. These mutants were isolated in the treatments with gamma rays in both the varieties and in combination treatments of 100 Gy gamma rays + 0.2% EMS in the variety BG-1053 (Fig. 6.3-3).

(v) Axillary branched mutants: Mutant plants were characterized by profuse axillary branches, short internodes, small pods, low seed setting and seeds were smaller in size. These mutants were noticed at higher doses of mutagens used in both varieties (Fig. 6.3-2).

III. Mutants with altered leaf morphology

(i) Broad/Giant leaves mutants: These mutants were tall and vigorous with large, thick leaflets. The size of stipules was larger than the normal stipules in control plants. Number of pods increased and they had bold seeds. These mutants were isolated at the lower and moderate doses of gamma rays + EMS employed alone or in combination in both varieties (Fig. 6.4-3).

(ii) Narrow leaves mutants: These mutants had small, narrow leaves with pointed tips. Pods were smaller in size, with a reduced number of seeds. Seed size was smaller and they were dark in colour. They appeared at higher doses of the mutagens in both varieties (Fig. 6.4-2).

(iii) Altered leaf architecture mutants: Mutant plants were characterized by vast variation in the leaf margins and apices. Variations in the plant heights were also noticed and such mutants were 5-7 cm shorter than the control plants. These mutants were isolated in almost all the mutagenic treatments (Figs. 6.4-4 and 6.4-5).

(iv) Elongated rachis: The mutants were characterized by elongated rachis with reduced number of leaves. These mutants were isolated at lower doses of mutagens in both varieties (Fig. 6.4-7).

Fig. 6.3: Morphological mutants (mutants with altered growth habit): **1.** Control; **2.** Axillary branched mutant; **3.** One sided branching mutant; **4.** Spreading mutant (Photographs by M I Kozgar)

IV. Mutants with altered flower characters

(i) Double flower mutants: These mutants were characterized by the presence of two flowers borne on the same stalk (peduncle) in contrast to one in control. The leaflets, pods and seeds were smaller in size (Fig. 6.5-3). The plants were normal in growth but late in flowering. These mutants appeared at 0.1% of EMS treatments in the variety Pusa-256.

(ii) Flower colour mutants: In the variety Pusa-256 white colour flower mutants were noticed (Fig. 6.5-2), in comparison to pink colour flower in the control, in 400 Gy gamma rays treatments. As compared to white colour flower in control, light blue

Fig. 6.4: Morphological mutants (mutants with altered leaf morphology): **1.** Leaf of control plant; **2.** Narrow leaf (small narrow leaflets with pointed tips); **3.** Giant leaf mutant (large and thick leaflets, larger size stipules); **4.** & **5.** Altered leaf architecture mutants; **6.** Rachis (Control); **7.** Elongated rachis mutant (Photographs by M I Kozgar)

Fig. 6.5: Morphological mutants (mutants with altered flower characters): **1.** Pink colour flower (Pusa-256-Control); **2.** White flower mutant of Pusa-256; **3.** A branch showing double flowers (Pusa-256); **4.** White colour flower (BG-1053-Control); **5.** Light blue flower mutant of BG-1053; **6.** Open flower mutant of BG-1053 (Androecium open); **7.** Open flower mutant of Pusa-256 (Androecium and Gynoecium open); **8.** Non-flowering/Vegetative mutant (Photographs by M I Kozgar)

colour flower mutants were found in combination treatments of 200 Gy gamma rays + 0.2% EMS in the variety BG-1053 (Fig. 6.5-5).

(iii) Open flower mutants: These mutants possessed bell-shaped flowers with open keel and wings. The androecium and gynoecium were exposed (Fig. 6.5-6 and 7). Flowers withered without fruiting. They appeared at the lower concentrations of EMS in both varieties.

(iv) Non-flowering/Vegetative mutants: These mutants did not bear flowers and continued to grow vegetatively by the time other plants were setting seeds. They had profuse branching, dark green foliage, short internodes and large leaves (Fig. 6.5-8). These mutants were noticed in lower doses EMS and combination treatments.

(v) Late flowering mutants: In these plants, the flowering started 8-10 days later and growth period was extended by a similar number of days from the expected normal 95 days. These mutants were isolated at the higher doses in both single and combination treatments.

(vi) Early maturing mutants: These mutants matured 10-15 days ahead of their respective controls, of around 145 days. Early maturing mutants were recorded at the lower doses of the mutagens in both varieties.

V. Mutants with altered pod characters

(i) Small/Narrow pods: These mutants were mostly found at the higher doses of mutagenic treatments, except gamma rays and combination treatments in the variety BG-1053. These were characterized by presence of narrow leaves. In these mutants yield per plant was lower than in the control.

(ii) Bold pod mutants: The mutant plants exhibited vigorous growth and pods were larger containing 2-3 normal seeds. As a result of bold pods, a significant increase in yield was recorded. These mutants were also evaluated up to M_3 generation. The mutants were observed at the lower doses of the single and combination treatments of gamma rays and EMS.

6.1.1.1 NRA, Chlorophyll and Carotenoid Contents of Morphological Mutants

The alterations in the NRA assay, chlorophyll and carotenoid contents due to the mutagenic treatments of gamma rays, EMS and their combinations in the leaves of morphological mutants indicate that the activity of nitrate redutase (NR) and the contents of the chlorophyll and carotenoid vary differently with the mutant type (unpublished data). Among the mutants the highest activity of NR and high carotenoid contents have been found in bold seed pod mutants in both varieties of chickpea. The high chlorophyll content was also detected in the bold pod mutants in the variety Pusa-256, whereas in the variety BG-1053 the high chlorophyll content was detected in early flowering mutants. The values of all the three parameters (NRA, chlorophyll and carotenoid contents) mostly differed significantly between the mutant types and also from the control values either in positive or negative directions.

6.1.2 Discussion

Enhancement of frequency and spectrum of mutations in a predictable manner for achieving desired plant characteristics is an important goal of induced mutagenesis programmes for the improvement of crop productivity. In the present study, the morphological mutations affecting different plant characteristics were isolated basing on the screening of M_2 population. These mutants differed from control in plant height, growth habit, leaf, pod and flowering habits. Although most of the morphological mutants were uneconomical, some mutants, nevertheless, could be used as a source of many beneficial genes in cross breeding programmes or for the improvement of some quantitative traits (Khan *et al.*, 2011) may be useful in mapping studies (Gaur and Gaur, 2003) and in determining the evolution of the crops (Toker, 2009). Such mutants might be either a result of pleiotropic effects of mutated genes or chromosomal aberrations or gene mutations (Wani *et al.*, 2011a). The morphological mutants differed not only in the two varieties of chickpea but also within the variety in different mutagenic treatments, suggesting that the varieties responded differently to the dose and type of mutagens employed. Based on morphological mutation frequency, Pusa-256 was proved to be the most mutable variety, while BG-1053 showed less mutability. The progenies of tall, dwarf, bushy, prostrate and bold seeded mutants bred true for the altered traits in M_3 generation. Several workers have reported that such mutant types were monogenic recessive (Jana, 1963; Sharma and Sharma, 1979; Reddy and Gupta, 1988; Satyanarayana *et al.*, 1989; Singh *et al.*, 1999; Talukdar, 2009). Konzak *et al.* (1969) in wheat and Shakoor *et al.* (1978) in triticale reported that semi dwarf character was controlled by polygenes. Qin *et al.* (2008) reported a dominant dwarf mutation, controlled by a single dominant gene in rice. EMS gave highest mutation frequency in both varieties. A high frequency and broad spectrum of morphological mutants induced by chemical mutagens has been reported in *Vigna mungo* (Arulbalachandran *et al.*, 2009; Goyal and Khan, 2010b), *Lens culinaris* (Tyagi and Gupta, 1991; Tripathy and Dubey, 1992; Solanki and Sharma, 1999), *Cicer arietinum* (Khan *et al.*, 2004a) and *Glycine max* (Khan and Tyagi, 2010b). Relative differences in the mutability of genes for different traits have been observed, as some of the mutant types appeared with higher frequencies in some mutagen treatments. The more frequent induction of certain mutation types by a particular mutagen may be attributed to the fact that the genes for these traits are probably more responsive to different mutagens with different modes of action. Nilan (1967) reported that different mutagens and treatment procedures might also change the relative proportion of different mutation types. Like chlorophyll mutations, combination treatments of gamma rays and EMS produced a negative synergistic effect on morphological mutations frequencies. Sharma (1970) in barley observed a synergistic effect for viable mutations at low doses and additive effect at higher doses of gamma rays + EMS.

Tall mutants, as observed in the present study, were also reported earlier by Solanki *et al.* (2004) in lentil and Kumar *et al.* (2009b) and Jana (1963) in blackgram using

different mutagens. In comparison to the control, plant height was reduced to different magnitude in dwarf mutants, as also seen earlier in barley (Sethi, 1974), grasspea (Talukdar and Biswas, 2006) and *Vigna* spp. (Wani *et al.,* 2011a). Dwarfness may result from reduced internodes length and/or internodes number (Sjodin, 1971). In the present study, reduction in internode length was mainly responsible for dwarfness. The mutant plants, which exhibited prostrate growth habit, had long internodes and weak stem. Prostrate mutants have been reported in *Phaseolus vulgaris* (Marghitu, 1972) and *Vigna radiata* (Wani *et al.,* 2011a). Bold seeded mutant is a useful variation and can be exploited in increasing the number of seeds per pod and seed size leading to increased genetic potential of the yield. Cytology of bold seeded mutants revealed normal meiotic divisions and 8 bivalents at metaphase-I. The present bold seeded mutants may be the result of gene mutations. Singh (1996) characterized bold seeded mutants in *Vigna mungo* as gene mutations as there were no visible chromosomal changes associated with them. A number of early maturing mutants were isolated at various mutagen treatments in the two varieties of chickpea. In these mutants, earliness was combined with normal seed yield. However, early mutants with altered agronomic characteristics like yield and growth habit were isolated in *Vigna mungo* (Thakur and Sethi, 1993; Kumar *et al.,* 2009b). Earliness is one of those characters of a crop that can be obtained reliably in mutation experiments (Gottschalk and Wolff, 1983a). Many such morphological mutants have been studied in different crop plants and attributed to the chromosomal breakage, disturbed auxin synthesis, disruption of mineral metabolism and accumulation of free amino acids (Gunkel and Sparrow, 1961; Blixt, 1972; Sharma and Sharma, 1979; Toker and Cagirgan, 2004a; Goyal and Khan, 2010b). Flower colour mutants can be exploited as genetic markers in different breeding experiments (Data and Senegupta, 2002; Atta *et al.*, 2003).

Morphological mutants were assayed for nitrate reductase activity (NRA), total chlorophyll and carotenoid contents, which differed from the control. The role of NRA and chlorophyll contents in promoting the growth and enriching the nutritional quality of the crops through induced mutagenesis have been reported in chickpea (Barshile *et al.*, 2009; Barshile and Apparoa, 2009), makhana (Verma *et al.*, 2010) and *Vigna* spp. (Kozgar *et al.,* 2011). In the present study, the isolated dwarf, bushy, early maturing and bold seeded mutants exhibited the higher values for total carotenoid contents with respect to the controls, depicting their role in stress physiology. A dwarf mutant of wheat, named s-dwarf, tolerant to varying level of stresses, was reported by Zhang *et al.* (2005). In higher plants, carotenoids protect the photosynthetic apparatus from excess of photons and oxidative stress, which are generated under stress (Siefermann-Harms. 1987; Panda and Biswal, 1989; Srichandran *et al.*, 1989; Demmig-Adams. 1990; Youmg, 1991).

Though, it is not easy to eliminate the negative traits of this pleiotropic spectrum from the positive ones, the pleiotropic pattern of mutant gene can be altered to some extent by transferring it into a specific genotypic background (Sidorova, 1981).

7 Induced Mutagenesis and Quantitative Traits

For the last four decades the practical value of induced mutagenesis, in creating successful genetic variability for several desired traits in plant improvement programmes, has been well established and has been demonstrated by many workers in different crop categories viz. pulses like pigeonpea (Rao, 1984; Srivastava and Singh, 1993), cowpea (Murugan and Subramanian, 1993; Gunasekaran *et al.*, 1998; Pandey, 2002), urdbean (Singh and Singh, 2001; Selvam *et al.,* 2010), lentil (Kumar and Lal, 2001; Solanki and Sharma, 2001, 2002; Khan *et al.*, 2006b), faba bean (Filippetti and De Pace, 1986; Verma and Rao, 1994; Joshi and Verma, 2004), mungbean (Khan, 1984; Singh and Yadav, 1991; Mathew *et al.,* 2005; Khan and Goyal, 2009), chickpea (Harer *et al.*, 1999; Kharkwal, 2001; Khan and Wani, 2005b, Kozgar and Khan, 2009), cereals like wheat (Scossiroli, 1964; Konzak, 1973; Siddiqui, 1983; Boreiko *et al.*, 1986; Khan, 1988; Nalini *et al.*, 1993; Kalia *et al.*, 2000; Jamil and Khan, 2002; Sakin and Yildirim, 2004), rice (Swaminathan *et al.*, 1969; Misra *et al.* 1973; Awan *et al.*, 1980; Shanthi and Singh, 2001; Ishiy *et al.*, 2006), barley (Gustafsson, 1963; Gaul, 1964; Bhargava and Khalatkar, 1986; Nalini *et al.*, 1993), triticale (Reddy, 1988, 1989; Viswanathan *et al.*, 1994), other ornamental and medicinal plants (Amarnath and Prasad, 2000; Cagirgan, 2006; Datta and Laxmi, 1992; Datta and Sengupta, 2002; Kumar *et al.*, 1996; Marry and Jayabalan, 1995). In all the cases, most of the plant attributes of interest to plant breeders were quantitative traits which are governed by the principles of quantitative genetics.

A common practice in mutation breeding programme is to advance only normal looking M_2 plants to M_3 generation, and apply the first dose of selection in M_2 generation (Brock, 1965; 1967). This methodology has been advocated by Gupta and Swaminathan (1967), Tickoo and Jain (1979), Sharma (1986) and Wani and Khan (2006), however, Jana and Roy (1973) selected M_2 families on the basis of significantly changed mean only. Since most of the desired combinations of favourable alleles are likely to be lost in advanced generations due to intensive or even no selection for other traits thus the selection for quantitative traits, such as yield and yield attributing characters, should preferably be carried out in early generation (Sneep, 1977; Saini and Gautam, 1990 and Sharma, 1997). The efficiency of early generation (M_2) selection in mutation breeding experiments has been reported in the crops like lentil (Solanki and Sharma, 2002), pea (Singh, 1988), mungbean (Tickoo and Chandra, 1999), sesame (Sheeba *et al.*, 2003), soybean (Pavadai *et al.*, 2010; Nakagawa, 2009 and Nakagawa *et al.*, 2011). Since the quantitative traits selected in early generations indicate the degree of stability to the environmental fluctuations, henceforth the potential transmissibility of these traits from parent to offsprings, and from generation to generation, are to be evaluated. For this, the estimates of heritability of various quantitative traits are essential (Mather and Jinks, 1971; Kaul and Garg, 1979, Scossiroli *et al.*, 1966; Ignacimuthu and Babu, 1993; Brunner, 1995; Mohanty, 2001; Chaudhary *et al.*, 2004; Khan *et al.*, 2006; Arulbalachandran *et al.*, 2010). Trivedi *et al.* (2006) clearly brought

out that, in the treated population, the estimates of heritability were larger and varied from trait to trait. Johnson *et al.* (1955) suggested that heritability in combination from genetic advance was more helpful in predicting the effect of selection. This has been advocated by many workers like Kaul and Garg (1979), Sakin, (2002) and Khan and Goyal (2009). Higher values of heritability and genetic advance over the control population for certain quantitative traits were reported by Kaul and Kumar (1983) and Rather *et al.* (1998) in rice, Sharma and Sharma (1984) in lentil, Mehetre *et al.* (1998) in soybean, Sakin and Yildirim (2004) in wheat, Khan and Wani (2006) in green gram and Mohammadi and Pourdad (2009) in safflower.

