Mutagenesis, Cytotoxicity and Crop Improvement

Revolutionizing Food Science

Edited by Tariq Ahmad Bhat

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Cambridge
Scholars
Publishing

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This book first published 2021

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-6296-4
ISBN (13): 978-1-5275-6296-7

This book is dedicated to

Sir Mohammad Iqbal (1877-1938)

A great poet, visionary, educationist, statesman and a revolutionist of the nineteenth century.

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PREFACE

The author takes great pleasure in presenting to the readers this enlarged and extensive book on mutagenesis, cytotoxicity and crop improvement in which special emphasis has been laid on induced mutagenesis, mutagenic effectiveness and efficiency, principles and prospects of mutagenesis, induced chromosomal aberrations, chromosomal analysis and improvement of quantitative traits. There is an emphasis on the improvement of agronomic characters by manipulating the genotype of plant species to increase productivity to combat world hunger by induced mutagenesis. The book is a valuable asset to all the stakeholders, including under-graduate students, post-graduate students, teachers and researchers.

During the last few decades, there has been remarkable progress in research on various aspects of mutagenesis and cytotoxicity and crop improvement geneticists, evolutionary biologists, ecologists, cell biologists, plant breeders etc. have been exploiting the various aspects of mutation research. Cytotoxicity and crop improvement have been used to understand the genetic architecture of organisms, prepare chromosome linkage maps, understand the evolutionary relationship among organisms and groups of organisms, and understand speciation, adaptation and modes of invasion of plant and animal species. The manipulation and engineering of chromosomes have facilitated their transfer across kingdoms for genetic improvement of crop and animal species which has led to crop improvement. The present book is intended to fulfill the needs of students, teachers, researchers and all stakeholders who are engaged in the study of evolution, molecular biology, biodiversity, mutation breeding, plant breeding, chromosome manipulation, genetic and physical mapping, cell biology, and crop improvement. The book comprises eighteen chapters. The first three chapters deal with the types of mutagens, their mechanism of action, applications of mutagenesis, and principles and future prospects of mutation breeding. The fourth chapter deals with cytotoxicity evolution and the fifth and ninth chapters are about induced chromosomal aberrations. The sixth, seventh, eighth and eleventh chapters deal with applications of individual and combined treatments and their outcomes in terms of increased crop productivity, particularly in pulses and oilseed crops. The tenth and fourteenth chapters deal with applications of mutagenesis and

isolation of promising mutants and their protein electrophoresis through SDS-PAGE. The twelfth chapter is about structural and numerical chromosomal changes. The thirteenth chapter deals with correlation analysis for biochemical aspects of isolated mutants of faba beans. The fifteenth chapter deals with site-directed mutagenesis in plants while the sixteenth looks at mutagenesis and plant breeding in the twenty-first century. The seventeenth and eighteenth chapters deal with cytogenetics of pill-millipedes. The chapters of the book are from eminent authors and researchers of the world scientific community who are working on different aspects of mutation research.

In presenting this book, I wish to express my gratitude to Prof. A. H. Khan and Prof. Samiullah, Department of Botany, Aligarh Muslim University, Aligarh, without whose help and encouragement, I would have never become a student of cytogenetics and mutation breeding. I am thankful to the authors who contributed chapters for this book which is the outcome of their decades of research work. I thank especially Prof. (Dr) Abdul Rauf Shakori, Distinguished National Professor, Director and Professor Emeritus for his significant inputs and encouragement which proved very fruitful in the formation of this book. Many of the ideas in the book are the outcome of teacher training programs, conferences, workshops and seminars. I wish to express my gratefulness to all teachers and researchers associated with these programs for their suggestions and advice, without holding them responsible for any shortcomings in the book.

I am also grateful to my students whom I taught all these years because it is through teaching them in the classroom that I learned much that I know.

I am thankful to the technical editors and board of Cambridge Scholars Publishing Co. for their wholehearted cooperation and sympathetic assistance whenever it was required.

Finally, I wish to acknowledge a debt to my family whom I left waiting on several evenings, nights and holidays while I was busy finalizing the manuscript or the illustrations for this book. I especially thank my wife for the technical setting of the material and giving special input for making this project successful.

Anantnag, Jammu and Kashmir, India
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FOREWORD

Mutation is an abrupt occurrence of a heritable alteration in the genomic make up of an organism, which acts as an indispensable evolutionary force in nature. Induced mutagenesis can create desirable traits at high rates for plant breeders to initiate crop improvement programmes and produce novel crop varieties through mutation breeding. Mutation breeding procedures have contributed enormously to crop improvement, by developing and officially releasing thousands of improved crop varieties. In mutation breeding various physical mutagens such as gamma-rays, X-rays, fast neutrons, and chemical mutagens such as EMS (ethyl-methane-sulphonate), MMS (methyl-methane-sulphonate), hydrazine hydrates and sodium azides were normally engaged.

The current book by Dr Tariq is a welcome addition to the field of plant breeding mutagenicity and how mutations prove beneficial in the enhancement of crop productivity. The book comprises eighteen chapters by well-known researchers and experts in the fields of plant breeding, cell biology, biotechnology and mutation sciences. Chapter 1 mentions the types and mechanism of action of mutagens with special emphasis on sodium azide and gamma radiation. This chapter provides information on the basis of classification of mutagens, mode of mutagenic action, characteristic features and landmark achievements of different mutagens. In Chapter 2 and 3, the principles and application of mutations and problems related to it are discussed in studies related to crop improvement. Chapter 4 gives evaluation studies about bioassay applications for mutagenicity and cytotoxicity. Chapter 5 provides a review of cytological aberrations through mutagenesis. Chapters, 6, 7, 8, 9, 10, 11, 13, 14 and 16 mention case studies of mutagenesis involving different crop and oil plants. Chapters 17 and 18 deal with the cytogenetics of two giant pill-millipedes of the genus *Arthrosphaera.* Chapter 12 provides the basis of physical and chemical agents which induce structural and numerical changes in chromosomes and chapter 15 mentions the procedures of site-directed mutagenesis in plants.

In short, a great emphasis has been laid on different topics related to the key principles influencing crop improvement together with an elucidation of the nature of new approaches in improvement. I am sure that a new generation of researchers will benefit greatly from this book and share the respect for

the crop plants we all live by and concern for the maintenance of diversity. I applaud the editor, Dr Tariq Ahmad Bhat as well as the book chapter contributors for successfully bringing together this volume.

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CHAPTER 1

MUTAGENS, THEIR TYPES AND MECHANISM OF ACTION WITH AN EMPHASIS ON SODIUM AZIDE AND GAMMA RADIATIONS

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Abstract: Mutation breeding techniques have contributed immensely to crop improvement, by developing and officially releasing thousands of improved crop varieties. In mutation breeding, various physical mutagens such as gamma rays, X-rays, and fast neutrons, and chemical mutagens such as EMS (ethyl methanesulfonate), MMS (methyl methanesulfonate), hydrazine hydrates and sodium azides, were employed. The sudden heritable change in the genetic constitution of an organism not caused by the normal process of genetic segregation is referred to as a mutation. Mutations are the ultimate source of variation in living organisms on planet earth. The knowledge of mutations in higher plants traces back to as early as 1590, however, the application of mutations for crop improvement has a history of eight decades only. Natural mutations occur spontaneously, however, the frequency is very low and inaccessible to

plant breeders. The low frequency necessitates the induction of artificial mutation through the use of agents capable of bringing new and heritable variations in the plant genomes. Based on the type of mutations induced, mutagens are classified into several classes, viz., physical mutagens that induce gross chromosomal breakages, chemical mutagens that are known to cause point gene mutations and biological agents that are able to disrupt the functional elements of genes thereby inducing a wide range of mutations. The use of mutagens has resulted in several thousand mutant varieties that have improved characters in more than two hundred fifty plant species across the globe. However, the maximum number of mutant varieties has been induced by physical mutagens followed by chemical and biological agents. This chapter briefly provide insights into the basis of the classification of mutagens, mode of mutagenic action, characteristic features of different mutagens.

Keywords: mutagens, mutagenic action, mutant varieties, mutagen types.

1. Introduction

Mutagens are chemical compounds such as ethyl methanesulfonate, methyl methanesulfonate, sodium azide or radiation such as gamma rays, ultraviolet light, X-rays that cause heritable alteration in the genome. Mutations occur when they remain undetected by cellular proofreading mechanisms, when the repair machinery gets compromised or when the repair machinery are overwhelmed by heavy damage. By the virtue of cellular replication, these mutations get fixed in the subsequent generations. Several factors influence the impact of mutation and these include target gene, mutation type, mutagen dose, the sensitivity of the target organism and compounding effects of pre-existing mutations (Bhat *et al*., 2005a; Khursheed *et al*., 2018b). Thus, a mutation in the induced untranslated region of DNA will have no effect (silent mutation), whereas a mutation in an actively transcribed region may influence gene expression and even lead to cell death (lethal mutation). The breakdown of phosphodiester bonds of DNA, constant production of free radicals, and miscopying of DNA replicative enzymes are the main sources of spontaneous mutations. However, the frequency of spontaneous mutation is extremely low and is not enough to achieve the desired aims of breeding. Therefore, different kinds of physical and chemical mutagens are used to treat different plant parts to increase the mutation rate.

2. Mutation and Mutagens

A mutation is a sudden heritable alteration in the genetic material of a living cell induced by mutagens (Bhat *et al*., 2006a; Raina *et al*., 2018). However, mutations may occur in nature without exposure to mutagens, and such mutations are said to be spontaneous. Transposons are mobile genetic elements that can migrate to any position within the genome and alter the expression of the gene(s) and eventually lead to a broader spectrum of spontaneous mutations, particularly deletions and insertions (Wessler, 2006). Retro-transposons are transcribed first to the RNA and then back to DNA by reverse transcriptase and then move into the genome in a "copy and paste" manner, while DNA transposons move directly in a "cut and paste" manner using a transposase enzyme, inducing different spontaneous mutations (Kidwell, 2005). The frequency of spontaneous mutations is very low and is not enough to keep pace with evolution. The spontaneous changes in the DNA may or may not become fixed in DNA. Even if such mutations would be fixed, they may not be apparent as most of the spontaneous mutations are recessive (Ranel, 1989). Additionally, spontaneous mutations are dependent on chance and the rate of spontaneous mutation breeding programs are very slow. Ahloowalia *et al*. (2004) and Wilde *et al.* (2012) reported that selection for economically important spontaneous mutants still occurs but with little success. Targeted mutagenesis is a much-desired option wherein the purposeful induction of a specifically desired mutation at a specific time and place is ensured (Bhat, 2007).

2.1. Mutation Induction

Any agent that alters the information encoded in the nucleotides of DNA and/or RNA and thus increases the frequency of mutations is considered a mutagen. The nature, properties, and underlying mechanisms of mutagens have been reviewed by Kaul (1989). The reviews of Gottschalk and Wolf (1983) and Kodym and Afza (2003) have enhanced our understanding of the subject of mutagens. DeVries (1905) employed radiation to induce mutations. Muller 1927 and Stadler 1928 discovered X-ray induced mutants in *Drosophila melanogaster* and in *Hordeum vulgare* respectively and established the field of X-ray-induced mutations for altering traits in a wide range of organisms, whereas Auerbach and Robson (1946) were pioneers in using chemicals such as mustard gas as mutagens. The core mutagens such as UV radiation, electromagnetic waves such as gamma rays, X-rays and cosmic rays; fast-moving particles such as α-particles, β-

particles and neutrons; and chemical agents such as alkylating agents, acridines, azides, and hydroxyl amides have been employed for induction of mutations by different workers from time to time. Ionizing radiations such as X-rays and gamma rays are preferred over chemical mutagens due to their good penetration, better reproducibility, and high mutation frequency. The main source of X-rays is from X-ray machines whereby tungsten or molybdenum is bombarded with electrons in a vacuum, whereas a gamma chamber fitted with radioisotopes such as ^{60}Co and ^{137}Cs emits gamma rays. UV radiation possesses less tissue penetration, lower linear energy transfer [LET] and induces comparatively lower damage and hence prolonged exposures are required to achieve the desired results (Kovacs and Keresztes, 2002). Induced mutations have wide applicability not only in crop improvement programs but also in basic research on the plant genome. Plant breeders were the pioneers in reporting the effectiveness and efficiency of mutagens compared with spontaneous mutations. In various crops, many of the mutants that had been treated with mutagenic treatments and showed improved traits were screened and selected in the second generation (M_2) and advanced to the subsequent generation for mutation fixation and officially released as new varieties. Several mutants with mutations in desired traits were also used in hybridization programs as a source of elite genes (Laskar *et al*., 2018a,b; Gulfishan *et al*., 2015). These mutants were improved and possessed genes for desired traits such as dwarf height, biotic stress resistance or enhanced oil quality (Bhat *et al*., 2007a; Laskar *et al*., 2019). These mutated plants with the desired genes have led to the development of officially released elite varieties of a wide range of crops. Across the globe, mutation breeding has led to the official release of 1540 cereal and 480 legume mutant varieties from 170 plant species (Table 1.1).

Induced mutations are known to increase the spontaneous mutation rates 10–100 fold, thereby enhancing the chances of isolating a higher number of mutants with improved traits. Plants are facing a wide range of environmental induced stresses and to overcome the deleterious effects of such stresses requires alterations in the secondary metabolites such as phenolics (Ahmad *et al*., 2019a,b; Niakoo *et al.,* 2019). Induced mutations can play a critical role in the development of plants that reveal improved tolerance to a range of biotic and abiotic stresses. In the recent era induced mutations have been considered as the better option available for enhancing genetic variation in crops with a narrow genetic base and also to serve as an alternative to conventional breeding approaches. Induced mutations are also used for discovering new genes, studying the structure and function of genes and their role in regulating the vital biochemical

processes (Micke *et al*., 1990). Mutagen induced genetic variation differs from natural genetic variation as it is not yet acted upon by nature or man and thus contains traits which were not favored during the course of evolution or previous plant breeding activities. Moreover, genes governing the agro-economically important traits may possess a complete linkage with undesirable genes and consequently recombination through hybridization is a rare event.

Mutation breeding is a coherent tool to understand genetic phenomena such as inheritance, genetic advance, genotypic and phenotypic coefficient of variability and mutagenic effectiveness and efficiency (Bhat *et al*., 2007b; Khursheed *et al*., 2016). Mutation breeding is widely employed for crop improvement programs of various crops (Bhat *et al*., 2005b; Adamu and Aliyu, 2007; Kharkwal and Shu, 2009). Treatment with mutagens distorts the normal DNA double-helical structure and eventually results in chromosome breaks and mutations. Most of these mutations are corrected by the cellular proofreading mechanism but some may escape detection and are transmitted to the next generation. Novak and Brunner (1992), Kozgar *et al*. (2012) and Bhat *et al.* (2006c) have reported that in order to enhance the existing variability, breeders have to rely on mutation breeding, as the existing genetic variation is very limited.

Mutagens have been employed to induce genetic variability in plants for more than seven decades and about 3275 mutant varieties have been developed in 60 countries across the globe (Bhat *et al*., 2006b; Raina *et al.*, 2016; 2018). Millions of hectares of cultivated land have been devoted to the higher-yielding or more disease-resistant mutant varieties across the globe. About 90% of the mutant varieties were produced using radiation as the mutagen. In mutation breeding for crop improvement programs, selection of an appropriate mutagen is necessary to achieve the desired frequency and spectrum of desirable mutations. Various chemical mutagens have been used for developing elite mutants in crops (Ganai *et al*., 2005; Khursheed *et al*., 2015). Recently, new physical mutagens, such as ion beam radiation and cosmic rays, have been proven to be effective for inducing mutations. However, several workers emphasize that artificial induction of mutation by ethyl methanesulfonate (EMS), sodium azide (SA), and maleic hydrazide (MH) also play a pivotal role in increasing the genetic variability in plants, particularly self-pollinated plants which possess narrow genetic variability (Gulfishan *et al*., 2013; Jafri *et al*., 2011; Khursheed *et al*., 2018a). Various factors such as choice of mutagen, duration of treatment, pH, pre- and post-treatment, and temperature all influence the effect of mutagens (Gulfishan *et al*., 2011;

Laskar *et al*., 2015; Tantray *et al*., 2017; Khursheed *et al*., 2019). The selection of an appropriate mutagen dose determines the success of mutation breeding (Gulfishan *et al*., 2012; Mensah and Obadoni, 2007; Gnanamurthy *et al*., 2012). Generally, it is reported that a higher dose of mutagen induces greater biological damage and is less effective, while a lower dose induces less biological damage and possesses more effectiveness. Several factors such as duration of mutagen treatment, mutagen dose, pH, temperature and plant material to be treated can all influence the effectiveness and efficiency of a mutagen (Gulfishan *et al*., 2010; Laskar *et al*., 2015). Single and combined mutagen treatments were employed for improving the agro-economic traits in various cereals, pulses and ornamental plants, since various physical and chemical mutagens used individually and/or in combination act in several ways to induce DNA breaks, modify DNA bases and distort the double-helical structure. In wheat, dose-dependent effects were observed with a combination treatment of UV and X-rays. Swaminathan and Natarajan in 1959 reported that the frequency of mutations at low doses of X-rays resulted in a lower frequency of mutation in UV pre-treated seeds as compared to higher doses of X-rays. Likewise, the effect of combined treatment of ethyl methanesulfonate (EMS) and hydroxylamine (HA) was studied in wheat. It was observed that EMS is a more effective mutagen in inducing chlorophyll and viable mutations as compared to HA, but when HA was administered after EMS treatment, mutation frequency reduced significantly, thereby revealing that HA plays a vital role in mutational repair processes (Chopra and Swaminathan, 1966).

2.2. Mutagens and Their Doses

The choice of an effective and efficient dose plays a critical role in determining the success of the breeding program. It is recommended to ascertain a relationship between the induced biological damage and the dose of radiation or chemical mutagens. Mutagen effectiveness is measured in terms of induced biological damage (Roychowdhury, 2011; Raina *et al*., 2018; Khursheed *et al*., 2016). In radiation biology, the 'simple dose' (D) is the quantity of energy absorbed per mass of irradiated matter. The special unit of D is the rad (1 rad =100 erg/g = 10^{-2} joule/kg), expressed in terms of time as rad/h, rad/min and rad/s. Therefore, any alterations in radiation dose and duration of exposure are critical parameters in mutation breeding. In the case of chemical agents, the mutagen dose is determined based on several parameters, viz., (i) concentration, (ii) duration of treatment and (iii) temperature during

treatment. The volume of the mutagen solution should ensure that each seed or organ absorbs the effective amount of mutagen. The dose which induces 30–40% growth reduction is considered an optimum dose for mutagenesis. The literature is scanty regarding the optimum mutation dose in a particular crop, however, LD50 (Lethal Dose-50), is a common parameter used to determine the effective doses of mutagens (Albokari *et al*., 2012). The LD50 is a dose which results in the death of 50% of treated seeds. With ionizing radiation, a dose which restricts survival to 50% (LD50) or growth to 50% (GR50) and 30% (GR30) is considered as the optimum dose. Long treatment, usually of six hours duration, is advisable, but it can be shortened to study the effect of treatment duration on the different traits of plants. For a short period, a high concentration is used after pre-soaking at high temperatures.

3. Physical Mutagens

The discoveries that radiation (X-rays) induced changes in the genome of fruit flies (Muller, 1927) and plants such as *Zea mays* and *Hordeum vulgare* (Stadler, 1928a; Stadler, 1928b; Stadler, 1930; Stadler, 1931) are considered as landmark achievements. These discoveries proved to be watershed moments in mutation breeding as they offered the impulsion for the successive widespread implementation of this technique in crop improvement and very recently as a tactic to ascertain genes and illuminate their roles. The ionizing radiations are the most widely used mutagens in addition to the alpha (α) and beta (β) particles and neutrons (Mba *et al*., 2012; Mba and Shu, 2012). These radiations are part of the electromagnetic spectrum (EM) and by virtue of their high energy levels dislodge electrons from the nuclear orbits of the atoms. Ultraviolet (UV) rays, classified as non-ionizing, are capable of penetrating tissues thereby inducing a high frequency of mutations. The mutagens induce nucleotide dimmers and reactive species formation which in turn cause deletion, insertion, substitution, gross chromosomal breakages and rearrangements (Table 1.2). Physical mutagens are used for the development and official release of more than 2500 mutant varieties (Table 1.3).

3.1. Ion Beam Mutagenesis

In China breeders employed low-energy ions as a part of ion beam mutagenesis in the late 1980s with the aim of improving food security. In the early 1990s, Japan employed heavy ions to improve floriculture. Ion beams cause mass deposition and charge exchange and differ from gamma

rays, X-rays and other physical mutagens which involve energy transfer (Hase *et al*., 2012) and thereby induce alterations in DNA and eventually result in DNA damage. Ion beams are emitted by particle accelerators such as cyclotrons. Neon-20, Nitrogen-14, Carbon-12, Lithium-7, Argon-40, and Iron-56 are some heavy ions that are employed for irradiation. The linear energy transfer (LET) is the energy deposited by ionizing particles onto the biological material. LET is expressed in kiloelectron volts per micrometer (keV/μm), which represents the average amount of energy lost per unit distance. Ion beams have a comparatively high LET (10–1,000 keV/μm or higher), while X-rays, gamma rays and electrons have low LETs (0.2 keV/μm). The high LET heavy-ion beam has lately been used on many plants, with ensuing productive achievements (Yu *et al*., 1991), such as for carnations (Okamura *et al.*, 2003), chrysanthemums (Yamaguchi *et al.*, 2010), wheat (Wei *et al*., 1998), buckwheat (Morishita *et al*., 2003) and *Arabidopsis* (Kazama *et al*., 2008), because of its higher frequency of mutation and wide spectrum of mutations with less damage to irradiated materials compared to the low LET radiation methods (Abe *et al*., 2000; Tanaka, 1999). Thus, more and more researchers pay attention to the application of heavy-ion beam irradiation in plant mutation breeding. Different ion beams have different energy levels and different linear energy transfer (LET), ionization densities and penetration which correlate to the induction of DNA damage. Once an accelerated particle encounters any substance it transfers a certain amount of energy onto the substance and loses an equal amount of energy and eventually stops at the region where the highest energy is transferred. LET reaches its maximum just before the ionizing particle stops. Hence, ion beams induce more severe damage to biological material compared to other radiations, resulting in high relative biological effectiveness (Blakely, 1992; Lett, 1992). The impact of biological damage induced by ion beam radiation is dependent on absorption doses and LET values irrespective of ion species (Kazama *et al*., 2008). The frequently induced DNA damage includes double-strand breaks and also deletions, insertions, inversions and translocations. Several workers have reported while studying the mutant gene alleles induced by ion beam radiation, that most mutations are deletions and that the size of deletion is LET-dependent. As compared to X-rays and gamma rays, heavy-ion beams (HIB) escape the repair machinery and are considered more effective in inducing the mutations. A wide spectrum mutation and less biological damage have been reported for ion beam radiation as compared to other mutagens. In China, ion beam technology has been employed and was successful in developing 23 new rice and wheat mutant varieties which benefited the country's revenue as

more than 1 million ha of crops per annum were grown and sold in large scale commercial production. The wheat variety 'Wanmai 54' showed more tolerance against head scab and rust disease and the total yield was improved by 7–10.6%. In Japan, ion beam technology has been used for the development and official release of mutants in a vast range of plant species.

3.2. Gamma Phytotron

Genetic improvement by chronic irradiation is one of the options of mutation breeding techniques, especially when a wide spectrum of mutants with the least biological damage is required. Hence, chronic irradiation of biological material has been favored to induce desired mutants in mutation breeding. However, chronic irradiation facilities are available in only a few Asian countries such as Malaysia, Japan and Thailand which have operational chronic irradiation facilities such as gamma greenhouses, gamma fields, and gamma phytotrons, respectively. Several security and management issues are taken care of while operating chronic irradiation experimentation. In the year 2010, Kang *et al.* (2010) designed a new gamma phytotron for pot plants or cultured callus to expose the material to lower doses of gamma rays for a longer duration. This phytotron uses ^{60}Co with 400 curies of radioactivity fitted in a three-room infrastructure, first for irradiation with an area of about 104.16 m^2, second for non-irradiation, and the third for operations and a glasshouse. The entire phytotron plant is covered with concrete walls of 1.2 m depth and possesses a lead shielded door between the operating room and the irradiation room. The temperature, humidity, and light flux are controlled during irradiation and non-irradiation conditions according to the requirements of the plants. A comparative study can be carried out between acute and chronic irradiation in such phytotrons. In the future, the heavy application of the chronic gamma phytotron will be extended to a wide range of crop plants for mutation research.

3.3. Gamma Rays

Gamma rays are the most favored physical mutagen by mutation breeders and are extensively used in crop improvement programs (Çelik and Atak, 2017). Gamma rays are ionizing rays with superb penetration power and energy levels ranging from ten kiloelectron volts (keV) to several hundred keV. When rays pass through tissue, ionization and excitation are created that affect the DNA and as a result, the chemical bonds of the bases as

well as the backbone of the DNA molecules rupture. Secondly, ionizing rays produce free radicals (H, OH are free radicals) from water. The free radicals attack the constituents of DNA, more vigorously in the presence of oxygen. Cobalt-60 (^{60}Co) and caesium-137 (^{137}Cs) are the major sources of gamma rays used in biological studies. The absorption of gamma rays and their impact on biological material are greatly influenced by species, varieties, plant age, genetic organizations and degree of irradiation (Çelik and Atak, 2017). Stimulatory, moderate, and damaging effects on plant growth are dependent on the dose employed and the duration of gamma rays and on the targeted crop. In their review, Han and Yu (2010) showed that the rays predominantly target the genetic material for the biological effects and lead to various alterations in structural and functional properties of DNA molecules such as substitutions, deletions and gross chromosomal abnormalities. These alterations are attributed to the appearance of macroscopic phenotypic variations (van Harten, 1998; Predieri, 2001; Oladosu *et al*., 2015) due to the gamma rays having higher penetrating power that can cause substantial damage on interacting with the tissues. During the process of irradiation treatment of the biological matter, these high-energy rays collide with atoms and emit electrons leaving positively charged ions or free radicals (van Harten, 1998). Reisz *et al.* (2014) reported that the gamma rays interact with cellular water quickly and produce positively charged free radicals such as reactive oxygen species (ROS), ionized water (H_2O+) hydroxyl radicals (·OH), as well as the reactive nitrogen species (RNS). In general, a "core" of ions is formed along the path of each high-energy ray as it passes through living tissues. The interaction between gamma rays and atoms or molecules of the biological material can be direct or indirect. In direct action, DNA is hit by the rays, thereby disrupting the molecular/genome structure, while in indirect action, the rays hit the water and cause radiolysis of water that eventually results in the generation of free radicals (Limoli *et al*., 2001; Desouky *et al*., 2015; Çelik and Atak, 2017). Saha (2013) reported that radiation generally causes damage via an indirect mode of action as water constitutes 70 per cent of the cell. Induced reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide alter the deoxyribose ring and bases, DNA-DNA, and DNA-protein crosslinks (Çelik and Atak, 2017). These alterations in the genetic material modify almost all vital structural and functional biomolecules such as lipids and proteins, these alterations eventually affect diverse morphological, anatomical, biochemical, developmental and physiological processes of crop species (Kebeish *et al*., 2015). In addition to the ROS generation, gamma rays also result in the formation of reactive nitrogen species (RNS)

and other species that may further enhance the cellular damage (Reisz *et al*., 2014; Wardman, 2009). Gamma rays have been the most successful mutagenic agent in inducing a broad spectrum of mutations in mustard (Javed *et al*., 2000), *Trigonella* (Parveen *et al*., 2006), chrysanthemum (Momin *et al*., 2012), chickpea (Raina *et al*., 2017), cowpea (Thimmaiah *et al*., 1998; Abu *et al.,* 2006; Badr *et al*., 2014), fenugreek (Hassan *et al*., 2018), black cumin (Amin *et al*., 2016, 2019; Tantray *et al*., 2017), faba bean (Khursheed *et al*., 2017; 2018b, c), mung bean (Wani *et al*., 2017) and lentil (Laskar *et al*., 2018a,b, Haneef *et al*., 2013), black cumin (Amin *et al*., 2016, 2019), pea (Shahab *et al*., 2018a,b), Coriander (Jafri *et al*., 2013), and *Capsicum* **(**Gulfishan *et al*., 2011).

4. Chemical Mutagenesis

In the beginning, mutation breeding was primarily based on physical mutagens. However, the continuously escalating level of knowledge in mutation breeding increased due to the discovery of chemicals with mutagenic potency. Auerbach and Robson (1942) were the first to provide a detailed account of chemical mutagens. They reported that mustard gas can cause mutations as well as chromosomal breaks in fruit flies. Since then several of these chemicals, for example, ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethylene imine (EI), diethyl sulfate (dES), N-nitroso-N-methylurea (NMU), N-nitroso-N-ethylurea (NEU), sodium azide (NaN_3), and hydrazine hydrate (HZ) among others, were reported to possess mutagenic efficiency on a level with physical mutagens (Sander and Muehlbauer, 1977). Chemical mutagens are easy to handle, readily available, less expensive, cost effective, more efficient and harbor more specificity than physical mutagens (Kharkwal, 1998; Khursheed *et al*., 2018; Hassan *et al*., 2018, Gulfishan *et al*., 2012a, b, 2013, Jafri *et al*., 2011). However, the chemical mutagens act as strong carcinogens and hence proper care is required at all steps of mutagenic treatment. Research on mutagenesis in crops has revealed that chemical mutagens are more advantageous than ionizing radiations due to milder effects on genetic constituents as compared to ionizing radiations which induce chromosome breaks. Rapoport (1966) reported an immensely increasing number of chemical mutagens were gaining applicability in crop improvement programs. Up to now, chemical mutagens have been successful for the development and official release of more than 390 mutant varieties (Table 1.5). Employing chemical mutagenesis in his research, Rapoport has made a remarkable contribution by conceptualizing the term “microgenetics,” which provides information about gene structure

and function, mode of action of the mutagen and mutation, mutation origin and their fixation in the progeny.

Even though there are quite a few unanswered questions on the subject of the action of mutagens, a more inclusive description of them has been provided by Sharma in 1985 (Table 1.4). Based on mutagenic action, several mutagens are classified as alkylating agents due to their ability to alkylate various sites of the genetic material (Table 1.4). EMS, MMS, dES, NMU, NEU are some of the widely used alkylating agents. Replacement of hydrogen in the nitrogenous bases with that of the alkyl group of the mutagen is referred to as alkylation (Sharma and Chopra, 1994). Ashburner (1989) and Sharma and Chopra (1994) reported the following effects of alkylation.