Both physical and chemical mutagens, alone or in combinations, were used in pulses for generating variability in quantitative traits like plant height, pods per plant, pod length, flowering and maturity period, seed weight, biological yield, number of fertile branches, number of seeds per pod, yield and harvest index (Kundu and Singh, 1981, Kundu and Singh, 1982; Reddy *et al.*, 1992; Srivastava and Singh, 1993; Kumar *et al.*, 1995; Waghmare and Mehra, 2000; Rehman *et al.*, 2001; Yaqoob and Rashid, 2001, Wani and Khan 2004, Wani *et al.*, 2011c). The important primary yield components in pulses are considered pods bearing branches, pods per plant, seeds per pod and seed weight and several studies (Bahl, 1988; Kumar and Arora, 1991; Rao, 1996; Khan and Siddiqui, 1997; Guler *et al.*, 2001; Kharkwal, 2003; Singh and Singh, 2003; Raut *et al.*, 2004; Khan and Wani, 2005b; Mubeen *et al.*, 2007; Makeen *et al.*, 2009; Giri *et al.*, 2010) have shown a close association between these components to the total plant yield in induced mutant lines. In chickpea, different workers have reported increased variability for various agronomic characters in mutagen treated population as observed by significant changes in the mean values and coefficient of variability as compared to control (Mandal, 1974; Nerker and Mote, 1978; Haq and Shakoor, 1980; Kharkwal, 1981; Kumar *et al.*, 1981; Kozgar and Khan, 2009; Barshille *et al.*, 2009; Kozgar *et al.* 2012).

7.1 Case Study 5

In M_2 generation, the observations were made on 25-30 normal looking plants of each progeny, for each treatment along with the control (see Appendix materials and methods). The progenies segregating for macromutations were not used for such analysis. The following eight quantitative traits were thoroughly studied in different generations:

(i) Days to flowering; (ii) Plant height; (iii) Days to maturity; (iv) Number of pods bearing branches; (v) Number of pods; (vi) Seeds per pod; (vii) 100-seed weight; (viii) Total Seed Plant Seed Yield.

For raising M_3 generation, two treatments of gamma rays and EMS alone and their combinations for each variety were used which gave the maximum total plant yield in M_2 generation.

7.1.1 Results

The availability of ample genetic variability particularly for quantitatively inherited traits is a pre-requisite for attempting selection in plant breeding to develop ideotypes in self pollinating crops like chickpea.

The genetic variability induced for eight quantitative traits by gamma rays, EMS and their combination treatments was studied in the two varieties of chickpea viz. Pusa-256 and BG-1053 in M_2 and M_3 generations. Since, the genetic parameters such as genotypic coefficient of variation (GCV), heritability in broad sense (h^2) and genetic advance as percentage of mean (GA) provide guideline for the improvement of quantitative traits, their estimates were determined for each trait in the mutagenized population. Results obtained for each trait are elaborated below and examplenary alterations in genetic advances are given in Table 7.1 with respect to mutagen type:

(i) Days to flowering

The procured results on mean values, shift in mean values, genotypic coefficient of variation, heritability and genetic advance for days to flowering in M_2 generation showed that the mean values shifted negatively in the mutagenized population. The mean days to flowering decreased significantly by three days with the treatments of 0.4% EMS in the Pusa-256. Though the doses were increasing in a linear order, yet the induced genotypic coefficient of variation did not show a consistent increase. The highest genotypic variability was recorded with 0.4% EMS treatments in the variety Pusa-256 while the highest genotypic variability was recorded with 100 Gy of gamma rays and 200 Gy gamma rays + 0.1% EMS combination treatments in the variety BG-1053 (unpublished data).

Heritability and genetic advance showed a considerable increase over the control in both varieties. In most of the mutagenic treatments, higher estimates of heritability are associated with maximum genetic advance. The highest heritability estimates were recorded in the variety Pusa-256 with 0.3% EMS, whereas in the variety BG-1053, it was the highest in combination treatment of 200 Gy gamma rays + 0.1% EMS.

The maximum genetic advance in the treated population of the variety Pusa-256 was with 0.4% EMS, while it was recorded to be the highest (12.34%) 200 Gy gamma rays + 0.1% EMS combination treatment in the variety BG-1053.

In M_3 generation the mean flowering time was reduced for all the treatments in both varieties. The mean values of the treated population differed significantly from the controls. However, 200 Gy gamma rays, 100 Gy gamma rays + 0.1% EMS and EMS treatments had no significant effect on the mean flowering time in the variety BG-1053.

The estimated values of genotypic coefficients of variation, heritability and genetic advance were slightly decreased as compared to M_2 generation but were still higher than the controls of both varieties.

Table 7.1: Tabulated format of Genetic Advance (as % of $\overline{X}$) alterations due to mutagen induced mutagenesis in two varieties of case studied chickpea in M_3 generation.

Feature	Pusa-256			BG-1053		
	gamma ray	EMS	gamma ray + EMS	gamma ray	EMS	gamma ray + EMS
Days to flowering	↑ (5.5%)	↑ (7.0%)	↑ (7.5%)	↑ (4.0%)	↑ (7.0%)	↑ (5.0%)
Plant height	↑ (8.0%)	↑ (6.0%)	↑ (5.5%)	↑ (15.0%)	↑ (6.5.0%)	↑ (6.0%)
Days to maturity	↑ (3.0%)	↑ (4.0%)	↑ (4.0%)	↑ (4.5%)	↑ (5.5%)	↑ (6.5%)
Number of Pods Bearing Branches	↑ (45.0%)	↑ (45.0%)	↑ (47.5%)	↑ (44.0%)	↑ (55.0%)	↑ (44.5%)
Number of pods per plant	↑ (41.0%)	↑ (50.0%)	↑ (40.5%)	↑ (39.5%)	↑ (41.0%)	↑ (40.5%)
Number of Seeds per Pod	↑ (23.0%)	↑ (28.0%)	↑ (33.0%)	↑ (23.0%)	↑ (28.0%)	↑ (24.5%)
100 Seed Weight	↑ (38.0%)	↑ (39.0%)	↑ (34.5%)	↑ (41.0%)	↑ (28.0%)	↑ (20.5%)
Total plant seed yield	↑ (29.0%)	↑ (27.0%)	↑ (36.0%)	↑ (28.0%)	↑ (35.5.0%)	↑ (33.0%)

(ii) Plant height (cm)

Results recorded on plant height indicate that the mean values shifted significantly in the negative direction in most of the treatments with gamma rays and EMS alone or in combination in M_2 generation. Plant height showed a greater reduction in the combined treatments compared to individual mutagenic treatments of gamma rays and EMS. The genotypic coefficient of variation was higher in all the treatments. The highest genotypic coefficient of variation was at 400 Gy of gamma rays and at 0.3% EMS as observed in the varieties Pusa-256 and BG-1053, respectively.

Heritability and genetic advance increased in both single and combined treatments in both varieties. The highest heritability for both varieties was recorded with the treatment of 400 Gy gamma rays. The highest values of genetic advance were recorded with 400 Gy gamma rays in the variety Pusa-256 and with the treatment of 0.2% EMS in the variety BG-1053.

The mean values for plant height in M_3 were significantly reduced in all the treatments of gamma rays and EMS employed alone or in combination in both varieties. The reduction in mean plant height was the highest in combination treatments of gamma rays + EMS.

The genotypic coefficient of variation, heritability and genetic advance increased in almost all treatments. The highest genotypic coefficient of variation was recorded in the gamma rays treated population. The highest heritability estimate was obtained with 300 Gy of gamma rays treatment in the variety BG-1053. The genetic advance was similarly affected. Genetic parameters were recorded lower in M_3 than in M_2 generation.

(iii) Days to maturity

The results obtained on the effect of single and combination treatments of gamma rays and EMS on days to maturity in M_2 generations revealed that the mean for days to maturity was shifted towards the negative side in all the mutagen treatments in both the varieties. The highest reduction in mean was observed in the variety Pusa-256 with 200 Gy gamma rays + 0.1% EMS treatment followed by 200 Gy gamma rays + 0.2% EMS treatment in the variety BG-1053. Reduction in the mean was higher in combination treatments as compared to individual mutagenic treatments of gamma rays and EMS.

The genotypic coefficient of variation increased considerably in most of the treatments. The estimated GCV was the highest with 200 Gy gamma rays + 0.1% EMS in the variety Pusa-256 whereas, in the variety BG-1053, the highest GCV was obtained with 0.2% EMS treatment.

The estimated heritability shows considerable variation for days to maturity and was the highest in under study varieties of chickpea at 200 Gy gamma rays + 0.1% EMS combined treatment. The highest genetic advance was recorded with 200 Gy gamma rays + 0.1% EMS treatment in the variety Pusa-256 and with 0.2% EMS in the variety BG-1053.

The days to maturity in M_3 generation in most of the single and combination treatments of gamma rays and EMS was significantly reduced. Early maturity by 4 days was recorded with 0.3% EMS and 100 Gy gamma rays + 0.2% EMS treatments in the variety Pusa-256.

The genotypic coefficient of variation, heritability and genetic advance were recorded higher in the treated population. The genetic parameters did not show much variation in any of the treatments used when compared to control. The values of genetic parameters were lower in M_3 than M_2 generation.

(iv) Number of pods bearing branches

The results recorded in M_2 generation on number of pods bearing branches per plant showed that the mean shifts in the positive direction in all mutagen treatments, except for the doses of gamma rays, EMS and their combination, in the variety BG-1053. In general, there was an increasing trend of mean values of number of pods bearing branches in almost all mutagens in both varieties.

The genotypic coefficient of variation increased with all the treatments in both varieties. The maximum GCV in both varieties of chickpea under consideration was recorded with 0.2% of EMS treatment.

High heritability coupled with high genetic advance was observed for number of pods bearing branches in both varieties. The heritability and genetic advance were maximum with 300 Gy gamma rays treatment in the variety Pusa-256. On the other hand, the 0.2% of EMS treatment gave the maximum heritability and genetic advance in the variety BG-1053.

The data on the number of the pods bearing branches per plant in M_3 generation showed significant increase in mean values of the pods bearing branches in all the mutagen treatments in both varieties.

The genotypic coefficient of variation increased in the mutagenized population. The most effective treatment with regard to heritability and genetic advance was 300 Gy gamma rays in the variety Pusa-256. As to the variety BG-1053 the most effective treatment was 0.2% EMS, which gave the maximum heritability and genetic advance.

(v) Number of pods per plant

The data recorded on pods per plant in M_2 generation revealed the mean value shifted in both positive as well as in negative direction. Decrease in mean values, compared to the control, was mostly found at the higher doses of the mutagens used alone or in combination. The lower and moderate doses of mutagens significantly increased the mean number of pods per plant. The mean values increased more in the variety Pusa-256 than in the variety BG-1053 (unpublished data).

The genotypic coefficient of variation was recorded to be higher with all the treatments of mutagens. The highest GCV was recorded with 100 Gy gamma rays + 0.1% EMS for variety Pusa-256 and 100 Gy gamma rays treatments for variety BG-1053.

The heritability and genetic advance showed considerable variation for the number of pods per plant in both varieties. The estimated heritability and genetic advance were the highest at 100 Gy gamma rays + 0.1% EMS in the variety Pusa-256. The variety BG-1053 showed the maximum heritability and genetic advance with 0.3% EMS treatment.

The mean values for the number of pods per plant increased over the controls in both varieties for all mutagenic treatments selected in M_3 generation. The mean values for the treated population differed significantly from the control mean.

The genetic parameters were elevated in all the treatments of gamma rays and EMS employed alone or in combination. The highest genotypic coefficient of variation, heritability and genetic advance was observed in the combination treatment of 100 Gy gamma rays + 0.1% EMS in the variety Pusa-256. The values of such genetic parameters were the highest with 0.2% EMS in the variety BG-1053. High heritability coupled with high genetic advance was recorded in most of the single and combination treatments of gamma rays and EMS.

(vi) Number of seeds per pod

The seeds per pod in the two varieties showed that most of the single and combination treatments of gamma rays and EMS were not capable of inducing significant differences in the mean number of seeds per pod. The mean number of seeds per pod for the varieties Pusa-256 and BG-1053 were the highest with 200 Gy gamma rays treatment.

The estimated genotypic coefficient of variation for the variety Pusa-256 was the highest in combination treatment of 100 Gy gamma rays + 0.1% EMS. GCV for the variety BG-1053 was the highest at 200 Gy gamma rays. It is interesting to note that in most of the

treatments the heritability percentage was lower than in the control in both varieties. However, the genetic advance increased for most of the treatments of gamma rays and EMS alone or in combination. The genetic advance estimates in the variety Pusa-256 were the highest with 100 Gy gamma rays + 0.1% EMS treatment whereas in the variety B-1053, the highest value was found in 200 Gy gamma rays treatments.

The mean values of the treated population did not differ significantly from the control in most of the mutagenic treatments of the M_3 generation raised plants.

As regards the genetic parameters, the variability was higher in the combined treatments of gamma rays + EMS followed by EMS and gamma rays treatments. However, estimated heritability was lower in comparison to control in number of treatments. The genetic advances increased in both single and combined treatments except 300 Gy gamma rays in the variety Pusa-256.

(vi) 100 seed weight (g)

The data recorded for 100 seed weight (g) after various mutagenic treatments depicts that the shift in mean values goes bi-directional, being more in the positive direction. Mean values for 100 seed weight increased significantly at almost all the lower doses of mutagens. However, the most effective treatments for increase in mean values of 100 seed weight were recorded for 200 Gy and 300 Gy gamma rays, 0.1% and 0.2% EMS and the combined treatments of 100 Gy gamma rays + 0.1% EMS and 100 Gy gamma rays + 0.2% EMS. There was a significant reduction in 100 seed weight at the higher doses of gamma rays and EMS employed alone, or in combination, with a few exceptions.

The genotypic coefficients of variation were higher in the treated population as compared to controls. The highest GCV was recorded in combination treatment of 100 Gy gamma rays + 0.2% EMS in the variety Pusa-256 while the highest estimated GCV was obtained at 0.3% EMS in the variety BG-1053.

The estimated heritability and genetic advance were the highest in combination treatments of 100 Gy gamma rays + 0.2% EMS in the variety Pusa-256 while the variety BG-1053 gave the highest estimated heritability and genetic advances with 0.3% EMS.

Results recorded for 100 seed weight showed that the mean values increased significantly with each treatment, except in the combined treatments of gamma rays + EMS in the variety Pusa-256, of both varieties in M_3 generations.

The genetic parameters increased in the treated population as compared to the controls. The values of genotypic coefficient of variation, heritability and the genetic advance were the highest at 0.2% EMS treatment in the variety Pusa-256, whereas in the variety BG-1053, the highest values of genotypic coefficient of variation, heritability and the genetic advance were recorded with 200 Gy gamma rays treatment.

(viii) Total plant seed yield (g)

The results recorded on seed yield per plant presented here revealed that the mean value shifted in both positive as well as in negative directions in the treated population.

In general, the lower and moderate doses of gamma rays and EMS and the lower doses of gamma rays + EMS combined treatments showed a significant increase in mean yield per plant over the controls in both varieties. The mean values increased more in the variety Pusa-256 than in the variety BG-1053.

The genotypic coefficient of variation, heritability and genetic advance increased over the controls with all the treatments in both varieties. In the variety Pusa-256, the highest GCV, heritability and genetic advance were recorded at 0.3% EMS, whereas GCV, heritability and genetic advance were the highest in combination treatments of 100 Gy gamma rays + 0.1% EMS in the variety BG-1053.

The high heritability coupled with high genetic advance was recorded for number of the treatments with gamma rays, and EMS alone, or in combination, indicating that significant gains could be expected from selection.

Mutagen treatments, selected for M_3 generation, exhibited an increase in the mean values for total plant seed yield in both selected varieties of chickpea in M_3 generations. The mean values in the treated population significantly differed from these in the control.

The genetic parameters for total plant yield increased in the treated population as compared to control. The highest genotypic coefficient of variation and genetic advance were observed with 300 Gy gamma rays treatment in the variety Pusa-256, whereas in the variety BG-1053, 100 Gy gamma rays + 0.1% EMS treatment gave the highest values of genetic parameters.

7.1.2 Discussion

It is now the known fact that the availability of the large genetic variability within the species is prerequisite for the improvement of the cultivated plants and the mutagenesis has proved to be a handy tool to enhance the mutation rate and thereby enlarging the genetic variability and increasing the scope for obtaining the desired selections. Particularly, induction of micromutations in the polygenic system, controlling the quantitative traits is important for crop improvement. From the work already reported by several authors in various crops (Gregory, 1965; Swaminathan, 1969; Borojevic and Borojevic, 1972; Ignacimuthu and Babu, 1993; Khan *et al.*, 1998; Joshi and Verma, 2004; Shin *et al.*, 2011) it is now quite clear that micromutations result in the release of considerable genetic variability in the mutagen treated population. In some studies on the use of mutations for quantitative traits improvement, it was found that different traits differ in their response to the mutagenic treatments. Variance level may be less responsive in one trait and highly responsive in other (Kaul and Kumar, 1983; Sharma, 1995; Khan and Wani, 2006a). Moreover, the direction of polygenic mutations depends on the genotypic background of the material under study (Loesch, 1964). Thus, the genetic improvement of such traits in turn depends upon the magnitude of genetic parameters and the breeding methodology adopted.

Estimates of genetic parameters like genotypic coefficient of variation, heritability and genetic advance are therefore, needed to formulate suitable breeding procedures and to foresee the possibility how particular trait could be improved.

In the present study, data on eight quantitative traits were analyzed to assess the extent of induced variability in M_2 and M_3 generations of the two varieties of chickpea and the control. The extent of variability induced by gamma rays and EMS alone or in combination differed from trait to trait. Study of the direction of shift in mean values of quantitative traits ascertains whether mutation breeding can be restored for the improvement of a trait under study. Although means shifted on either side of the control mean, most of them went towards the positive side for yield and yield contributing traits. Opinions differ regarding the direction of the mutations. Most quantitative traits have a complex genetic determination involving large number of genes interacting with one another, consequently, variation in both the directions is expected. From the results of the present study, it could be concluded that the range of quantitative characters through induced mutations are random, bi-directional and the direction of the mutation depends upon the genotype/character under study and the dose applied. This postulation was also reported by Rao *et al.* (1988), Waxman and Peck (2003) and Siddiqui *et al*. (2009). Enlargement in range of variability for yield and its attributes such as pods bearing branches, pods per plant and 100 seed weight for the two varieties of chickpea in M_2 and M_3 generations is indicative of the wider scope for selection.

The mean flowering time decreased significantly (approximately by 15 days) with an increase in variability in some mutagenic treatments. The reduction in flowering time accompanied by increase in variability (approximately by 35%) indicated that variability had been induced in desired direction and would offer the possibility for selecting early flowering mutants in such treatments. Kaul (1980b) suggested that the mutation of two dominant genes to their recessive forms makes for an early flowering in peas. The adverse effect of mutagen treatments on plant height was observed in both varieties. The reduction in plant height was greater in combination treatments as compared to individual mutagenic treatments of gamma rays and EMS in both varieties. The reduction in plant height was reported by Rajput (1974), Yaqoob and Rashid (2001), Khan *et al.* (2005d) and Wani *et al.* (2011a) in different crops after mutagenic treatments. However, Singh *et al.* (2000b) and Arulbalachandran and Mullainathan (2009b) reported an increase in plant height after treatments with gamma rays and EMS in *Vigna mungo*. Decrease in plant height may result from the inhibition of mitotic divisions (Subba Rao, 1988).

Use of mutations for obtaining early maturing varieties has been a frequent breeding objective (Micke, 1979). The data obtained on days to maturity resulted in a gain in reducing the maturity period by 4 days with 0.3% EMS and 100Gy gamma rays + 0.2% EMS treatments in M_3 generation. Being cultivated as a winter season crop in Northern India, chickpea faces chilling and freezing temperatures during vegetative and reproductive growth. Cold stress during reproductive growth of chickpea is

detrimental to flowering and pod set. Early maturity would be ideal for chickpea to avoid severe cold and attain maximum production. Yaqoob and Rashid (2001) in mungbean, Wang *et al.* (2003b) in soybean, Shamsuzzaman *et al.* (2005) in chickpea, Singh *et al.* (2006) in lentil and Arulbalachandran *et al.* (2009) in urdbean reported a significant reduction in days to maturity after mutagenic treatments.

The mean number of pods bearing branches and pods per plant increased simultaneously in M_2 and M_3 generations, suggesting close correlation between these two traits. The author is of the view that the increase in the number of pods per plant in the present study is obviously due to an increase in the number of flowers. The number of pod sets was higher in the lines, which produced large number of flowers. All these three traits namely, number of pods bearing branches, number of pods per plant and number of flowers seem to be highly correlated. It has been suggested (Rajput, 1974; Sangwan and Singh, 1977; Khan, 1982; Khan and Goyal, 2009; Hiremath *et al.*, 2010; Giri *et al.*, 2010; Wani *et. al,* 2011b) that an increase in the yield of pulses could be achieved by enhancing pod number. The mean number of seeds per pod seems to be a stable character as most of the treatments with gamma rays and EMS alone, or in combination, did not make much difference in the mean values in both varieties of chickpea. This is probably due to the fact that only 1-2 seeds can be accommodated in the pod of chickpea. Kumar and Sinha (1989) and Giri *et al.* (2010) in pigeon pea, Khan *et al.* (2005d) in chickpea recorded a non-significant difference for the number of seeds per pod after mutagenic treatments.

The character of 100 seed weight is a reliable source of measuring yielding ability in pulses. In the present study, 100 seed weight has shown a very significant increase (approximately by 2 g/100 seed weight) from the control with most of the treatments of gamma rays and EMS used either singly or in combination in both the varieties of chickpea. This character has been reported to be governed by a relatively smaller number of genes, unlike other polygenic traits (Ghose *et al.*, 1960; Khan, 1990). On the contrary, Jana and Roy (1971), Tickoo and Chandra (1999), Waghmare and Mehra (2000) and Giri *et al.* (2010) reported the reduction in the mean 100 seed weight.

Seed yield in pulses is a complex trait and is influenced by many other quantitative traits such as pods bearing branches, pods per plant, seeds per pod and 100 seed weight. Seed yield increased by 2-4 g in the variety Pusa-256 (normal yield was 23 g) and 3-5 g in the variety BG-1053 (normal yield was 20 g) after various mutagen treatments. The mean plant yield in M_2 generation, except for higher doses of gamma rays and EMS, employed alone or in combination, and in M_3 generation showed complete positive trend in the mean values of seed yield per plant with almost all the selected mutagen treatments in both the varieties.

In the present study, increase in mean seed yield in M_3 over M_2 generation and the controls could be attributed to effective selection adopted for various yield-contributing traits in M_2 generation. Ramulu (1974) concluded that the mean of the treated population, where no selection had been applied with regard to the specific character under study, tended to go down in comparison to the control. In the present

investigation, increase in mean seed yield per plant may result from the selection of normal looking plants in M_2 which led to elimination of aberrant plants and also due to changes induced at genetic level. Kumar (1972), Jana and Roy (1973) and Tar'an *et al.* (2004) suggested that the selection process should be delayed until M_3 or later generations following mutagenic treatment. However, the selection of progenies on the basis of desirable mean and greater variance in M_2 was found to be highly useful in the present study. Many other workers have also proposed that effective selection for polygenic traits can be done in early generations even in M_2 itself (Sneep, 1977; Kaul and Matta, 1985; Sarker and Sharma, 1988; Tickoo and Chandra, 1999; Singh *et al.*, 2001; Solanki and Sharma, 2002; Sheeba *et al.*, 2003; Arulbalachandran and Mullainathan, 2009b; Giri *et al.*, 2010).

The magnitude of the phenotypic variation, however, does not reveal the relative amounts of heritable (genetic), and non-heritable (non-genetic) components of variation. This was ascertained with the help of some genetic parameters like genotypic coefficient of variation (GCV), heritability in broad sense (h^2) and genetic advance (GA) as percent of mean. Since the genotypic coefficients of variation and heritability of various quantitative traits indicate the degree of stability to the environmental fluctuations and the potential transmissibility of a trait from parent to offspring and from generation to generation hence their estimates are essential (Kaul and Garg, 1979; Sakin and Yildirim, 2004; Kozgar and Khan, 2009). It is clearly evident from the data that considerable amount of genotypic coefficient of variation was induced by different treatments of gamma rays and EMS alone or in combination. The genotypic coefficient of variation was recorded to be high for yield and yield components, except for seeds per pod, while it was comparatively low for other traits like days to flowering, plant height and days to maturity. Such differential behaviour of different traits was reported earlier by Patel and Shah (1982), Kumar and Dubey (2001), Arulbalachandran and Mullainathan (2009b) and Arulbalachandran *et al.* (2010). The genotypic coefficient of variation for days to flowering, plant height and days to maturity was higher in M_2 than M_3 generation. Shakoor and Haq (1980) reported negligible increase in variability, due to generation advance for some quantitative traits, as a result of stabilization of trait in early generation.

The genetic coefficient of variation measures the range of genetic variability shown by the plant character and helps to compare the genetic variability present in various characters. However, with the help of genetic coefficient of variation alone it is not possible to determine the amount of variation that is heritable. Heritability is of interest to plant breeder as an index of transmissibility and a measure of the value of selection for particular trait. The value of heritability depends on the magnitude of all the components of variance and change in any one of the variance values may affect it. The traits such as the number of pods bearing branches, number of pods, 100-seed weight and total plant yield were found to have a high heritability (21% as compared to normal value 2%). However, these estimates were from low to moderate for days to flowering, plant height, days

to maturity and seeds per pod. In the present study, heritability estimate for yield was high. However, low heritability for yield was reported by various workers in rice (Kaul and Kumar, 1983) and in mungbean (Khan *et al.*, 2004b). The disparity in results could be because heritability is an attribute not only of a trait but also of the population, environment and the circumstances to which the genotypes are subjected. The heritability estimates for number of pods bearing branches, number of pods, 100 seed weight and seed yield per plant in M_3 generation were higher than in M_2 generation of the treated population of the two varieties of chickpea. The heritability estimate for seeds per pod was low than the control in both the varieties. The decrease in heritability for seeds per pod indicates that, although genetic variance has increased with the mutagenic treatment, the ratio of its increase was not at par with the total phenotypic variance, which also increased. A rational approach towards the improvement of any crop plant involves selection. Genetic advance is an indicator of the expected genetic progress for a particular trait under suitable selection procedure and consequently carries much significance in self-pollinated crops like chickpea. Estimates of heritability along with genetic advance are usually more helpful than the heritability alone in predicting the resultant effects of selection. This is because the heritability estimates are subjected to interactions between the genotype and environment (Lin *et al.*, 1979; Sakin, 2002; Toker, 2004). The estimated values of genetic advance differed in different mutagenic treatments and also from one trait to another. The traits like pods bearing branches, pods per plant, 100 seed weight and total plant yield have shown a high heritability and genetic advance. The traits possessing a high heritability along with a high genetic advance are more responsive to the effective selection and improvement. Induced mutations in polygenes governing characters can best be inferred by the estimation of heritability and genetic advance, in addition to mean and coefficient of variation, in successive generations of mutagen treated population (Siddiqui *et al.* 2009).

Results indicate that different traits of the same variety exhibit different response to various mutagens and also their heritable proportion of variance and genetic advance show differential patterns. It is possible that each trait of plant is mutagen specific responsive. This was found in the traits like days to flowering, plant height, days to maturity and seeds per pod where the variability was lower when compared to the traits like yield and yield components. Furthermore, the variability for days to flowering, plant height and days to maturity was lower in M_3 when compared to M_2 generation. However, there was an increase in the variability for yield and yield components in M_3 comparing to M_2 generation. These findings implicate that some traits may have a tendency to stabilize sooner than others, depending on selection of mutatants and strategies adopted. Therefore, it is suggested that in the two varieties of chickpea, selection for days to flowering, plant height and days to maturity could be confined to M_2 generation. The yield and yield components, which have shown increased variability in M_3, provide further scope to select more promising lines in later generations.