1. **Alkylation of the DNA phosphate groups**: Alkylation results in the formation of highly unstable phosphate tri-esters and tends to liberate the alkyl group. Nevertheless, several alkyl groups remain bonded to the phosphate groups and obstruct the process of DNA replication. Sometimes the DNA backbone gets broken down due to the hydrolysis of phosphate tri-ester bonds.

2. **Alkylation of bases:** The most preferred site for alkylation is the seventh position in the guanine, but it has been well-known that the main mutagenic effects take place due to alkylation at the sixth position of guanine, i.e., O^6 alkyl-guanine. O^6 alkyl-guanine can then enhance the frequency of transition as the O^6 alkyl-guanine can pair with thymine.

3. **Depurination:** The separation of alkylated guanine from the DNA sugar-phosphate backbone resulting in an error-prone gap leading to transitions or transversions.

EMS is among the frequently used chemical mutagens due to its higher effectiveness and efficiency and ability to induce a higher frequency and a broader spectrum of mutations. The mutagenic action of EMS was investigated previously in *Drosophila* (Fahmy and Fahmy, 1957), bacteriophages (Loveless, 1959), *Escherichia coli* (Strauss, 1964), barley (Jafri *et al*., 2012) and *Arabidopsis* (Greene *et al*., 2003).

Gruszka *et al*. (2012) reported that sodium azide (NaN_3) is a potent mutagen in microorganisms and a very efficient mutagen in cereals and pulses. Sodium azide (SA) has been documented as an effective mutagen in black gram (Misra, 1995) and lentil (Gaikwad and Kothekar, 2004). The first report on the mutagenicity of SA was documented by Wyss *et al*.

(1948) while studying the role of peroxides in rays-induced mutagenesis. Berger *et al.* (1953) noticed that sodium azide enhanced the frequency of streptomycin and penicillin-resistant *Staphylococcus aureus* mutants. They reported that this mutagenicity was attributed to SA induced inhibition of catalase and peroxidase that led to the build-up of hydrogen peroxide (H_2O_2). The H_2O_2 was supposed to be the actual mutagen. Meanwhile (Spence, 1965), inadvertently discovered SA mutagenicity in barley. Again, the azide induced inhibition of peroxidase and catalase was supposed to be the result of elevated peroxide concentration and, hence, the mutagenic effect. Ando and Neto (1979) and Hasegawa and Inoue (1980) have reported that SA can induce a higher frequency of mutation in different crops.

Studies on the genotoxicity of SA in different organisms confirmed the induction of gene mutation, AT→GC base pair transition and transversion (Khan *et al*., 2009; Gruszka *et al*., 2012), chromosome aberrations (Gruszka *et al*., 2012), and DNA damage (Veleminsky *et al*., 1987). The underlying mechanism of sodium azide mutation occurs through the production of β-azidoalanine moiety [N3–CH2–CH(_NH2)–COOH], an organic metabolite first recognized in bacteria and barley, as an amino acid analog L-azidoalanine (Gruszka *et al.,* 2012). The mutagenic effect of SA requires acidic pH of the treatment solution. A free amino acid group is vital for mutagenic activity in comparison to the carboxyl group (Nilan *et al*., 1973; Szarejko *et al*., 2017). The β-azidoalanine interacts with genetic material and induces point mutation in the genome (Khan *et al*., 2009). SA tends to reduce the level of a cellular calcium-binding protein, calmodulin (Osborn and Weber, 1980) thereby affecting signal transduction and cell division. It also acts as an inhibitor of the proton pump (Kleinhofs *et al*., 1978) that stops secretion and accumulation of cAMP in the cell (Dinauer *et al*., 1980). Being an effective and efficient chemical mutagen, it affects diverse developmental, physiological and metabolic activities in plants.

The early assessments on the toxicity of sodium azide for different plant species such as *Hordeum vulgare* (Conger, 1973), *Arabidopsis thaliana* (Gichner and Velemínský, 1977), *Oryza sativa* (Awan *et al*., 1980), *Allium cepa* (Adegoke, 1984), *Linum usitatssimum,* (Bertagne-Sagnard *et al*., 1996), *Vigna radiata* (Khan *et al.,* 2004a), *Striga hermonthica,* (Kiruki *et al.,* 2006), *Zea mays* (He *et al.,* 2009), *Eruca sativa* (Al-Qurainy, 2009), and *Vigna unguiculata* (Mensah *et al.,* 2005) have documented suppression of germination, foliar dehydration without chlorosis and necrosis, withered leaves and death at different concentrations of SA. Awan *et al.* (1980) reported a dose-dependent decrease in M_1 germination

rate and seedling height in *Oryza sativa*. The decrease in seed size, seed germination and induction of stunted and deformed plants was reported by Sander and Muehlbauer (1977), in *Pisum sativa*, Khan and Shoukat (1987), in *Vigna radiata,* Mahna *et al.* (1989), in *Vigna mungo,* Conger and Carabia (1977), in *Zea mays*, and Srivastava *et al.* (2011) in *Triticum aestivum*. Prina and Favret (1983), in *Hordeum vulgare* reported the chlorophyll mutation and ovule sterility after sodium azide treatment. Mensah and Obadoni (2007) have reported that higher sodium azide doses induced a reduction in plant survival percent in *Arachis hypogea*. The morphological aberrations induced by sodium azide such as deformed leaf forms and puffy and/or short internodes in *Arachis hypogea* have been reported by Mensah and Obadoni (2007) having a linear relationship with increasing concentration of sodium azide.

A wide range of morphological, physiological, cytological, agronomical and color mutants induced by sodium azide have been reported in several crops viz., lentil (Ali *et al*., 2014), wheat (Srivastava *et al*., 2011), groundnut (Mensah and Obadoni, 2007), common bean (Silva and Barbosa, 1996), sorghum (Seetharami-Reddi and Prabhakar, 1983), barley (Vagera *et al.,* 2004), nightshade (Kopecky and Vagera, 2005), rice (Hasegawa and Inoue, 1980b), tomato (Adamu and Aliyu, 2007), faba bean (Gulfishan *et al*., 2010), garlic (Mahajan *et al*., 2015), calendula (El-Nashar and Asrar, 2016), chickpea (Raina *et al*., 2017) and wheat (Dubey *et al*., 2017). Kleinhofs *et al*. (1978) reported that sodium azide induced a high frequency of mutations and less biological damage makes SA a predominantly efficient mutagen for breeding programs (Salvi *et al*., 2014).

There is definite evidence about the mutagenic action of hydrazine in both prokaryotes and eukaryotes. It was sometimes classified mainly as an inactivating agent with weak mutagenic activity (Fishbein *et al*., 1970), but studies with bacterial species advocate that it can be an effective mutagen with slight toxic effect. A useful review of the previous work with particular emphasis on the chemical basis for mutagenesis of hydrazine was given by Brown *et al*. (1966). Hydrazine was reported to result in a wide range of morphological, chlorophyll, yield, physiological, and color mutants in various crop plants such as chickpea (Khan *et al.,* 2005) and mung bean (Wani *et al*., 2011b, c, 2017). In general, hydrazine in these studies appeared to be as successful as other potent mutagens. However, it appeared to differ in two ways:

1. High frequency of mutations in M_1 generation as compared to other mutagens.

2. Hydrazine reacts with the cytosine to form an altered base i.e., N^4-amino-cytosine and also causes depyrimidation via the formation of H_2O_2 (Kimball, 1977).

The chemical mutagens induce a wide range of damage in both somatic and germline cells, however, after mutagenic treatment, most of the damage in germline cells recover through repair mechanisms and only a few mutations remain undetected and get fixed in the subsequent generations. The other mutations that occur in somatic cells such as mitotic chromosomal anomalies, anomalies of cytosolic components, changes in plant growth and development are termed as the somatic effect of mutagen. The general steps involved in chemical mutagenesis are pre-soaking in distilled water for about 9 hours, mutagen treatment for about 6 hours, rinsing in tap water to remove excess mutagen adhered to the surface and immediate sowing. Pre-soaking duration varies from species to species depending on the biology of germination. For instance, 8–10 hours and 9 hours of pre-soaking are required for cereals and pulses, respectively. For the dose and duration of mutagen laboratory temperature is included in the term "dose" in chemical mutagenesis. A temperature of a mutagenic solution of 22–24 °C is most often applied for the seed treatment of various crop species. The enhanced temperature will substantially reduce the half-life of the chemical mutagen and create hydrolysis products that can enhance the undesired somatic effect of a mutagen as observed with alkylating mutagens. To ensure uniform mutagen penetration through the cells of a seed embryo, it is imperative to treat seeds in a water solution of the mutagen for 3–6h. The dose of the mutagen has direct correlation with the duration of the treatment. A shorter duration treatment with a higher dose of mutagen can enhance somatic effects and could be insufficient to penetrate uniformly through all cells in the plant material. Longer treatment duration and a lower dose are advisable to obtain a wide spectrum and higher frequency of mutations.

5. Molecular Mechanisms of Mutagenesis

5.1. DNA Damage

DNA is a double-helical structure made up of paired heterocyclic nitrogenous purine and pyrimidine bases attached to a backbone of deoxyribose and phosphoric acid. The purine bases (guanine and adenine)

and pyrimidine bases (cytosine and thymine) are complementarity in base pairing under normal conditions (guanine/cytosine and adenine/thymine) stabilized by hydrogen bonding. The specificity of base pairing and sequence of bases along the DNA double helix forms the foundation of the genetic code, and any modification results in mutations manifested as alterations in gene expression or protein structure and function.

5.2. Spontaneous Damage

The instability of a variety of parts of the DNA molecule in aqueous solution results in spontaneous damage to DNA and leads to the induction of mutations. In addition to base deamination, the bonds between purines or pyrimidines and deoxyribose can spontaneously hydrolyze, forming purinic or pyrimidinic (AP) sites, leaving the sugar-phosphate backbone susceptible to strand breakage. Also, the bases can exist in several (tautomeric) forms, which can lead to mispairing by altered hydrogen bonding characteristics.

5.3. Chemical Adducts

Numerous dietary and environmental factors that react with various reactive sites within the DNA structure can lead to the induction of mutations. Several chemical mutagens act as electrophiles and are very reactive, electron-deficient species able to form covalent adducts with nucleophilic sites within DNA. Binding of chemical adducts to the DNA bases can stabilize tautomeric forms and/or alter their structural and hydrogen bonding characteristics. These modifications alter the standard base-pairing rules and induce mismatches during replication and transcription and enhance increased base hydrolysis and AP site formation. Also, some chemical agents produce inter and intra-strand cross-linking of DNA, which impedes strand separation and imposes a barrier to the replication, transcription, and repair processes.

5.4. Oxidative Damage

Some mutagens work by causing oxidative stress and induce oxygen or nitrogen radical species such as hydroperoxide, hydroxyl and superoxide. Transition metal ions such as iron and copper can catalyze free radical formation (the Fenton reaction). The free radicals interact with DNA and lead to single- and double-strand breaks, hydroxylated derivatives of bases, and several other lesions in DNA.

5.5. DNA Intercalation

Some mutagens alter the double-helical structure by intercalating between the DNA bases especially the GC pair, thereby hampering hydrogen bonding between base pairs. The presence of such compounds causes misreading during replication and transcription, leading to the generation of mutations.

5.6. Conclusions

The traditional plant breeding approaches are time-consuming while induced mutagenesis serves as a coherent tool and is widely employed in crop improvement programs to enhance crop quality and quantitative traits. Induced mutagenesis is much easier to apply on crops and a cost-effective way to create better agro-economical traits, higher yields and stress resistance in several crops.

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Table 1.1. Number of mutant varieties developed in cereals and legumes. Data source: FAO mutant variety database - 2018.

	Number of mutants released
Cereals	
Avena sativa (Oats)	23
Hordium vulgare (Barley)	309
Oryza sativa (Rice)	821
Secale cereal (Rye)	4
Triticum aestivum (Bread wheat)	255
Triticum turgidum (Durum wheat)	31
Zea mays (Maize)	96
Total	**1539**
Legumes	
Arachis hypogea (Groundnut)	76
Cajanus cajanus (Pigeon pea)	7
Cicer arietinum (Chickpea)	27
Dolichus lablab (Hyacinth bean)	1
Lathyrus sativus (Grass pea)	3
Lens culinaris (Lentil)	18
Glycine max (Soybean)	173
Phaseolus vulgaris (French bean)	59
Pisum sativum (Pea)	34
Trifolium alexadrinum (Egyptian clover)	1
T. incarnatum (Crimson clover)	1
T. pratenus (Red clover)	1
T. subterraneum (Subterranean clover)	1
Vicia faba (Faba bean)	20
Vigna angularis (Azuki bean)	3
V. mungo (Black gram)	9
V. radiata (Mung bean)	39
V. unguiculata (Cowpea)	13
Total	**486**

Table 1.2 Characteristics of physical mutagens.

Mutagen	Characteristics
X-rays	Electromagnetic radiation, ionizing, penetrate tissues from a few millimeters to many centimeters
Gamma rays	Electromagnetic radiation, ionizing, very penetrating into tissues; sources are ^{60}Co and ^{137}Ce
Neutrons	Uncharged particles; penetrate tissues to many centimeters; source is ^{235}U
Beta particles	Negatively charged electrons; ionize; shallowly penetrating; sources ^{32}P and C
Alpha particles	A helium nucleus capable of heavy ionization; very shallowly penetrating
Protons	Positively charged particles; penetrate tissues up to several centimeters

Table 1.3. Number of mutant varieties developed by physical mutagens. Data source: FAO mutant variety database - 2018.

Physical mutagens	No. of mutants
Gamma rays	163
X-rays	561
Gamma rays chronic	89
Fast Neutrons	54
Thermal Neutrons	50
Aerospace	46
Laser	26
Neutrons	24
Ion beams	17
Beta rays	16
Gamma and X-rays recurrent	9
Soft X-rays	6
X-rays or fN	5
Carbon ion beams	3
Accelerated aging	3
Gamma rays and microwave	3
32P	2
Electrons	2
32P and Gamma rays	1
Gamma rays and mixed neutrons	1
Gamma rays and neutrons	1
Gamma rays recurrent	1
protons	1
ultrasound	1
UV-rays	1
Gamma rays+ fN	1
Chronic X-rays	1
Total	**2555**

Table 1.4: Chemical mutagen types and their mode of action. Source: Redrawn from van Harten, 1998.

Mutagen Group	Examples	Mode of Action
Base analogs	5-bromouracil, 5-bromodeoxyuridine, 2-aminopurine (2AP)	Incorporates into DNA in place of the normal bases during DNA replication thereby causing transitions and tautomerization. This eventually leads to deletion, addition, frame shift.
Alkylating agents:		They react with bases and add methyl or ethyl groups and, depending on the affected atom, the alkylated base may then degrade to yield a baseless site, which is mutagenic and recombinogenic, or miss pair to result in replication.
Sulfur mustards	Ethyl–2-chloroethyl sulfide	
Nitrogen mustards	2-chloroethyl-dimethyl amine	
Epoxides	Ethylene oxide	
Ethyleneimines	Ethyleneimine	
Sulfonates, etc	Ethyl methanesulfonate (EMS),	
	diethylsulfonate (DES)	
Diazoalanes	Diazomethane	
Nitroso compounds	N-ethyl-N-nitroso urea	
Azide	Sodium azide	Production of an organic metabolite, β-azidoalanine moiety. This organic metabolite enters into the nucleus, interacts with DNA and induces point mutation in the genome.
Hydroxylamine	Hydroxylamine	Hydrazine reacts with the cytosine and thymine in DNA to saturate the 5, 6 double bond, particularly of thymine, to form N4 -amino-cytosine and to open up the pyrimidine ring with consequent loss of pyrimidines from DNA or via intermediate radical reactions including the formation of hydrogen peroxide

Nitrous acid	Nitrous acid	Acts through deamination, the replacement of cytosine by uracil which can pair with adenine and thus from subsequent cycles of replication leading to transitions such as AT→ GC and GC→ AT
Intercalating agents	ethidium bromide, acridine orange, proflavine and daunorubicin	They insert between bases of DNA thereby causing a "stretching" of the DNA duplex and the DNA polymerase in turn recognizes this stretch as an additional base and inserts an extra base opposite this stretched (intercalated) molecule. This results in frameshifts i.e., an alteration of the reading frame since codons are groups of three nucleotides.
Miscellaneous group of agents; large molecules referred to as "bulky" lesions	N-acetoxy-N-2-acetyl-aminofluorine (NAAAF)	They bind to bases in DNA and cause them to be noncoding thereby preventing transcription and DNA replication; They cause intra- and inter-strand crosslinks (e.g., Psoralens); They also cause DNA strand breaks (e.g., peroxides).
Antibiotics	Actinomycin D, mitomycin C, streptonigrin	Chromosome breaking
Related compounds	Maleic hydrazide (MH), 8-ethoxy caffeine	Chromosome breaking

Table 1.5: Number of mutant varieties developed by chemical mutagens. Data source: FAO mutant variety database - 2018.

Chemical mutagens	No. of mutants
EMS	106
NEU	57
Colchicine	46
NMU	46
EI	36
DMS	19
dES	14
NaN3	11
MNU	7
MNH	6
NENG	5
Tissue culture induced	4
NMU+dioxane	3
Arsenic-q	2
NMU +NEU	2
NMU+ DAB +dioxane	2
DES+PYM	2
PMS	2
NMH	2
Ethyleneoxide	2
AA+DAB	1
BEO(Beryllium Oxide)	1
Bromodeoxyuridine	1
DMS+DAB+dioxane	1
DMSO	1
Diazoacetylbutane	1
EMS+DAB	1
ENH	1
Extract of Datura seeds	1
NDEU	1
NDMU	1
NEU+dioxane	1
NEU+NMU+DES+DMS+EI	1
NMU+EI+DMS	1
NTMU	1
NMU and EI	1
Sodium azide	1
Total	392

CHAPTER 2

PLANT MUTAGENESIS: PRINCIPLE AND APPLICATION IN CROP IMPROVEMENT

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Abstract: A mutation is a sudden occurrence of a heritable alteration in the genomic makeup of an organism, which acts as an indispensable evolutionary force in nature. Induced mutagenesis plays a vital role in creating crop varieties with improved traits. Spontaneous mutation occurs due to replication errors, tautomeric shifts, exposure of an organism to certain radiations and chemicals, however, the frequency of these mutations is very low and hence insufficient to fulfil the demands of the present situation. This low frequency of spontaneous mutations necessitates the use of artificial mutagenic agents to enhance the frequency of mutations. A wide range of mutagenic agents has been employed for the development and release of mutant varieties. In the world, more than 3278 mutant varieties in 261 plant species have been developed, with improved traits such as yield, adaptability, disease resistance, quality and tolerance to abiotic stresses. Mutation breeding has played a key role in improving one or two traits without altering the entire genetic constitution of the crop

species. This chapter provides detailed aspects of present and future prospects of mutation breeding and highlights the role of mutation breeding, its impact, challenges and landmark achievements. The various mutagens such as physical, chemical, and biological, their effects, mode of action and contribution in the development of mutant varieties are described. Additionally, standard procedures are described for the optimization of mutagenesis and subsequent handling of mutated materials.

Keywords: mutation breeding, mutagens, improved traits, mutant varieties.

2.1 Background of Mutation Breeding

The ancient book "Lulan" mentioned the existence of spontaneous cereal mutants in China around 300 BC (van Harten, 1998). The concept of the mutation using induced mutations in plant breeding programs to create novel varieties was reported by Hugo de Vries (1901), but its practical significance was subsequently demonstrated by Stadler (1928) in barley and Goodspeed (1929) in *Datura* and *Nicotiana*. A definite authentication that X-rays can induce mutations in *Drosophila* and maize was advocated by Muller (1927) and Stadler (1928) respectively and concluded that it was possible to enhance the rate of spontaneous mutations through irradiation. Similar results have been reported by Ganger and Blakeslee (1927) in *Datura stramonium* and Goodspeed (1929) in *Nicotiana.* The first example was a mutant of *Nicotiana tabacum* called "Chlorina" that was generated through the treatment of floral buds with X-ray irradiations in the 1930s (Tollenaar, 1934; 1938; Konzak, 1957; Coolhaas, 1952). However, Gustafsson (1947) was among the pioneers to report the utility of mutations for the genetic improvement of various crop plants. Thereafter, with the development of international coordination and availability of some financial assistance by the FAO/IAEA to the research workers/organizations, systematic work started on mutation breeding in various crops. Many workers supplemented comprehensive knowledge on the function and applicability of induced mutations for the improvement of genetic resources in several crop plants across the globe (Gaul, 1965; Brock, 1965; Kharkwal, 1996; Rajput *et al.*, 2001; Chhun *et al.*, 2003; Ilbas *et al.*, 2005; Wani and Khan, 2006; Bhat *et al.*, 2006a,b,c,d; Talame *et al.*, 2008; Toker, 2009; Goyal and Khan, 2010a; Nakagawa *et al.*, 2011; Kozgar *et al.*, 2014a; Amin *et al.*, 2016; Khursheed *et al.*, 2018a; Oladosu *et al.*, 2015; Mondal *et al.*, 2017; Raina *et al.*, 2017). Micke (1995) reported that the continued efforts of numerous workers over the years

ultimately converted the randomness of the induced mutation into a targeted event for specific economic benefits. Widespread use of induced mutations in crop improvement programs has led to the official release of 3275 mutant varieties in 170 different plant species worldwide. This advantage of mutation breeding has been exploited in both cereals and legumes, as is evident from the list of mutant cultivars developed in legumes and cereals (Table 2.1, Figs. 2.3–5).

2.2 Introduction

Induced mutations have led to the development of varieties with high genetic variability, good quality and high yield, thus enhancing agronomic inputs and wide farmer acceptance **(**Bhat *et al.*, 2007; Sharma *et al.*, 2009; Ahloowalia *et al.,* 2004; Javed *et al.*, 2016; Khursheed *et al.*, 2016; Lakar *et al.*, 2018a). Mutations may occur spontaneously in natural populations without any prior treatment or can be induced artificially with the help of mutagenic agents. The underlying mechanisms of mutations have been attributed to alteration in the double-helical structure of DNA, deletion, addition or substitution of one or more bases in one DNA strand and inversion of a sequence of base pairs within the DNA molecule (Acquaah, 2012). The frequency of spontaneous mutations is very low, therefore, insufficient to meet the human need for speedy improvements in crop plants. Due to the slow pace of spontaneous mutations, plant breeders are forced to induce mutations by artificial means. Various reports suggest that the mutation frequency can be increased greatly by mutagen treatments. The primary strategy in mutation-based breeding has been to improve a single trait of an otherwise outstanding crop variety with higher yield and wide adaptability. Several mutants of economic significance and also of academic interest have been identified and isolated in different pulse crops by various workers (Maluszynski *et al.,* 1995; Bhat *et al.*, 2005a; Bhat *et al.*, 2007b; Bhat *et al.*, 2005b; Dixit *et al.*, 2000; Shu and Lagoda, 2007; Sestili *et al.*, 2010; Tomlekova, 2010; Haidar and Aslam, 2016; Raina *et al.*, 2017; Laskar *et al.*, 2015 Taziun *et al.*, 2017; Khursheed *et al.*, 2019). Mutation breeding offers the unpredictable possibility of inducing desired attributes in the crop that either cannot be found in nature or have been lost during evolution and also supplements the conventional methods of crop improvement favourably by upgrading a specific character without altering the original genetic makeup of the variety (Gottschalk, 1986; Novak and Brunner, 1992; Toker *et al.*, 2007; Bhat *et al.*, 2007a; Bhat *et al.*, 2007b). The basic components of mutation breeding are (a) mutation induction (b) mutation detection and (c) mutant

testing and official release. Mutation induction is carried out by exposing biological material to mutagens. Mutation induction is quick, taking minutes or a few hours while mutation detection takes a few months or even years, but this could be accelerated through high-throughput screens. Mutant release, on average, takes about ten years but can be accelerated using marker-assisted selection and other emerging biotechnologies (Joung and Sander, 2013; Zheng *et al.*, 2013). Gaul (1964) classified mutations phenotypically into two groups:

- Macromutations—A macromutation is a mutation phenotypically visible and morphologically distinct that produces a phenotype well outside the range of variation previously existing in the population.
- Micromutations—A mutation with a small effect that can be detected only by the help of statistical analysis such as character mean, variance, heritability etc. The majority of such mutations are in polygenic traits, they are of the greatest value to plant breeders since most of the economically useful traits are polygenically controlled.

The importance of micromutations for increasing polygenic variability increased after Brock (1965) proposed the hypothesis of induction of quantitative variability through mutagen treatment. Micromutation produces genetic variability in quantitative traits of the crop plants and therefore deserves the full attention of plant breeders. Such mutations might be useful for improving quantitatively inherited traits (e.g. seed yield) without disturbing the overall genomic makeup of crops for isolating the best mutants with intact basic phenotypic architecture. Globally, continued efforts are ongoing to evaluate mutagen induced genetic variability in quantitative traits across a wide range of pulse crops. Over the years, induced mutagenesis has been advocated as a valuable tool for plant breeding to circumvent the genetic bottleneck of cultivated germplasm and create novel allelic combinations for agro-economic trait(s) (Maluszynski *et al.,* 1995; Micke, 1999; Branch, 2002; Canci *et al.*, 2004; Toker, 2009; Tomlekova *et al.*, 2009; Kumar *et al.*, 2010; Tomlekova, 2010; Ganai *et al.*, 2005; Bhat and Wani, 2017a; Nakagawa *et al.*, 2011; Ranalli, 2012; Wani *et al.*, 2014a; Amin *et al.*, 2016; Xiong *et al.*, 2016; Raina *et al.*, 2017; Gulfishan *et al.*, 2012; Khursheed *et al.*, 2018c) which facilitates the selection of improved mutant varieties through mutation breeding. The scarcity in genetic variability represents a limitation for cowpea breeding programs. The availability of genetic variability in the traits of interest is the prerequisite for any crop improvement program. Micke (1999) suggested that the mutation

approach is superior to other methods of crop improvement as it can generate a good amount of genetic variability in a short period. Unlike the conventional approaches of breeding, such as hybridization and backcrossing, mutation breeding is very quick in broadening the genetic base of the target crop (Gulfishan *et al.*, 2010; Gulfishan *et al.*, 2013; Tomlekova *et al.*, 2016). In addition to mutation breeding, there are other options such as transposon or T-DNA insertional mutagenesis for creating genetic variability. However, these approaches lead to complete disruption of gene function, thereby hampering the chances of obtaining desirable traits (Chopra, 2005; Parry *et al.*, 2009). Also, the insertion sites may not be distributed randomly within the genome (Zhang *et al.*, 2007), hence increasing the number of insertion lines required for full genome coverage to an unrealistic level (Parry *et al.*, 2009). Conversely, mutation breeding provides a number of advantages over modern breeding approaches such as the random mutations induced by mutagens throughout the genomes that would generate a wide range of mutations in all target genes (Toker *et al.*, 2007; Parry *et al.*, 2009; Tomlekova, 2010).

In addition to crop improvement, induced mutations are coherent tools to study the nature and function of genes that regulate diverse developmental processes (Adamu and Aliyu, 2007). The great advantage of mutation breeding is the scope it has of improving only one or two traits without affecting the entire genetic constitution of crop plants (Gulfishan *et al.*, 2011; Khan *et al.*, 2009; Shu *et al.*, 2012). Many economically important varieties have been developed and released through mutation breeding (Parveen *et al.*, 2006; Ahloowalia *et al.*, 2004; Khan *et al.*, 2009; Raina *et al.*, 2018). In the genetic improvement of crops, induced mutations have shown promising results and offer a great potential to serve as a complementary approach (Gulfishan *et al.*, 2015; Mahandjiev *et al.*, 2001). There have been a great number of mutant varieties developed around the world in the last few decades (Figs. 2.1 and 2.2), which are playing a vital role in the agricultural system and food production.