8 High Yielding and Productive Mutants

Most of the attributes of interest to a plant breeder are quantitative traits which are mostly controlled by polygenic interactions and in such situations, the efficiency of selecting a desirable mutant is generally lower than for specific trait controlled by a single gene. Emphasizing the significance of micro-mutations in plant breeding, Gaul (1965) stated that "*there appears to be no doubt that micro-mutations may affect virtually all morphological and physiological characters as do large mutations and they might have higher mutation rate than the macromutations*". However, much difference in opinion exists among the breeders on the relative incidence of induced polygenic variations (through induced mutagenesis) in negative or positive direction and shift of the mean in M_2 and later generations (Brock, 1965; Gaul and Aastveit, 1966; Goud, 1967; Rao and Siddiq, 1976; Faulkner, 1978; Rao *et al.*, 1988; Kaneri, 2008; Siddiqui and Singh, 2010). Since mutagen derived variability for quantitative traits in crop plants is heritable and the response of the selection seems good, henceforth, many workers hold the view that induced mutations can be used to generate useful variation in the quantitatively inherited traits where appropriate selection is applied for improvement (Scossiroli, 1964; Lawrence, 1965; Frey, 1969; Brock, 1970; Chakrabarti, 1975; Khan, 1984; Kaul and Kumar, 1983; Tickoo and Chandra, 1999; Khan *et. al*, 2004; Kozgar and Khan, 2009; MacKay, 2011; Hadi and Fuller, 2013; Mba, 2013) and for the expression of mutated gene homozygosity is required because induced mutations occur more or less randomly in the genome and cannot be directed, only one of the two or more alleles of a locus is affected, inheritance is almost never recessive (Micke, 1999).

8.1 Productive Mutants: Enhanced Micronutrients

Increased food insecurity and malnutrition conditions, especially in the developing countries like India, have forced the plant breeders to chalk out the policies of increasing the micronutrinets in the crop in addition to the other quantitative traits, which contribute to yield. Bouis (1996) is of the opinion that mineral and vitamin deficiencies affect a greater number of people in the world and if farmers could be induced to grow commonly eaten food staple crops, like chickpea, that would fortify their seeds with essential vitamins and minerals, and because of high consumption of dietary food, any increase in mineral concentration might as well have a significant effect on human nutrition and health. Induced mutagenesis can be potent methodology for balanced increase in mineral elements (Wang *et al.*, 2003a), in addition to yield and its attributing characters. The impact of mutagens on the trace elements like that of iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) and their genetic variation has not been extensively studied. Mutants with altered seed mineral profiles have been identified in pea (Wang *et al.*, 2003). The Consultative Group on

International Agriculture Research Micronutrient Project (CGIARMP) indicated that it is possible to combine the high micronutrient trait, as Fe, Zn, Mn and Cu contents, with high yield, unlike protein content and yield that are mostly negatively correlated through breeding strategies (Gregoria, 2002). The report also showed that it would be possible to improve the content of several limiting micronutrients together, thus pushing populations toward nutritional balance.

8.2 Productive Mutants: Seed Protein Variants

The improvement of seed protein content, coupled with high grain yield of cereals and legumes the genetic fortification through induced mutagenesis was done in the past and in fact at the end of late 1960s an international research programme was started by the I.A.E.A. in Vienna to improve the cereals and legumes seed protein quantity and quality by means of mutations (Gottschalk, 1986). Several workers put forward contradictory reports on the extent of success of induced mutations for high grain yield coupled with high protein contents of the mutants. Some research works by Hartwig (1979), Abo-Hegazi (1980), Gottschalk and Muller (1982), Matta and Gatehouse (1982), Gottschalk and Wolff (1983a), Gottschalk (1986) and Rehman *et al.* (2001) are of the view that high protein content is difficult to combine with high yield as these two traits reveal to some extent a negative correlation. However, high yielding mutants coupled with high protein contents were reported by Misra *et al.* (1973) and Borah and Goswami (1995) in rice, Olejniczak (1986) in maize, Kalia *et al.* (2000) in wheat, Blixt (1979) in operate legumes, Ignacimuthu and Babu (1989a) in urdbean, Naik *et al.* (2002) in mungbean, Hasan *et al.* (2001) in chickpea and Hiremath *et al.* (2010) in groundnut. Gottschalk (1990) explained that there is no doubt that these traits, i.e. protein content and yield, are controlled by genes and mutations in these genes and can alter the protein make up of the genotypes. It is, however, very difficult to discern their action reliably because protein content is known to be influenced by various endogenous and exogenous factors, like stem height, leaf area, time of maturation, seeds size, seed number, temperature, water stress, nitrogen feeding levels and other environmental factors (Gottschalk and Wolff, 1983b). The protein production in plants is highly influenced by the interaction of gene(s) and environmental factor(s) (Gottschalk, 1990; Singh *et al.*, 1990).

The main difficulty in assessing the high seed protein mutant concurrent to high yield is that no handy field screen methods are available, by which mutagenically treated plants can be analyzed biochemically especially with regard to the composition of their seed proteins. Gottschalk (1986) suggested that it is possible to analyze the seed proteins of mutants, quantitatively and qualitatively, which had been selected with regard to other useful traits but not with regard to improved seed proteins. In this way, it is possible to obtain genotypes with increased protein production per plant, but they do not represent "protein mutants", for example, the bold seeded mutants of

Vigan mungo obtained by Singh (1996), with gamma rays treatment, showed a slight increase in protein content over the control.

For the last couple of years the genetic diversity studies through protein profiling have interestingly increased and now the change in protein profiling among the isolated mutants is of paramount interest. Profiling of proteins in the isolated mutants was done via electrophoresis (Gepts *et al.*, 1986; Anitha *et al.*, 2008). The protein profile could be used as molecular marker to distinguish them from other mutants and also from the controls (Auti and Apparao, 2009) and to indentify the mechanism therein involved. Protein profiling in the isolated mutants was reported by Prasad *et al.* (1986) and Belele *et al.* (2001) in *Phaseolus vulgaris*, Mahmoud and Al-Twaty (2006) in *Lycopersicon esculentum*, Zamani *et al.* (2009) and Das and Bhagwat (2009) in *Triticum aestivum*, Auti and Apparao (2009) in *Vigna radiata*, Barshille *et al.* (2009) in *Cicer arietinum*, Nakagawa (2009) and Nakagawa *et al.* (2011) in *Glycine max* and Khadke and Kothekar (2011) in *Vigna acontifolia*.

In addition to the estimation of genetic variability of characters, such as yield and yield-contributing traits, there is imperative need to undertake studies on correlation coefficients in the mutation breeding programmes. Bahl (1988) studied the change in correlations between various character pairs after mutagen treatments. Kumar and Arora (1991), Rao (1996), Guler *et al.* (2001), Kharkwal, (2003) and Raut *et al.* (2004) studied relationships among various plant characteristics and yield in chickpea.

8.3 Case Study 6

The mutants of chickpea varities Pusa-256 and BG-1053, based on previous chapters' case studies, were screened and illustrated. The parameters such as yield, some minerals and total seed protein contents were analyzed for the isolated high yielding mutants. All these parmeters were assessed to link up probable role in curbing food insecurity and malnutritions conditions.

8.3.1 Results

8.3.1.1 Screening and Isolation of High Yielding Mutants

The details of the mutants isolated in M_3 generation and of their parents are given in Table 8.1. Since yield per plant is the most desirable character, certain mutants which were distinctly much superior to the others with regard to seed yield per plant were selected in M_2 generation and grown in plant progeny rows in M_3 generation. The frequency of occurrence of such mutants was rather low, considering the large size of the M_2 population raised (unpublished data).

Table 8.1: Brief description of the mutants isolated in M_3 generation of chickpea.

Strain Number	Origin/Treatment	Remarks
Var. Pusa-256	Pusa-256 (Control)	-
1. Pusa-256-A	300 Gy gamma rays	High yield
2. Pusa-256-B	0.2% EMS	High yield
3. Pusa-256-C	0.3% EMS	High yield
4. Pusa-256-D	100 Gy gamma rays + 0.2% EMS	High yield
Var. BG-1053	BG-1053 (Control)	-
5. BG-1053-A	200 Gy gamma rays	High yield
6. BG-1053-B	0.3% EMS	High yield

The mean values of quantitative traits of the isolated mutants, such as pods bearing branches per plant, pods per plant and seed yield per plant, showed a significant increase over the control. The range was fairly wide among mutant progenies and differed from trait to trait. Among the mutants of the variety Pusa-256, the highest increase in mean plant yield associated with the mean number of pods bearing branches and mean number of pods per plant was exhibited by the mutant Pusa-256-D isolated at 100 Gy gamma rays + 0.2% EMS treatment. In the variety BG-1053, the mutant BG-1053-A gave the maximum mean plant yield and the mean number of pods bearing branches and mean number of pods per plant at 200 Gy gamma rays treatment.

Genotypic coefficient of variation, heritability and genetic advance for the number of pods bearing branches, number of pods and the total plant yield were also recorded to be higher in all these mutants. This suggests that gains from selection based on these traits would be possible in later generations.

8.3.1.2 NRA, Chlorophyll and Carotenoid Contents

Estimated values for the assay of nitrate reductase activity (NRA), chlorophyll and carotenoid contents of the isolated high yielding mutants of chickpea in M_3 generation showed a significant increase relative to control values. The highest values of NRA were recorded for the mutants Pusa-256-C and BG-1053-A, respectively. The chlorophyll and carotenoid contents also showed significant difference between the controls and the high yielding mutants isolated in the two varieties of chickpea. From the results obtained on the activity of NR and the contents of the chlorophyll and carotenoids, it may be inferred that the mean values of such parameters differed with the mutant type.

8.3.1.3 Seed Protein and Mineral Element Contents

The mean seed protein content of the mutants showed a slight but insignificant improvement over the controls except in the mutant Pusa-256-C (0.3% EMS), where it was lower than in the control (variety Pusa-256). The highest increase in seed protein content was observed in the mutant Pusa-256-B (0.2% EMS) in comparison to the control of the variety Pusa-256. In the variety BG-1053, the maximum increase in the mean protein content was observed in the mutant BG-1053-B (0.3% EMS) as compared to the control value.

The mean values for the mineral elements like iron, manganese, zinc and copper were shifted towards the positive side, with few exceptions, in seeds of the isolated mutants. The mean for mineral elements showed a significant improvement over the controls in the mutants viz. Pusa-256-C, Pusa-256-D and BG-1053-A for iron contents and Pusa-256-B and BG-1053-B for manganese and copper contents, respectively. The highest iron content was recorded in the mutants Pusa-256-D and BG-1053-A, respectively. The manganese content was the highest in the mutants Pusa-256-B and BG-1053-A. The mean values for zinc contents were found to be the highest in Pusa-256-D and BG-1053-A, respectively. The highest copper content was recorded in the mutants Pusa-256-A, Pusa-256-C and in the mutant BG-1053-B.

The values of the coefficient of variation (C.V., %) for seed protein contents of the mutants did not differ much from the control values, indicating that no further improvement in seed protein contents is possible. However the values of C.V. (%) for mineral elements of few mutants differed from the control values, which indicates that the improvement of the mineral elements through induced mutagenesis may be possible (unpublished data).

8.3.1.4 SDS-PAGE

Electrophoretic analysis, through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of seed storage proteins of the high yielding mutants isolated in M_3 generation and the controls of both the varieties of chickpea, revealed the number of polypeptides that were resolved for all the seed protein extracts.

The SDS-PAGE gel of the mutants of Pusa-256 and the control, showed polymorphism patterns among proteins with respect to the control mainly in the region of high molecular weight poylpeptiedes (> 29 kDa) and in the region of polypeptides of low molecular weight (< 14.3 kDa). However, the banding patterns were mostly similar among the mutants isolated in the variety Pusa-256 (Fig. 8.1).

The polymorphisms were also noted in protein polypeptides in the region of high molecular weight poylpeptiedes (> 29 kDa), and in the region of polypeptides of low molecular weight (< 14.3 kDa) in the mutants BG-1053-A and BG-1053-B. The polymorphisms in polypeptides were also present in the mutant BG-1053. The banding patterns also differed among the mutants of the variety BG-1053.

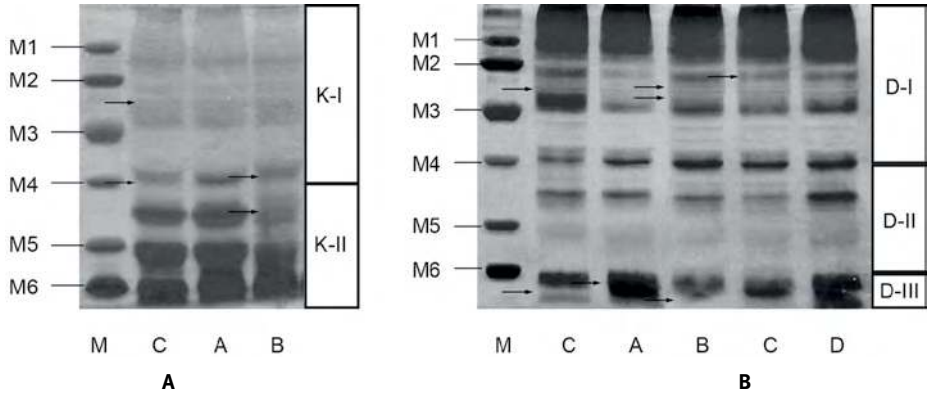

Fig. 8.1: Seed storage proteins of the high yielding mutants of M_3 generation isolated *via* SDS-PAGE form **(A) BG-1053** and isolated mutants BG-1053 (A) and BG-1053-B (B); **(B) Pusa-256** and isolated mutants Pusa-256-A (A), Pusa-256-B (B), Pusa-256-C (C) and Pusa-256-D (D). **Abbreviations**: C – control; D-I: polypeptides of the region-I (> 29 kDa), D-II: polypeptides of the region-II (14.3 to 29 kDa); D-III: polypeptides of the region-III (< 14.3 kDa); K-1: polypeptides of the region-I (> 29 kDa); K-II: polypeptides of the region II (< 29 kDa); M – marker line (M1: phosphorylase b (97.4 kDa); M2: bovine serum albumin (66 kDa); M3: ovalbumin (43 kDa); M4: carbonic anhydrase (29 kDa); M5: soybean trypsin inhibitor (20.1 kDa); M6: lysozyme (14.3 kDa)).

In general, the appearances of new bands of different intensities in the protein polypeptide banding patterns were different in the mutants and the controls.

8.3.1.5 Correlation Studies

The results of correlations among various pairs of yield attributing traits, protein and mineral contents (Fe, Mn, Zn, Cu) of the high yielding mutants isolated in M_3 generation showed significant improvement in positive correlations between the number of pods bearing branches and pods per plant, between the number of pods bearing branches and the total plant yield and between the number of pods per plant and the total plant yield. The exceptions are Pusa-256-C (0.3% EMS), and BG-1053-B (0.3% EMS), where no significant correlation was observed between the number of pods bearing branches and the total plant yield and the number of pods bearing branches and pods per plant, respectively.

A negative correlation was found between protein content and the total yield in the mutants, except Pusa-256-A (300 Gy gamma rays) and Pusa-256-B (0.2% EMS) where insignificant correlations were observed. The results suggest that it is not possible to increase the protein content concurrent with an increase in yield through induction of mutations in chickpea.

Correlation trend between mineral elements and yield per plant, mineral elements and protein, and among mineral elements themselves in some high-yielding isolated mutants was altered significantly positively. A significant increase in positive correlation between Fe and Mn (BG-1053-A), Mn and Zn (Pusa-256-C) and Zn and Cu

(Pusa-256-C) was observed. The correlations between Fe and Mn and Zn and Cu were found to be positive, whereas the correlations between Fe and Zn, Fe and Cu, Mn and Zn and Mn and Cu were found to be negative in most of the treatments. Seed protein content showed a significant correlation with the mineral elements in some of the mutants. Positive and significant correlation between the total plant yield and Fe (Pusa-256-A, Pusa-256-B, Pusa-256-D and BG-1053-A), the total plant yield and Mn (Pusa-256-B), the total plant yield and Zn (Pusa-256-A and Pusa-256-B) and the total plant yield and Cu (Pusa-256-A, Pusa256-C and BG-1053-A) were observed.

8.3.2 Discussion

In the present study, a wide range of variability was observed for the number of pods bearing branches, number of pods and total plant yield of the mutants isolated in M_3 generation. Mean values of these traits increased significantly in different mutant lines compared to the control. Selection for number of pods bearing branches, number of pods per plant and seed yield per plant in M_3 generation was found to be effective in mutants Pusa-256-A (300 Gy gamma rays), Pusa-256-B (0.2% EMS), Pusa-256-C (0.3% EMS), Pusa-256-D (100 Gy gamma rays + 0.2% EMS), BG-1053-A (200 Gy gamma rays) and BG-1053-B (0.3% EMS) as is evident from the manifold increase in the values of the genotypic coefficient of variation, heritability and genetic advance, compared to the controls and rest of the M_3 population. Therefore, these three traits have a high selection value and breeding significance.

8.3.2.1 Physiological Parameters

The study of physiological parameters like nitrate reductase activity (NRA), chlorophyll and carotenoid contents have been advocated as an efficient tool to screen the mutants of interest (de Ronde *et al.*, 2009; Verma *et al.*, 2010). Estimation of NRA, chlorophyll and carotenoid contents in the isolated high-yielding mutants of chickpea show higher values than the control plants. Pandey and Singh (1991), Lal and Tomer (2009) and Kozgar *et al.* (2011) reported that the NRA is strongly and positively correlated to the yield. The NRA could be used as a tool to correlate with protein content and overall productivity of the mutants in early stages of mutation breeding strategies (Barshille *et al.*, 2009). Tomlekova *et al.* (2009) suggested that the increase in total chlorophyll and the carotenoid contents mainly results from the increase in chlorophyll-a type and β-carotene levels, respectively, in the screened mutants. Increase in chlorophyll and carotenoid contents accompanied by an increase in yield has been reported by Hou *et al.* (2009) in *Arthrospira platensis* and Borzouei *et al.* (2010) in *Triticum aestivum*. The chlorophyll and carotenoid estimation also gave an idea about the resistance and tolerance level against different types of stresses. Borzouei *et al.* (2010) reported that up-regulation of some physiological characteristics following mutagenic treatments

may be used for abiotic control such as drought and salt stress. Gechev *et al.* (2009) reported that the chlorophyll contents in the EMS mutated *Arabidopsis* was higher in the strain which was tolerant to the paraquat herbicide. Lee *et al.* (2011) successfully isolated the imidazolinone-resistant barley mutant through induced mutagenesis. Similarly, moderate salt tolerance plants of rice were obtained by Saleem *et al.* (2005). This indicates that high-yielding mutants may be tolerant to many abiotic and biotic stresses and the character stabilization may be screened for subsequent generations.

8.3.2.2 Mineral Elements Composition

Increased micronutrient (mineral elements) density in seed destined for human consumption may alleviate micronutrient deficiencies in human population around the world (Rengel *at. al.,* 1999). Present study has shown that the contents of the total seed Fe, Mn and Cu significantly increased in some of the high yielding mutants isolated from the two varieties of chickpea in M_3 generation. It is possible to combine the high micronutrient traits with high yield, unlike protein content and yield that are negatively correlated, through breeding strategies (Gregoria, 2002). Alikamanoglu *et al.* (2011) reported that the gamma radiation doses increased the concentrations of the Fe, Zn and Cu in soybean plant variants. White and Broadley (2009) suggested that two complementary approaches have been successfully adopted to increase the concentrations of bioavailable mineral elements in food crops. First, agronomic approaches optimizing the application of mineral fertilizers and/or improving the solubilization and mobilization of mineral elements in the soil. Secondly, crops been developed with: (i) increased abilities to acquire mineral elements and accumulate them in edible tissues; (ii) increased concentrations of "promoter" substances, such as ascorbate, β-carotene and cysteine-rich polypeptides which stimulate the absorption of essential mineral elements in the gut; and (iii) reduced concentrations of "antinutrients", such as oxalate, polyphenolics or phytate, which interfere with their absorption. In the present study increased concentrations of promoter substances like β-carotene may be the cause of increase in the mineral elements in the high-yielding mutants. Induction of mutations in chickpea, through chemical and physical mutagens has been investigated but assessing the impact of mutagens on the mineral elements like Fe, Mn, Zn and/or Cu has not been extensively studied. Increase in mean values and variability for the total seed Fe, Mn, Zn and Cu contents of the isolated mutant lines is an indication of the wider scope for genetic improvement of this crop for mineral elements.

8.3.2.3 Seed Protein Content and SDS-PAGE

The mean seed protein content of high yielding mutants showed a slight but insignificant improvement over the controls. Radiation induced effects on amino acid and edible seed protein profiles were also reported by Khattak and Klopfenstein

(1989) and Maity *et al.* (2009) in legumes. Arulbalachandran and Mullainathan (2009c) reported no significant increase in total seed protein content of some mutants induced by gamma rays and EMS. Ignacimuthu and Babu (1989a) and Rehman *et al.* (2001) reported a negative shift of mean values in seed protein content of blackgram mutants. The increase in the protein content might result from the increased activity of nitrate reductase (NR) in the isolated mutants. Similar reports have been made earlier by Lal and Tomer (2009). Coefficient of variation for total seed protein content did not differ from the controls, indicating that further improvement in seed protein is difficult to achieve. Seed protein content is influenced greatly by the interactions of genetic and environmental factors as was reported in chickpea (Singh *et al.*, 1990), mungbean (Ignacimuthu and Babu, 1989a), rice (Kaul, 1980a) and pea (Gottschalk and Wolff, 1983a; Santalla *et al.*, 2001).

SDS-PAGE analysis of the seed storage proteins of high-yielding mutants of chickpea revealed the polymorphic changes in polypeptide chains, in terms of variation in the number and intensity of bands, from the control as well as amongst themselves. Variations are being clearly seen through the surface graph and densitogram output of the SDS-PAGE gels among the mutants. Since proteins are direct gene products, mutation in gene(s) responsible for its synthesis may be reflected in its polypeptides. Electrophoresis (SDS-PAGE) of seed storage proteins via its profiling can economically be used to assess genetic variation and relation in germplasm and the specific bands of seed storage protein profiles may also be used as markers for identification of the mutants (Hameed *et al.,* 2009). Protein profiling through SDS-PAGE analysis may be useful in determining the response of any mutant genotype towards the abiotic and biotic stresses (Hong *et al.,* 2007) and hence, the tolerant one with high yield potential may be screened. Prasad *et al.* (1986) and Belele *et al.* (2001) in *Phaseolus vulgaris*, Mahmoud and Al-Twaty (2006) in *Lycopersicon esculentum*, Auti and Apparao (2009) in *Vigna radiata*, Khadke and Kothekar (2011) in *Vigna acconitifolia* and Nakagawa *et al.* (2011) in *Glycine max* also observed alterations in the electrophoresis patterns of the seed proteins after physical and chemical mutagenic and/or their combination mutagen treatments.

8.3.2.4 Correlation Studies

Knowledge about correlations between quantitatively inherited traits is essential for designing an effective breeding programme (Kaul and Grag, 1982). A number of significant changes towards the desirable side were induced in the correlation coefficient of various pairs of traits in the mutants isolated in M_3 generation. Since the number of pods bearing branches per plant, and the number of pods per plant have shown a significant relationship with total plant yield, it would be desirable to direct selection for these traits. The usefulness of mutations in weakening, strengthening or altering characters associations was reported earlier (Reddy and Khan, 1984; Agarwal *et al.*, 2001; Yadav *et al.*, 2002; Kharkwal, 2003; Toker and Cagirgan, 2004b; Khan and

Wani, 2005b and Shin *et al*., 2011). If the nature of selection practiced in the control and treated populations is the same, any differential correlation between the two populations will result from the effect of mutagens or altered pleiotropic effects of newly mutated genes. Seed protein contents in the isolated high yielding mutants showed negative and non-significant correlations with yield, except for the mutants Pusa-256-A and Pusa-256-B where the correlations between seed protein contents and yield were positive but non- significant. It indicates that simultaneous improvement of yield and protein is not possible in this crop. Similar results on correlation between yield and seed protein have been reported in pulses (Bliss *et al.*, 1973; Kaul and Matta, 1976; Blixt, 1979; Imam, 1979; Gottschalk and Muller, 1982; Karjalainen and Kortet, 1987; Khan and Wani, 2005b). Gottschalk (1986) showed that climatic factors can influence a pleiotropic pattern positively or negatively. The correlations of the mineral elements studied in the present study were positive and significant with that of protein and yield in most of the isolated high yielding mutants, which indicates an added nutritional advantage of the screened mutants. The positive and significant correlations were reported between mineral elements and high yield (Gregoria, 2002) and mineral elements and proteins (Dikeman *et al.*, 1982). Correlations among the mineral elements were found to be either in positive or in negative directions. This might be due to the agonistic and antagonistic metabolic pathways followed (Graham *et al.*, 1981; Loneragan *et al.*, 1982; Kabata-Pendias, 2011) which leads to differential response in terms of correlation output values.

The induction of mutations in the major pulse crops like chickpea for generating high yielding mutants and assessing the genetic variability for protein and mineral element contents and correlations between different character pairs could be a novel strategy to combat the concurrent problems of food insecurity and malnutrition in countries such as India. In the present study, the variability in the treated population was higher than the control for almost all the traits. The results suggested that 200 Gy and 300 Gy of gamma rays, 0.2% and 0.3% of EMS and combination treatment of 100 Gy gamma rays + 0.2% EMS proved to be highly effective in increasing the genetic variability not only for yield-oriented selection in the two varieties of chickpea but also for the mineral composition. The high yielding mutants isolated in M_3 generation can be evaluated in future generations and after multi-location trials they may be released as new varieties. Thus the genetic variability induced by gamma rays, EMS and their combination treatments can effectively be exploited not only for the improvement of chickpea for yield and nutritional factors, but also can act as a novel tool to combat present scenario of hunger in a very sustainable manner.

9 Recent Technologies and Future Strategies

A high rate of population growth, particularly in the developing countries like India, is the principle factor behind increased demand for food and its reduced per capita availability (Shapouri and Rosen, 2001; Tonukari and Omotor, 2010). The genetic improvement of crops primarily for higher production, in order to ensure welfare of the human race, has to play a pivotal role in these challenging conditions. The main and basic component involved in this activity is the creation of genetic variability, which otherwise, can be lost either by too rigid selection or by narrow base of germplasm of a crop plant under improvement. Kumar *et al.* (2011) advocates that mutation breeding can constitute a valuable tool to the conventional breeding methods in widening the genetic base of cultivated germplasm in crops through creation of some useful mutants, henceforth, mutation breeding finds a prominent place in the augmentation and recreation of genetic variability and has played a significant role in the development of many crop varieties (Micke, 1988; Maluszynski *et al.*, 1995; Jain, 2002; Branch, 2002; Canci *et al.*, 2004, Nakagawa *et al.* 2011). The developed mutant varieties increase biodiversity, and thus, directly contribute to the conservation and use of plant genetic resources (Rannali, 2012).

The potentiality of mutations for this purpose, however, depends upon the efficiency of induction of mutations to be aimed at efficiency of screening of the mutants and at the nature of induced mutation (Siddiqui and Yousufzai, 1988; Manju and Gopimony 2009). The enhancement of mutation frequency and the alteration of mutation spectrum in a predictable manner remain constantly important aspects of induced mutagenesis research activities. To ensure a speedy generation of variability for plant traits to be improved, a mutation breeder has to go through all basic events met in the methodologies viz. bio-morpho-physiological, cytological and genetical studies in order to document the reliable information about the mutagen dose/concentration, mutagenic sensitivity of biological material, the extent of effectiveness and efficiency of a mutagen in question and the environmental impact on the induced mutation strategies. Mutagens vary in their mode of action, effectiveness, efficiency and the spectrum of mutations induced in varying environmental conditions. Similarly, genotypes show differential sensitivity towards mutagens even at varietal level.

Among the mutagen types, ionizing radiations still remain the most employed tool of inducing mutations, however a number of chemicals have been found to be equal or higher in potency in their mutagenic effects. On an economical basis, 90% of the officially released mutant varieties were produced using radiation and contribute billions of dollars of additional income to farmers annually (Ahloowalia *et al.*, 2004). However, combination of different mutagens, if their mutation induction process is independent and capable of interaction, should increase mutation frequency and alter the mutation spectrum (Siddiqui and Singh, 2010).

9.1 Modern Techniques

With the recent advances in genomics it has been documented that the use of high throughput platforms, such as TILLING (Targeted Induced Local Lesions in Genomes) and EMAIL (Endonucleolytic Mutation Analysis by Internal Labelling) in the rapid evaluation of mutant stocks for specific genomic sequence alterations can be very much helpful in studying the genetic variability at molecular level. With the advancement of molecular techniques, the mutation breeding comes into the era that is called the "Molecular Mutation Breeding". Ranalli (2012) defines the plant molecular mutation breeding as:

> "Mutation breeding, in which molecular or genomic information and tools are used in the development of breeding strategies, screening, selection and verification of induced mutants, and in the utilization of mutated genes in the breeding process".

The key techniques and resources in molecular mutation breeding revolves around the mutagenic treatment, super-mutable genetic lines, molecular markers and high throughput DNA technologies (Shu, 2009).

Some of the modern techniques presently used for induction and study of mutation breedig parameters are:

9.1.1 High Hydrostatic Pressure (HHP)

High hydrostatic pressure (HHP) is defined as an extreme thermo-physical factor that affects the multiple cellular processes like synthesis of DNA, RNA, proteins, cell survival (Ishii *et al.*, 2004) and has been very effective in inducing mutagenesis in microorganisms (Zhang *et al.*, 2013). The usage of HHP in mutation breeding has started last few years and one of the examples is the creation of mutant varities of rice (Zhang *et al.*, 2013). The most important aspect of HHP is cost-effectiveness and ease of use. There is a hope that within the next years the HHP technique will be used in other crops and even in chickpea studies for betterment and creation of new mutant varities.