2.3 Mutagens

Mutagenic agents are capable of altering the genetic material of an organism and thus increasing the frequency of mutations above the natural background level. Based on characteristics, mode of action and type of mutation induced, the mutagenic agents are broadly classified into physical (Table 2.1) and chemical (Table 2.2) mutagens. Physical and chemical mutagens have been successfully employed in mutation breeding

programs to artificially generate variations for the development of new varieties with improved traits, such as higher yield, reduced plant height and resistance to disease (Reddy and Annadurai, 1992; Verma *et al*., 1999; Khan *et al.,* 2005a; Khan *et al.,* 2006a; Bhat *et al.,* 2007; Talame *et al.,* 2008; Mohan and Mathur, 2015; Laskar *et al.,* 2015; Bhat and Wani, 2017a; Bhat and Wani 2017b). In the last few decades around the world, many mutant pulses and cereal varieties have been derived from the application of both physical and chemical mutagens (Table 2.3 and Table 2.4). Among the physical mutagens, gamma rays followed by X-rays have been most widely used to develop high yielding varieties in various crop plants. Gamma rays are high energy electromagnetic radiation that can ionize atoms in molecules with which they interact. Gamma rays interact with cellular water quickly and produce positively charged free radicals such as reactive oxygen species (ROS), ionized water (H_2O+), and hydroxyl radicals (·OH) as well as reactive nitrogen species (RNS) (Reisz *et al*., 2014). Gamma rays are the most favoured physical mutagen by mutation breeders and are extensively used in crop improvement programs (Çelik and Atak, 2017). Gamma rays are ionizing rays with superb penetration power and energy levels ranging from 10 kilo electron volts (keV) to several hundred keV. When rays pass through tissue, the result is ionization (ejection of electrons from molecules) and excitation (a process of raising electrons to a higher energy state) that affects the DNA. As a result of this, there is a rupture of chemical bonds of the bases as well as the backbone of DNA molecules. Secondly, ionizing rays produce free radicals (H and OH are free radicals) from water. Therefore, free radicals attack the constituents of DNA, more vigorously in the presence of oxygen. Cobalt-60 (Co-60) and cesium-137 (Cs-137) are the major sources of gamma rays used in biological studies. Cesium-137 is more favoured since its half-life is longer than that of cobalt-60. Gamma ray absorption and the resulting impact on biological material are greatly influenced by species, variety, plant age, physiology, and morphology of the plants, as well as their genetic organization and the degree of irradiation (Çelik and Atak, 2017). Stimulatory, moderate, and damaging effects on plant growth and development depend on the employed dose and duration of the gamma rays and the targeted crop. The Han and Yu (2010) review showed that the rays predominantly target the genetic material for the biological effects and lead to various alterations in the structural and functional properties of DNA molecules. The types of DNA modifications include base substitutions, base alterations, base deletions, and chromosomal abnormalities. These modifications are attributed to the appearance of macroscopic phenotypic variations (van Harten, 1998; Predieri, 2001;

Oladosu *et al.,* 2015) advocated that the gamma rays have higher penetrating power that can cause substantial damage on interaction with the tissues. During the process of irradiation treatment of the biological matter, these high energy rays collide with atoms and emit electrons, leaving positively charged ions or free radicals (van Harten, 1998). Reisz *et al*., (2014) reported that the gamma rays interact with cellular water quickly and produce positively charged free radicals such as reactive oxygen species (ROS), ionized water (H_2O+) and hydroxyl radicals ($^{\cdot}OH$), as well as the reactive nitrogen species (RNS). In general, a "core" of ions is formed along the path of each high energy ray as it passes through living tissues. The interaction between gamma rays and atoms or molecules of the biological material can be direct or indirect. In direct action, DNA is hit by the rays, thereby disrupting the molecular/genome structure, while in indirect action, the rays hit the water and cause radiolysis of water that eventually results in the generation of free radicals (Limoli *et al.,* 2001; Desouky *et al*., 2015; Çelik and Atak, 2017). Saha (2013) reported that the majority of rays induced damage results from the indirect mode of action because water constitutes nearly 70 percent of the cell. Induced reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide can cause changes in the deoxyribose ring and the structures of bases, DNA-DNA cross-links, and DNA-protein cross-links (Çelik and Atak, 2017). These alterations in the genetic material modify almost all vital structural and functional biomolecules such as lipids and proteins, and eventually affect diverse morphological, anatomical, biochemical, developmental and physiological processes of crop species (Kebeish *et al*., 2015). In addition to the ROS generation, gamma rays also result in the formation of reactive nitrogen species (RNS) and other species that may further enhance the cellular damage (Reisz *et al.,* 2014; Wardman, 2009). Gamma rays have been the most successful mutagenic agent in inducing a broad spectrum of mutations in mustard (Javed *et al*., 2000), chrysanthemum (Momin *et al*., 2012), chickpea (Raina *et al*., 2017), cowpea (Thimmaiah *et al*., 1998; Abu *et al*., 2006; Badr *et al*., 2014; Raina *et al.,* 2018), faba bean (Khursheed *et al.,* 2015), fenugreek (Hassan *et al*., 2018), lentil (Laskar *et al.,* 2018a) black cumin (Amin *et al.,* 2019) and broad bean (Bhat and Wani, 2015).

Table 2.1 Physical mutagens and their mode of action

Physical mutagens (Radiation)	Mode of action
Ionizing radiation Non-particulate radiation (X-rays and gamma rays) Particulate radiations (α-rays, β-rays, thermal neutrons)	Breakage of hydrogen bond and a sugar phosphate moiety
Non-ionizing radiation (UV rays)	Formation of purine and pyrimidine dimers

Source: Toker *et al.,* 2007.

Initially, mutation breeding was based largely upon physical mutagens such as X-rays, gamma rays, thermal neutrons and radioisotopes of certain heavy elements, however, the discovery of chemicals with mutagenic potential was considered to be another milestone in the history of mutation breeding. Chemical mutagens have gained popularity since they are readily available, easy to use, do not require any specialized equipment and can induce a high frequency of mutations. Unlike physical mutagens, which induce gross chromosomal mutations, chemical mutagens tend to induce single-gene mutations, or single-nucleotide polymorphisms (SNPs) (Sikora *et al*., 2011). In *Drosophila,* iodide and copper sulphate chemicals were shown to possess mutagenic potential and the use of the chemical urethane during the Second World War were the initial demonstrations of chemical mutagenic activity (Donini and Sonnino, 1998). Indications about the likelihood of induction of mutations by the use of chemicals appeared within a decade after the discovery of this phenomenon. The first elaborated description of chemical mutagenesis was presented by Auerback and Robson (1942), who documented that mustard gas induces mutations as well as chromosomal breaks in *Drosophila*. A wide range of chemical mutagens, employed singly or in combination, successively or simultaneously with physical mutagens, are now known to induce mutations in various crop plants (Konzak *et al.*, 1965; Ahloowalia and Maluszynski, 2001; Saleem *et al.*, 2005; Encheva, 2009; Goyal and Khan,

2010b; Khan *et al*., 2011). The comprehensive accounts of Sharma (1985) and later by van Harten (1998), Micke (1999) and Kodym and Afza (2003) on the basic mutational process and mode of action of mutagens extensively contributed to our present knowledge of the subject. Application of induced mutagenesis on higher plants revealed that chemical mutagens have a much greater advantage over ionizing rays, owing to their relatively low cost, easy handling, milder effect and greater specificity (Auerbach, 1965; Handro, 1981; Salnikova, 1995; Wani *et al.,* 2014a). An overwhelming majority of strong chemical mutagens were discovered by Rapoport (1966) which have been widely used in genetic and breeding research programs. Rapoport has made a significant contribution to the theory, having developed the broader concept of "microgenetics," and provides a brief account of the structure and function of genes, mechanisms of mutagen action, their origin, and manifestation in the mutagenized population. The nature, fundamental properties, and mode of action of several physical and chemical mutagens have been reviewed by Kaul (1989). The conceptual knowledge of the fundamental aspects of mutational processes and the possible mechanism of action of several mutagens was widened with the reports and reviews of Gottschalk and Wolf (1983) and Kodym and Afza (2003). Even though there are a few unanswered queries regarding the underlying mechanism of mutagenic action, a more inclusive account of them has been given by Sharma (1985). The chemical mutagens can be further classified into three classes:

A) Alkylating agents: These include ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), ethyl ethanesulfonate (EES), ethylene imines, diethyl sulphate (DES), and nitroso compounds. Alkylating agents, the most widely used group of chemical mutagens, are broadly classified as monofunctional and bi- or polyfunctional ones, depending upon the number of alkyl groups present in the compound (Natarajan, 2005). Sharma and Chopra (1994) have defined mutagen induced alkylation as the replacement of an alkyl group (e.g., C_2H_5 of EMS; CH_3 of MMS) for hydrogen in the nitrogenous bases. These mutagens induce every kind of point mutations, transitions, transversions, deletions, and frameshifts and a comparatively low frequency of the chromosome breakages that cause several chromosomal rearrangements (Maluszynski *et al*., 2003). Ashburner (1990) and Sharma and Chopra 1994 have reviewed that the alkylation of DNA leads to the following effects:

1. **Alkylation of the phosphate groups of DNA:** Alkylation results in the formation of phosphate tri-esters which are very unstable and liberate the alkyl group. However, if adequate alkyl groups remain

unreleased, then the attached alkyl groups obstruct DNA duplication (Wani *et al.,* 2014a). At times the phosphate tri-ester bond between the sugar and the phosphate group gets hydrolyzed and results in the breakage of the DNA strand.

2. **Alkylation of bases:** The seventh position in the guanine (N^7) is a favoured site for alkylation, however, it has been well-known that the major mutagens alkylate O^6 position of guanine. O^6 alkyl-guanine then pairs with thymine and leads to AT→GC transition.
3. **Depurination:** The alkylated guanine can detach from the deoxyribose leaving behind a depurination site. The site may be filled up by any random base during DNA replication and result in transition or transversion type of mutation (Wani *et al.,* 2014a).

B) Base analogs: The base analogs such as 5-bromo uracil and 5-Bromo deoxyuridine have been shown to have mutagenic effects due to their close resemblance to some of the naturally occurring bases in the DNA molecule. For example, the natural base thymine (T), a 5-methyl uracil, has a structural analog, 5-Bromouracil (5-BU). The two bases are so similar in structure that 5-BU can substitute for T during DNA duplication, leading to transition type of mutation.

C) Miscellaneous mutagens: This group of mutagens comprises azides (sodium azide), antibiotics, acridines, nitrous acid and hydroxylamine.

Table 2.2 Chemical mutagens and their mode of action:

Chemical mutagens	**Mode of action**
Alkylating agents EMS, DES, NMU and ethylene amine	Alkylate phosphate groups and bases. Mispairing and loss of bases
Acridines Ethidium bromide, acridine orange and proflavin	Intercalate between bases and results in deletion and addition of bases
Base analogs 2-Amino purine, hypoxanthine	Base pair substitution
Nitrous acid Hydroxyl amine, sodium azide	Replacement of amino group with a hydroxyl group and conversion of cytosine to a modified base

Source: Toker *et al.,* 2007.

2.4 Mutagen Dose and Genotypic Sensitivity

The optimization of a mutagen dose before conducting field experiments is critical for successful mutagenic treatment. Simple B.O.D/pot tests for seedling emergence and growth reduction are routinely used to select a critical dose, i.e., a dose that induces 50 percent seedling growth reduction. Such a dose induces a very high degree of lethality of M_1 plants, and hence, for plant breeding research that is aimed at enhancing only one or two traits in an otherwise outstanding variety, doses of a mutagen that induce below 50 percent growth reduction should be employed. When high mutagen doses are applied high lethality in the M_1 population is likely to occur. Whereas, if low doses of mutagens are applied a low frequency of mutations will be induced (Brown and Caligari, 2011; Szarejko *et al.,* 2017). It is therefore imperative to use appropriate doses of mutagen for the desired results. Acquaah (2007) stated that the optimum dose rate of a mutagen can be determined with careful experimentation. According to Solanki and Waldia (1997) an optimum dose is the one that induces the highest frequency of mutations with the least degree of lethality. However, the optimum dose varies from plant to plant, plant part exposed to the mutagen and the physiological state of the plant and the frequency and spectrum of mutations differ depending on the mutagen employed and the dose applied. Polyploid species are slightly less affected by the mutagenic treatments as compared to diploid species, and thereby depicting that the optimum dose of mutagen is likely to vary in an individual crop (Wani *et al.,* 2014a). van Harten (1998) documented that it is recommended to carry out a "seedling growth test" with a range of doses to establish the optimal dose for a specific variety. To enhance the mutation rate and to broaden the spectrum of mutations, several different treatment procedures have been employed by different workers. Many workers have opined that a dose close to lethal dose 50 (LD50), a dose that would kill 50 percent of the treated individuals, should be considered optimum. The LD50 of a particular genotype varies to a great extent and this is attributed to the fact that the genetic constitution of an organism is a vital factor in determining the genotypic difference toward the mutagens.

Several workers have reported the inter-varietal differences in LD50 values and dose-dependent effectiveness of mutagens in different crops such as Khan (1988) in *Vigna mungo*, Kharkwal (1981a,b) in *Cicer arietinum*, Salim *et al.* (1974) in *Pisum sativum*, Singh and Chaturvedi (1980) and Khan and Wani (2004) in *Vigna radiata,* and Kamau *et al.* (2011) in *Lablab purpureus*. Since the sensitivity of different genotypes varies toward the same mutagen, as a result, the LD50 values of the

mutagen also vary greatly from one genotype to another (Sharma and Sharma, 1979; Khan, 1990). Khan *et al.* (1998), while studying the mutagenic effect of maleic hydrazide (MH) in two varieties of *Vigna radiata,* noticed inter-varietal differences, the var. PS-16 was found to be more sensitive than the var. K-851. Wani (2007) in *Vigna radiata,* Khursheed *et al.,* 2018b in *Vicia faba,* Laskar *et al.*, 2018b in *Lens culinaris* and Raina *et al.,* 2018 in *Vigna unguiculata* have also demonstrated the varied inter-varietal response toward mutagenic concentrations. Bykovets and Vasykiv (1971) carried out mutation studies in leguminous crops such as *Glycine max, Pisum sativum*, and *Lathyrus* with three chemical mutagens namely, NMU, NEU and DES. It was noticed that all the three crops were not equally mutable and that the most mutagenic effect appeared in *Pisum*, followed by *Glycine max* and *Lathyrus*. Even though the precise reasons for the differential sensitivity of genotypes toward mutagen doses are under speculation, quite a few probable explanations have been postulated from time to time. Akbar *et al.* (1976), while working on rice, concluded that the variations in radiosensitivity among rice varieties could be attributed to the difference in their recovery processes involving enzyme activity. Khamankar (1984) from his studies on tomato, considered that differences in the rate of mutations at certain loci with different physical and chemical mutagens was possibly because some of the gene loci were affected by one mutagen but not by the other. Thus, differential sensitivity of genotypes toward different types of mutagen doses needs to be explored for a better understanding of the mutational process.

The appropriate selection of not only mutagen and dose but also the variety exposed to mutagenic treatment is the key to obtaining success in mutation breeding programs. At least two varieties, well adapted to a range of environmental conditions should be considered for improvement through mutagenesis. Since the potency of a mutagen varies from variety to variety, it will allow comparative analysis and also since the recommended varieties have best-adapted characters, it will be useful to improve one or two specific traits. The effectiveness and efficiency of a chemical mutagen mainly depend on (i) concentration (ii) duration of treatment and (iii) temperature during treatment (Toker *et al*., 2007). Modifying factors are: (i) pre-soaking, (ii) pH of the solution, (iii) metallic ions, (iv) carrier agents, (v) subsequent washing of seeds (post-washing), (vi) post-drying and (vii) storage of treated seeds. The use of chemical mutagens requires several procedures such as (i) preparation of seeds, (ii) pre-soaking, (iii) mutagen treatment such as duration of treatment and temperature during treatment, (iv) post-washing and (v) post-drying.

Additionally, chemical mutagenic treatments require the proper disposal of leftover mutagen which can be very toxic (Toker *et al*., 2007). On the contrary, treatment with physical mutagens is a two-step process (i) preparation of seeds and (ii) mutagen treatment.

Table 2.3 Officially released some mutant varieties of cereals and pulses in the FAO/IAEA Mutant Varieties Database: (Chemical Mutagens, 2000–2019).

Common Name	Variety Name	Country	Date of Release
Barley	Gama	Ukraine	2000
Lentil	Binamasur-1	Bangladesh	2001
Rice	DT22	Viet Nam	2002
Groundnut	Mutant 28-2	India	2003
Mungbean	Binamoong-7	Bangladesh	2005
Rice	LGC- Jun	Japan	2006
Rice	Miya-yutaka	Japan	2007
Chickpea	CM-2008	Pakistan	2008
Maize	Kneja 546	Bulgaria	2009
Wheat	Deada	Ukraine	2017

Table 2.4 Officially released some mutant varieties of cereals and pulses in the FAO/IAEA Mutant Varieties Database: (Physical Mutagens, 2000-2019).

Common Name	Variety Name	Country	Year
Rice	IIyou 3027	China	2000
Rice	IIyou 949	China	2001
Cowpea	COCP 702	India	2002
Rice	Chiyo-no-mochi	Japan	2003
Rice	Asa-tsuyu	Japan	2004
Lentil	Binamasur-2	Bangladesh	2005
Wheat	Fumai 2008	China	2006
Black gram	DU-1	India	2007
Rice	Liangyouhang 2	China	2008
Soyabean	Hefeng 57	China	2009
Soyabean	Mutiara 1	Indonesia	2010
Soyabean	DT 2008	Viet Nam	2011
Wheat	Leroy	Ukraine	2017
Cowpea	Lukusuzi	Zambia	2018

COUNTRY MUTANTS

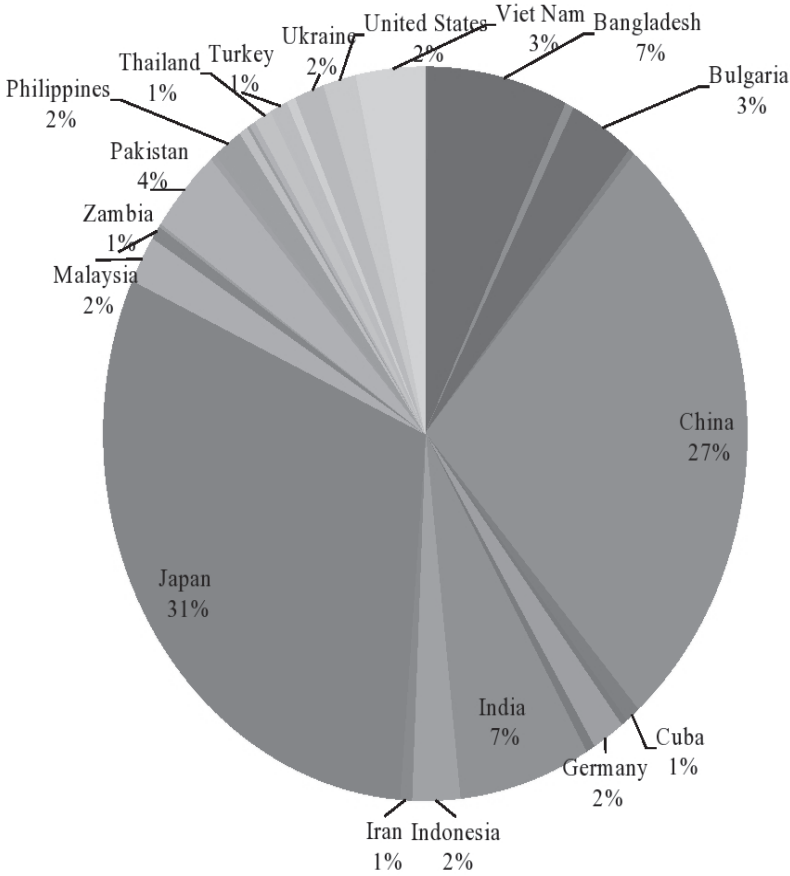

Figure 2.1 Officially released mutant varieties in the years 2000–2019 across the countries (Data source: FAO mutant variety database).

CONTINENT MUTANTS

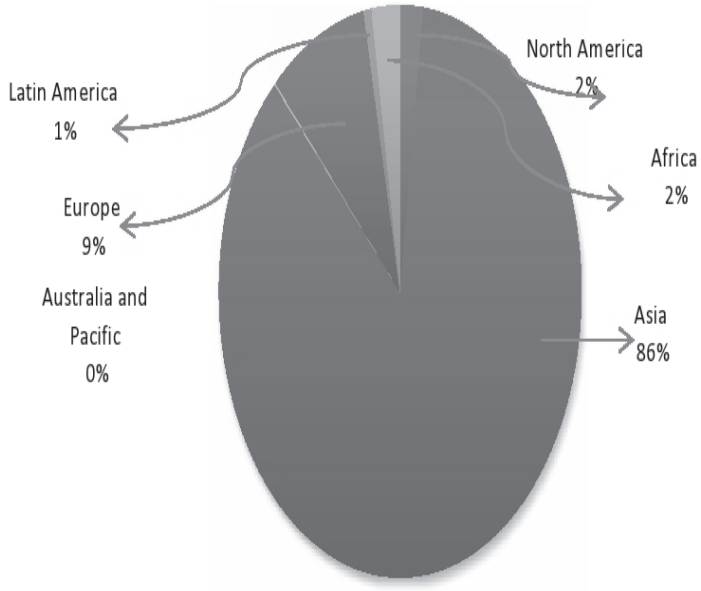

Figure 2.2 Officially released mutant varieties in years 2000–2019 across the continents (Data source: FAO mutant variety database).

2.5 Conclusions

Genetic variability has been narrowed using conventional breeding approaches for a long time. Induced mutagenesis is one of the most important approaches for broadening the genetic variation and diversity in crops to circumvent the bottleneck conditions. Induced mutagenesis, albeit almost a seven decades-old technique, demonstrably can contribute to unleashing the potential of plant genetic resources and thereby avail plant breeders the raw materials required to generate the envisaged smart crop varieties. Crop varieties generated through the exploitations of mutation breeding are significantly contributing to global food and nutritional security and improved livelihoods.

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CHAPTER 3

NEW PLANT BREEDING TECHNIQUES: PROBLEMS AND PROSPECTS

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Abstract: Mutation breeding aims to increase and/or restore genetic diversity in crop plants, which has been eroded under continued artificial selection. The development of new cultivars resistant to pests and diseases, salinity and acidity remain the major goals of mutation breeding, as well as improving yield quality and quantity. Traditionally, X-rays, gamma rays and chemicals mutagens such as ethyl methanesulfonate, etc. were used for inducing trait variation in the plants of agricultural importance. However, newer methods became available with developments in the fields of functional genomics, proteomics and metabolomics. Advancements in the fields of molecular biology and biotechnology have also positively influenced plant mutation breeding. Many of these potential breeding methods are currently under investigation and may need some more time before they could be perfected for use at a commercial level. The use of these methods under laboratory conditions has achieved results that would require considerable time as well as skilled manpower using conventional breeding techniques. However, since these techniques involve modifications in the genome and some fall under the purview of GMO legislation, commercial utilization of some of these techniques may have legal impediments. Some of the molecular methods for increasing trait variation in plants are discussed in the present chapter.

Keywords: Mutation breeding, Molecular methods, Gene editing

3.1 Introduction

Mutation is a prerequisite for species evolution. Man has exploited variation in traits of species (plants or animals) in the processes of their domestication and crop improvement. Mutation breeding is the process of the deliberate introduction of mutations in crop plants to improve any of their qualities of human interest. The goal of mutation breeding is to cause maximal genomic variation, to increase the agronomic and nutritional quality traits of plants while maintaining their viability (Sikora *et al.*, 2011). Mutation breeding may play a critical role in ensuring food security to the burgeoning global human population which is expected to cross the 9 billion mark in 2050. Plant breeding became popular from the 1950s (Nencheva, 2010; Oldach, 2011; Leitao, 2012; Oladosu *et al.*, 2015) and since then it has immensely contributed to the development of various crop species, vegetables and ornamental plants as well as medicinal herbs,especially in terms of their productivity. Mutation breeding has not only resulted in the improvement of the existing varieties but has also helped develop new edible products (Dribnenki and Green, 1995; Dribnenki *et al*., 2005, 2007; Pathirana, 2011). It also provided solutions against the havoc-wreaking crop diseases, including powdery mildew of barley, verticillium wilt and black spot disease (Mejlhede *et al.*, 2006; Micke *et al.*, 1993; Murray and Todd, 2013; Pathirana, 2011). Mutation breeding also developed plants resistant to abiotic environmental stresses including salinity, acidic and toxic soils, and drought,besides affecting the photo-periodic and thermo-periodic responses of plants (Pathirana, 2011). Itincreased returns on crops thereby affectingagricultural economy (Ahloowalia *et al.*, 2004; Parveen *et al.*, 2006; Kharkwaland Shu, 2009; Pathirana, 2011). However, present-day plant breeding has different challenges; better nutritional quality and tolerance to abiotic stresses including salinity and water stress have become more prevalent under the changing climate conditions, in comparison to increase in yield, resistance to disease and pests, etc. (Kharkwal, 2011; FAO/IAEA. 2018).

The establishment of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture with a Plant Breeding and Genetics Section, in 1964 at the IAEA, Vienna, provided the impetus to use mutation technology in plant breeding. With its services of information, irradiation, training and forging international collaboration in plant mutation breeding projects, the use of mutation breeding took off, so much so, that 3,100 mutant varieties in about 190 plant species were listed in FAO /IAEA Database of Mutant Variety and Genetic Stock (http://mvgs.iaea.org) by 2009 (Forster and Shu, 2011). As per its recent (2018) public information

and communication document, the number is over 3,222 with the highest number in small grain cereal crops in Asia.

During the early days and until recently only a select group of methods were used in plant mutation breeding which included radiation-based and chemical mutagen based methods. For example, of the 3,222 mutant varieties officially released to date as per the FAO data, about 70% have been developed using ionizing radiations only (Mba *et al.*, 2012; Mba, 2013; Sharma *et al.*, 2009; Oladosu *et al.*, 2016). However, advancements in areas such as biotechnology, plant molecular biology, and plant genomics have broadened the horizons of mutation breeding; understanding the mechanism of DNA damage and repair transformed mutation induction from a chance based event to a method based one. A better understanding of mutation technologies increased their applications during the early years of the twenty-first century. Mutant screening and characterization benefited from the use of molecular and genomic tools, besides developments and innovations in plant genomics and molecular biology (Gulfishan *et al.*, 2015; Forester and Shu, 2011). Today plant mutation research has moved from fundamental studies of mutagenesis in exclusively the forward direction to methods using reverse as well as forward genetics approaches, with the result mutation breeding is more sophisticated and efficient now and is reciprocally benefiting the related disciplines including plant genomic research (Kharkwal, 2011; Forester and Shu, 2011). Mutationscan can now be detected at the level of DNA sequence. However, techniques that lead to modification of the genome of crops have been the subject of much debate, owing to the general belief that the processes of genetic modifications are unsafe inherently. Notwithstanding this, the involvement of biotechnological methods in plant breeding is on an upward trend due to their technological, economic and crop improvement advantages. Some of the new plant breeding techniques (NPBTs) are discussed below:

3.2 Reverse breeding

Reverse breeding is a novel plant breeding technique in which homozygous parental lines are produced from a heterozygous parent (Dirks *et al.,*2009; Kumari *et al.*, 2018). Many crops are products of a cross between carefully selected parents. In reverse breeding, these elite heterozygotes are used to generate homozygous parental lines which can regenerate the original heterozygotes on the subsequent crossing. To develop these lines, plants with desirable characters are selected and

normal genetic recombination is suppressed in them. Thus, the essential measure in reverse breeding is silencing or down-regulating the expression of *dmc1* and *spo11* genes which are involved in meiotic recombination (COGEM Report).This is achieved by various methods including using RNAi (RNA interference), siRNA (short interfering RNA), Virus-Induced Gene Silencing (VIGS), or by using the exogenous supply of compounds which cause inhibition of recombination (Siaud *et al*., 2004; Higgins *et al.*, 2004). Alternatively, silencing molecules travel through graft union (Shaharuddin *et al*., 2006; Dirk *et al.,* 2009). The use of any of these methods may allow very low crossover, which is then not an issue, it may aid in the regular separation of the homologs. Residual crossovers, with the frequency of one per bivalent, increase the incidence of double haploid spores with recombinant chromosomes, but still, 50% spores with non-recombinant chromosomes are produced which can be selected by using molecular markers (Kumari *et al.*, 2018). Presently, plants with small chromosome numbers (twelve in a haploid set) are amenable to reverse breeding technology. The genes used for the genetic modification are crossed out in the last breeding steps, thus, the end products do not contain DNA sequences with genetic modification (Schaart and Visser, 2009). Microspores of plants subject to the down regulation of *dmc1* and *spo11* genes involved in suppressing meiotic crossover have been grown in tissue culture and their haploid chromosome complement doubled by double haploid technology. From the homozygous double haploid plants so produced, the genetic composition of the original heterozygous elite plant can be reconstituted by selecting the appropriate parental lines (Schaart and Visser, 2009). The product, the resulting F_1 is genetically non-modified. This technique makes it possible to recreate any heterozygous line through the creation of parental lines from it, and then using them in the cross.

Reverse breeding considerably shortens the time to produce a new hybrid (van Dun *et al.*, 2005; Dirks *et al.*, 2006; Schaart and Visser, 2009). It accelerates the breeding process thereby enabling a breeder to respond to the needs of plant growers much more quickly, besides, it eliminates the trial and error part of conventional hybrid breeding. Reverse breeding also fixes desirable characters in a species by suppressing crossover during the meiotic prophase which leads to the development of homozygous double haploid lines. This technique, by constructing complementing homozygous lines, provides a strategy to fix the hybrid vigor which has been difficult to achieve in conventional breeding (Kumari *et al*., 2018). The final product of the breeding does not fall in the category of GM crops which is an advantage of this technique.

3.3 Cis-genesis and intra-genesis

In this approach, a gene from the same species (as the plant to be transformed) or from a cross-compatible species is introduced into a species to improve any of its traits (Schouten *et al.*, 2006). The two techniques differ from each other as, in cis-genesis, alteration of the genome is not allowed, whereas intra-genesis allows redesigning of the gene in vitro. In cis-genesis, the gene of interest is used in its natural form and has its regulatory elements—promoter and terminator, introns and 3 and 5 untranslated regions—whereas in intra-genesis, the gene of interest is modified by combining it with regulatory sequences from the species itself or a cross-compatible species (Lusser *et al.*, 2011). Intra-genesis, therefore, offers more options for altering gene expression. Expression of the new gene, which is inserted at a random location in the genome as an extra copy of an already existing gene, may be altered as compared to its other copy due to its position. Genes of disease resistance or those with other beneficial effects may be transferred from wild relatives of the species or members of the same species with a better genotype. Selection markers or the vector backbone are not present in the progeny in plants developed by cis or intra-genesis. *Agrobacterium* sp. is commonly used in producing cis- and intra-genic plants (Lusser *et al.*, 2011; Ribarit s*et al.*, 2014). Cis and intra-genesis are carried out by the same techniques as those used in genome transformation, the introduced DNA though, in principle, is free from vector DNA, yet as a consequence of the technique used for the transformation, the end products may contain T-DNA border sequences flanking the gene sequences (Schaart and Visser, 2009).

With the techniques of isolating, characterizing and improving the genes, cis-genesis seems to look promising as the future plant breeding technique. The plants obtained via cis-genesis can be obtained through conventional breeding also; however, this is time-consuming and there is always a linkage drag (Jacobsen and Schouten, 2007; Schaart and Visser, 2009). Linkage drag is essentially problematic in plants with a long generation time, such as fruit trees, and in vegetatively propagated and polyploid plants, in other cases it may be removed by repeated back-crossing (Schaart and Visser, 2009). The new insert can have variable effects on the recipient genome, ranging from silencing their expression to enhancing it (Tani *et al.*, 2004; Schaart and Visser, 2009). Due to the increased copy number of a particular gene in the recipient plant genome in cis- or intra-genesis, the gene expression can be modified immensely due to copy number per se, or due to position effects. Random integration of a gene into another gene may form a new chimeric protein, or it may even result

in disruption of gene function (Forsbach *et al.*, 2003; Schaart and Visser, 2009). The position effects of the inserted genes are explained based on the effect of regulatory sequences of the nearby genes which may influence the expression positively or negatively in the new location. If cis-genesis is mediated by *Agrobacterium* sp. there is a possibility of insertion of border sequences (left border sequence (LBS) and right border sequence (RBS)) into the genome of the recipient along with the intended gene. The integration of these sequences into a protein is undesirable (Schaart and Visser, 2009). PCR (polymerase chain reaction) can be used to detect the genetic modification changes including the insertion of any undesirable sequence into the recipient genome.