9.1.2 Ion Beam Technology (IBT)

Plant breeding with heavy ion beams is an unique technology wherein heavy ions beams are generated by accelerating atomic ions using a particle accelerator. Ion Beam Technology was found to show high relative biological effectiveness (RBE) as compared to low linear energy transfer (LET) radiations, such as gamma rays, x-rays and electrons (Blakely, 1992). Although the basic research on plant mutation

by ion beams began in 1991 (Goodhead, 1995), it has been utilized in full zeal only since last decade and promising achievements have been made in agriculture. Datta (2012) arguments that ion beam technology has been further modified as ion beam implantation into organisms, and energy deposit, mass deposit, charge transfer of the implanted ions into target organisms, long distance systemic effects in intact organisms, generation of reactive oxygen species etc. are the main topic of research (Feng *et al.*, 2009; Datta, 2012).

9.1.3 Space Breeding

The experiments in space show that the growth cycle of the seeds is shortened and the effective components are strengthened, thus space breeding is a proven way and can be applicable in modern mutation breeding strategies. The two parameters in combination i.e., presence of cosmic rays and microgravity, affect the genetic diversity of the crop and thus they are the main causes of the changes in breeding new crop varieties (Mei *et al.*, 1998; Gu and Shen, 1989). China is the leader in the field of space breeding, having more than 66 new varities developed in the space breeding programme (Lie *et al.*, 2005). One of the important features of space breeding is that the growth cycle of the seeds could be shortened, as reported by Li (2013).

9.1.4 Targeting Induced Local Lesions in Genomes (TILLING)

Targeting Induced Local Lesions in Genomes (TILLING) is a method that allows directed identification of the mutations in a very specific gene. It is one of the most high-throughput, non-transgenic reverse-genetic approaches, which combines mutagenesis (chemical) with a sensitive DNA screening-technique and enables the recovery of individuals carrying allelic variants of candidate genes (Colbart *et al.*, 2001). TILLING combines traditional mutagenesis followed by high-throughput mutation discovery, which can improve the efficiency of using induced mutations and nucleotide polymorphism discovery methods for a reverse genetic strategy that is high in throughput, low in cost and applicable to most organisms, and in less than a decade will help to develop crops with improved traits (Colbert *et al.*, 2001; Ranalli, 2012). It is an efficient early-screening tool for specific point mutations in genes of interest from a small population and enables geneticists to analyze gene function and associate genotype with phenotype. It is useful in scanning gamma-irradiated mutant populations (Sato *et al.*, 2006). The TILLING has moved from a proof of concept to a well-accepted reverse genetic method that has been applied to over 20 different species (Ranalli, 2012). The advancements in new mutation discovery techniques have promised to increase further the efficiency and applicability of the TILLING method (Till *et al.*, 2009).

9.1.5 Endonucleolytic Mutation Analysis by Internal Labelling (EMAIL)

Endonucleolytic Mutation Analysis by Internal Labelling (EMAIL) has been developed by Cross *et al.* (2008) for detecting rare mutations in specific genes in pooled samples using capillary electrophoressis. The inventors i.e., Cross *et al.* (2008) advocate that this technique, EMAIL, is an alternate approach to mismatch detection, in which amplicon labeling is achieved by incorporating flourescently labeled deoxynucleotides. The strength of the EMAIL assay was demonstrated in the reclassification of a rice line as being heterozygous for the starch gene, which in previously used sequence studies had been described as being homozygous. Thus, this technique offers increased sensitivity in gene-specific mutant detection in pooled samples, enbling enlarged pool sizes and improving throughput and efficiency (Lee *et al.*, 2009). Prior to these methodologies, the principle of capillary electrophoressis was used in ecotilling processes (Cordeiro *et al.*, 2006). Some workers are of the opinion that this technique is highly improved over TILLING approach and offers the plant breeder a new tool for efficient screening of induced point mutation at an early stage for variants in genes of specific interest before taking plants to field trial (Lee *et al.*, 2009: Datta, 2012).

9.2 Future Strategies

The crisis of food insecurity which denied the access to food to millions of humans, particularly in early 1970s) and especially in Indian sub-continent, in the past has erupted again due to which hundreds of millions of people go hungry (Kozgar *et al.*, 2012). Henceforth, a new "Green Revolution" and in fact an "Evergreen Revolution" is desperately needed to solve the two problems viz. food insecurity and malnutrition which are constituting great threat for global development. In this regard, induced mutagenesis is gaining importance in plant molecular biology as a tool to identify and to isolate genes. It also serves to study their structure and function and has become a more powerful and effective tool in breeding the new crop varities, once integrated with modern technologies (Ranalli, 2012). Mutation breeding has clearly entered into a new era of molecular mutation breeding. Therefore, induced mutations will continue to play a significant role for improving world food security in the coming years and decades (Ranalli, 2012; Kozgar, 2012; Husain *et al.*, 2013; Mba, 2013).

Ranalli (2012) has quoted in his paper:

> "Development of novel and more efficient genomic tools have become routine and the pursuit of new physical mutagens continues. Some technologies are already in place and when integrated into mutation research, they will greatly increase the efficiency and application of mutation techniques in plant research. For example, the next generation sequencing technologies, e.g. Roche 454 Genome Sequencer-FLXF™ and Applied Biosystems SOLiDF™ instruments, have the potential to reduce the cost of genome sequencing by several magnitudes, and simplify the

process of mutation detection, the key point in mutation research and application programmes. In particular, they will enable the identification of mutant genes underlying important quantitative traits such as drought tolerance and yield, something that is still very difficult, if not impossible with traditional means"

In this persuit, the global integration, cooperation and collobrative research works have to be enhanced in order to make the applications useful in practical life and move from labs to fields for betterment of human race.

All the applications of modern mutation breeding are to be applied on the crops which have been neglected in past era, like pulse crops and particularly the crop which has the potential to be a part of reducing the hunger to large extent as chickpea. Due to less genetic diversity and self-pollinating nature of number of pulse crops including chickpea, induced mutation breeding is of paramount importance (Kozgar *et al.*, 2013). Although the mutation breeding technique has been serving the humankind in many forms but the breakthrough in Indian sub-continent in this particular area is still awaited. There is a desperate need for cooperation of countries, such as India with the associations which are working within countries or outside on mutation breeding methodologies like Forum for Nuclear Cooperation in Asia, FNCA (Japan), International Atomic Energy Agency, IAEA (Vienna, Austria), Bhabha Atomic Research Centre, BARC (Mumbai, India), etc.

In the present documentation, the exemplary study was undertaken to estimate the extent of induced genetic variability for quantitative traits, the bio-physiological damages, cytological aberrations and alterations in certain biochemical indices of the seed components induced and/or altered by gamma rays (physical mutagen), EMS (chemical mutagen) and their combination (gamma rays + EMS) treatments in the two varieties viz. Pusa-256 and BG-1053 of chickpea (*Cicer arietinum* L.). Chickpea possesses low genetic variability due to cleistogamous nature of their flowers. The observations made during the course of case studies have been discussed in the respective chapters.

The survey of important literature on induced mutations of pulse crops in general and of chickpea in particular, revealed that: a suitable character combination that affects plant yield to the maximum extent is important in formulating an effective selection programme; correlation between yield and yield components are known in chickpea, however, correlations of yield to protein and mineral elements (iron, manganese, zinc and copper) are not equally known. In the light of the above facts, it felt desirable to use gamma rays, EMS treatments and their combination for generating polygenic variability for quantitative traits in chickpea, a self-pollinated crop, for getting maximum economical use of the plant in the era where food insecurity and malnutrition groom over developing countries like India for the past several years. However, the study of molecular parameters of one of the major pulse crops (chickpea), to make its molecular mutation breeding a reality, has to be enhanced at every level and it has to begin with the drafting of chickpea genome sequencing, as a resource of traits for improvement that provides insights into both genome diversity and domestication (Varshney *et al.*, 2013).

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Appendix

1 Materials and Methods

Information on the materials used and methods applied during the present study as summarized in case studies from chapters 3-8 are provided in this Appendix.

1.1 Agroclimatic Conditions of the Site of Study

The site of present study viz. Aligarh has the characteristic semi-arid and sub-tropical climate with hot dry summers and cold winters. The average rainfall in this district is 847.30 mm. More than 85% rainfall occurs from mid July to September. During the summer, the average temperature is 35 °C and during the winter, the average temperature is 15 °C. The soil of Aligarh is sandy loam and alkaline.

1.2 Materials

1.2.1 Varieties used

Two varieties of chickpea (*Cicer arietinum* L.) namely Pusa-256 (desi) and BG-1053 (kabuli) were used in the present study. The kabuli variety, BG-1053, is also commonly known as Pusa Chamatkar. These varieties are released at I.A.R.I, New Delhi. Seeds of both varieties were procured from the Government Seed Store, Aligarh. Both varieties are well adapted to agroclimatic conditions of Uttar Pradesh (including the site of study). A brief description of both varieties is given below:

Pedigree	Year of release	Distinguishing characters
Variety Pusa- 256		
Double cross of (JG-62 x 850- 3/27) x (L. 550 x H. 208) involving three *desi* and one *kabuli* types	1985	Matures in 135-145 days; erect with medium height; seeds light brown and medium bold; moderately resistant to wilt, stunt and *Ascochyta* blight; average yield is 18 kg/ha
Variety BG-1053		
ICCV x FLIP88-120	2000	Matures in 145-150 days; semi-erect; resistant to soil born diseases; bold seeded with the average yield of 20 kg/ha

Source:
Department of Information and Communication, Ministry of Agriculture, Government of India.

1.3 Experimental Procedures

1.3.1 Mutagens Used

Two varieties of chickpea were selected for irradiation and chemical mutagen treatments as detailed below:

1.3.1.1 Irradiation: Gamma rays (γ rays)

Gamma irradiation was chosen as a physical mutagen. Dry seeds of each variety, with moisture content 12%, were irradiated with 100, 200, 300 and 400 Gy of gamma rays with a radioisotope $^{60}C_0$ source (Gamma chamber Model-900, supplied by Bhabha Atomic Research Centre, Mumbai, India) at the National Botanical Research Institute, Lucknow, Uttar Pradesh, India.

1.3.1.2 Chemical Mutagen: Ethylmethane sulphonate (EMS)

EMS ($C_3H_8O_3S$), a monofunctional alkylating agent, having molecular weight 124.16, manufactured by Sissco Research Laboratories Pvt. Ltd., Mumbai, India was used as a chemical mutagen. For chemical mutagen treatments, healthy seeds of uniform size of each variety were presoaked for 9 h in distilled water and treated with 0.1, 0.2, 0.3 and 0.4% of EMS for 6 h with intermittent shaking at room temperature of 25 ± 2 °C. The solution of EMS was prepared in the phosphate buffer of pH 7. The pH of the solution was maintained by using buffer tablets (MERCK, Mumbai, India). After treatment the seeds were thoroughly washed in running tap water to remove the excess of mutagen.

1.3.1.3 Combination Treatment: Gamma rays + EMS

For combination treatments, dry seeds of each variety were first irradiated with gamma rays at 100 and 200 Gy doses and then treated with 0.1 and 0.2% EMS. (i.e. 100 Gy + 0.1% EMS, 100 Gy + 0.2% EMS, 200 Gy + 0.1% EMS and 200 Gy + 0.2% EMS). The procedure adopted was similar to that for the individual treatment.

1.3.2 Sample Size

400 seeds were used for each treatment and control.

1.3.3 Controls

For each variety, 400 pre-soaked seeds were again soaked in phosphate buffer for 6 h to serve as controls.

1.4 Handling and Selection of the Treated Material in Different Generations

1.4.1 M_1 Generation

Three replications of 100-seeds each, were sown for every treatment and control in each variety in a randomized complete block design (RCBD) at the Agriculture Farm, Aligarh Muslim University, Aligarh. The spacing was maintained at 30 cm (seed to seed in a row) and 60 cm (between the rows) in the field. The experiment was conducted during winter season of 2008. Recommended agronomic practices were employed for preparation of field, sowing and subsequent management of the population of chickpea.

The remaining lot of 100 seeds was grown in two sets (50 seeds in each) on moist cotton in petriplates. One set was used for determining percentage of seed germination and measuring the seedling height i.e. root and shoot lengths and another set of 50 seeds was used to study the nitrate reductase activity (NRA) and estimation of chlorophyll and carotenoid contents. For this, the petri plates were kept in the B.O.D. incubator at 27 ± 1 °C.

1.4.2 Studies in M_1 generation

1.4.2.1 Seed Germination

After recording germination counts, the percentage of seed germination was calculated on the basis of total number of seeds sown in the petri dishes and in the field.

$$\text{Germination (\%)} = \frac{\text{No. of seeds germinated}}{\text{No. of seeds sown}} \times 100$$

1.4.2.2 Seedling Height

Seedling height was recorded after 10 days by measuring the root and shoot lengths for each treatment and control. Seedling injury was measured in terms of reduction in seedling height with respect to control.

1.4.2.3 Estimation of Nitrate Reductase Activity (NRA), Chlorophyll and Carotenoid Contents

The estimation of NRA, chlorophyll and carotenoid contents was recorded from secondary emergent leaflets of the seedlings raised from mutagen treated seeds and from control seeds in petri dishes. The procedure was as follows:

1.4.2.3.1 Assay for Nitrate Reductase Activity (NRA)

The activity of nitrate reductase (EC 1.7.7.2) was measured as the described by Jaworski (1971) in fresh leaf samples.

The leaves were cut into small pieces avoiding the mid veins. 200 mg of chopped leaves were weighed and transferred to plastic vials. To each vial 2.5 ml of 0.1 M phosphate buffer pH 7.4 (Appendix I-A) and 0.5 ml of 0.2 M potassium nitrate solution (Appendix I-B) were added, followed by the addition of 2.5 ml of 5% isopropanol (Appendix I-C). These vials were incubated in B.O.D. incubator for 2 h at 30 ± 2 °C in dark. 0.4 ml of incubated mixture was transferred to a test tube (borosil) to which 0.3 ml each of 0.1% sulphanilamide solution (Appendix I-D) and 0.02% NED-HCL (Appendix I-E) were added. The test tube was left for 30 min, for maximum colour development. The mixture was diluted to 5 ml with double distilled water (DDW). The absorbance in terms of optical density was read at 540 nm on spectrophotometer (Spectronic 20D, Milton Roy, USA). A blank consisting of 4.4 ml DDW and 0.3 ml each of sulphanilamide and NED-HCL were used simultaneously for comparison with each sample. A standard graded concentration of sodium nitrite ($NaNO_2$) from standard aqueous solution of the salt was used. The absorbance of each sample was compared with that of the calibration curve and NRA (nmol NO_2^- $g^{-1}h^{-1}$FW) was computed on fresh mass basis.

1.4.2.3.2 Estimation of Chlorophyll and Carotenoid Contents

The chlorophyll and carotenoid contents of leaves on which measurement were made was estimated by the method of MacKinney (1941).

1 g of finely cut fresh leaves was ground to a fine pulp using a mortar and pestle after pouring 20 ml of 80% acetone (Appendix I-F). The mixture was centrifuged at 5,000 rpm for 5 min. The supernatant was collected in 100 ml volumetric flask. The residue was washed three times, using 80% acetone. Each washing was collected in the same volumetric flask and volume was made up to the mark (100 ml) using 80% acetone. The absorbance in terms of optical density was read at 645 and 663 nm for chlorophyll and 480 and 510 nm for caretenoid against the acetone (80%) as blank on spectrophotometer (Spectronic 20D, Milton Roy, USA).

The chlorophyll and carotenoid contents present in the extracts of leaves were calculated according to the equation given by Arnon (1949).

$$\text{Total chlorophyll (mg g}^{-1}\text{leaf fresh mass)} = (20.2(OD_{645}) + 8.02(OD_{663})) \times \frac{V}{1000 \times W}$$

$$\text{Carotenoid (mg g}^{-1}\text{ leaf fresh mass)} = \frac{7.6\,(OD_{480}) - 1.49\,(OD_{510})}{d \times 1000 \times W} \times V$$

where,

OD_{645}, OD_{663}, OD_{480}, OD_{510} = Optical densities at 480, 663, 480 and 510 nm, respectively

V = Volume of an extract

W = Mass of leaf tissues

d = Length of light path (d = 1.4 cm)

1.4.2.4 Pollen Fertility

Pollen fertility was determined basing on 30 randomly selected plants (10 plants from each replicate) from each treatment and control for both varieties at the time of flowering. Pollen grains were stained with 1% acetocarmine solution on glass slides and covered with cover slips. Pollen grains which took stain and had a regular outline were considered as fertile, while the shrunken and unstained ones as sterile.

1.4.2.5 Plant Survival

The surviving plants in different treatments and control were counted at the time of maturity and the survival was computed as percentage of the germinated seeds in the field.

The following formula was used to calculate the percentage of inhibition, injury or reduction.

$$\begin{matrix}\text{Percentage inhibition} \\ \text{or} \\ \text{Percentage injury} \\ \text{or} \\ \text{Percentage reduction}\end{matrix} = \frac{\text{Control - Treated}}{\text{Control}} \times 100$$

1.4.2.6 Meiotic Analysis

Meiotic studies were conducted on 30 randomly selected plants from each treatment and control for both varieties. For meiotic studies, young flower buds were fixed in Carnoy's fluid (1 : 3 : 6, glacial acetic acid : chloroform : ethyl alcohol, respectively) for 24 h. Ferric chloride was added to the fixative to get better staining. After 24 h of fixation, flower buds were transferred to 70% alcohol. Anthers were smeared in 1% acetocarmine solution and pollen mother cells were examined under compound microscope for their behaviour at various stages of microsporogenesis.

1.4.3 M_2 Generation

25 healthy seeds from each normal looking M_1 plant of all different treatments with their respective controls of both varieties were planted in plant progeny rows in M_2 generation during winter season of 2009. Different treatments and controls comprised of 50 progenies. The distance between seeds in a row and between the rows was kept 30 × 60 cm, respectively. Three replications were done for each treatment.

1.4.4 Studies in M_2 Generation

1.4.4.1 Chlorophyll Mutations

Chlorophyll mutations were scored when the seedlings were 10-20 days old. They were identified and classified according to Gustafsson (1940). The frequency of chlorophyll mutations was calculated by the following formula:

$$\text{Mutation frequency (\%)} = \frac{\text{Number of mutant seedlings}}{\text{Total number of } M_2 \text{ seedlings}} \times 100$$

The effect of combined treatments on chlorophyll mutations frequency was analysed following the method of Sharma (1970):

$$\text{Coefficient of interaction (k)} = \frac{(a+b)}{(a) + (b)}$$

where,
(a + b) = The mutation frequency induced by the two mutagens in combination treatments
(a) + (b) = The mutation frequencies induced by the two mutagens when applied alone
k = Hypothetical interaction coefficient.

The “k” value should be one, if the interaction is additive. Any deviation from this value would show synergistic or less than additive effects.

1.4.4.2 Mutagenic Effectiveness and Efficiency

Mutagenic effectiveness is a measure of the frequency of mutations induced by unit dose of a mutagen, while mutagenic efficiency represents the proportion of mutations in relation to biological damage. Formulae suggested by Konzak *et al*. (1965) were used to evaluate the mutagenic effectiveness and efficiency of the mutagens used.

$$\text{Mutagenic effectiveness (Gamma rays)} = \frac{\text{Rate of mutation (Mp)}}{\text{Dose in Gray (Gy)}}$$

$$\text{Mutagenic effectiveness (EMS)} = \frac{\text{Rate of mutation (Mp)}}{\text{Concentration} \times \text{duration of treatment}}$$

$$\text{Mutagenic effectiveness (Combination)} = \frac{\text{Rate of mutation (Mp)}}{\text{Dose of physical mutagen (Gy)} \times \text{concentration of chemical mutagen} \times \text{duration of treatment}}$$

$$\text{Mutagenic efficiency} = \frac{\text{Rate of mutation (Mp)}}{\text{*Biological damage in } M_1 \text{ generation}}$$

*Biological damage: For measuring the biological damage, three different criteria were used;
(i) Injury - i.e. percentage reduction in seedling height (Mp/I)
(ii) Sterility - i.e. percentage reduction in pollen fertility (Mp/S)
(iii) Meiotic abnormalities - i.e. percentage of meiotic abnormalities (Mp/Me)

1.4.4.3 Morphological Mutations

The frequency of morphological mutations was calculated on the basis of M_2 plants.

1.4.4.3.1 Estimation of NRA, Chlorophyll and Carotenoid Contents

The estimation NRA, chlorophyll and carotenoid contents were recorded from various isolated morphological mutants and controls from the upper most leaf tissues of the plant twig, at flowering stages[1]. The procedure of their estimation is same as described earlier in section 1.4.2.3 of this appendix.

1 In case of isolated non-flowering mutants the NRA assay, chlorophyll and carotenoid estimation was done when the leaves show a tinge of maturation, this delay was to confirm their non-flowering nature.

1.4.4.4 Quantitative Traits

Observations were made on 25-30 normal looking plants of each progeny, for each treatment along with the control. The progenies segregating for macromutations were not used for such analysis. The following eight quantitative traits were thoroughly studied in different generations:

(i) Days to flowering: Number of days taken by the plant from date of sowing to the date of opening of first flower bud.

(ii) Plant height (cm): Plant height was measured at maturity in centimeters from the base up to the apex of the plant.

(iii) Days to maturity: Days to maturity were noted as the number of days taken by the plant from the date of sowing to the date of harvesting of the plant.

(iv) Number of pods bearing branches: Number of pods bearing branches was counted at maturity as the number of branches which bore more than one pod.

(v) Number of pods: Number of pods were counted at maturity and noted as the number of pods borne on the whole plant.

(vi) Seeds per pod: The best pods were threshed and number of seeds per pod was counted. The mean was calculated for each plant.

(vii) 100-seed weight (g): It was taken as a weight of a random sample of 100 seeds from each plant.

(viii) Total plant yield (g): Plant yield was the weight of total number of seeds harvested per plant and the yield of each plant was recorded in grams.

1.4.5 M_3 Generation

For raising M_3 generation, two treatments of gamma rays and EMS alone and their combinations for each variety were used which gave the maximum total plant yield in M_2 generation. The selected treatments were 200 Gy and 300 Gy of gamma rays, 0.2% and 0.3% of EMS and 100 Gy + 0.1% EMS and 100 Gy + 0.2% EMS of combination treatments. For each of these treatments, 10 M_2 progenies were selected which showed significant deviations in mean values in the positive direction from the mean values of control, particularly for the yield components under study in M_2 generation. Seeds from each selected M_2 progeny were sown in plant progeny rows. M_3 generation was raised during winter season 2010. Seeds were taken only from the normal looking M_2 plants. Plants showing chlorophyll, morphological and other variations were discarded from each progeny. The plant to plant and row to row distance was kept the same as in earlier experiments. Quantitative traits studied in M_3 generation were the same as in M_2 generation.

1.4.6 Bio-physiological Studies of Isolated Mutants

1.4.6.1 Estimation of NRA, Chlorophyll and Carotenoid Contents

Although, NRA, chlorophyll and carotenoid contents were estimated for the plants for each treatment in M_3 generation plants, but the documentation has been given for the isolated high yielding mutants in the present study. The procedure of their estimation is same as elaborated in earlier section 1.4.2.3 of this Appendix.

1.4.6.2 Mineral Element Estimation

Estimation of important mineral elements concentrations viz. iron (Fe), manganese (Mn), zinc (Zn) and copper (Cu) in the seeds of high yielding mutants isolated in M_3 generation and controls of chickpea were determined using an atomic absorption spectrophotometer (AAS) after wet digestion as given by Gupta (2004). For mineral element analysis dried seed samples were homogenized by grinding in a stainless steel blender and then passed through sieves of 2 mm mesh size and kept at room temperature for further analysis.

1.4.6.2.1 Digestion of Samples

0.5 g grinded seed samples of mutants and controls were weighed and put into 50 ml digestion tube. 5 ml of acid mixture (Appendix I-G) were added into each sample. The pyrex funnel was placed into the tubes and put into the block digestor. Samples were heated initially at 60 °C for 15 min till reaction was completed which was confirmed on fumes eruption. Samples were then heated to 120 °C until samples became colourless. Once the samples got transparent, the tubes were removed from the digestor and cooled down. The blank was also prepared consisting only of acid mixture, without sample contents.

1.4.6.2.2 Estimation of Mineral Elements

Digested and blank samples were diluted with 50 ml deionized water and mineral elements (Fe, Mn, Zn, Cu) contents were detected using AAS (GBC Scientific Equipment Ptv. Ltd., Australia). The standard solution (MERCK, Mumbai, India) for each mineral element was used for calibration. The absorbance was read at the cathode lamp set up with slit width of 0.5 nm for the Cu and Zn and 0.2 nm for Fe and Mn estimation. The wavelengths of the lamp were kept 372.0, 403.1, 307.6 and 327.4 nm for the estimation of Fe, Mn, Zn and Cu, respectively. The same lamp wavelengths and width of the slit were kept for their respective standards and blank samples. The statistical data output for total Fe, Mn, Zn and Cu contents were directly assessed by the computer software AVANTA 2.0 version preinstalled on AAS computer equipment.

1.4.6.3 Seed Protein Estimation

Seed protein content of the mutants isolated in M_3 generation was estimated following the method of Lowry *et al*. (1951). For extraction of soluble and insoluble protein, seed powder was kept in an oven at 80 °C overnight. Then it was cooled and 50 mg of sample were transferred to a mortar and ground by a pestle with 5 ml of double distilled water (DDW). The ground material was collected in a centrifuge tube. The tube was centrifuged at 4000 rpm. The supernatant was collected in a 25 ml volumetric flask using 2-3 washings with DDW. Volume was made up to the mark with DW and kept for estimation of soluble protein. The residue was used for the estimation of insoluble protein.

1.4.6.3.1 Insoluble Protein Estimation

5 ml of 5% trichloroacetic acid (TCA) were added to the residue. The solution was shaken thoroughly and allowed to stand at room temperature for 30 min. It was then centrifuged at 4000 rpm for 10 min and the supernatant was discarded. 5 ml of 1N sodium hydroxide was added to the residue and mixed well and kept for 30 min. The residue was allowed to stand in a water bath at 80 °C for 30 min. Then it was cooled and centrifuged at 4000 rpm. The supernatant together with three washings with 1N sodium hydroxide was collected in a 25 ml volumetric flask. The volume was made upto the mark with 1N sodium hydroxide.

For the estimation of seed insoluble protein, 1 ml of sodium hydroxide extract was transferred to a 10 ml test tube and 5 ml of reagent D (Appendix II) were added and allowed to stand for 10 min.

0.5 ml of reagent E (Appendix II) was added rapidly with immediate mixing. After 30 min, the solution turned blue. The optical density (O.D.) of the solution was read at 660 nm on "Spectronic-20D" (Milton Roy, USA) spectrophotometer. A blank was run with each sample. The optical density of this solution was compared with standard curve, used for soluble protein.

1.4.6.3.2 Soluble Protein Estimation

For the estimation of soluble protein, 1 ml of water extract from supernatant was transferred to a 10 ml test tube. 5 ml of reagent C (Appendix II) were added. The solution was mixed and allowed to stand for 10 min at room temperature. 0.5 ml of reagent E (Appendix II) was added rapidly with immediate mixing.

After 30 min, the blue coloured solution was transferred to a colorimetric tube and its intensity was measured by reading its optical density at 660 nm, using a "Spectronic-20D" spectrophotometer.

A blank was run simultaneously. The soluble protein content was estimated by comparing the optical density of each sample with a calibration curve plotted by taking known dilutions of a standard solution of egg albumin.

1.4.6.3.3 Standard for Protein

50 mg of egg albumin was taken in a 100 ml volumetric flask, to which 1-2 ml of 0.1N NaOH was added. The flask was rotated carefully placed on a water-bath for a short period (5-10 min) for heating. After the albumin became solubilized, the volume of the flask was made up to the mark by DDW. From this solution a range of 10 volumes (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml) was pipetted out to ten different test tubes. The solution in each test tube was diluted to 1 ml by adding 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, 0.3, 0.2, 0.1 and 0.0 ml of DDW, respectively.

In each test tube 5 ml of reagent C (Appendix II) was mixed and allowed to stand for 10 min at room temperature. 0.5 ml of reagent E (Appendix II) was then added rapidly with immediate mixing. The optical density of the solution was read at 660 nm using a "Spectronic-20D" spectrophotometer. A blank was also run simultaneously and a calibration curve was plotted.

The soluble protein content was estimated by comparing the optical density of each sample with a calibration curve plotted by taking known dilutions of a standard solution of egg albumin.

1.4.6.3.4 Total Seed Protein Estimation

The total protein content of the seeds was obtained by adding the value for the soluble and insoluble protein content.

1.4.6.4 SDS- PAGE Analysis

1.4.6.4.1 Protein Extraction

Total storage protein from the isolated high yielding mutant seeds of M_3 generation and control plants were extracted following the method of Alsohaimy *et al.* (2007). Seeds of the mutants and controls of both varieties were ground into fine powder using mortar and pestle. 50 mg fine powder was weighted into a fresh eppendorf. Powder was dissolved in 600 µl of reverse osmosis (RO) water. Sample pH was adjusted to 11 using 0.1N NaOH and made up the volume to 1 ml using RO water. Sample was incubated at room temperature for 1 h on a rocker and centrifuged at 12,000 rpm for 5 min. Supernatant was transferred into a fresh eppendorf. pH was gradually brought down to 4.5 using 1 M HCl, with proper mixing at each step. Sample was centrifuged at 12,000 rpm for 2 min. Supernatant was discarded and the pellet was dissolved in 200 µl of re-suspension buffer (Appendix III A), which acted as the total protein sample for SDS-PAGE.