In contrast to cis-genesis, intra-genesis involves modification of the genetic elements coming from the same or crossable species and integration of this modified genetic element into the genome. The intra-genesis gene insert is thus tailor-made to suit the requirements, it is carried out *in vitro* by combing elements such as promoters, coding parts with or without their introns and terminators, etc. The genetic modification may also involve the use of inverted DNA repeats which leads to gene silencing by way of RNA interference (Rommens *et al.*, 2004, 2006, 2007; Conner *et al.*, 2007; Schouten and Jacobsen, 2008; Schaart and Visser, 2009). With the discovery of T-DNA like elements in the plant genome, Rommens *et al*. (2004) proposed "all-native DNA transformation" wherein agrobacterial T-DNA was proposed to be replaced by the T-DNA like elements in the plant genome called P-DNA. Species-specific vectors have been constructed from functional plant DNA sequences from the recipient genome (Conner *et al.*, 2007). Given the genetic modification that the DNA fragment undergoes in intra-genesis, the end product may or may not be necessarily similar to the ones achieved by conventional breeding and the level and pattern of gene expression may differ considerably in comparison to the non-modified plants. As happens in cis-genesis, the DNA fragment may be inserted within another gene which may lead to its disruption, usually known as insertional mutagenesis (Forsbach *et al.*, 2003), or the formation of a chimeric protein if the insert becomes a part of the reading fragment of this gene. Besides, these inserts are also subject to the positional effects just as in the case of the cis-genes and to the insertion of border sequences in *Agrobacterium*-mediated transformations. However, these effects of intragenic integration also occur naturally during transposon transition (Greco *et al*., 2001) and translocation breeding (Papazova and Gecheff, 2003; Schaart and Visser, 2009).

The overall aim of these techniques is to improve the crop by introducing better genes from the species with which the recipient crop can easily interbreed. The application of the cis- and intra-genesis techniques, however, is limited by the variable expression of the gene inserts due to incomplete information about the promoter sequences. Promoter sequences are dispersed upstream of the gene and the length of promoters is not known in the majority of the genes, which makes promoters difficult to characterize (Barta *et al.*, 2005; Goñi *et al.*, 2007; Szankowski *et al.*, 2009; Schaart and Visser, 2009). Intra-genesis has been used for the production of non-browning apples by silencing the polyphenol oxidase gene (Schaart and Visser, 2009). Three genes were silenced in potato through RNAi by combining functional elements of seven genes into a transformation construct (Rommens *et al*., 2006). In New Zealand, this technique has been used to develop drought-tolerant ryegrass (*Lolium prenne*) that over-expresses a native salt-tolerant gene. Efforts are underway to transfer disease resistance into apples and potatoes from their respective wild crossable species via these techniques, pointing toward the potential of these techniques as the major plant breeding techniques of the future.

3.4 Grafting

Grafting is an age-old technique of plant breeding where the rooted stem of one plant is fused with the stem or bud of another plant. The former is called the rootstock and the latter is known as the scion. Grafting is an important breeding technique in commercially cultivated fruit trees such as apples, pears, cherries, etc. as well as being practiced in some flower-bearing and vegetable plants. Transport between the rootstock and the scion is essential, therefore vascular connection must be established between these two partners in the graft union (Schaart and Visser, 2009). Grafting is carried out to achieve various ends including better rooting ability, e.g., in heavy soils, resistance to soil-borne pathogens, etc. It is also used in silencing flowering repressing genes (via RNAi), thereby inducing early flowering (Flachowsky *et al.*, 2007).Grafting can also be used to suppress recombination in reverse breeding (Dirks *et al.*, 2006; Schaart and Visser, 2009), and endogenous plant genes can also be suppressed by RNAi in grafting. The mobility of RNAi within the plant is well established (Palauqui *et al.*, 1997), and graft union may become a conduit for its transfer (Sonoda *et al.*, 2000; Crété *et al.*, 2001; Shaharuddin; *et al.*, 2006; Tournier *et al.*, 2006) which makes RNAi induced gene silencing transferable across many graft unions and a reason

for gene silencing in non-modified scions (Schaart and Visser, 2009). The gene silencing phenotype inherited by a scion is maintained even after re-grafting such scions on genetically non-transformed rootstocks (Sonada *et al.*, 2000; Schaart and Visser, 2009). Thus, a genetically non-modified scion grafted on to a genetically modified rootstock can be permanently altered by RNAi silencing and the produce harvested from it may have an altered phenotype traceable to the parent genetically modified rootstock (Schaart and Visser, 2009). RNAi silencing can also cause RNA-directed DNA methylation (Mathieu and Bender, 2004), which in turn (as discussed in the next section), may cause silencing (Hohn *et al.*, 1996; Schaart and Visser, 2009) as well as upregulation of gene functions (Li *et al.*, 2008; Shibuya *et al.*, 2009; Schaart and Visser, 2009). Following generations may also inherit the altered phenotype (Park *et al.*, 1996; Schaart and Visser, 2009). However, results to the contrary have also been reported by Sonada *et al*. (2000) wherein no transference of the silencing signal to the progeny in a graft union involving genetically modified (silencing) rootstock and non-genetically modified scion was reported, indicating the source of the signal was not permanently integrated into the scion genome. Thus, the offspring in a breeding procedure involving RNAi silencing can be regarded as genetically non-modified due to their similarity (genetic as well as phenotypic) to their genetically non-modified counterparts (Schaart and Visser, 2009). Besides, it is also possible that there is no transference of any molecules across the graft union. The products harvested from the genetically non-modified scion in a graft union with a genetically modified rootstock are usually free from modified DNA sequences, however, metabolites, proteins and small RNA molecules may travel across the graft union. The transport of metabolites and proteins from the rootstock to the scion has not been fully explored and more work needs to be done before conclusions can be drawn (Schaart and Visser, 2009). The chimeric plants (plants with graft union) overall, have better agronomic characters.

3.5 Agro-infiltration

In agro-infiltration foreign genes are inserted into a plant species using *Agrobacterium* sp. for their local temporary expression (Schaart and Visser, 2009). This technique has been in use for many years now (Lee *et al.*, 2001; Lusser and Davies, 2013). It is used for leaf tissue and protoplast transformation and to study functions of gene constructs and the impact of gene knock-outs, etc. The protoplast cultures are difficult to work with because of their fragile nature and contamination issues, besides, getting

rid of the cell walls also involves tedious procedures (Belhaj *et al.*, 2013). Agro-infiltration, *sensu stricto* involves infiltrating a suspension of *Agrobacterium* sp. carrying a gene of interest, into a non-germline tissue, usually leaves, for its temporary and localized expression in the infiltrated part of the leaf. Full plants or their detached parts may be used in agro-infiltration (Van der Hoorn *et al.*, 2000; Belhaj *et al.*, 2013). During this transient expression integration of genes into the host the genome does not occur, but the infiltrated genes remain as free DNA molecules expressing rapidly into either transcribable or double-stranded RNAs (RNAi) as the case may be. To transfer the genes plant tissues including leaves or stems are pierced by needleless syringes. In a similar technique, flowers are dipped into a suspension of *Agrobacterium* sp. containing a transformed T-DNA. This technique called floral dip is used for obtaining GM seeds, as here female gametophytes are subject to transformation.

Agro-infiltration is also used for studying the plant's defense response against pathogenic proteins. Foreign DNA is inserted into the cell and on its transient expression proteins are produced which interact with plant disease resistance genes to produce a defense response. The plant may turn out to be susceptible or resistant to the disease. Resistant plants may be selected for breeding purposes or evaluation of the mechanism underlying the resistance (Schaart and Visser, 2009). The method is also helpful in studying down regulation of genes in which case dsRNA producing gene constructs may be introduced into the plants to silence gene/s by degrading mRNA (RNAi). RNAi is not only capable of down regulation of genes but is also capable of RNA-directed DNA methylation causing changes in DNA at the chromatin level (Mathieu and Bender, 2004; Schaart and Visser, 2009).

To avoid secondary infection during crop development arising due to persistence of the *Agrobacterium* sp. products are harvested from a site other than the site of inoculation in the agro-infiltrated plants (Moligner *et al.*, 1993). However, *Agrobacterium* sp. has been reported to travel through the conduction tissue xylem which may aid in its spread to the unintended sites and may also lead to the stable transformation of plants (Tarbah and Googman, 1987; Schaart and Visser, 2009). Therefore, the possibility of the stable integration of DNA intended for a transient effect cannot be ruled out (Schaart and Visser, 2009). Conclusive information is not available on the fate of *Agrobacterium* sp. in the infiltrated plants as only a few studies to that effect have been conducted. Careful screening of the plant parts for the absence of the *Agrobacterium* sp. chromosomal agrobacterial DNA and the T-DNA used for the agro-infiltration,

especially if further propagation of the plant is intended, is thus necessary (Schaart and Visser, 2009; COGEM Report).

Agro-infiltration may be useful in molecular farming (e.g., for producing pharmaceutically important proteins, etc.), analyzing gene function (e.g., as in the case of defense interactions of plants with their pathogens), besides it can also be used to study tolerance to various kinds of stresses and in resistance breeding (Ribartis *et al.*, 2014). In some cases, the gene construct may, however, be a stably integrated ion into the plant genome. Not all species are amenable to agro-infiltration (Belhaj *et al.*, 2013). Agro-infiltration, despite having great potential for disease resistance to viral, bacterial and fungal pathogens, has not yet been applied at the commercial level.

3.6 RNA-directed DNA methylation (RdDM)

RNA-directed DNA methylation (RdDM) is a small RNA-mediated epigenetic process (Cogoni, 2001; Chicas and Macino, 2001; Hutvágner and Zamore, 2002; Aufsatz *et al.*, 2002; Matzke *et al.*, 2015), in which RNA induces gene silencing by methylation of cytosine residues in the region of sequence homology between the triggering RNA and the target DNA (Aufsatz *et al.*, 2002). DNA methylation influences chromatin accessibility, which in turn affects replication and repair of the DNA, expression of genes and activity of transposable elements in plants as well as in animals (Law and Jacobsen, 2010; Allis and Jenuwein, 2016). Plant and animal whole-genome methylation patterns differ and animals are commonly more methylated than plants. Regulation of stress responses and developmental patterns by DNA methylation in plants is very well known (Lanciano and Mirouze, 2017). Methylation targets in plants are CG, CHG, CHH where H may represent A, T or C (Lanciano and Mirouze, 2017). Since epigenetic regulatory mechanisms are known to affect plant stress responses, reproductive development (regulation of flowering, including timing to flower, development of gametophyte and seed/embryo, etc.), such pathways may play an important role in crop improvement (Springer, 2013; Zemach *et al.*, 2010; Shi *et al.*, 2014; Xing *et al.*, 2015). Variations in DNA methylation patterns lead to altered transcription of genes and may give rise to new adaptive traits. Indeed, analysis of 1,107 DNA methylation pattern variants of *Arabidopsis thaliana* from 1001 genome collections revealed that intraspecific epigenomic diversity extent in this plant was correlated with climate and geographic origin (Kawakatsu *et al.*, 2016; Lanciano and Mirouze, 2017).

Drought sensitive and resistant variants of rice differ in their methylome under drought conditions with the latter having more stable methylome than the former (Wang *et al.*, 2016; Lanciano and Mirouze, 2017). In rice, the methylation pattern is influenced by the use of pesticides and heavy metals (Feng *et al.*, 2016; Lu *et al.*, 2016; Lanciano and Mirouze, 2017). Methylation in transposable elements keeps them silent. Epigenetic mutations can be heritable or reversible (Weigel and Colot, 2012; Quadrana and Colot, 2016; Zheng *et al.*, 2017). Transposable elements have been reported to have influenced the course of the evolution of plants and their potential role in affecting traits of agricultural importance has also been acknowledged (Martin *et al.*, 2009; Lisch, 2013; Ong-Abdullah *et al.*, 2015).

RdDM involves sequence-specific changes at the genome level (Wassenegger, 2000; Aufsatz *et al.*, 2002). Several proteins work in a coordinated manner to bring about RdDM (Mahfouz, 2010). The double-stranded RNA which is at the core of RdDM is enzymatically cleaved into short RNA molecules about 21-24 nucleotides in length (siRNA) which cause methylation of DNA in the target region (Mette *et al*., 2000; Aufsatz *et al.*, 2002). Though the target site in the DNA is approximately 30 bp and coincides well with the length of the small RNA molecules, it is not known whether the short RNA or the dsRNA itself is responsible for guiding methylation of the homologous DNA sequence (Pélissier and Wassenegger, 2000; Aufsatz *et al.*, 2002). Methylation in the promoter can lead to complete prevention of transcription (Mette *et al*., 2002; Schaart and Visser, 2009). Methylation in the transcribed region is believed to have not effect but some workers believe that it may lead to contrasting results including silencing (Hohn *et al.*, 1996; Schaart and Visser, 2009) or upregulation of the gene (Li *et al.*, 2008; Shibuya *et al.*, 2009; Schaart and Visser, 2009). RdDM does not involve any change in the nucleotide sequence of the DNA and is heritable in the absence of the original trigger. It provides not only a mechanism of gene silencing for use in plant breeding but also a pathway of suppressing transposable elements and viruses reproducing via dsRNA (Vance and Vaucheret, 2000; Waterhouse *et al.*, 2001; Voinnet, 2001; Aufsatz *et al.*, 2002; Lanciano and Mirouze, 2017).

3.7 Oligonucleotide-Directed Mutagenesis (ODM)

ODM is a technique where in a certain nucleotide length is synthesized to share sequence homology with a target sequence with exception of a few nucleotides for its use in generating site-specific mutations, including

changing one or a few base pairs, reverting an existing mutation or introducing short deletions (Lusser *et al.*, 2012). This technique thus targets the endogenous genes. This base pair-specific precision gene-editing technique does not involve transgenesis and has been used to achieve new and commercially important traits in agriculturally important crops (Sauer *et al.*, 2016).The oligonucleotides are rapidly degraded and are not themselves inherited but cause heritable changes in the genome of the recipient. This technique is also known by other names including targeted gene repair, oligonucleotide-directed gene targeting, genioplasty and chimeraplasty. It is a promising technique both for knocking out or adapting gene function in crops (COGEM; Sauer *et al.*, 2016). ODM is commercially available and is a reliable technique (Ribartis *et al.*, 2014). It is a method which entails site-specific gene modification that alters the gene sequence precisely without incorporation of any foreign genes. It uses small 70bp chemically synthesized, chimeric (composed of DNA as well as RNA) oligonucleotides to induce mutations (substitutions, insertions or deletions). These oligonucleotides are inserted into the cell by electroporation or by particle bombardment. After entering the cell these hybridize with the target sequence causing a mismatched base pairing which is corrected by a cell's gene repair system which introduces a mutation while making corrections. The oligonucleotides are degraded afterward within the cell. Oligonucleotides may be directed either to create gene mutation or modify the regulatory elements changing the gene expression. The ODM technique has been successfully used not only in plants but in bacterial, yeast, and mammalian systems also (Sauer *et al.,* 2016). Although no foreign DNA has been integrated into the genome of a plant yet, unintended mutations may be induced in the adjacent genes in addition to the intended ones (Kochevenko and Willmitzer, 2003). Besides, there is a possibility of chimeric nucleotides stably integrating into the DNA. Moreover, this technique has low efficiency, therefore, has not been used much except for the induction of herbicide-resistance in plants (Schaart and Visser, 2009). Herbicide-resistant phenotypes are created by modifying the acetolactate synthase gene (Beetham *et al.*, 1999; Kochevenko and Willmitzer, 2003; Okuzaki *et al.*, 2004; Schaart and Visser, 2009; COGEM Report), also its use has been documented in the repair of a mutated reporter gene in a transgenic plant (Dong *et al.*, 2006; COGEM Report) and in developing a herbicide-resistant *Brassica* line by altering the *a/s* gene (Schaart and Visser, 2009).

3.8 ZFN technology

Zinc Finger Nucleases (ZFNs) are synthetic restriction endonucleases used in site-directed mutagenesis. ZFNs consist of two domains—a zinc finger domain and a nuclease domain, a specific DNA sequence is recognized by the former and cut by the latter and this two domain system is delivered into the cells in an expression vector. The expression plasmid also carries a short template or DNA sequence to be incorporated into the genome. The cellular DNA repair machinery, the process of homologous recombination and the insertion of DNA all are stimulated by a double-strand break (DSB) introduced by the ZFNs in DNA at a specific site. ZFNs technology is a new and attractive method for creating trait variation (Zhang *et al.*, 2010; Curtin *et al.*, 2011). ZFNs are a tool for targeted gene alteration with great potential in plant mutation breeding. Gene alteration has been achieved in *Zea mays* and *Nicotiana tabacum* besides *Arabidopsis thaliana*, a high-frequency heritable transmission has been reported in the latter case (Shukla *et al.*, 2009; Townsend *et al.*, 2009; Zhang *et al.,* 2010; Curtis *et al.*, 2011). ZFN technology can be used for altering single or a few nucleotides in the genome (ZFN-1 and ZFN- 2) or it can also be used for site-specific insertion of DNA into the genome (ZFN-3) (Ribartis *et al.*, 2014). ZFN-1 and ZFN-2 along with ODM are specific as these target homologous sequences in the DNA; their effects include deletions, alterations, reversions and gene silencing (Ribartis *et al.*, 2014). In ZFN-1, ZFN is delivered into a cell without a repair template and the plant cells, while repairing the DBS, give rise to site-specific mutations (change of a single or a few base pairs, short deletions or insertions) (Lusser *et al.*, 2012). In ZFN-2, the plasmid carries the ZFN and a short DNA sequence containing a point mutation homologous to the target area, which is intended to be used as a repair template. The plant cell machinery intervenes to repair the DBS generated by the ZFN; copying the repair template causes it to generate a site-specific point mutation (Lusser *et al.*, 2012). In ZFN-3, ZFN is delivered into the plant cell along with a long stretch of DNA which may be the gene of interest. Since the sites flanking the DBS are homologous to the ends of the DNA stretch, the latter is inserted into the genome in a site-specific manner. The ZFN technology can be used to generate site-specific mutations or to inactivate agene thereby producing the preferred phenotype. Thus, targeted addition of genes of interest, replacement of genes and trait stacking can be achieved by the ZFN-3 approach. Not only this but also the position effect caused by the insertion of genes randomly in the genome can be

prevented by gene-specific targeting using this technique (Lusser *et al.*, 2012).

The zinc finger arrays (ZFA) in the ZFNs contain an engineered Cys2-His2 zinc finger and a non-specific DNA-cleavage domain of the restriction enzyme FokI (Urnov *et al*., 2010; Curtis *et al.*, 2011). A spacer 5-7bp separates 9bp binding sites of a three-finger ZFA to allow the dimerization of the FokI nuclease. Heterodimer formation is required for binding and cleaving the target site. Correct dimer formation requires independent binding of both ZFAs separated by the appropriate spacer. As the FokI monomers dimerize successfully, cellular DNA repair by non-homologous end joining (NEHJ) and the homology-directed repair (HDR) pathways are triggered by the introduction of DBS in the spacer sequence between ZFA binding sites. NEHJ introduces indels while ligating the DBS (Curtis *et al*., 2011).

Recently the Zinc Finger Consortium developed a rapid and easy to perform method to design and construct ZFNs which is called context-dependent assembly (CoDA). CoDA has created novel ZFNs by assembling arrays from optimized two-finger units (Sander *et al.*, 2011; Curtis *et al.*, 2011). In the transformed soybean roots mutations in the target site have been created using CoDAZFNs (Sander *et al.*, 2011; Curtis *et al.*, 2011). Apart from CoDA, other methods of designing ZFNs include modular assembly and OPEN (Oligomerized Pool Engineering) (Wright *et al.*, 2006; Maeder *et al.*, 2009; Joung *et al.*, 2010; Kim *et al.*, 2010; Sander *et al.*, 2011; Curtis *et al.,* 2011), but screening combinatorial libraries is labor intensive and also requires expertise which has limited the broader adoption of OPEN (Kim *et al.*, 2010; Sander *et al.*, 2011). CoDA shows a success rate comparable to selection-based methods, such as OPEN. It is a simple to use publicly available platform of software and reagents (Sander *et al.*, 2011). Despite being more efficient than modular assembly, CoDA cannot target many sites which modular assembly can potentially do (Kim *et al.*, 2009; Lee *et al.*, 2010; Sander *et al.*, 2011). CoDA is rapid and does not require specialized expertise, which is prompting broader adoption of ZFN technology, especially for projects focused on multi-gene pathways or genome-wide alterations that have been evasive so far (Sander *et al.*, 2011). In plants such as soybean with highly duplicated genomes, a series of unique allelic combinations can be introduced by ZFN site-directed mutagenesis, besides its ability to generate heritable mutations (Curtis *et al.*, 2011). Therefore, this technology raises hope for other crop species with polyploidization and with homologous and paralogous copies of

many genes (Blanc and Wolfe, 2004; Schmutz *et al.*, 2010; Curtis *et al.*, 2011). Furthermore, it can be useful for plant functional genomics.

3.9 CRISPR/Cas

The Clustered Regularly Interspaced Short Palindromic Repeats, (CRISPR) and CRISPR-associated (Cas9) gene-editing system provides the most fascinating opportunities for increasing trait variation in plants. It is a versatile, reliable, easy to apply and robust genome editing tool for basic and applied research in the plant sciences. Contrary to the ZFNs, in the CRISPR/Cas system assembly of individual DNA-binding proteins is not required. Before this system, obtaining knockout lines through conventional genetic methods was much dominated by chance. Small sizes of the genome also lowered the probability of mutation or insertion of a T-DNA construct. Therefore, targeted mutagenesis using this technology has opened new vistas in the field of plant genetic research and plant breeding (Li *et al.*, 2013; Mao *et al.*, 2013; Belhaj *et al.*, 2013).The CRISPR/Cas gene-editing tool is simpler than ZFNs and is a preferred choice for gene editing in plants (Nekrasov *et al*., 2013; Shan *et al.*, 2013; Li *et al.*, 2013; Feng *et al.*, 2013; Xie and Yang, 2013; Miao *et al.*, 2013). The CRISPR/Cas system of bacteria has recently been used in plant gene editing. Mutations induced by NHEJ as well as HDR methods have been reported. Whole plants carrying mutations have been generated in some cases.

To put it simply, the CRISPR/Cas system consists of two components; an endonuclease (Cas9) and an RNA that guides it to the target DNA sequence. The specificity of the endonuclease can be reprogrammed using customizable small non-coding RNAs (Jinek *et al.*, 2012, 2013; Mali *et al.*, 2013; Cong *et al.*, 2013; Jiang *et al.*, 2013; Belhaj *et al.*, 2013). Cas9 is guided to the DNA target sequence adjacent to the protospacer adjacent motif (PAM) by a complex of two non-coding RNAs, viz. CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) (Deltcheva *et al.*, 2011; Jinek *et al.*, 2012; Sorek *et al.*, 2013 Belhaj *et al.*, 2013). crRNA and tracrRNA have been combined to give rise to a single guide RNA (sgRNA), reducing the number of components in the CRISPR/Cas system to two i.e., Cas9 and sgRNA (Jinek*et al.*, 2012; Belhaj *et al.*, 2013). Cas9 nuclease introduces double-stranded (blunt) cuts in the target DNA specified by sgRNA (Mussolino and Cathomen, 2013). A 20bp guide sequence located at the 5′ end of sgRNA is responsible for its target specificity; sgRNA with different specificities can be created by altering

the guide sequence (Jinek *et al.*, 2012; Belhj *et al.*, 2013). Target recognition of Cas9 may be abolished by the mismatch between the target and the sgRNA guide sequence contained within the last 8-10bp of the 20bp sequence, however mismatches toward the 5′ end of a target are tolerated. The 20bp target must essentially be followed a PAM motif (NGG) for activity, although the latter with non-canonical NAG sequences does retain some activity (Hsu *et al.*, 2013; Belhaj *et al.*, 2013). The tolerance to the number and position of mismatches between the DNA and the guide sequence varies among targets (Fu *et al.*, 2013; Hsu *et al.*, 2013; Belhaj *et al.*, 2013). The availability of only limited data on off-site targets in CRISPR/Cas at the moment hinders drawing any conclusions; comprehensive whole-genome sequencing of the mutant plants will help make the picture clearer. However, in general, off-site target mutations can be easily crossed out in plants, therefore, do not pose many problems. Besides, algorithms can be used to choose the specific sites, as has been done in *Arabidopsis* and rice (Li *et al.*, 2013; Xie and Yang, 2013; Miao *et al.*, 2013; Belhaj *et al.,* 2013). For designing CRISPR targets with a very low off-target effect, online tools analogous to http://crispr.mit.edu/ are also being developed (Belhaj *et al.*, 2013). The component of Cas9 homologous to HNH nuclease nicks the complementary DNA strand, while the non-complementary strand is cleaved by the component homologous to Ruvc nuclease (Jinek *et al.*, 2012; Belhaj *et al.*, 2013). sgRNA has been expressed in plants using U6 and U3 promoters of RNA polymerase III. Different configurations of Cas9 with different promoters have different success rates.

CRISPR/Cas generated mutations can be detected by loss of restriction site if the restriction site overlaps the PAM motif. The restriction site will be disrupted by faulty repair of DBS in DNA via the HHEJ mechanism, and this mutation can be detected by restriction digestion of the DNA across the target site after its PCR amplification (Nekrasov *et al*., 2013; Jiang *et al*., 2013; Belhaj *et al*., 2013). Another method of mutation detection is the survey or assay in which PCR amplification, denaturation and annealing of the Cas9/sgRNA treated sample is followed by treatment with endonucleases cleaving hetero-duplexes formed by mutated DNA. This assay can be applied to any target sequence but requires a higher mutation rate, however, this assay is not as sensitive as the restriction enzymes assay (Voytas *et al.*, 2013; Mao *et al.*, 2013; Xie and Yang *et al.*, 2013; Belhaj *et al.*, 2013). DBS introduced by Cas9 nuclease guided by an sgRNA may be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) mechanisms. Following cuts generated by CRISPR/Cas, NHEJ can be exploited to generate not only single and

multiple gene knock-outs but also large chromosomal deletions (Belhaj *et al.*, 2013). Creating large deletions by introducing two DBSs greatly improves the efficiency of mutagenesis, as in the case of *Arabidopsis* (Mao *et al.*, 2013; Li *et al.*, 2013; Belhaj *et al.*, 2013). HDR can be used for targeted gene insertions or gene replacements (Voytas *et al.*, 2013; Belhaj *et al.*, 2013). In *Nicotiana benthamiana* (Li *et al.*, 2013) and rice (Shan *et al.*, 2013) HDR-dependent genome editing has been achieved using the CRISPR system; a single (Shan *et al.*, 2013) or double-stranded (Li *et al.*, 2013), DNA fragment served as the repair template. Regenerating whole plants from protoplasts would be the next level which so far has been possible in the cases of a few species only (e.g., *N. benthamiana* and *Arabidopsis*). In I-SceImeganuclease the *A. tumefaciens* delivery system has been used to deliver all the components, viz. the DNA repair template, the Cas9 and sgRNA (Fauser *et al.*, 2012). However, in the case of plants, HDR using the CRISPR/Cas system has not yet been achieved. Callus bombardment is the method of choice for delivering CRISPR/Cas9 components into the cells of plants that do not regenerate from protoplasts or have problems with *Agrobacterium* transformations. This method has been already used in cotton (D'Halluin *et al.,* 2013; Belhaj *et al.*, 2013). Homologous recombination has very low efficiency which may make the HDR-mediated genome editing problematic (Li *et al.*, 2013; Belhaj *et al.*, 2013). However, this can be overcome by using NHEJ DNA repair pathway mutants. Using *ku70* and *lig4* mutants increased efficiency by 5 to 6 fold and 3 to 4 fold respectively in *Arabidopsis* (Qi *et al.*, 2013; Belhaj *et al.,* 2013). Mutants can be later crossed out when the desired gene targeting event has been accomplished. A codon-optimized version of bacterial (*Streptococcus pyogenes*) Cas9 is being used in eukaryotic cells. Wild Cas9 of *S. pyogenes* is useful in rice protoplast against the OsSWEET14 target (Jiang *et al.*, 2013; Belhaj *et al.*, 2013). Mutant plants with high success rates have been recovered using plant codon-optimized and human codon-optimized versions of Cas9 (Shan *et al.*, 2013; Li *et al.*, 2013; Miao *et al.*, 2013; Jiang *et al.*, 2013; Belhaj *et al.*, 2013), for example ,biallelic mutations have been recovered in the case of *Arabidopsis* (up to 89%) and rice (up to 92%) (Belhaj *et al.,* 2013).

CRISPR technology is one of the new plant breeding technologies (NPBTs) and regulatory and advisory authorities in Europe and throughout the world are discussing this and other NPBTs in relation to the GMO legislation (Lusser and Davies, 2013; Lusser *et al.*, 2012; Pauwels *et al.*, 2013; Kuzma and Kokotovich, 2011; Belhaj *et al.*, 2013). These technologies aim to introduce mutations in plants of interest which are the

same as those introduced by conventional plant breeding methods so that crops can fall under the non-GMO category which can enormously boost plant biotechnology and plant breeding. CRISPR/Cas technology seems to be the future of the targeted single and multiple gene knock-outs, for SNPs introduction into genes and for expressing proteins tagged with affinity or fluorescent tags, etc.