1.4.6.4.2 Electrophoresis

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of total proteins extracted from seed samples was performed by using the methodology of Sadasivam and Manickam (2008). Dry and cleaned SDS-PAGE apparatus (mold) were placed in a proper position for casting using clips. Then 10 ml resolving gel mixture (Appendix III B) was poured between the plates till its level was 2 cm below from the top edge of the plate. In order to smooth the upper surface of the resolving gel 1 ml butanol solution was poured at the top to ensure the even surface. After the gel settled down (after 30-40 min), it was washed properly to remove unpolymerized acrylamide. Then the comb was placed, leaving 1 cm gap between resolving gel and comb. After that 2.5 ml stacking gel mixture (Appendix III C) was poured on the top level of the resolving gel directly. Once the stacking gel was set, comb was removed very carefully without disturbing the wells. Then the wells were washed with RO water to remove unpolymerized acrylamide, the glass plates were fixed to the running tray carefully and the reservoir was filled with 1X running buffer (Appendix III D). Then 2 µl of total protein sample with 8 µl RO water and 5 µl loading dye (Bromophenol blue) were taken and carefully injected in each well. A protein molecular weight marker (GENEI, Bangalore) was also incorporated into the gel. The established mold was then connected with power supply and the gel was ran at 45 mA till the dye reached the bottom of the glass plate. After that the glass plates were removed and carefully separated with a spatula.

1.4.6.4.3 Staining

After electrophoresis, stacking gel was removed and the resolving gel was washed in a washing solution (Appendix III E) in a clean plastic container with slow shaking for 10 min. Wash solution was discarded and gel was rinsed with water for 2 min. Then sodium thiosulphate solution (Appendix III E) was used to soak the gel for 1-2 min. The gel was washed again twice with water and then stained with silver nitrate solution (Appendix III E) for 10 min with gentle shaking and poured developer (Appendix III E) to the plastic container and shaked till yellow to dark brown colour bands appeared and when the band developed the reaction was stopped by adding acetic acid solution (Appendix III E) and photographed.

1.4.6.4.4 Gel Documentation

Stained gels were documented using SONY digital camera (Model HS-10, Zoom-10X, 8.2 Mega pixels). Gels were also documented by scanning on an all-in-one HP Deskjet (F370) computer assembly. These documented gels were used for further analysis of the data.

1.5 Statistical Analysis

Data collected for eight quantitative traits in M_1, M_2 and M_3 generations were subjected to statistical analysis in order to assess the extent of induced variation, as indicated below:

1.5.1 Mean ($\overline{X}$)

The mean was computed by taking the sum of a number of values (X_1, $X_{2,}$ .... X_n) and dividing by the total number of values (N) involved, thus;

$$\overline{X} = \frac{X_1 + X_2 + \ldots\ldots X_n}{N}$$

where,
X_1, X_2,.....X_n = Observations
N = Total number of observations involved

1.5.2 Standard Deviation (S.D.)

The Standard deviation was calculated by the following formula for each parameter of study.

$$S.D. = \sqrt{\frac{(\overline{X} - X_1)^2 + (\overline{X} - X_2)^2 + \ldots\ldots\ldots(\overline{X} - X_n)}{N}}$$

where,
($\overline{X}$) = Mean of the observations involved
$X_{1,}$ X_{2}.....X_n = observations
N = Total number of observations

1.5.3 Coefficient of Variation (C.V.)

It measures the relative magnitude of variation present in observations relative to magnitude of their mean. It is expressed as the percentage ratio of standard deviation to corresponding mean, i.e.

$$C.V.\ (\%) = \frac{\text{Standard deviation}}{\overline{X}} \times 100$$

1.5.4 Standard Error (S.E.)

$$\text{S.E.} = \frac{\text{S.D. of sample}}{\sqrt{N}}$$

where,
S.D. = Standard deviation
N = Number of observations

1.5.5 Components of Variance

Analysis of variance was done according to Singh and Chaudhary (1985) to find out the variance between the families and within the families. The components of variance considered were:
(i) within–family variation in the control and in the treated material which was an estimate of environmental variation,
(ii) between–families variation which was an estimate of the between families genetic variation.

1.5.5.1 Genotypic Variance ($\sigma^2 g$)

Genotypic variance ($\sigma^2 g$) was estimated by the following formula:

$$\sigma^2 g = \frac{(MS_{Bf} - MS_e)}{N}$$

where,
MS_{Bf} and MS_e = Mean sum of squares for between families and within families or error, respectively
N = Number of replications

1.5.5.2 Genotypic Coefficient of Variation (GCV)

$$GCV(\%) = \frac{\sqrt{\sigma^2 g}}{\bar{X}} \times 100$$

1.5.5.3 Phenotypic Variance ($\sigma^2 p$)

Phenotypic variance was estimated by summing the estimated genotypic variance ($\sigma^2 g$) and the environmental variance (MS_e or $\sigma^2 e$).

$$\sigma^2 p = \sigma^2 g + \sigma^2 e$$

1.5.5.4 Heritability (h^2)

It is the ratio of genotypic variance to the phenotypic variance. The broad-sense heritability (h^2) was estimated by the following formula:

$$h^2\,(\%) \;=\; \frac{\sigma^2 g}{\sigma^2 p} \times 100$$

1.5.5.5 Genetic Advance (GA)

The estimates of genetic advance (GA) with 1% selection intensity were based on the formula derived by Allard (1960).

$$GA = k \times \sigma p \times h^2$$

where,

h^2 = Broad-sense heritability

σp = Phenotypic standard deviation of the mean performance of treated population

K = 2.64, constant for 1% selection intensity

$$GA\ (\%\ \text{of}\ \bar{X}) \;=\; \frac{GA}{\bar{X}} \times 100$$

1.5.6 Test of Significance

In order to compare the means of various treatments, critical (least significant) difference was applied and computed as follows:

Step-1 Construction of Data Table

The data were compiled such that each treatment occupies a column and their replicates were arranged in rows.

Rows (Replicates)	Column (Treatments) Number					Total of Rows (Replicates) (Σ)	Squares of total of rows
	T_1	T_2	T_3	T_4	T_5		
R_1	A_1	B_1	C_1	D_1	E_1	A_1+..E_1=X_1	$(X_1)^2$
R_2	A_2	B_2	C_2	D_2	E_2	A_2+..E_2=X_2	$(X_2)^2$
R_3	A_3	B_3	C_3	D_3	E_3	A_3+..E_3=X_3	$(X_3)^2$
Total of column (Σ)	$A_1 + A_3 = Y_1$	B_1+.$B_3 = Y_2$	C_1+$C_3 = Y_3$	D_1+.$D_3 = Y_4$	E_1+..$E_3 = Y_5$	$(X_1)^2$+.$(X_3)^2$	= Wr
						Y_1+Y_5 or X_1+X_3	=W
Squares of total of columns $(\Sigma)^2$	$(Y_1)^2$	$(Y_2)^2$	$(Y_3)^2$	$(Y_4)^2$	$(Y_5)^2$	$(Y_1)^2$+..$(Y_5)^2$	= Wy
Sum of square of total of columns (Σ^2)	$(A_1)^2 + (A_3)^2 = Z_1$	$(B_1)^2 + (B_3)^2 = Z_2$	$(C_1)^2 + (C_3)^2 = Z_3$	$(D_1)^2 + (D_3)^2 = Z_4$	$(E_1)^2 + (E_3)^2 = Z_5$	Z_1+..Z_5	= Wz

Step-2 Correction Factor (CF)

$$CF = \frac{(\text{Grand total})^2}{\text{t.r.}}$$

OR

$$CF = \frac{(W)^2}{\text{t.r.}}$$

where,
W = Grand total
t = Number of treatments
r = Number of replicates

Step-3 Total Sum of Squares (SSQT)

This is the sum of squares of all the values in the table, minus the correction factor.

$$SSQT = [(Z_1 + Z_2 + \ldots..Z_5] - CF$$

OR

$$SSQT = Wz - CF$$

Step-4 Sum of Squares of Treatments (SSQt)

$$SSQt = \frac{(Y_1)^2 + (Y_2)^2 + \ldots. (Y_5)^2}{r} - CF$$

OR

$$SSQt = \frac{Wy}{r} - CF$$

where,
r = Number of replicates

Step-5 Sum of Squares of Replicates (SSQr)

$$SSQr = \frac{(X_1)^2 + (X_2)^2 + (X_3)^2}{t} - CF$$

OR

$$SSQr = \frac{Wr}{t} - CF$$

where,
t = Number of treatments

Step-6 Sum of Squares of Error (SSQ_e)

$$SSQe = SSQT - (SSQt - SSQr)$$

Step-7 Estimated Variance of Error (MS_e)

$$MS_e = \frac{SSQ_e}{(t-1)(r-1)}$$

Step-8 Critical (least significant) Difference

$$\text{CD at 5\% (p=0.05) level} = \sqrt{\frac{2\ MS_e}{r}} \times (t - \text{value at 5\% level})$$

$$\text{CD at 1\% (p=0.01) level} = \sqrt{\frac{2\ MS_e}{r}} \times (t - \text{value at 1\% level})$$

If the difference between any two treatment means exceeds the CD values obtained at 5% and/or 1% level, the difference between the two means is said to be significant.

1.5.7 Correlation Coefficient (r)

Correlation coefficient, a statistical measure which indicates association between two or more than two traits (say number of pods and plant yield), generally denoted by symbol "r", involves the following steps in computation.

Step-1 Construction of Data Table

Observations of pair of quantitative traits were arranged as per the following table:

S.No. of pairs of observation	Trait 1 (X)	Trait 2 (Y)	X^2	Y^2	XY
1	X_1	Y_1	$(X_1)^2$	$(Y_1)^2$	X_1Y_1
2	X_2	Y_2	$(X_2)^2$	$(Y_2)^2$	X_2Y_2
3	X_3	Y_3	$(X_3)^2$	$(Y_3)^2$	X_3Y_3
4	X_4	Y_4	$(X_4)^2$	$(Y_4)^2$	X_4Y_4
5	X_5	Y_5	$(X_5)^2$	$(Y_5)^2$	X_5Y_5
.	.	.	.	.	.
.	.	.	.	.	.
.	.	.	.	.	.
15	X_{15}	Y_{15}	$(X_{15})^2$	$(Y_{15})^2$	$X_{15}Y_{15}$
Total	**$\sum X$**	**$\sum Y$**	**$\sum X^2$**	**$\sum Y^2$**	**$\sum XY$**

Step-2 Computation of the following

(i) Sum of X-values '$\sum X$'
(ii) Sum of Y-values '$\sum Y$'
(iii) Square of each X-value and their sum '$\sum X^2$'
(iv) Square of each Y-value and their sum '$\sum Y^2$'
(v) Product of each pair (X and Y) and their sum '$\sum XY$'

Step-3 Computation of "r" Values as Follows

$$r = \frac{\sum XY - (\sum X)\ (\sum Y)/ N}{\sqrt{[\sum X^2 - (\sum X)^2/ N]\ [(\sum Y^2 - (\sum Y)^2/ N]}}$$

where,
N = Number of pairs of observations

Test of significance of "r"

The *t* test for correlation coefficient was applied to determine whether the relationship between two traits is really significant or merely due to chance. It was computed as follows:

$$t = r\sqrt{\frac{N-2}{1-(r)^2}}$$

where,
r = Correlation coefficient
N = Total number of observations

The observed value of correlation coefficient is compared with the tabulated value for (N-2) degree of freedom. If the observed value is more than the table value of *t*, the relationship is said to be significant.

Appendix I

Reagents used for the estimation of NRA, chlorophyll and carotenoid contents and mineral elements.

(A) 0.1 M phosphate buffer (7.4 pH)

27.2 g of KH_2PO_4 and 45.63 g of $K_2HPO_4 \times 7H_2O$ were dissolved separately in 1000 ml of DDW.

The above solution of KH_2PO_4 and $K_2HPO_4 \times 7H_2O$ were mixed in the ratio of 36:64, respectively.

(B) 0.2 M potassium nitrate

20.2 g of KNO_3 was dissolved in sufficient DDW and final volume was made up to 1000 ml using DDW

(C) Isopropanol (5%)

5 ml of isopropanol was pipetted into sufficient DDW and final volume was made up to 100 ml, using DDW.

(D) Sulphanilamide (1%)

1 g of sulphamnilamide was dissolved in 100 ml of 3N HCL.

3N HCl was prepared by dissolving 25.86 ml of HCl in sufficient DDW and final volume was maintained to 100 ml, using DDW.

(E) N-1-nehthyl-ethylenediamine dihydro chloride-HCl (NED-HCl) (0.02%)

20 mg of NED-HCl was dissolved in sufficient DDW and final volume was made up to 100 ml, using DDW.

(F) Acetone (80%)

80% acetone was prepared by mixing 80 ml of acetone with 20 ml of DDW.

(G) Acid mixture

The acid mixture was made by nitric, sulfuric, and perchloric acids in the volume ratio 10:1:4, respectively.

Appendix II

Reagents used for the estimation of seed protein.

Reagent A:
2% of sodium carbonate in 0.1N NaOH (1:1 ratio)

Reagent B:
0.5% of $CuSO_4$ in 1% of sodium tartrate (1:1 ratio)

Reagent C:
Alkaline $CuSO_4$ in solution obtained by mixing 50 ml of reagent A with 1 ml of reagent B

Reagent D:
Carbonate copper sulphate solution same as reagent C except for omission of NaOH

Reagent E:
Folin's phenol reagent; Folin phenol reagent was made after diluting it with DDW in the ratio of 1:2

Reagent F:
1N NaOH

Appendix III

Reagents and components used in SDS-PAGE.

(A) Re-suspension buffer:
- Tris (pH 6.8) – 50 mM
- DDT – 100 mM
- 2% SDS

(B) Resolving gel: (10% gel)
- Distilled H_2O – 12.3 ml
- 1.5 M Tris HCl (pH 8.8) – 7.5 ml
- 20% SDS – 0.15 ml
- 30% Acrylamide solution – 9.9 ml
- 10% ammonium persulphate (APS) – 0.15 ml
- TEMED – 0.015 ml

(C) Stacking gel: (4% gel)
- Distilled H_2O – 3.075 ml
- 0.5 M Tris HCl (pH 6.8) – 1.25 ml
- 20% SDS – 0.025 ml
- 30% Acrylamide solution – 0.67 ml
- 10% ammonium persulphate (APS) – 0.025 ml
- TEMED – 0.005 ml

(D) 5X Running buffer:
- Tris base – 15 g
- Glycine – 72 g
- SDS – 5 g
- Distilled H_2O – make volume up to 1 litre
  (Diluted to 1X before use)

(E) Staining:
- Washing solution - 1ml formaldehyde + 40 ml methanol + 60 ml distilled water
- Sodium thiosulphate - 200 mg in 1 l water
- Silver nitrate solution – 0.1%
- Developer - sodium carbonate (3 g) in 80 ml water + sodium thiosulphate solution (1 ml) and formaldehyde (1 ml) and make the volume up to 100 ml with water
- Stopper - acetic acid solution (5%)

**APS and TEMED were added just prior to pouring the gel*

***All the components have been added sequentially*

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