3.10 Conclusion

The use of innovative and new methods in agriculture is indispensable because of the global challenge of population growth, especially under changing climate conditions which threaten crop productivity. New plant breeding techniques, as discussed above, are much less time-consuming in comparison to the traditional agricultural methods. However, despite their potential for rapid crop improvement, these techniques have general safety and legal concerns besides their low efficiency. There is a considerable debate on the regulation of NPBTs and whether some of them should be considered as GMOs. Thus, their legal classification is uncertain. Some of the methods discussed above fall under the European Directive (2001/18/EC). The plants developed via ZFNs, RdDM, agro-infiltration and reverse breeding do not contain any foreign DNA element at the end. The genetic information coding for the desired trait occurs either only ephemerally in the plants or is integrated into the genome in plants that act only as intermediates, during segregation new lines are created which do not contain the transgene. However, the organisms which involve genetic modification at some stage of their development are covered under the European Directive which means that even if the part of the DNA which is genetically modified does not exist in the final product of breeding, the product will still be considered as genetically modified. Crops designated as GMOs require additional time on regulatory research associated with them (Bradford *et al.*, 2005; McElroy *et al.*, 2003), which delays the launch of a new variety, besides generating data on the regulatory dossier also costs about $35 million per GM event, which restricts the use of such techniques to the high-value crops (Kalaitzandonakes *et al.*, 2007; Miller *et al.*, 2010). Delaying the launch of a new variety again causes losses (Lusser *et al.*, 2011). Moreover, detection and identification of the modified sequences are not always feasible. To detect and identify the modified gene in plants developed via ZFN-3, or for that matter, cis- and intra-genesis information about the flanking sequences is important. Using standard methods for GMO detection can detect and identify modification in cases where a graft union is established between a non-modified scion

on a GM rootstock. DNA sequences are not modified in RdDM but the gene is silenced through DNA and/or histone methylation and differentiating deliberately induced methylation patterns from the naturally occurring ones cannot be routinely identified or detected. Similarly, in agro-infiltrated plants standard methods of GMO detection can be used to detect and identify the modified gene construct if it stably integrates into the genome, or it can be detected in the agro-infiltrated tissue only in cases where integration into the genome does not take place (Lusser *et al.*, 2012).

In the adoption and acceptance of the mutant plant species, some countries are showing a way forward, e.g., herbicide-tolerant canola developed by Cibus through ODM is exempted from biotechnology regulation under 7CFR Part 340 as per the Animal Plant Health Inspection Service (APHIS) of the US Department of Agriculture (Sauer *et al.,* 2016). In Canada, the method of producing a variety with a new trait is not taken into account for approving its release and the cases of mutant varieties are taken on a case to case basis (Shearer, 2015; Sauer *et al.*, 2016). Many workers have proposed that ODM be regarded as a mutagenesis technique outside the scope of the directives governing GMOs (Sauer *et al.*, 2016).

The efficiency of the different techniques discussed varies with the crop, the method used, the genes involved and the marker genes, where used, but the low efficiency of many of the above techniques has limited their use (Li *et al.*, 2007; Shukla *et al.*, 2009; Lusser *et al.,* 2012). In *Arabidopsis* 2% frequency (de Pater *et al.*, 2009) and for tobacco, 40% (Townsend *et al.*, 2009) was reported for mutations induced via ZFN. Besides, the occurrence of non-target, unintended mutations cannot be ruled out (Durai *et al.*, 2005; Pattanayak *et al.*, 2011; Lusser *et al.*, 2012). The use of RdDM at the commercial level is marred by the instability of the methylation status. In cis- and intra-genesis also the efficiency of the mutation effort is determined by the crop species and cultivars. Both these techniques also require removing promoters and the selectable marker genes in the final breeding steps (Schaart *et al.*, 2013; Lusser *et al.*, 2012). More research and development efforts are required to develop efficient methods of delivering the gene constructs into the cell for perfecting these techniques for commercial use. Despite the shortcomings related to the legal aspects, cost, and efficiency, the new breeding techniques still offer potential technical and economic advantages over conventional breeding methods which is the reason for their increased popularity in the industry. Improved technical efficiency and regulatory framework in which crops

developed via such methods are seen will determine the future of these techniques.

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CHAPTER 4

BIOASSAYS APPLICATIONS FOR MUTAGENICITY AND CYTOTOXICITY EVALUATION

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Abstract: Mutagenicity is the extent a physical or chemical agent induces permanent transmissible variations in the genetic content of a cell or organism. These genetic alterations, referred to as mutations, can be either qualitative or quantitative. They can affect a chromosome, a group of genes, a gene segment or a single gene. Mutagenicity leads to harmful mutations responsible for cell death, several human genetic disorders and cancer. In plants, mutagenicity can also hamper crop productivity by reducing growth, resistance to diseases and photosynthesis ability. The agents responsible for these variations are called mutagens, whereas cytotoxicity is the state of being toxic to the cells and can be due to environmental, physical or chemical factors. Cytotoxicity normally induces necrosis, apoptosis or cytostatic effects. Cytotoxicity can be determined by measuring changes in cell proliferation, cell viability, phospholipidosis and steatosis, mitochondrial function, cell cycle and DNA damage. Both mutagenicity and cytotoxicity mechanisms are routinely used to evaluate a wide variety of compounds. However, in contrast to cytotoxicity, mutagenicity is also known to play a vital role in the evolution, development and diversity of living organisms. Mutagenicity leading to DNA duplication or due to transposons has evolved new genes, genomes and genetic diversity in both plants and animals (Aminetzach *et al*., 2005, Hastings *et al*., 2009, Aminetzach *et al*., 2005). Apart from the natural mechanisms, mutagenicity has been widely adopted and is the preferred technology in crop improvement.

Techniques to screen, identify and evaluate mutagenicity and cytotoxicity are of prime importance. A bioassay is an analytical technique used for the qualitative or quantitative estimation of potential or concentration of a compound *in vitro* (tissues, cells) or *in vivo* (live organisms). Multiple bioassays are designed specifically to evaluate either mutagenicity or cytotoxicity of a living organism or a compound. These bioassays have widespread practical applications. For example, mutagenicity assays can help to screen mutations involved in different human diseases, identify novel and enhanced phenotypes for plant breeding, crop improvement and defense against pathogens (Bhat *et al*., 2007; Bhat *et al*., 2006; Bhat, 2007; Parry *et al*., 2009). Whereas, bioassays used for cytotoxicity are useful for the identification of compounds cytotoxic to humans, drug discovery, anticancer agents and cytotoxic genetic alterations. These assays can also help to understand biological processes leading to cell proliferation, growth and death. In this chapter we will discuss bioassays available for both mutagenicity and cytotoxicity and their practical applications.

Keywords: Mutagenecity, cytotoxicity, bioassays, DNA damage, genetic alterations

4.1 Ames Test

The Ames test is a biological assay devised to test the mutagenic potential of chemical compounds. Bruce N. Ames developed this test in the 1970s on the principle of back or reverse mutation in bacteria. Therefore, bacterial reverse mutation is another name used for the Ames test. Different mutant bacterial strains such as *Escherichia Coli* and *Salmonella* are being used to detect mutagenicity. These auxotrophic mutant strains carry alterations in the histidine synthesis gene (his–) and are unable to produce histidine using components of cell culture medium. Therefore they require additional histidine in the media for normal growth. However, in the presence of a test chemical, if the his– strains start to grow, even in histidine free media, it shows the capability of the test compound to revert it to the his+ strain which starts synthesizing histidine (Ames *et al*., 1973). Figure 4.1 explains the protocol of the Ames test. By specific construction of test strains, the Ames test can also show the exact mechanism of mutation such as point or frameshift.

The Ames test has broad practical applications. It helps to screen the mutagenic potential of chemicals that are readily soluble in liquid suspensions, such as pesticides, wastewater, cosmetics, reagents, dyes and drugs. Different flavoring agents (safrole), food additives (AF-2),

antibacterial additives (furylfuramide), drugs (anti TB isoniazid), flame retardants (tris-BP), chemicals in commercial hair dyes and pesticides (DDT) have all been found to cause mutation and cancer using the Ames test. Also, the test is sensitive enough to identify suitable mutants from large bacterial populations. This test has been included in the USA Toxic Substances Control Act and Pesticides Act. However, the Ames test has certain limitations. To test mutagenic compounds for humans, *Salmonella* is not the finest test organism. Initially, many chemicals are safe for humans. However, when metabolized they can be converted to mutagens, for example, in the stomach, HCl acts on sodium nitrate and converts it to an effective mutagen, i.e., nitrous oxide.

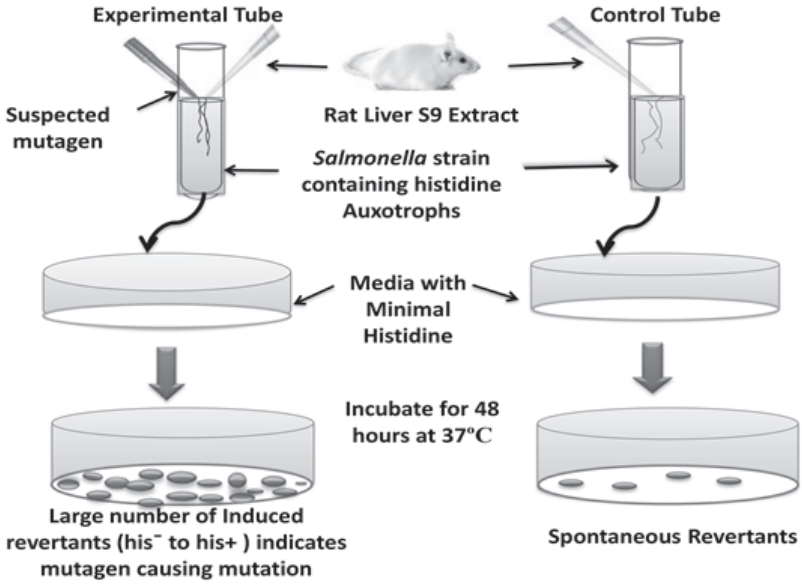

Figure 4.1: A schematic diagram of the Ames test. S9 extract of rat liver and *Salmonella* strain having his auxotrophs were added in experimental and control tubes. The suspected mutagen was added in an experimental tube with histidine free media. In the control, *Salmonella* was unable to grow due to histidine absence, whereas in an experimental plate, a large number of *Salmonella* colonies grew, showing that the suspected mutagen has reverted the *Salmonella* to His+ strain. S9 extract was added to mimic the metabolism of mammals as it contains the enzymes involves in mammal's metabolism (Vijay *et al*., 2018).

4.2 *Umu* Chromotest

The *umu* chromotest is a highly sensitive and convenient method used for the evaluation of mutagenicity of a compound based on its ability of DNA damage dependent activation of *umu* operon expression. This test is closely related to the Ames test and can detect different genetic damage using a single bacterial strain. A genetically engineered bacterial strain, *Salmonella typhimurium* TA 1535/pSK 1002 with fused *umu*C'-'*lac*Z gene (Figure 4.4) has been used to access the damage. Induction of SOS response results in activation of the *umu*C'-'*lac*Z gene region in the plasmid and can be monitored by the activity of *lac Z* gene product, β-galactosidase. β-galactosidase converts colorless ONPG into o-nitrophenyl, a yellow product which is colorimetrically recorded at OD420. This assay gives an indirect measurement of DNA damage due to a test compound. (Oda *et al*., 1985; Yasunaga *et al*., 2004).

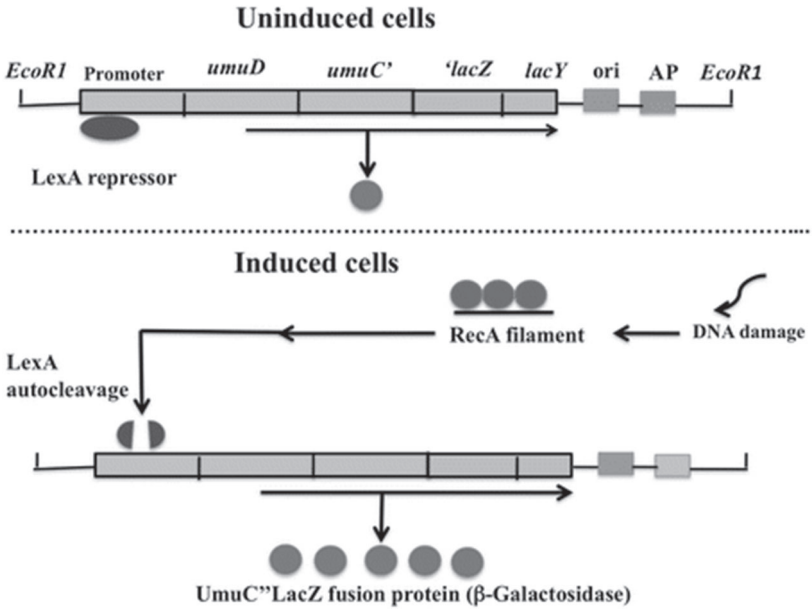

Figure 4.2: The principle of the *umu* test. Upstream from *umu* operon LexA repressor bind to SOS box operator sequences. When DNA is damaged, the cell produces an SOS signal. This signal activates protease activity of RecA protein, as a result, LexA repressor is autocleavaged. This allows expression of fused *umu*C'-'*lac*Z gene and production of β-galactosidase (Oda, 2016).

This test is highly reproducible, simple, and rapid with significantly reduced labor and material expenses. Practically, it has been applied to detect the mutagenetic, cytotoxic and carcinogenic potential of chemicals present in complex raw mixtures, water-soluble air pollutants, potable water supplies, surface and groundwater, municipal discharges and industrial effluents (Oda, 2016; Nie *et al*., 2017).

4.3 *Allium cepa* test

The *Allium cepa* test is used to chemically screen and monitor mutagenicity and cytotoxicity of environmental pollutants. It has also been frequently used to evaluate the mutagenicity of pesticides and insecticides present in vegetables and fruit and known to pose serious health risks (Feretti *et al*., 2007). As the root meristem of *A. cepa* is grown in direct contact with the test compound this test is an excellent *in vivo* model to detect DNA damage in eukaryotes (Tedesco and Laughinghouse, 2012).

Figure 4.3 explains the *Allium. cepa* test in a schematic diagram. Concisely, in the *A. cepa* test, uniformly sized young onion bulbs were selected which could fit in the holes of a rack or in the mouth of a glass jar container. Loose outer scales were removed to uncover the bulbs and expose the root primordia. One quarter of the bulbs were submerged in normal growth water and allowed to grow for 2–5 days at 20 °C. The bulbs were then transferred to a similar container containing test solution for 2–24 h for treatment in a time-dependent manner. Mitotic delay can also be induced chemically, to allow one complete cell cycle and analysis of chromosomal aberrations at each cell cycle stage (Grant, 1982). The root tips were cut up to 10 mm and placed in a glass tube with HCl/ acetic acid solution. To fix and macerate the root cells, the tips were further heated for 5 minutes, placed on a microscope slide and stained with a few drops of orcein solution. Microscopic analysis was performed to calculate the mitotic index and detect chromosome aberrations (Feretti *et al*., 2007). Rodríguez *et al*. (2015) detected the cytotoxicity of an insecticide, imidacloprid, through a modified version, where seeds of *Allium cepa* were germinated at 23 ºC in Petri dishes lined with paper filters moistened with the test compound. Imidacloprid caused chromosomal aberrations and micronuclei in the tested seed samples (Rodríguez *et al*., 2015). This test has also been employed for the evaluation of cytotoxicity of medicinal plants (Fachinetto *et al*., 2007; Fachinetto *et al*., 2009).

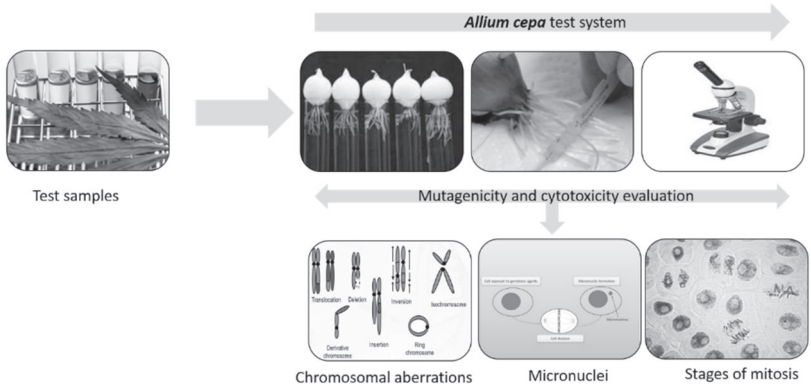

Figure 4.3: Schematic diagram of the *Allium cepa* test.

The *Allium cepa* test has broad practical applications and has already assessed many chemical agents to monitor the environment and effects caused by xenobiotics. This test has also detected various environmental pollutants such as metals, aromatic hydrocarbons, disinfectants of drinking water and radioactive contaminants in soil samples. Due to its low cost, easy handling and short time, this test is preferred over other such tests with complicated experimental setups. This test also plays an important role in the evaluation of the action mechanisms of a compound and its effect on genetic content (Leme and Marin-Morales, 2009). However, using a plant test system for carcinogens shows certain restrictions as it is not suitable for carcinogen classes which require complex metabolic systems to become activated and cause mutagenicity (Tedesco and Laughinghouse, 2012).

4.4 Chromosomal Aberration Test

The chromosomal aberration test is based on the extent a test sample induces chromosomal abnormalities in *in vitro* cultured mammalian cells. In many human genetic diseases, alterations in tumor suppressor genes and oncogenes result due to chromosomal aberrations. This test is very useful in detecting the possible compounds responsible for these aberrations *in vitro*. A test can be performed with either primary cells, or secondary established cell lines to identify the agents responsible for chromosomal aberrations. Compounds can be tested both with and without metabolic activation (Hilliard *et al*., 1998). In brief, cells are exposed to different concentrations of the test substance and further treated with colchicine to arrest chromosomes at the metaphase. These cells are harvested and stained with

Giemsa dye to microscopically visualize the chromosomes at the metaphase stage. Numerical aberrations are detected by observing an increase or decrease in numbers due to a test compound (Mayshar *et al*., 2010). This test has several applications such helping to evaluate the mutagenic potential of pesticides. Chlorthiophos, an organophosphate pesticide responsible for gene mutation, chromosome damage and aneuploidy in human peripheral lymphocytes has been identified using this test (Akyıl and Konuk, 2015). However, false-positive results can also occur due to extreme osmolality and pH conditions (Scott *et al*., 1991).

4.5 *Tradescantia* Micronucleus Test

Tradescantia pallida, commonly known as walking jew or wandering jew is an ornamental plant and one of the best genetic models for identifying mutagens in the environment. This bioassay evaluates genetic damage induced by aneugenic or clastogenic agents in dividing cells and was first established in 1976 (Suyama *et al*., 2002). On exposure to mutagenic or genotoxic agents, the process of nuclear fragmentation occurs in reproductive cells due to chromosomal breaks or loss (Carneiro and Takayanagui, 2009).

Inflorescences of *T. pallida* are collected and maintained to perform this assay. They are further treated with chemical or physical test agents, microscope slides prepared and stained with a drop of acetic carmine (2%) for the demonstration of micronuclei. Slides are further examined under a light microscope and results are compared with the control (Rodríguez *et al*., 2015).

This bioassay is used conventionally used for monitoring water (Cassanego *et al*., 2014), chemical contaminants, insecticides (Rodríguez *et al.,* 2015, Mendonça *et al.,* 2019), and pollutants in the environment (air, soil) (Pereira *et al*., 2013, Batalha *et al*., 1999). Hundreds of chemicals in the food and drug industries are being tested using this bioassay. Genotoxicity of gaseous emissions and leachates from landfills and emissions from incinerators are also identified with the *Tradescantia* micronucleus test (Mielli *et al*., 2009).

4.6 Brine Shrimp Lethality Bioassay

This bioassay is a basic, high throughput test for the evaluation of cytotoxicity of bioactive chemicals. The test is based on the ability of test compounds to kill the zoological organism *Artemia salina,* also called brine shrimps, sea monkeys or fairy shrimps (Harwig and Scott, 1971). It was first

suggested in 1956 by Michael *et al.,* and was later developed. Figure 4-4 explains the basic protocol of the brine shrimp bioassay. Briefly, brine shrimp eggs are incubated in brine for 48 hours and produce many larvae (nauplii). These larvae are long enough, approximately 22 mm, to be seen without using high magnification microscopes. Larvae are further incubated with a test compound for 24 hours and analyzed for mortality rate. The cytotoxicity effect of a compound can also be monitored in a dose-dependent manner.

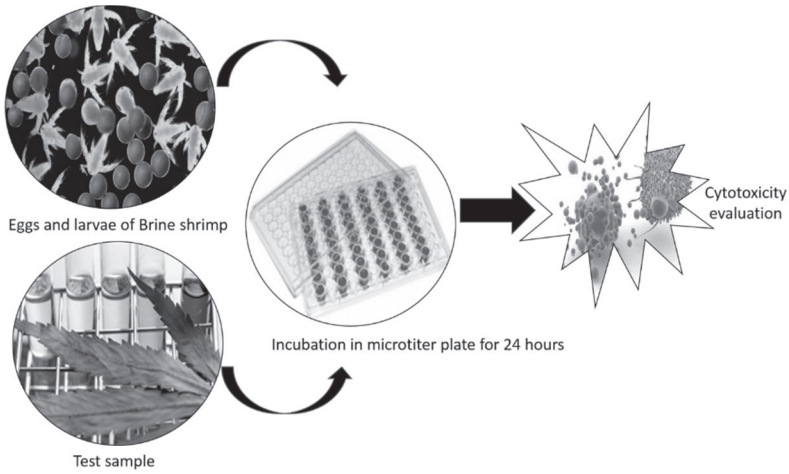

Figure 4-4 Schematic diagram of Brine shrimp lethality bioassay.

Brine shrimp are used for screening preliminary toxicity before performing experiments on animal models. It has been used to evaluate the toxicity of nanoparticles (Maurer-Jones *et al.,* 2013), pesticides (Michael *et al.,* 1956), heavy metals (Saliba and Krzyz, 1976), dental materials (Pelka *et al.,* 2000) and medicines, especially those from natural plant extracts (Sufian and Haque, 2015, Mwiti and Afolayan, 2016, Ghosh *et al*., 2015).

4.7 Micronucleus Assay

The micronucleus is the third or erratic nucleus originating during the anaphase of meiosis or mitosis. These are small cytoplasmic bodies containing the whole chromosome, a chromosome portion or an acentric chromosome that will not be able to move toward opposite poles at anaphase. This chromosomal region later develops a nuclear membrane

around it and becomes a micronucleus. After cytokinesis, one daughter cell will be normal with one nucleus and the other has two, one large and another small nucleus (micronucleus). The number of micronuclei could be more than one, depending on genetic damage. This assay can be performed both *in vivo* and *in vitro*. Usually, mouse bone marrow is used *in vivo* (Hayashi, 2016). Giemsa staining has been routinely used to score micronuclei. This assay has been considered as the most reliable and successful for the toxicological screening of potentially genotoxic compounds, carcinogens and chemical testing. This test is much simpler and easier to perform than a chromosomal aberration test (Picot, 2012).

4.8 Combined Dye-based Live:Dead Cell Assay

A combination of multiple dyes with different colors which label live and dead cells separately can be used in a single live:dead cell assay. The dyes used are usually esterase cleave dye for the live cells and ethidium homodimer for labeling dead cells in the live:dead cell assay. The viable cells have an intact plasma membrane and the ubiquitous intracellular-esterase activity. Cytotoxicity can be evaluated by live cells stained with the green fluorescent calcein-AM indicating intracellular-esterase activity and the red fluorescent ethidium homodimer 3 which indicates the loss of integrity of the plasma membrane. The assay can be used with many fluorescence detection methodologies such as fluorescence microscopy, flow cytometry, and fluorescence microplate readers. It is a fast, safe, sensitive and less expensive method to use for intracellular staining in flow cytometry for yeast, bacteria, fungi and mammalian cells.

4.9 Comet Assay (Single Cell Gel Electrophoresis)

Comet assay (Single Cell Gel Electrophoresis) is an assay that primarily identifies the integrity of DNA. It is known as the comet assay as the pattern of the DNA migration through gel electrophoresis looks like a comet. As a cell breaks there is a loss of supercoiling and DNA can freely migrate. Therefore, when a charge is applied, the damaged DNA leaves the nucleus and moves toward the anode due to its negative charge, forming tail-like structures called comets. In contrast, the undamaged DNA remains at its place giving a head like structure (Figure 4.5). Length, intensity and brightness of the comet tail about the head is an indicator of the number of DNA breaks.

Nandhakumar, 2011 explained the protocol of comet assay in detail to test the mutagenicity and cytotoxicity of any agent (Nandhakumar, 2011). In brief, cell samples (peripheral blood, *in vivo,* derived, *in vitro* cultures, sperm, tumors, bacteria and yeast cells) are dispersed to individual cells and suspended in molten, low melting point, agarose at 37 °C. This mono suspension is further cast on a microscope slide and allowed to polymerize at 4 °C. Agarose allows carbohydrate fiber matrix formation which encapsulates cells and anchors them in their place. As agarose behaves as an osmotic neutral, therefore, any test solution will penetrate the gel and affect encapsulated cells without shifting their position. To visualize DNA, slides are immersed in lysis solution and all other cellular contents such as RNA, proteins, nucleoplasm and cytoplasmic contents are broken down and diffused in an agarose matrix. Slides are further submerged in electrophoresis solution for 25 minutes and an electric field (usually 1 V/cm) is applied for about 20 minutes. At the end, slides are neutralized, stained with DNA specific dyes and visualized under a microscope.

Comet assay has been used for both prokaryotic cells and eukaryotic cells. This test has been widely adapted in bio testing and biomonitoring of several physical and chemical agents (Tice, 2000). This test has also been extensively employed in oncology research for screening genotoxicity (damage to the genetic materials), gauging the effectiveness of chemoprevention, and determination of the effectiveness of specific agents in halting the progression of invasive cancers in the human body.

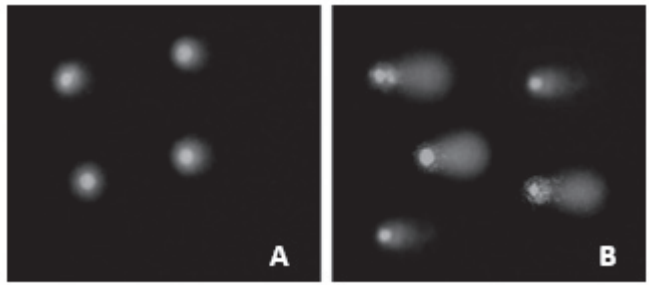

Figure 4.5 A) Undamaged DNA, B) Damaged DNA.

4.10 TUNEL Assay

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay is another method used to detect DNA fragmentation caused by a test

compound both *in vitro* (cells) and *in vivo* (plants and animals). This assay is based on terminal deoxynucleotidyl transferase (TdT) which catalyzes deoxynucleotide attachments to 3' hydroxy terminals of double-strand breaks of DNA. Therefore, it labels 3'-hydroxyl terminals of double-stranded DNA breaks generated during apoptosis.

4.11 Lactate Dehydrogenase (LDH) Assay

The lactate dehydrogenase (LDH) assay is a well-known assay for detecting cytotoxicity. LDH is an enzyme found in the cytoplasm of all cells. It is rapidly leaked in the supernatant of cell culture upon damage to the plasma membrane during necrosis, apoptosis, and some other types of cellular damage. Therefore, LDH is known as an indicator of cytolysis and cytotoxicity. An enzyme coupled reaction is carried out to measure LDH release by the colorimetric assay as shown in Figure 4.6. LDH normally reduces NAD^+ to NADH during lactate to pyruvate conversion. These assay measures reduce yellow tetrazolium salt INT into red water-soluble dye formazan-class. The quantity of formazan is directly proportional to the LDH amount in culture, which in turn is directly proportional to the number of damaged or dead cells. Therefore, cytotoxicity can be evaluated by measuring the activity of LDH by quantifying formazan at an absorbance of 492 nm. (Kumar *et al*., 2018)

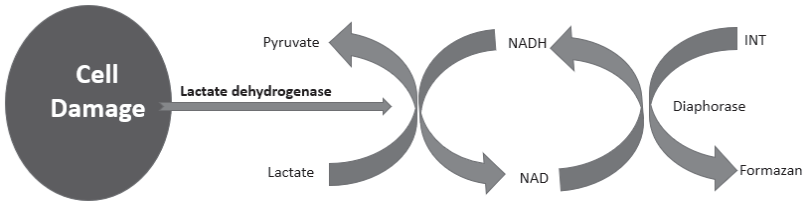

Figure 4.6 Chemical reaction of lactate dehydrogenase assay.

4.12 Adenylate Kinase (AK) Assay

AK is the phosphotransferase enzyme which is ubiquitously found in every cell of prokaryotes and eukaryotes. As a result of cytotoxic compounds, the plasma membrane becomes damaged and AK is rapidly released in the culture medium. AK has the property of helping to convert ADP to ATP, which can be detected by luciferase assay. The scheme in Figure 4.7 shows the reactions of the AK assay. The end-product can be easily quantified

colorimetrically at an absorbance of 570 nm or fluorometrically at an excitation/emission maxima = 535/587 nm (DiDone *et al*., 2010).

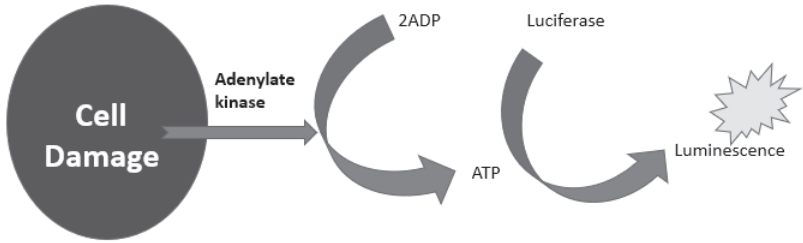

Figure 4.7 Chemical reaction of adenylate kinase assay.

4.13 Membrane-Impermeable Dye-based Assays

These assays are used to measure cytotoxicity using different membrane-impermeable, DNA binding fluorescent dyes which selectively stain cells with damaged membranes. These dyes can cross the membranes of live cells, making it a useful assay for differentiation of apoptotic, necrotic, and healthy cells.

4.13.1 Propidium Iodide (PI) is one such dye which binds to the DNA by intercalating between bases with no preference of sequence. In the aqueous solution, this dye has excitation/emission maxima of 493/636 nm. Once bound with the dye, fluorescence is increased about twenty to thirtyfold, resulting in the excitation maximum at about 535 nm and the fluorescence emission maximum at about 617 nm.

4.13.2 Ethidium Homodimer-1 is another membrane-impermeable, DNA binding fluorescent dye used to detect damaged or dead cells. When cell samples are stained with ethidium homodimer, dead cells can be observed and can be counted under a UV-light microscope.

4.13.3 7-Aminoactinomycin D (7-AAD) Assay is a third such kind of fluorescent dye that intercalates in the DNA of damaged cells and undergoes a spectral shift. The 7-AAD/DNA complex when excited by a laser of 488 nm has an emission maxima of approximately 647 nm. 7-AAD is also used for the analysis of the cell cycle by flow cytometry. 7-AAD binds specifically to the GC regions of the DNA which yields a distinct pattern of bands in the polytene chromosomes. It can also be used to stain chromatin in chromosome banding studies.

4.13.4 Trypan Blue Exclusion Assay is the fourth dye-based assay, it uses Trypan blue, a non-fluorescent cell stain. This is a classical cell viability assay by which the number of the viable cells in suspension can be visualized and calculated by a dye exclusion test. Cells that retain a clear cytoplasm are the viable cells and cells which have a blue-stained cytoplasm are the nonviable cells in this assay (Strober, 2015). All these assays are practiced in fluorescence microscopy, flow cytometry, confocal laser scanning microscopy and fluorometry.

4.13.5 Amine-Reactive Dye-based Assays

Amine-reactive dyes intensely stain damaged or dead cells with compromised membranes due to their strong interactions with free intracellular amines. In contrast, live cells exclude these dyes because of their intact cell membranes, and free dye is washed away after staining (Figure 4.8). Unlike other viability dyes, the amine-reactive dye-based reaction is irreversible, therefore, this assay is also suitable for the identification of dead cells in samples which are later fixed and permeabilized. Since amine-reactive dyes are fluorescent when excited by lasers, dead cells can be identified by flow cytometry (Perfetto *et al*., 2010).

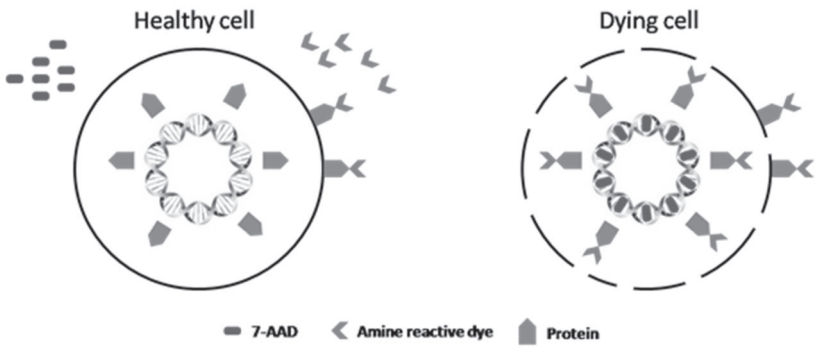

Figure 4.8 Principle of amine-reactive dyes.

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CHAPTER 5

INDUCED CYTOLOGICAL ABERRATIONS THROUGH MUTAGENESIS: A REVIEW

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Abstract: Estimation of cytological abnormalities and their magnitude during mitosis or meiosis is the most important index for evaluating the effect of a mutagen. It also provides a considerable clue to assess the radiosensitivity of plants to both physical and chemical mutagens. Mutagen-induced chromosomal aberrations have been reported by many workers in different plants such as in pea (Kallo, 1972), triticale (Pushpalatha *et al.,* 1992), lentil (Reddy and Annadurai, 1992), fenugreek (Anis and Wani, 1997), *Capsicum annum* (Anis *et al.,* 2000) and broad bean (Bhat, *et al.,* 2007). The mutants are an important source of valuable

genes that are used for crop improvement (Gulfishan *et al.,* 2015). Most of these workers observed dose-dependent increases in the frequency of chromosomal abnormalities due to mutagenic treatments. In the present review, a case study has been taken as an example in *Vicia faba* L. The two varieties of *Vicia faba* L. were exposed to three mutagens, i.e., EMS, gamma rays, methyl methanesulfonate (MMS) and their combination treatments. A detailed meiotic study was undertaken, and chromosomal aberrations were recorded. The chromosomal aberrations recorded were univalents, multivalents, stickiness, bridges, laggards, precautious separation, micronuclei, multinucleate condition, cytomixis, non-orientation, fragments, unequal separation, etc. The metaphase showed more chromosomal aberrations than anaphase followed by telophase and the aberrations increased with an increase in the dose of mutagens. The combined doses were more effective than individual doses in inducing chromosomal aberrations. In the combined doses, MMS+gamma rays were more effective than EMS+gamma rays. In the individual doses, MMS was more effective than gamma rays, followed by EMS. *Vicia faba* var. minor showed more chromosomal aberrations than *Vicia faba* var. major. The chiasmata frequency also showed a dose-dependent decrease and combined doses were more effective than individual doses.

Keywords: Mutagenesis, cytological aberrations, cytomoxis, mutagens.

5.1 Review of Mutagen-induced Chromosomal Aberrations

Laxmi *et al.* (1975) reported different meiotic abnormalities such as chromatin bridges, laggards, fragments, cytomixis, tripolar division, inversion, micronuclei and unequal separation of chromosomes in pearl millet following treatments with gamma rays and EMS. Lagging chromosomes and unequal separation of chromosomes were more frequent than other anomalies. They further reported that gamma rays were more effective than EMS or combination treatments in inducing chromosomal anomalies. An increase in the frequency of meiotic anomalies with the increase in dose and duration of mutagens was reported by Suganthi and Reddy (1992). Gamma rays induced meiotic aberrations have also been reported in chickpea by Ahmed (1993) and in broad bean by Bhat *et al.,* (2007). The meiotic aberrations increased with an increase in the dose of gamma rays as reported by Ganai *et al.* (2005) in chickpea and by Parveen *et al.* (2006) in *Trigonella.*

Subhash and Nizam (1977) reported that increasing the dose of X-rays resulted in the formation of an increased number of multivalents, fragments, bridges and micronuclei in *Capsicum annuum.* Katiyar (1978a) has reported chromosomal aberrations such as stickiness, altered association, breakage, bridges, laggards and abnormal microspores after gamma irradiation in chili. Pollen sterility increased with the increase in the dose of gamma rays and abnormalities were comparatively more in M_1 than in M_2 generations. Similar results were also reported by many workers (Rao and Laxmi, 1980; Tarar and Dnyansagar, 1980; Subhash and Venkatrajam, 1983; Bhat *et al., (*2007a), Bhat and Wani (2017)).

Katiyar (l978) investigated *Capsicum annum* L. plants grown from gamma-irradiated and control seeds for meiotic aberrations and pollen sterility in M_1 and M_2 generations. Chromosomal aberrations included stickiness, altered association, breakage, bridges, unequal segregation, laggards and abnormal microspores and their frequencies were dose-dependent. Pollen sterility showed a dose-dependent increase. The percentages of chromosomal aberrations were more in M_1 than M_2 generations as reported by Bhat *et al.,* (2007).

Rao and Kumar (1983) isolated three desynaptic mutants in a population of a local cultivar of chili. Meiotic studies showed reduced chiasma frequency and pollen fertility in desynaptic mutants compared with normal plants. The desynaptic plants were weak and medium-strong types. The mutant showed a monogenic pattern of inheritance as reported by Bhat and Wani (2015) in broad bean and by Gulfishan *et al.* (2013) in *Capsicum*.

Datta and Biswas (1986) detected the occurrence of cytomixis in the M_2 generation of a lax branching type mutant of *Nigella sativa* L. through EMS treatment. The presence of cytomixis was restricted only in the prophase-I cells of meiotic division. Transfer of nuclear materials from one pollen mother cell (PMC) to the adjacent PMCs occurred at random and in a specific direction within the PMCs. Cytomixis resulted in the formation of aneuploid and polyploid PMCs. Multivalents were absent in the mutant plant which indicated that the migration phenomenon was initiated after chromosome pairing in this material. Pollen fertility was reduced in the mutant (6.74%) but normal seed setting was not affected in the lax branching mutant. Aneuploids were detected among 0.65% individuals of the M_3 population following the selfing of the mutant, one of which was a trisomic. Phenotypically the trisomic plant was weak with a slender stem and drooping lamina. The extra chromosome in the

trisomic plant was present mostly as a univalent and rarely as trivalent. The plant was completely seed-sterile due to the unbalanced condition of the chromosomes in the zygote. The trisomic possibly arose through a union of normal and aneuploid gametes, the latter being the consequences of cytomixis. Therrien and Grant (1984) treated the seeds of *Lotus corniculatus* L. (Leguminoceae) with different concentrations (0.001, 0.01, 0.1 and 1.0%) of ethyl methanesulphonate, selfed, selectively crossed and open-pollinated lines were assessed for meiotic chromosome aberrations and quadrivalent formation in plants derived from treatment over four generations. The effect of EMS was confined largely to the M_1 generation with some persisting to the M_3 and M_4 generations after out-crossing. A significant increase in chromosome aberrations was not recovered in the progeny, except for a single dose in the M_3 generation after open pollination.

Datta and Biswas (1985) isolated a bushy mutant with desynaptic meiotic behavior from the M_2 population of *Nigella sativa* L. (black cumin) following 2 hours treatment with 0.5% EMS. A single pair of recessive genes (bu/bu) was ascribed for bushiness and the mutant bred true in the subsequent generations. The bushy mutant was characterized by delayed germination, flowering and maturity, high frequency of pollen sterility, poor seed setting and desynaptic behavior of chromosomes, possibly due to the pleiotropic effect of the mutant gene, as reported by Bhat *et al.* (2007).

Kumar and Rao (1985) isolated one desynaptic plant in a population of *Capsicum frutescence.* Meiotic studies in the normal and the desynaptic plants showed reduced chiasma frequency and pollen fertility. Chromosome pairing at pachytene was normal and complete in the normal plants, while it was partial in the desynaptic plants. Twelve bivalents were regularly formed both at diakinesis and metaphase-I in the normal, while univalents ranging from 12-24 were recorded at the corresponding stages in the desynaptic. At anaphase-I the chromosome segregation was regular (12:12) in the normal and irregular in the desynaptic.

Verma *et al.* (1986) observed cytomixis in triploid mulberry. It has been attributed to initial tetraploid and triploid hybrid nature. The cytomixis involved many cells at a time and was observed in all the stages of meiosis, contrary to the common belief that only earlier stages are favorable. In some cases, two different stages of meiosis were also involved in cytomixis. Cytomixis resulted in other abnormalities such as

anucleate cells, cells with more or fewer chromosomes, multinucleate cells, unusually large cells, triads, pentads and hexads. Cytomixis leads to sterility in mulberry, resulting in higher leaf yield and nutritiveness, useful traits for silkworm feeding.

Jayabalan and Rao (1987a) reported more meiotic abnormalities and pollen sterility in the combination than in the individual treatments in tomato. Venkateshwarlu *et al.* (1988) studied the effect of single and combined treatments with gamma rays, EMS and hydroxylamine (RA) in *Catharanthus roseus.* Besides various meiotic aberrations, tetrad abnormalities such as monads, dyads, triads and polyads were also observed. A dose-dependent decrease in pollen fertility was reported in *Vigna radiata* (Ignacimuthu and Sakthivel, 1989) following treatments with gamma rays and EMS.

Sapre and Deshpande (1987) reported a change in the chromosome number of some PMCs in an interspecific hybrid of *Coix* L. (*C. gigantean* x *C. aquatica,* 2n = 16) through cytomixis. In the cytomictic PMCs the chromosomal changes were 2n=13 and 2n=17 instead of the usual 2n=16, i.e. having a variable number of parental chromosomes instead of the usual 10+6 *C. gigantea* and *C. aquatica* combination.

Soodan and Walai (1987) detected cytomixis in some individuals of almond *(Prunus amygdalus* Baisch) and peach *(Prunus persica* Batsch), while others growing in their neighborhood and undergoing meiosis almost simultaneously did not exhibit the anomaly. This indicates the involvement of specific genes that are expressed only under particular environmental conditions. The fact that all pollen mother cells present inside the microsporangium are not involved in cytomixis indicates that they either vary in their cytomictic potential or fail to get exposed to the specific conditions required to trigger the process.

Lakshmi *et al.* (1989) recorded cytomixis, between adjacent PMCs in a sterile plant screened in the population of the Sindhur variety of *Capsicum annum* L. In 36.5% of cells cytomixis was affected through cytoplasmic bridges, resulting in PMCs with a variable number of chromosomes ranging from 4-36. Interestingly here, the phenomenon of cytomixis was associated with medium-strong desynapsis. It was also observed that cytomixis has some sort of negative effect on desynapsis resulting in increased pairing in the cells involved in cytomixis. Rao *et al.* (1990) isolated a desynaptic mutant in a progeny of pearl millet (*Pennisetum americanum*), the mutant showed dissociation of bivalents into univalents

and formation of non-specific congregations of chromosomes at diakinesis, shrinkage of cytoplasm and occurrence of unoriented sticky chromatin masses at metaphase-I, relaxation of stickiness, unbalanced chromosome numbers at the poles and laggards at anaphase-I and presence of other irregularities in subsequent stages. Male and female sterility was high. This meiotic mutant thus has multiple effects and is inherited as a monogenic recessive and designated as such. Reddy and Annadurai (1992) studied the effect of gamma rays, EMS, sodium azide and their combination on various cytological parameters in the M_2 generation in lentil variety PL-639. The mean values of quadrivalents, rod bivalents, univalents, fragments, bridges and pollen sterility showed an increase in the mutagenic treated population, while the chiasma frequency was decreased.

Velazquez (1994) isolated a desynaptic mutant in a wild population of *Rhoeo* which showed a high frequency of univalents at metaphase-I. The seedlings were found to be diploid with 2n = 2x=12. The mutant showed 6-12 univalents per cell and had a karyotype 8M+4SM, differing from that of the other desynaptic plants referred to in other work by the same author, which had 7M+5SM. The mutant had a high pollen fertility (65.7%). The microspores showed chromosome numbers from n=5 to n=14, unreduced pollen grains were formed at telophase second via second division restitution and comprised 34.14% of the pollen grains.

Mitra and Bhowmik (1996) reported radiosensitivity in two cultivars of black cumin (*Nigella sativa*) after treatments with gamma rays and EMS. Mitotic index was found to decrease with an increasing dose of mutagens, but the mitotic and meiotic abnormalities showed an increasing trend with mutagen doses. They observed no varietal differences in the mitotic index as well as cytological abnormalities.

Maria de Souza and Pagliarini (1997) investigated the meiotic behavior of eleven canola cultivars *(Brassica campestris* and *Brassica napus),* eight showing cytomixis between sporocytes. The percentage of cells with cytomixis was low. In two cultivars, cytoplasmic bridges without chromatin transfer between cells were higher than cytomixis. All meiotic phases from pachytene to tetrads were affected. Chromosome transfer was observed between cells in the same phase of division and also between cells in different phases of division. Poddar *et al.* (1998) isolated five medium-strong desynaptic mutants (DM1, DM2, DM3, DM4 and DM5) in the M_2 generation from gamma irradiated seeds of *Rhoeo spathacea* var. concolor. The desynaptic plants varied morphologically

and showed very high to complete pollen and ovule sterility. Crossing experiments were only successful between the pollen parent of DM3 and standard normal, which revealed monogenic recessive inheritance of the mutant trait. The manifestation of the intensity of desynaptic expression was also evidenced. Microspores and pollen grains showed hypo- and hyper-diploid chromosome number.

Singh *et al.* (1999) studied the effect of gamma rays, EMS and epichlorhydrin (ECH) on meiosis in *Vigna radiata* cv. PS 16. Meiotic studies showed chromosomal aberrations such as univalents, multivalents, ring chromosomes and laggards in treated populations. The frequency of cells showing chromosomal aberrations showed a linear increase with increasing dose as reported by Sharma *et al.,* (2009) and Golfishan *et al.,* (2010) in broad bean.

Saha and Datta (2000) observed the gamma irradiation-induced 3 translocation heterozygotes in the seeds of *Nigella damascena* L. These exhibited the formation of either a ring or a chain of four chromosomes in meiocytes. The predominance of rings or an equal proportion of rings and chains occurred in translocation heterozygotes. The rings showed a preponderance of adjacent orientation and the chains demonstrated frequent alternate orientation, though pollen fertility showed a reduction.

Kumar and Sharma (2001) isolated two desynaptic mutants at 8h of treatment with 0.5% of EMS in chickpea. The average number of univalents and bivalents per cell was 10.56 and 2.72 in mutant-1, 10.21 and 2.89 in mutant-2, respectively. The desynaptics obtained was medium-strong with reduced pollen fertility.

Kumar and Srivastava (2001) noticed cytomixis in plants of *Plantago ovata* Forsk raised from seeds treated with three different treatment durations, viz., 3, 5 and 7 hrs of 0.5% EMS. Chromosome transfer between microsporocytes occurred from the pachytene to telophase-II stages of meiosis. Two, three or a series of several cells were involved in cytomixis.

Kumar and Sharma (2002) observed cytomixis at different stages of meiosis in *Cicer arietinum* L. treated with three different concentrations viz., 0.2%, 0.4% and 0.6% of sodium azide. It has been observed at different stages of meiosis and may occur between two or more pollen mother cells and caused various types of irregularities in meiosis, resulting in hypo or hyperploid gametes.

Kumar and Tripathi (2002) isolated a strong type tetraploid desynaptic mutant of *Aloe barbadensis* (4n=28) during microsporogenesis. As a consequence of the formation of univalents, the meiosis was frequently disturbed by such irregularities as defective congression, unequal chromosome segregation, laggards and multipolarity leading to high pollen sterility.

Saha and Datta (2002) induced 5 translocation heterozygotes (P-14 and P-26 from 5kR and P-32, P-36 and P-37 from 10kR) in gamma-irradiated seeds of *Nigella sativa* L. (black cumin). P-14, P-32 and P-36 were viable translocations, while P-26 and P-37 yielded only abortive seeds at M_1 following selfing and on open or controlled pollination. The translocation heterozygotes exhibited the formation of either a ring or a chain of four chromosomes in 38.71% to 77.72% meiocytes.

Kumar *et al*. (2003) isolated one desynaptic mutant in *Vigna mungo* at 7h treatment of 0.5% EMS. At metaphase-I abnormal chromosomal configurations with a variable number of univalents and bivalents were frequently observed. Bridges, laggards and unequal separation of chromosomes were also observed at anaphase-I.

Kumar and Singh (2003) isolated a plant having a double interchange from the population raised from gamma ray irradiated seeds in *Pennisetum typhoids.* Diakinesis and Metaphase-I configurations revealed the predominance of a hexavalent along with four bivalents. In a few PMCs univalents along with a variable number of bi and multivalents could also be observed. Anaphase-I studies exhibited various anomalies as well, such as unequal separation, bridge and lagging chromosomes.

Kumar and Singh (2003) provided a relative account of cytological and development effects of gamma rays and EMS on meiotic features and gametic fertility in *Hordeum vulgare.* Studies undertaken in the M_1 generation on the variety K-12 of this species showed that both the physical and chemical forms of mutagens elicit various kinds of chromosomal abnormalities and reduction in pollen and seed fertility. Such effects were dose dependent. However, the percentage of abnormalities induced by gamma rays was higher than that induced by EMS, suggesting that gamma rays could be more effective in inducing genetic changes compared to EMS in this crop.

Tyagi (2003) recorded cytomixis during microsporogenesis in a tetraploid (2n=4x=48) genotype of spearmint *(Mentha spicata* L.), an aromatic plant

of the family Lamiaceae. The phenomenon of cytomixis was observed in leptotene to pachytene stages of the first meiotic prophase. The migration of nuclear content involved all the chromosomes or some of the chromosomes of the donor cell.

Sharma *et al.* (2004) studied the effect of different doses of gamma rays and EMS on meiotic behavior of chickpea. A dose-dependent increase in meiotic irregularities and sterility was observed at all the individual and combined dose treatments; however, combined treatments proved to be more efficient. Radiation-induced sterility was attributed to cryptic deletions and specific gene mutations, while the sterility caused by EMS and combined treatments could be attributed to chromosomal aberrations as reported by Golfishan *et al.* (2012) in *Capsicum.*

Verma *et al.* (2004) induced in broad bean *(Vicia faba* L.) seven translocation heterozygotes (5 with gamma rays and two with EMS treatment) and two paracentric inversion heterozygotes (in gamma ray treated materials). Three of the translocation heterozygotes involved the metacentric chromosome pair, whereas the remaining four had translocation in the acrocentric chromosome pairs. The induced translocations showed a ring or chain of four chromosomes in most of the PMCs at diakinesis/metaphase-I. The paracentric inversions were detected as a distinct bridge and fragment or loop-fragment observed at meiotic anaphase-II.

Bhat *et al.* (2005a) provided a relative account of the cytological and developmental effects of gamma rays, EMS and MMS on meiotic features and pollen fertility in *Vicia faba* L. Studies undertaken in M_1 generation on the variety minor of this species showed that both the physical and chemical mutagens elicit various kinds of chromosomal aberrations and reduction in pollen fertility. Such effects were dose-dependent and positively correlated with dose/concentration. However, the induction of meiotic aberrations was observed to be higher under MMS treatments followed by gamma rays and EMS, suggesting that MMS could be more effective in inducing genetic variability followed by gamma rays and EMS in this crop.

Bhat *et al.* (2005b) studied the relative effects of EMS and MMS on meiosis and pollen sterility in *Vicia faba* L. var. major in M_1 generation. Meiotic studies revealed various aberrations such as stickiness, laggards, bridges, precocious separations, disturbed polarity, cytomixis and non-synchronization. The stickiness of chromosomes was the most common

aberration, followed by bridges and precocious separation. Among the different stages of meiosis, the frequency of chromosomal aberrations was maximum at the metaphase-I stage and showed a linear increase with the increase in concentration, with both the mutagens. However, MMS induced a higher frequency of aberrations than EMS. Pollen sterility was the cumulative result of various meiotic aberrations as reported by Golfishan *et al.* (2011) in *Capsicum.*

Joshi and Verma (2004) isolated one medium-strong asynaptic mutant of *Vicia faba* L. (2n=12) from the M_2 population of 0.2% EMS treated seeds. At diakinesis/metaphase-I of meiosis the number of univalents ranged from 2-8 in 90% PMCs in M_2 and 2-4 in 44% of PMCs in M_3. A significant decrease in the number of chiasmata in this plant as compared to the control was found. The metacentric chromosome pair did not show univalents.

Mukherjee and Datta (2005) performed a meiotic analysis in *Ocimum basilicum* L. (2n=72) and *O. tenuflorum* L. (2n=36) and demonstrated the persistent presence of secondary association of chromosomes in 94.74 and 85.16 % of metaphase-I cells respectively.

Bhat *et al*. (2007) studied the comparative analysis of meiotic aberrations induced by DES and sodium azide (SA) in *Vicia faba* L. Stickiness, stray bivalents, univalents, multivalent, laggards, bridges, cytomixis, micronuclei, and disturbed polarity were the main chromosomal aberrations and these aberrations increased with the increase in the concentration of each mutagen. The DES was more effective than SA as it induced more chromosomal aberrations. Seeds of *Capsicum annuum* L. varieties CO-1 and jwala were treated with the potent chemical mutagen, EMS, resulting in various types of meiotic chromosomal aberrations such as multivalents, stickiness, clumping, bridges, laggards, micronuclei, tripolar orientation, pentad, non-synchronous separation, etc. Varietal response to the chromosomal aberrations was very pronounced, i.e., the variety jwala was more sensitive and the frequency of aberrations was comparatively high at all the mutagenic concentrations (Salam and Thoppil, 2010).

Seeds of *Capsicum annuum* L. var. G4 were subjected to different concentrations of MMS and diethyl sulfate (DES). Various types of meiotic aberrations such as univalents, multivalents, stickiness, bridges, laggards, cytomixis etc. were observed in all the treatments. However, the MMS treatments proved to be more effective in inducing meiotic aberrations as compared to DES. The frequency of meiotic aberrations was

maximum at metaphase followed by anaphase and telophase stages. As the concentrations increased, reduction in chiasma frequency and pollen fertility was observed in all the treatments and MMS was again found to be more effective than DES treatments (Gulfishan *et al*., 2012 and Bhat and Sharma (2007)).

Bhat *et al*. (2006b) studied the relative effects of EMS on meiosis and pollen fertility in *Vicia faba* L. var. 'minor' in the M_1 generation. Meiotic studies revealed various types of chromosomal aberrations such as stickiness, laggards, bridges, precocious separations, disturbed polarity, cytomixis and non-synchronization. The stickiness of chromosomes was the most common aberration, followed by bridges and precocious separation. Among the different stages of meiosis, the frequency of chromosomal aberrations was maximum at the metaphase-I stage and showed a linear increase with an increase in the concentration of the mutagen. Pollen sterility was the cumulative result of various meiotic aberrations.

Bhat *et al.* (2006c) carried out the meiotic studies in two varieties of *Vicia faba* L. viz., major and minor, after treatment with different concentrations of EMS. Different types of meiotic abnormalities such as stickiness, univalents, multivalents, unorientation of chromosomes, precocious separation of chromosomes at metaphase, bridges, laggards and unequal separation of chromosomes at anaphase were recorded. The meiotic aberrations in both the varieties were dose-dependent, however, *Vicia faba* L. variety minor showed more chromosomal aberrations as compared to *Vicia faba L.* variety major with the same treatment.

Bhat *et al.* (2006a) studied the relative effects of EMS on meiosis and pollen fertility in *Vicia faba* L. var. minor in the M_1 generation. Meiotic studies revealed various aberrations like stickiness, laggards, bridges, precocious separations, disturbed polarity, cytomixis and non-synchronization. The stickiness of chromosomes was the most common aberration followed by bridges and precocious separation. Among the different stages of meiosis, the frequency of chromosomal aberrations was maximum at the metaphase-I stage and showed a linear increase with the increase in the concentration of the mutagen. Pollen sterility was the cumulative result of various meiotic aberrations.

Bhat *et al*. (2006d) reported cytomixis during microsporogenesis in various stages of meiosis in MMS treated populations of *Vicia faba* L. Cytomixis was observed to occur through various methods, i.e. by

forming cytoplasmic channels and direct fusion of pollen mother cells. The migration of nuclear content involved all the chromatin/chromosomes or part of them from donor to recipient cell/cells. The occurrence of PMCs with chromosome numbers deviating from diploid number (2n=12) through the process of cytomixis leads to the production of aneuploid cells in all the populations treated with various concentrations of MMS. Increasing concentration of MMS had a positive effect on the percentage of PMCs showing cytomixis. The level of pollen fertility was found to be affected by cytomixis and chromosome stickiness.

Kumar and Gupta (2009) induced karyomorphological variations in three pheno deviants of *Capsicum annuum* L. Seeds were treated with 0.5% solution of EMS for 3, 5, and 7 h durations and genetic segregation was closely observed. Many chromosomal anomalies such as stickiness, bridges, and multivalents, secondary associations, laggards, and precocious movement were observed in all three durations of treatment as reported by Bhat *et al.,* (2006) in broad bean. These anomalies showed a dose-dependent increase in frequency. The morphological parameters showed a decreasing trend along with the increasing doses of treatments. However, with the 7 h dose, three morphological variants were isolated which varied in plant height, the number of nodes, leaf area, 100-seed weight (g), vigorousness and days to maturity, from other sib plants and also from control plants.

Kumar and Verma (2011) treated the seeds of *Vigna unguiculata* (cowpea) with gamma rays and sodium azide. They observed chromosomal aberrations such as unorientation, multivalents, laggards, bridges, micronuclei, stickiness and precocious movements, etc. Chromosomal aberrations were found to be correlated with the concentrations of both the mutagens individually as well as in combination. The combined treatment proved to be more effective in inducing chromosomal aberrations and sterility as compared to individual treatment sets.

Jafri *et al*. (2011a) carried out cytogenetical investigations in *Cichorium intybus* plants grown from seeds treated with EMS concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5%. Different meiotic irregularities were observed which were dose-dependent with increasing concentrations of mutagen.

Jafri *et al*. (2013) investigated the mutagenic effects of individual and combination treatment of gamma rays and EMS on *Coriandrum sativum* L. Results revealed the meiotic malfunctioning of pollen mother cells, (PMCs) that had shared various types of cytological abnormalities such as

univalents, multivalents, stickiness, precocious movement, stray bivalent, non-orientation, cytomixis, laggard, bridges, unequal separation, micronuclei and disturbed polarities.

Husain *et al.* (2013) studied the effect of two chemical mutagens viz. hydrazine hydrate (HZ) and maleic hydrazide (MH) on two varieties (NDF-1 and HB-405) of *Vicia faba* in terms of meiotic behavior and pollen sterility. They concluded that meiosis is a complex process that coordinates activity involving several genes and that mutation in any of these genes leads to irregularities.

Kolar *et al*. (2014) attempted to understand the effects of EMS, SA and gamma rays on the meiotic configuration of *Delphinium malabaricum*. The results demonstrated that the mutagens caused various types of cytological aberrations, such as univalents, chromatin bridges, laggards, fragments, stickiness and multinucleated cells. The maximum aberrations were found at higher doses/concentrations of the mutagens. The highest percentage of pollen mother cells showing abnormalities was induced by EMS followed by gamma rays and SA.

5.2 Induced Chromosomal Aberrations in Broad Bean

The control plants of var. minor and var. major revealed six perfect bivalents at diakinesis and metaphase-1 which separated into 6:6 at anaphase-I. Telophase-1, anaphase-2 and telophase-2 were normal stages.

The microsporogenesis of plants showed a range of chromosomal abnormalities which were more or less similar in both the varieties, but their frequencies were different (Tables1-6). The chromosomal aberrations were dose/concentration-dependent. The most frequent aberrations were univalents, multivalents, stickiness, precocious separation, non-orientation of bivalents and fragments at metaphase-I/II and laggards, bridges, unequal separation, non-disjunction and cytomixis at anaphase-I/II. The dominant meiotic aberrations at telophase I/II were disturbed polarity, micronuclei, multinucleate condition and cytomixis. Representative cytological features are shown in plate1 and 2 (Figs. 5.1-16).

The data recorded in the tables shows dose-dependent chromosomal aberrations. The combined treatments showed more frequency of chromosomal aberrations than individual treatments. Gamma rays + MMS induced more abnormalities than gamma rays + EMS followed by MMS, gamma rays and EMS. The total meiotic aberrations at metaphase-I/II for

var. minor ranged from 4.26-9.24% in EMS, 5.04-10.46% in gamma rays, 5.22-11.15% in MMS, 5.22-12.19% in gamma rays + EMS and 5.60-13.46% in gamma rays + MMS. The total meiotic aberrations at anaphase-I/II for var. minor ranged from 1.32-5.76% in EMS, 2.18-6.56% in gamma rays, 2.74-7.58% in MMS, 3.00-8.29% in gamma rays + EMS and 3.39-9.56% in gamma rays + MMS. The total meiotic aberrations at telophase I/II for var. minor ranged from 1.32-5.10% in EMS, 2.06-6.53% in gamma rays, 3.90-6.75% in MMS, 3.71-7.57% in gamma rays + EMS and 4.19-8.60% in gamma rays + MMS.

The total meiotic aberrations at metaphase-I/II for var. major ranged from 3.40-8.27% in EMS, 3.60-9.28% in gamma rays, 3.51-10.74% in MMS, 3.88-11.56% in gamma rays + EMS and 4.67-12.20% in gamma rays + MMS. The total meiotic aberrations at anaphase-I/II for var. major ranged from 1.13-4.14% in EMS, 1.34-5.01% in gamma rays, 1.85-6.13% in MMS, 2.22-6.97% in gamma rays + EMS and 2.82-8.30% in gamma rays + MMS. The total meiotic aberrations at telophase I/II for var. major ranged from 1.14-4.40% in EMS, 1.36-5.21% in gamma rays, 2.59-5.92% in MMS, 2.80-6.66% in gamma rays + EMS and 1.50-7.60% in gamma rays + MMS.

5.3 Chiasma Frequency

The number of PMCs from treated as well as untreated (control) plants were studied and chiasmata per cell were calculated (Tables 5.7 and 5.8). Results showing the frequency of chiasmata per cell in the treated plants as compared to their respective controls indicated that the chiasmata per cell decreased more in combined treatments with the increase of univalents and rod bivalents than the individual mutagenic treatments. The pooled mean of chiasmata per cell showed 19.27 (EMS), 19.12 (gamma rays), 18.80 (MMS), 18.30 (gamma rays + EMS), 17.80 (gamma rays + MMS) in var. minor. The pooled mean of chiasmata per cell showed 19.50 (EMS), 19.00 (gamma rays), 18.70 (MMS),18.35 (gamma rays + EMS), 17.92 (gamma rays + MMS) in var. major.

The pooled mean frequency of univalents were 0.45 (EMS), 0.55 (gamma rays), 0.65 (MMS), 0.75 (gamma rays + EMS) and 0.85 (gamma rays + MMS) in var. minor. The pooled mean frequency of univalents were 1.02 (EMS), 0.45 (gamma rays), 0.55 (MMS), 0.65 (gamma rays + EMS) and 0.75 (gamma rays + MMS) in var. major. The pooled mean frequency of multivalents were 0.55 (EMS), 0.67 (gamma rays), 0.77 (MMS), 0.85 (gamma rays + EMS) and 0.95 (gamma rays + MMS) in var. minor. The

pooled mean frequency of multivalents were 0.45 (EMS), 0.55 (gamma rays), 0.65 (MMS), 0.75 (gamma rays + EMS) and 0.82 (gamma rays + MMS) in var. major.

The pooled mean frequency of rod bivalents was 1.10 (EMS), 1.22 (gamma rays), 1.42 (MMS), 1.02 (gamma rays + EMS) and 1.27 (gamma rays + MMS) in var. minor. The pooled mean frequency of rod bivalents was 1.22 (EMS), 1.35 (gamma rays), 1.02 (MMS), 1.32 (gamma rays + EMS) and 1.42 (gamma rays + MMS) in var. major. The pooled mean frequency of ring bivalents was 3.52 (EMS), 3.70 (gamma rays), 3.17 (MMS), 3.19 (gamma rays + EMS) and 3.52 (gamma rays + MMS) in var. minor. The pooled mean frequency of ring bivalents was 3.55 (EMS), 3.97 (gamma rays), 3.22 (MMS), 3.14 (gamma rays + EMS) and 3.40 (gamma rays + MMS) in var. major.

In the present investigation, a dose-dependent decrease in the chiasmata frequency was observed in various treatments. The average frequency of chiasmata per cell decreased with an increase in univalents and rod bivalents in each treatment. Similar results were reported by Bhat and Wani (2015) and Gulfishan *et al.* (2013).

Chiasma frequency as influenced by each of the mutagens exhibited marked differences among the mutagens. However, there was a reduction in chiasma frequency at all treatment doses. The reduction was greater in combination treatments than individual mutagenic treatments as reported by Bhat and Wani (2015).

Table 5.1 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. minor in M_1 generation at metaphase-I/II (%).

Treatments	Total Number of PMCs Scored	Metaphase-I/II (%)						
		Univalents	Multivalents	Stickiness	Precocious Separation	Non-Orientation	Fragments	Total abnormalities
Control	550	-	-	-	-	-	-	-
EMS								
0.1(%)	540	0.74	1.11	0.92	0.55	0.94	-	4.26
0.2(%)	545	0.92	1.29	1.11	0.74	1.30	-	5.36
0.3(%)	550	1.32	1.64	1.46	1.12	1.46	0.56	7.56
0.4(%)	535	1.72	2.06	1.88	1.52	1.32	0.74	9.24
Gamma rays								
10KR	544	0.92	1.32	0.93	0.75	1.12	-	5.04
20KR	538	1.12	1.52	1.31	0.93	1.52	0.42	6.82
30kR	548	1.46	1.66	1.65	1.32	1.66	0.74	8.49
40KR	540	1.86	2.04	2.05	1.72	1.86	0.93	10.46

MMS (%)								
0.01(%)	543	0.93	1.29	0.93	0.74	1.11	0.22	5.22
0.02(%)	546	1.30	1.48	1.30	1.10	1.65	0.38	7.21
0.03(%)	548	1.66	2.04	1.83	1.46	1.84	0.74	9.57
0.04(%)	535	1.92	2.06	2.06	1.91	2.06	1.13	11.15
Gamma rays + EMS								
10kR+0.1%EMS	544	0.92	1.31	0.92	0.74	1.11	0.22	5.22
10kR+0.2%EMS	548	1.46	1.65	1.45	1.31	1.82	0.56	8.25
20KR+0.3%EMS	542	1.86	2.04	2.05	1.71	2.06	0.93	10.65
20KR+0.4%EMS	546	2.03	2.41	2.41	2.01	2.22	1.11	12.19
Gamma rays +MMS								
10kR+0.01%MMS	548	1.11	1.32	0.92	0.92	1.11	0.22	5.60
10KR+0.02%MMS	546	1.52	1.84	1.66	1.52	2.11	0.74	9.39
20KR+0.03%MMS	540	2.04	2.22	2.24	1.86	2.24	1.14	11.74
20KR+0.04%MMS	538	2.24	2.62	2.62	2.24	2.42	1.32	13.46

Table 5.2 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. minor in M_1 generation at Anaphase-I/II (%).

Treatments	Total Number of PMCs Scored	Laggards	Bridges	Unequal separation	Non- disjunction	Cytomixis	Total abnormalities
Control	-	-	-	-	-	-	-
EMS							
0.1(%)	540	-	-	1.12	0.20	-	1.32
0.2(%)	545	0.72	0.74	1.30	0.38	0.36	3.50
0.3(%)	550	0.90	0.92	1.46	0.55	0.54	4.37
0.4(%)	535	1.32	1.34	1.72	0.76	0.62	5.76
Gamma rays							
10KR	544	0.20	0.22	1.32	0.22	0.22	2.18
20KR	538	0.92	0.94	1.32	0.56	0.55	4.29
30kR	548	1.10	1.11	1.46	0.74	0.56	4.97
40KR	540	1.48	1.52	1.86	0.94	0.76	6.56

MMS (%)							
0.01(%)	543	0.40	0.42	1.32	0.22	0.38	2.74
0.02(%)	546	1.09	1.11	1.32	0.35	0.74	4.69
0.03(%)	548	1.09	1.11	1.64	0.72	0.74	5.30
0.04(%)	535	1.71	1.72	2.06	1.14	0.95	7.58
Gamma rays +EMS							
10kR+0.1%EMS	544	0.40	0.42	1.32	0.42	0.44	3.00
10kR+0.2%EMS	548	1.30	1.32	1.32	0.92	0.74	5.60
20KR+0.3%EMS	542	1.30	1.32	1.85	0.93	0.94	6.34
20KR+0.4%EMS	546	1.82	1.84	2.22	1.30	1.11	8.29
Gamma rays +MMS							
10kR+0.01%MMS	548	0.52	0.55	1.34	0.42	0.56	3.39
10KR+0.02%MMS	546	1.54	1.56	1.30	1.11	0.92	6.43
20KR+0.03%MMS	540	1.54	1.56	2.05	1.14	0.93	7.22
20KR+0.04%MMS	538`	2.04	2.06	2.42	1.52	1.52	9.56

Table 5.3 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. minor in M_1 generation at Telophase I/II (%).

Treatments	Total Number of PMCs Scored	TelophaseI/II (%)				
		Disturbed Polarity	Micronuclei	Multinucleate condition	Cytomixis	Total abnormalities
Control	550	-	-	-	-	-
EMS						
0.1(%)	540	1.32	-	-	-	1.32
0.2(%)	545	1.48	0.55	0.36	-	2.39
0.3(%)	550	1.82	0.90	0.54	0.54	3.80
0.4(%)	535	2.26	1.12	1.12	0.60	5.10
Gamma rays						
10KR	544	1.31	0.20	0.55	-	2.06
20KR	538	1.72	0.74	0.74	0.74	3.94
30kR	548	2.01	1.09	0.72	0.72	4.54
40KR	540	2.41	1.70	1.50	0.92	6.53

MMS						
0.01(%)	543	1.52	0.36	0.92	1.10	3.90
0.02(%)	546	1.66	0.91	1.09	0.73	4.39
0.03(%)	548	1.84	1.30	1.09	1.09	5.32
0.04(%)	535	2.43	1.50	1.70	1.12	6.75
Gamma rays +EMS						
10kR+0.1%EMS	544	1.49	0.40	0.91	0.91	3.71
10kR+0.2%EMS	548	1.85	1.09	1.30	1.09	5.33
20KR+0.3%EMS	542	2.04	1.50	1.30	1.30	6.14
20KR+0.4%EMS	546	2.64	1.64	1.83	1.46	7.57
Gamma rays +MMS						
10kR+0.01%MMS	548	1.47	0.54	1.09	1.09	4.19
10KR+0.02%MMS	546	2.02	1.28	1.50	1.28	6.30
20KR+0.03%MMS	540	1.72	1.70	1.50	1.50	6.42
20KR+0.04%MMS	538	2.82	1.85	2.23	1.70	8.60

Table 5.4 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. major in M_1 generation at Metaphase-I/II (%).

Treatments	Total Number of PMCs Scored	Metaphase-I/II (%)						
		Univalents	Multivalents	Stickiness	Precocious Separation	Non-Orientation	Fragments	Total abnormalities
Control	555	-	-	-	-	-	-	-
EMS								
0.1(%)	550	0.56	0.92	0.74	0.44	0.74	-	3.40
0.2(%)	552	0.74	1.10	0.92	0.55	0.92	-	4.23
0.3(%)	540	1.13	1.32	1.32	0.94	1.32	0.42	6.45
0.4(%)	550	1.46	1.83	1.65	1.33	1.46	0.54	8.27
Gamma rays								
10KR	540	0.76	0.94	0.74	0.42	0.74	-	3.60
20KR	548	0.92	1.13	1.12	0.74	1.11	0.38	5.40
30kR	546	1.12	1.46	1.48	1.11	1.47	0.38	7.02
40KR	542	1.68	1.86	1.84	1.48	1.86	0.56	9.28

MMS								
0.01(%)	547	0.74	0.92	0.74	0.37	0.74	-	3.51
0.02(%)	545	1.12	1.30	1.30	0.92	1.12	0.56	6.32
0.03(%)	548	1.28	1.66	1.66	1.30	1.84	0.57	8.31
0.04(%)	543	1.66	2.04	2.04	1.85	2.22	0.93	10.74
Gamma rays +EMS								
10kR+0.1%EMS	553	0.92	1.10	0.74	0.38	0.74	-	3.88
10kR+0.2%EMS	546	1.30	1.30	1.48	0.92	1.11	0.56	6.67
20KR+0.3%EMS	547	1.48	1.84	1.84	1.30	2.02	0.73	9.21
20KR+0.4%EMS	548	1.84	2.20	2.20	2.02	2.38	0.92	11.56
Gamma rays +MMS								
10kR+0.01%MMS	542	0.94	1.12	0.75	0.74	1.12	-	4.67
10KR+0.02%MMS	543	1.48	1.66	1.66	1.48	1.86	0.56	8.70
20KR+0.03%MMS	545	1.66	2.04	2.03	1.48	2.04	1.12	10.37
20KR+0.04%MMS	546	2.04	2.21	2.40	2.20	2.21	1.14	12.20

Table 5.5 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. major in M_1 generation at Anaphase-I/II (%).

Treatments	Total Number of PMCs Scored	Anaphase-I/II (%)					
		Laggards	Bridges	Unequal separation	Non-disjunction	Cytomixis	Total abnormalities
Control	555	-	-	-	-	-	-
EMS							
0.1(%)	550	-	0.91	0.22	-	-	1.13
0.2(%)	552	0.56	1.07	0.42	-	-	2.05
0.3(%)	540	0.76	1.32	0.56	0.22	0.22	3.08
0.4(%)	550	1.11	1.45	0.74	0.42	0.42	4.14
Gamma rays							
10KR	540	-	1.12	0.22	-	-	1.34
20KR	548	0.74	1.11	0.56	0.38	0.38	3.17
30kR	546	0.92	1.32	0.56	0.38	0.38	3.56
40KR	542	1.29	1.68	0.92	0.56	0.56	5.01

MMS							
0.01(%)	547	0.18	1.11	0.36	-	0.20	1.85
0.02(%)	545	0.92	1.13	0.73	0.56	0.56	3.90
0.03(%)	548	0.93	1.46	0.73	0.56	0.57	4.25
0.04(%)	543	1.48	1.86	1.10	0.93	0.76	6.13
Gamma rays +EMS							
10kR+0.1%EMS	553	0.19	1.10	0.54	0.19	0.20	2.22
10kR+0.2%EMS	546	1.11	1.12	0.73	0.56	0.56	4.08
20KR+0.3%EMS	547	1.12	1.66	0.92	0.74	0.74	5.18
20KR+0.4%EMS	548	1.65	2.02	1.28	1.10	0.92	6.97
Gamma rays +MMS							
10kR+0.01%MMS	542	0.38	1.12	0.74	0.20	0.38	2.82
10KR+0.02%MMS	543	1.28	1.13	1.12	0.92	0.74	5.16
20KR+0.03%MMS	545	1.29	1.85	1.47	0.92	0.74	6.27
20KR+0.04%MMS	546	1.84	2.21	1.65	1.30	1.30	8.30

Table 5.6 Frequency of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. major in M_1 generation at Telophase I/II (%).

Treatments	Total Number of PMCs Scored	Telophase I/II (%)				
		Disturbed polarity	Micronuclei	Multinucleate condition	Cytomixis	Total abnormalities
Control	555	-	-	-	-	-
EMS						
0.1(%)	550	0.92	-	0.22	-	1.14
0.2(%)	552	1.10	0.42	0.42	-	1.94
0.3(%)	540	1.50	0.76	0.92	0.42	3.60
0.4(%)	550	1.82	0.94	0.92	0.72	4.40
Gamma rays						
10KR	540	0.94	-	0.42	-	1.36
20KR	548	1.34	0.56	0.56	0.56	3.02
30kR	546	1.66	0.92	0.56	0.56	3.70
40KR	542	2.04	1.12	1.30	0.75	5.21

MMS						
0.01(%)	547	1.10	0.20	0.74	0.55	2.59
0.02(%)	545	1.30	0.92	0.92	0.57	5.68
0.03(%)	548	1.46	1.30	0.92	0.93	4.61
0.04(%)	543	2.04	1.46	1.48	0.94	5.92
Gamma rays +EMS						
10kR+0.1%EMS	553	1.12	0.20	0.74	0.74	2.80
10kR+0.2%EMS	546	1.50	0.94	1.11	0.93	4.48
20KR+0.3%EMS	547	1.66	1.32	1.12	1.12	5.22
20KR+0.4%EMS	548	2.20	1.50	1.66	1.30	6.66
Gamma rays +MMS						
10kR+0.01%MMS	542	1.12	0.38	0.94	0.94	1.50
10KR+0.02%MMS	543	1.66	1.13	1.30	1.12	5.21
20KR+0.03%MMS	545	1.84	1.48	1.30	1.30	5.92
20KR+0.04%MMS	546	2.40	1.68	2.04	1.48	7.60

Table 5.7 Comparison of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. minor in M_1 generation.

Treatments	Total Number of PMCs Scored	Total abnormalities at Metaphase-I/II(%)	Total abnormalities at Anaphase-I/II(%)	Total abnormalities at Telophase I/II(%)	Total abnormalities (%)
Control	555	-	-	-	-
EMS					
0.1%	550	4.26	1.32	1.32	6.90
0.2%	552	5.36	3.50	2.39	11.25
0.3%	540	7.56	4.37	3.80	15.73
0.4%	550	9.24	5.76	5.10	20.10
Gamma rays					
10KR	540	5.04	2.18	2.06	9.28
20KR	548	6.82	4.29	3.94	15.05
30KR	546	8.49	4.97	4.54	18.00
40KR	542	10.46	6.56	6.53	23.55
MMS					
0.01%	547	5.22	2.74	3.90	11.86
0.02%	545	7.21	4.69	4.39	16.29
0.03%	548	9.57	5.30	5.32	20.19
0.04%	543	11.15	7.58	6.75	25.41

EMS + gamma rays					
0.1%+10KR	553	5.22	3.00	3.71	11.93
0.2%+20KR	546	8.25	5.60	5.33	19.18
0.3%+30KR	547	10.65	6.34	6.14	23.13
0.4%+40KR	548	12.19	8.29	7.57	28.05
MMS + gamma rays					
0.01%+10KR	542	5.60	3.39	4.19	13.18
0.02%+20KR	543	9.39	6.43	6.30	22.12
0.03%+30KR	545	11.74	7.22	6.42	25.38
0.04%+40KR	546	13.46	9.56	8.60	31.62

Table 5.8 Comparison of Meiotic aberrations induced by EMS, gamma rays, MMS and their combination treatments in broad bean (*Vicia faba* L.) var. major in M_1 generation.

Treatments	Total Number of PMCs Scored	Total abnormalities at Metaphase-I/II(%)	Total abnormalities at Anaphase-I/II(%)	Total abnormalities at Telophase I/II(%)	Total abnormalities (%)
Control	555	-	-	-	-
EMS					
0.1%	550	4.26	1.32	1.32	6.90
0.2%	552	5.36	3.50	2.39	11.25
0.3%	540	7.56	4.37	3.80	15.73
0.4%	550	9.24	5.76	5.10	20.10
Gamma rays					
10KR	540	5.04	2.18	2.06	9.28
20KR	548	6.82	4.29	3.94	15.05
30KR	546	8.49	4.97	4.54	18.00
40KR	542	10.46	6.56	6.53	23.55
MMS					
0.01%	547	5.22	2.74	3.90	11.86
0.02%	545	7.21	4.69	4.39	16.29
0.03%	548	9.57	5.30	5.32	20.19
0.04%	543	11.15	7.58	6.75	25.41

EMS + gamma rays					
0.1%+10KR	553	5.22	3.00	3.71	11.93
0.2%+20KR	546	8.25	5.60	5.33	19.18
0.3%+30KR	547	10.65	6.34	6.14	23.13
0.4%+40KR	548	12.19	8.29	7.57	28.05
MMS + gamma rays					
0.01%+10KR	542	5.60	3.39	4.19	13.18
0.02%+20KR	543	9.39	6.43	6.30	22.12
0.03%+30KR	545	11.74	7.22	6.42	25.38
0.04%+40KR	546	13.46	9.56	8.60	31.62

Table 5.9 Chromosomal associations and chiasma frequency in M_1 generation in broad bean (*Vicia faba* L.) var. minor.

Treatments	Chromosomal association per cell				Chiasma frequency per cell
	Univalents Mean	Multivalents Mean	Rod Bivalents Mean	Ring Bivalents Mean	
Control	0.00	0.00	1.00	5.00	21.00
EMS					
0.1(%)	0.30	0.40	0.90	3.80	20.00
0.2(%)	0.40	0.50	1.00	3.90	19.30
0.3(%)	0.50	0.60	1.20	3.10	19.00
0.4(%)	0.60	0.70	1.30	3.30	18.80
Pooled Mean	**0.45**	**0.55**	**1.10**	**3.52**	**19.27**
Gamma rays					
10KR	0.40	0.50	1.00	4.60	19.50
20KR	0.50	0.60	1.20	3.50	19.20
30kR	0.60	0.70	1.30	3.60	19.00
40KR	0.70	0.90	1.40	3.10	18.80
Pooled Mean	**0.55**	**0.67**	**1.22**	**3.70**	**19.12**

MMS					
0.01(%)	0.50	0.60	1.00	3.40	19.00
0.02(%)	0.60	0.70	0.70	3.20	18.80
0.03(%)	0.70	0.80	1.00	3.10	18.60
0.04(%)	0.80	1.00	1.10	3.00	18.80
Pooled Mean	**0.65**	**0.77**	**1.42**	**3.17**	**18.80**
Gamma rays + EMS					
10kR+0.1%EMS	0.60	0.70	0.40	3.33	18.80
10kR+0.2%EMS	0.70	0.80	1.10	3.20	18.60
20KR+0.3%EMS	0.80	0.90	1.20	3.24	18.00
20KR+0.4%EMS	0.90	1.00	1.40	3.00	17.80
Pooled Mean	**0.75**	**0.85**	**1.02**	**3.19**	**18.30**
Gamma rays + MMS					
10kR+0.01%MMS	0.70	0.80	1.10	3.30	18.40
10KR+0.02%MMS	0.80	0.90	1.20	3.20	18.00
20KR+0.03%MMS	0.90	1.00	1.30	4.50	17.80
20KR+0.04%MMS	1.00	1.10	1.50	3.10	17.20
Pooled Mean	**0.85**	**0.95**	**1.27**	**3.52**	**17.85**

Table 5.10 Chromosomal associations and chiasma frequency in M_1 generation in broad bean (*Vicia faba* L.) var. major.

Treatments	Chromosomal association per cell				Chiasma frequency per cell
	Univalents Mean	Multivalents Mean	Rod Bivalents Mean	Ring Bivalents Mean	
Control	0.00	0.00	1.00	5.00	21.00
EMS					
0.1(%)	0.20	0.30	1.00	3.40	20.00
0.2(%)	0.30	0.40	1.20	3.80	20.00
0.3(%)	0.40	0.50	1.30	3.90	19.30
0.4(%)	0.50	0.60	1.40	3.10	19.00
Pooled Mean	**1.02**	**0.45**	**1.22**	**3.55**	**19.5**
Gamma rays					
10KR	0.30	0.40	1.20	4.20	19.00
20KR	0.40	0.50	1.30	4.60	19.50
30kR	0.50	0.60	1.40	3.50	19.20
40KR	0.60	0.70	1.50	3.60	19.00
Pooled Mean	**0.45**	**0.55**	**1.35**	**3.97**	**19.00**

MMS					
0.01(%)	0.40	0.50	0.70	3.20	18.40
0.02(%)	0.50	0.60	1.00	3.40	19.00
0.03(%)	0.60	0.70	1.10	3.20	18.80
0.04(%)	0.70	0.80	1.30	3.10	18.60
Pooled Mean	**0.55**	**0.65**	**1.02**	**3.22**	**18.70**
Gamma rays + EMS					
10kR+0.1%EMS	0.50	0.60	1.10	2.80	18.00
10kR+0.2%EMS	0.60	0.70	1.20	3.33	18.80
20KR+0.3%EMS	0.70	0.80	1.40	3.20	18.60
20KR+0.4%EMS	0.80	0.90	1.60	3.24	18.00
Pooled Mean	**0.65**	**0.75**	**1.32**	**3.14**	**18.35**
Gamma rays + MMS					
10kR+0.01%MMS	0.60	0.60	1.20	2.60	17.50
10KR+0.02%MMS	0.70	0.80	1.30	3.30	18.40
20KR+0.03%MMS	0.80	0.90	1.50	3.20	18.00
20KR+0.04%MMS	0.90	1.00	1.70	4.50	17.80
Pooled Mean	**0.75**	**0.82**	**1.42**	**3.40**	**17.92**

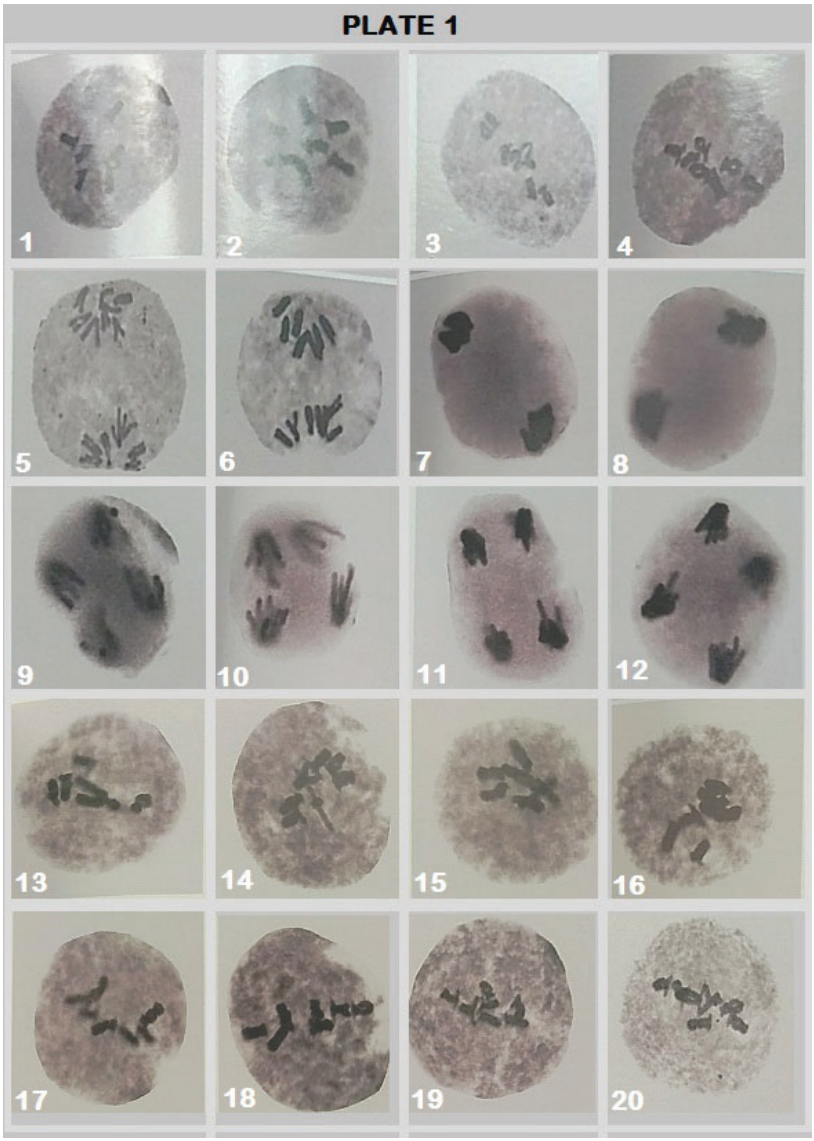
PLATE 1
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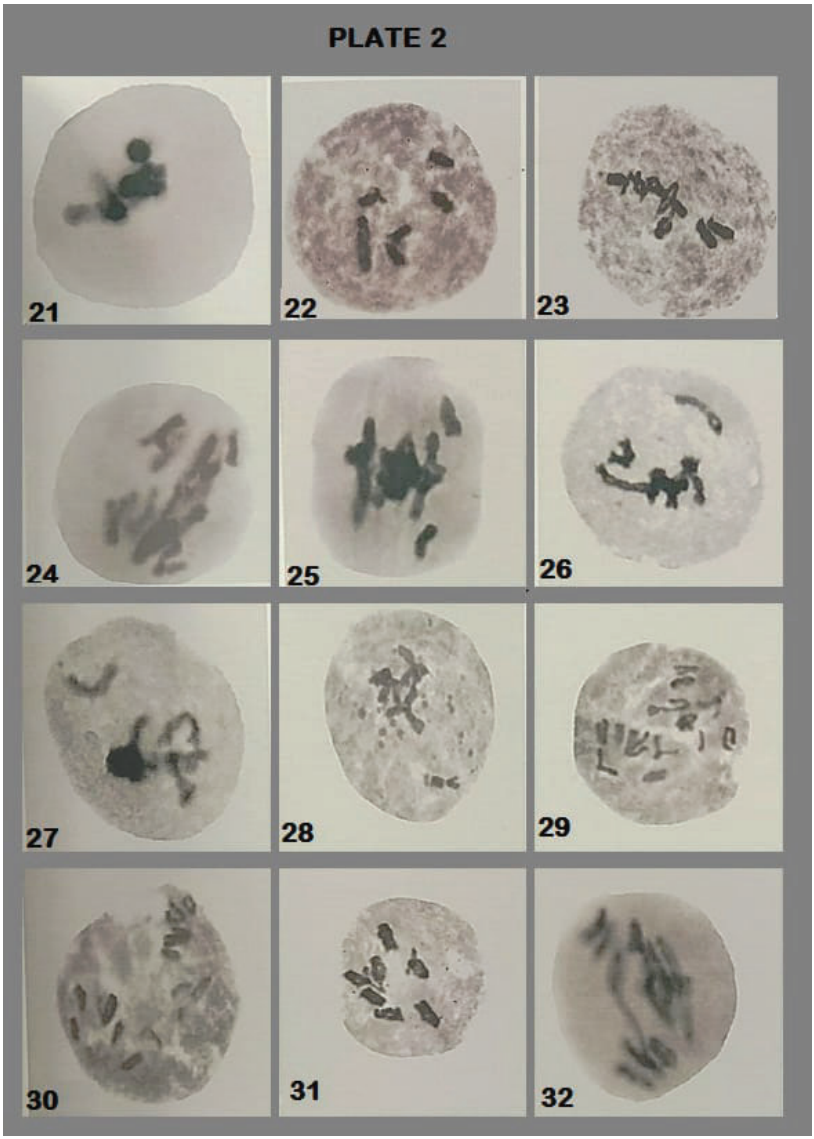
PLATE 2
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Description of figures of Plate 1 and 2

Fig. 1 PMC showing 6 perfect bivalents at diakinesis (control minor).
Fig. 2 PMC showing 6 perfect bivalents at diakinesis (control major).
Fig. 3 PMC showing 6 perfect bivalents at metaphase-I (control minor).
Fig. 4 PMC showing 6 perfect bivalents at metaphase-I (control major).
Fig. 5 PMC showing segregation of 6:6 chromosomes at early anaphase-I (control minor).
Fig. 6 PMC showing segregation of 6:6 chromosomes at early anaphase-I (control major).
Fig. 7 PMC showing Telophase I (control minor).
Fig. 8 PMC showing Telophase I (control major).
Fig. 9 PMC showing the separation of chromosomes in four groups at anaphase-II (control minor).
Fig. 10 PMC showing the separation of chromosomes in four groups at anaphase-II (control major).
Fig. 11 PMC showing normal telophase-II (control minor).
Fig. 12 PMC showing normal telophase-II (control major).
Fig. 13 PMC showing I^{IV} and 4^{II} at metaphase-I. (var. minor, 0.1% EMS).
Fig. 14 PMC showing 2^{IV} and 2^{II} at metaphase-I. (var. minor, 0.2% EMS).
Fig. 15 PMC showing secondary association at metaphase-I (var. major, 0.01% MMS).
Fig. 16 PMC showing stickiness of four bivalents and two separate bivalents (var. major, 0.02% MMS).
Fig. 17 PMC showing 2^{IV} and 2^{II} at metaphase-I. (var. minor, 10 Kr gamma rays).
Fig. 18 PMC showing 1^{IV} and 4^{II} at metaphase-I. (var. minor, 20 Kr gamma rays).
Fig. 19 PMC showing secondary association at metaphase-I (var. major, 10 kR +0.1% EMS).
Fig. 20 PMC showing secondary association at metaphase-I (var. major, 10 kR +0.2% EMS).
Fig. 21 PMC showing stickiness at metaphase-I (var. minor, 0.1% EMS).
Fig. 22 PMC showing 1^{IV} and 4^{II} at metaphase-I. (var. major, 30 kR gamma rays).
Fig. 23 PMC showing 2^{IV} and 2^{II} at metaphase-I. (var. minor, 40 Kr gamma rays).
Fig. 24 PMC showing 2 bridges at anaphase-I (var. minor, 0.4% EMS).
Fig. 25 PMC showing two stray bivalents at metaphase-I (var. minor, 0.2% EMS).
Fig. 26 PMC showing one stray bivalent at metaphase-I (var. major, 40 Kr gamma rays).

Fig. 27 PMC showing one stray bivalent at metaphase-I (var. major, 30 Kr gamma rays).
Fig. 28 PMC showing one stray bivalent and fragments at metaphase-I (var. minor, 20 Kr + 0.1% EMS).
Fig. 29 PMC showing twelve univalents at metaphase-I (var. minor, 10 Kr +0.2% EMS).
Fig. 30 PMC showing twelve univalents at metaphase-I (var. major, 20 Kr +0.03% MMS).
Fig. 31 PMC showing five bivalents and two fragments at metaphase-I (var. major, 40 Kr gamma rays).
Fig. 32 PMC showing bridges at anaphase-I (var. minor, 0.04% MMS).

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CHAPTER 6

INDIVIDUAL AND SIMULTANEOUS TREATMENTS OF GAMMA RAYS AND ETHYL METHANE SULFONATE INDUCED GENETIC VARIABILITY FOR PLANT HEIGHT IN URDBEAN (*VIGNA MUNGO* (L.) HEPPER)

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Abstract: Genetic variability is a pre-condition for every successful breeding program. The present study was designed to determine the extent of induced genetic variability for plant height in M_2 and M_3 generations of urdbean following mutagenesis with individual and combination treatments of gamma rays and EMS. Mean plant height diverted in a negative direction with most of the mutagenic treatments in both T-9 and Pant U-30 varieties. The genetic parameters were recorded to be higher in M_2 as compared to M_3 generations, indicating that prospective gain could be realized in an early generation.

Keywords: Mutagenesis, Gamma rays, EMS, Genetic variability, Plant height, Urdbean.

6.1 Introduction

Genetic variability works as the breeder's treasure for improving crop species through selection. Mutagenic treatments may create new variability through genetic alterations besides inducing new recombinations due to breakage of linkage (Bhat *et al.,* 2005a; Bhat *et al.,* 2006a; Bhat *et al.,* 2006b; Bhat *et al.,* 2007b; Gottschalk, 1986; Goyal *et al.,* 2019; Amin *et al*., 2019). Induced mutations may create different mutant alleles with varying degrees of trait modification (Bhat *et al.,* 2005b; Bhat *et al.,* 2006d; Bhat *et al.,* 2007a; Chopra, 2005).

The significance of micromutations for generating polygenic variability received an impetus after Brock's hypothesis in 1965 (Brock, 1965). Micromutations produce genetic variability for quantitatively inherited traits without disturbing the genotypic and phenotypic architecture of the crop, thus deserving the absolute attention of plant breeders. Lately, several efforts in support of mutagen induced genetic variability for quantitative traits in crop plants have been undertaken (Bhat *et al.,* 2007; Bhat 2007; Bhat and Wani, 2015; Usharani and Kumar, 2015; Khursheed *et al*., 2018; Wani, 2018; Amin *et al*., 2019), nevertheless little knowledge exists vis-à-vis the impact of mutagens on plant height in the urdbean. Against this backdrop, an attempt has been made to appraise induced genetic variability for a quantitative trait, viz., plant height in M_2 and M_3 generations after mutagenesis with individual and in combination treatments of gamma rays and EMS.

6.2 Materials and Methods

Seeds of varieties T9 and Pant U-30 of the urdbean (*Vigna mungo* (L.) Hepper) were irradiated with 100 to 400 Gy doses of gamma rays from the cobalt-60 source at NBRI Lucknow, India. For chemical treatments, seeds of both the varieties after 9 hours of presoaking in distilled water were treated with 0.1 to 0.4% of EMS for 6 hours with sporadic shaking at 25±1 ^{o}C. The seeds were scrupulously washed in running tap water to eliminate the left-over mutagen from seed surfaces after completion of the treatment period.

For combination treatments, seeds of each variety were firstly irradiated with 200 and 300 Gy doses of gamma rays and afterward treated with 0.2% and 0.3% of EMS. One hundred seeds were sown for every treatment and a control in a complete randomized block design in three replicates to raise the M_1 generation. Seed to seed distance in a row and

between the rows was kept at 30 and 60 centimeters respectively. Twenty-five healthy seeds from each normal-looking M_1 plant were sown in plant progeny rows to raise the M_2 generation. Observations for plant height were made on 25-30 normal-looking plants of each progeny from each treatment.

For raising the M_3 generation, two treatments of gamma rays and EMS alone as well as in combination were used. The selected treatments were 200 and 300 Gy doses of gamma rays, 0.2 and 0.3% of EMS and 200 Gy+0.2% EMS and 300 Gy+0.2% EMS. Data collected for plant height in M_2 and M_3 generations were subjected to statistical analysis according to the methods of Singh and Chaudhary (1985).

6.3 Results and Discussion

Data recorded on plant height in M_2 and M_3 generations are presented in Tables 1-3. The shift in mean was toward the negative direction with almost all the individual and combination treatments of gamma rays and EMS in both M_2 and M_3 generations. More reduction in plant height was noticed with combined treatments of gamma rays and EMS in both the varieties. The decline in plant height following mutagenic treatments was previously reported in mungbean (Rajput, 1974; Yaqoob and Rashid, 2001; Khan and Wani, 2006), broad bean (Bhat *et al.,* 2006, 2007; Bhat and Wani 2017a, 2017b), and chickpea (Ganai *et al.,* 2005; Khan *et al.,* 2005). However, Singh *et al*. (2000) and Arulbalachandran and Mullainathan (2009) in *Vigna mungo*, Ismail *et al.* (1977) in *Vicia faba* and Khan *et al.* (2006) in *Lens culinaris* reported an increase in plant height after mutagenic treatments. A decrease in plant height could be due to the inhibition of mitotic divisions as reported by Subba Rao (1988) in chickpea. These results are in conformity with the results reported by **(**Gulfishan *et al.,* 2013, 2012, 2010, 2011 in *Capsicum* and broad bean).

The highest genotypic (10.20% and 10.75%) coefficients of variation were observed with 200 Gy gamma rays and 200 Gy+0.3% of EMS in the varieties T-9 and Pant U-30 respectively in the M_2 generation. Heritability increased substantially in mutagen treated populations in both the varieties. For gamma ray treatments, in the M_2 generation, the highest values of heritability were recorded to be 48.59% in the var. T-9 and 51.93% in the var. Pant U-30. The highest heritability in the material treated with EMS was 55.80% and 57.44% in the varieties T-9 and Pant U-30, respectively. In combination treatments of gamma rays + EMS, it was 47.54% in the var. T-9 and 45.89% in the var. Pant U-30. The highest

values of genetic advance were recorded with 200 Gy gamma rays in the var. T-9 and 200 Gy+0.3% EMS in the var. Pant U-30.

Genotypic coefficients of variation and heritability are indispensable (Kaul and Garg, 1979) for representing the degree of stability to environmental fluctuation and probable trait transmissibility from parent to offspring. The genotypic coefficient of variation was higher in the M_2 as compared to M_3 generation and did not increase with an increasing dose of the mutagens. Lack of a consistent dose-dependent relationship may be due to additional uncontrolled environmental variation (Conger *et al*., 1966).

In this study, the heritability estimates were observed to be low to moderate in both the varieties. High heritability estimates for plant height and other traits have been reported by earlier workers in *Vigna radiata* (Vyas and Chauhan, 1994), *Lathyrus sativus* (Kumar and Dubey, 2001) and *Vigna mungo* (Deepalakshmi and Anandakumar, 2004). High heritability in the M_2 as compared to M_3 generation signifies that induced variability has been fixed in the early generation by selection process (Sharma *et al.,* 2009; Gulfishan *et al.*, 2015; Parveen *et al.*, 2006; Ibrahim and Sharaan, 1974).

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Table 6.1 Estimates of mean values ($\overline{X}$), shift in $\overline{X}$ and genetic parameters for plant height (cm) in M_2 generation of urdbean var. T-9*

Treatment	Mean ± S.E.	Shift in $\overline{X}$	PCV (%)	GCV (%)	h^2 (%)	GA (% of $\overline{X}$)
Control	35.24±0.31	-	3.23	1.33	16.92	1.36
Gamma rays						
100 Gy	34.16±0.32	-1.08	3.62	1.56	18.18	1.73
200 Gy	33.64±0.47	-1.06	16.78	10.20	36.91	15.93
300 Gy	31.10±0.43	-4.14	9.79	6.83	48.59	12.38
400 Gy	30.36±0.60	-4.88	10.15	6.39	39.62	10.44
CD (p = 0.05)		0.48				
EMS						
0.1%	35.45±0.42	+0.21	6.33	4.10	41.83	6.83
0.2%	33.70±0.53	-1.54	8.87	6.35	51.23	11.93
0.3%	31.61±0.24	-3.63	8.78	6.58	55.80	12.72
0.4%	30.99±0.24	-4.25	9.45	4.38	21.58	5.23
CD (p = 0.05)		0.74				
Gamma rays+EMS						
200 Gy+0.2%	32.56±0.46	-2.68	8.04	5.18	41.69	8.69
300 Gy+0.2%	31.02±0.42	-4.22	8.83	6.09	47.54	10.93
200 Gy+0.3%	30.15±0.15	-5.09	8.80	5.60	40.56	9.25
300 Gy+0.3%	28.16±0.16	-7.08	10.06	6.33	39.50	10.33
CD (p = 0.05)		1.84				

*SE= Standard error; PCV=Phenotypic coefficient of variation; GCV=Genotypic coefficient of variation; h^2 =Heritability; GA=Genetic advance; CD=Critical difference; Gy=Gamma rays; EMS= Ethyl methanesulfonate

Table 6.2 Estimates of mean values ($\overline{X}$), shift in $\overline{X}$ and genetic parameters for plant height (cm) in M_2 generation of urdbean var. Pant U-30

Treatment	Mean ± S.E.	Shift in $\overline{X}$	PCV (%)	GCV (%)	h^2 (%)	GA (% of $\overline{X}$)
Control	35.64±0.36	-	4.01	2.22	30.39	3.17
Gamma rays						
100 Gy	34.43±0.35	-1.21	8.81	6.05	47.17	10.92
200 Gy	34.12±0.37	-1.52	7.57	5.08	44.89	8.76
300 Gy	32.76±0.47	-2.88	8.24	5.93	51.93	12.57
400 Gy	31.75±0.29	-3.89	5.28	3.29	38.29	5.29
CD (p = 0.05)		0.51				
EMS						
0.1%	35.99±0.39	+0.35	6.32	4.12	42.52	7.00
0.2%	34.81±0.36	-0.83	6.87	5.20	57.44	10.31
0.3%	33.20±0.44	-2.44	4.61	3.18	47.01	5.69
0.4%	32.39±0.32	-3.25	14.98	9.20	37.75	14.63
CD (p = 0.05)		1.22				
Gamma rays+EMS						
200 Gy+0.2%	33.08±0.46	-2.56	14.62	8.38	32.73	12.36
300 Gy+0.2%	31.90±0.33	-3.74	8.93	5.76	41.62	9.65
200 Gy+0.3%	30.66±0.38	-4.98	15.11	10.75	45.89	20.84
300 Gy+0.3%	28.96±0.22	-6.68	9.20	5.45	34.79	8.21
CD (p = 0.05)		2.19				

Table 6.3 Estimates of mean values ($\overline{X}$), shift in $\overline{X}$ and genetic parameters for plant height (cm) in M_3 generation of urdbean

Treatment	Mean ± S.E.	Shift in $\overline{X}$	PCV (%)	GCV (%)	h^2 (%)	GA (% of $\overline{X}$)
Var. T-9						
Control	35.86±0.22	-	3.12	1.17	14.40	1.14
Gamma rays						
200 Gy	34.15±0.53	-1.71	6.35	3.52	30.57	5.00
300 Gy	31.93±0.42	-3.93	7.89	5.14	42.36	8.74
CD (p=0.05)		0.97				
EMS						
0.2%	34.27±0.69	-1.59	8.32	6.16	54.80	11.85
0.3%	33.32±0.35	-2.54	7.44	4.89	43.25	8.43
CD (p=0.05)		0.86				
Gamma rays+EMS						
200 Gy+0.2%	33.20±0.62	-2.66	7.36	4.25	33.27	6.38
300 Gy+0.2%	31.95±0.49	-3.91	8.79	4.94	31.64	7.20
CD (p=0.05)		0.55				
Var. Pant U-30						
Control	36.01±0.31	-	3.95	2.14	29.35	2.99
Gamma rays						
200 Gy	34.42±0.62	-1.59	7.35	4.76	42.03	8.13
300 Gy	33.00±0.51	-3.01	8.21	5.42	43.60	9.33
CD (p=0.05)		1.41				
EMS						
0.2%	35.12±0.49	-0.89	6.52	4.38	45.08	7.68
0.3%	33.90±0.35	-2.11	4.00	2.30	32.41	3.36
CD (p=0.05)		0.78				

Gamma rays+EMS 200 Gy+0.2%	33.48±0.22	-2.53	10.20	7.28	41.16	13.71
300 Gy+0.2%	32.21±0.54	-3.80	8.50	5.34	39.73	8.72
CD (p=0.05)		1.30				

CHAPTER 7

MUTATION BREEDING FOR QUALITY IMPROVEMENT: A CASE STUDY FOR OILSEED CROPS

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Abstract: Oilseed crops are a large and diverse group of economically important plants. Vegetable oil has one of the highest trade shares (42%) of the production of all agricultural commodities. Oilseeds represent a rich source of dietary protein and fat that make them an ideal complement to root crops. They are used in different forms such as whole seed, vegetable oil and cake oil and their by-products are used for fuelwood and industrial purposes. Major oilseed crops of the world include soybean, rapeseed, cottonseed and groundnut, while minor oilseed crops such as castor, linseed, and fenugreek are also being exploited in medicines and pharmaceuticals, industries, biodiesel, pet foods and are a component of many other products. The major countries in oilseed production are the United States of America, Brazil, Argentina, China and India. The improvement of oil quantity and quality by employing mutation breeding approaches is considered to be the most viable method across the globe. The utilization of chemical, physical and combination mutagens has undoubtedly led to the increase in the nutritive parameters in diverse crops. Costs have risen considerably in countries that typically rely on revenues from oil and other primary commodity exports to finance food

imports and subsidies. Possibly the most global impact of mutation breeding is observed in oilseed crops, mainly in rapeseed and sunflower, to enhance yield and oil contents in combination with conventional breeding methods. Oil crops are an essential ingredient for food supply for the growing world population. With the human population expected to reach 9 billion by 2050, it has been estimated that 44% of the required additional calories will come from oil crops, however, we could reach the goal of global food security by improving food security and nutrition sensitivity and by making rural livelihoods more resilient. Mutation breeding is entering into a new era: molecular mutation breeding. Therefore, induced mutations will continue to play a significant role in improving world food security. The chapter provides a detailed study of mutation breeding in fenugreek and linseed, limitations and landmark achievements and prospects.

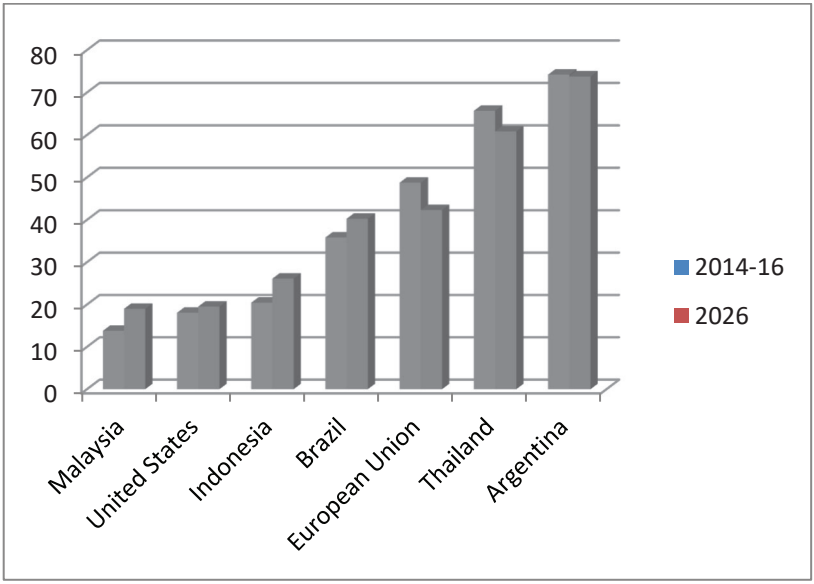
Keywords: oilseed, mutation breeding, quality improvement, food security

7.1 Introduction

The earliest farmers with limited understanding of genetics made far-reaching changes in the genetic makeup of their crops simply by choosing, based on their phenotypical characters, which plants had the best qualities to sow as seed. Yes, we owe a debt to our ancestors for developing such plants according to their knowledge which helps to sustain modern civilization. In the past decade in developing countries such as India, China and Southeast Asia, calorie availability has increased and in the coming decades it is expected to increase further. We know that the main source of calories is cereals, but in India, cereal calorie availability decreased from 60% to 55% during 2006-2016 and it may further decrease in the coming years. We have to look toward other sources of calories, and these could be from vegetable oil, dairy and sugar (OECD-FAO Agricultural Outlook 2017-2026). Oilseed crops belong to a very large and diverse group of economically important plants. Vegetable oil has one of the highest trade shares (42%) of the production of all the agricultural commodities. A large number of various kinds of oil crops are grown worldwide. Some of them such as soybean, peanut, rapeseed mustard, sunflower, safflower, linseed, castor and cottonseed are widespread oil crops (Yadava *et al.,* 2011). Because of high consumer demand and health and safety measures, fast food chains are now switching to vegetable oils to replace animal fat (lard) for making French fries and various other

snack foods. Lipids that have saturated fatty acids as major components that are solid at room temperature, for example, coconut oil and palm kernel oil that remain solid at room temperature are known as fats, although oils remain liquid at room temperature.

Figure 7.1 Share of vegetable oil used for biodiesel production (Dataset: OECD-FAO Agricultural Outlook 2017-2026).



Due to the high demand for fuel in the modern era, people switched from fossil fuels to other sources of fuel for their vehicles and transport. Biofuel is now one of the main sources. Maize, sugarcane and vegetable oil are important commodities of global biofuel production. But now growth in biofuels production is slowing down. Many countries show the growth in demand for maize and vegetable oil in the last decade and over the projection period, by use. Reflecting the overall trend toward a slowdown, biofuel use practically disappears as a source of demand growth over the outlook period for both commodities. In the last decade, vegetable oil showed an additional consumption of 64 Mt, but consumption in the next decade will only increase by 40 Mt. Most of this slowdown is explained by biofuels. Although on a global scale biofuel use shrinks in its importance as a driver of demand growth, this net effect masks shifts among countries that reduce demand for feedstock for biofuels and others

that increase their use over the outlook period. By 2026, it is estimated that the total use of vegetable oil for biofuels is to be around 26 Mt. with developed and developing countries (mostly Latin American and Asian countries) each accounting for half of the demand (OECD-FAO Agricultural outlook 2017-2026).

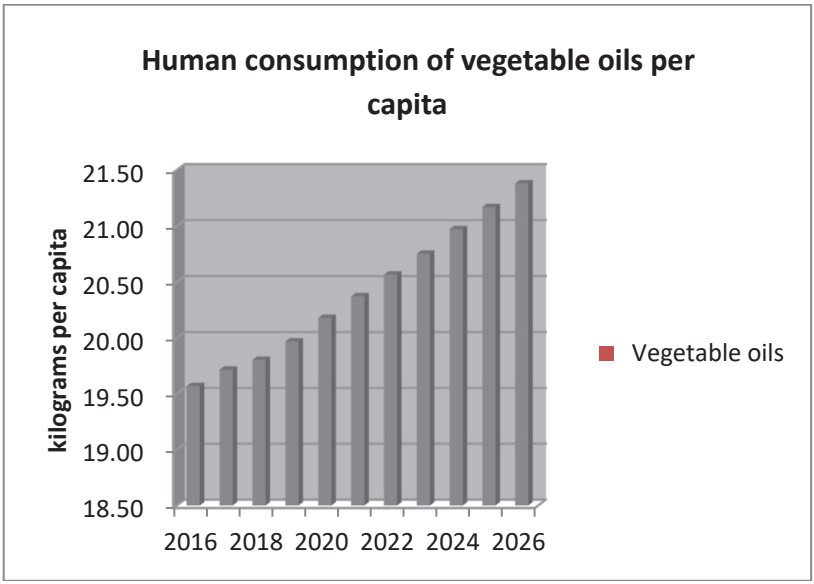
Soybean is the most important oil crop and ranked first in 2017 of the top oil-producing crops. About half of the total world production of soybean is used in trading, while this trade share is much lower for other oilseed crops, at about 13% of global production. The largest soybean exporting countries are Canada, Australia and Ukraine and, according to the report, it is assumed that by 2026 these countries will export more than 75%. Canada and Australia also export other oilseed crop products, for example rapeseed. Rapeseed mustard is also one of the most important sources of vegetable oil in the world and is grown in more than fifty countries across the globe. China, Canada, India, Germany, France, the UK, Australia, Poland and the USA are the major cultivators of different species. Sunflower is grown on all continents. It is estimated that around 70% of the total area and 80% of total production is solely accounted for by Europe and America (Damodaran and Hegde, 2007).

It is evident that due to expanding populations in India and Sub-Saharan Africa, the global demand will increase. China has a large cultivation area so that it will continue to contribute to supply the demand for many other key commodities. It is estimated that by 2026 the total demand for food consumption is to increase by 338 Mt. About 38% of this demand will come from three countries: China, India and Sub-Saharan Africa. But this share is higher for rice in India and lower for wheat and maize.

Looking at coconut cultivation we see the highest commercial production is found in Southeast Asia and about 44% of global production is accounted for in the Philippines, which is followed by a 28% share from Indonesia. About 25 million people in the Philippines rely solely on the coconut industry. This industry is very large, and it is the socio-economic pillar of many countries, including mostly rural areas, where about 80-90% of people have smallholdings producing coconuts and indeed it significantly contributes to agri-food sectors of these countries. We all know that palm oil is the main raw material for the chemical industry. It is also known that coconut is used at a large scale in various industries, meaning there is a global demand for highly valuable coconut products, such as coconut water, coconut sugar and virgin coconut oil. Because of these valuable products, the global demand for coconut products has

greatly increased in recent years. Additional demand for vegetable oil (23%) is expected to come from India and other Southeast Asian countries such as Thailand, Việt Nam, Lao PDR, Indonesia, Philippines, Malaysia, Myanmar and Cambodia in the coming decade (OECD-FAO Agricultural outlook 2017-2026). The demand for vegetable oil for human consumption is increasing with the human population. We know that human consumption per capita in 2017 was 19.72 kg per capita and in 2026 it is expected to increase up to 21.38 kg per capita (Figure 7.2). The increase in human consumption is not just due to vegetable oil for culinary purposes but also for industrial purposes such as snack food factories, fast food restaurants and also in the cosmetics industry.

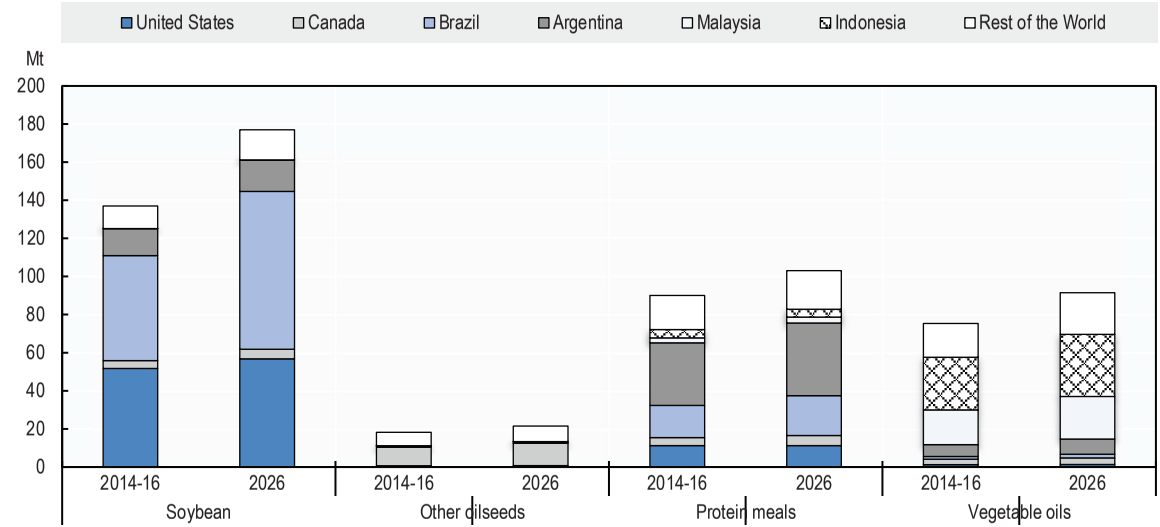
Figure 7.2 Vegetable oil human consumption per capita from 2016 to 2026 (expected).



Dataset: OECD-FAO Agricultural Outlook 2017-2026

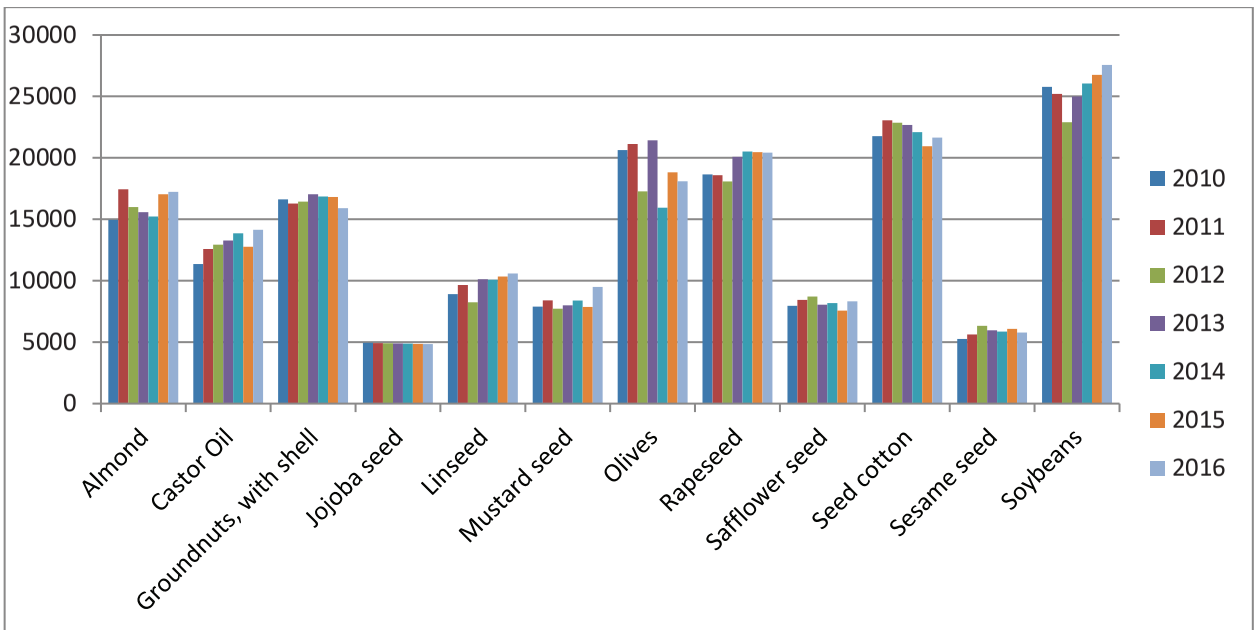
It is shown that by 2026 the demand for soybean, other oilseeds, protein meals and vegetable oils is expected to increase. A significant role in oilseed production of soybean is played by two main countries, the United States and Brazil which is expected to remain the same in the coming years (Figure 7.3) while vegetable oilseed products are dominated by Indonesia.

Figure 7.3 Exports of oilseeds and oilseed products by region (Source: OECD/FAO 2017).



If we compare worldwide yield of oil crops it is shown that the yield of many oil crops increased significantly from the year 2010 to 2016, for example, almond, castor oil, linseed, rapeseed mustard, safflower, sesame seed and soybeans but some crops also showed a decrease in yield, for example, groundnut, jojoba seed, olives and seed cotton. Among these crops, soybean showed the highest yield from 2010-2016 and, in between these years, soybean showed the lowest yield of 22894 metric tons in 2012 and the highest yield of 27556 metric tons in 2016 (Figure 7.4). It is clear that not every crop is showing a gradual increase in yield, while sustainable agriculture and global food demand increases in yield every coming year so genetic modification of oil crops is a must to avoid a food crisis in the coming years due to population increases.

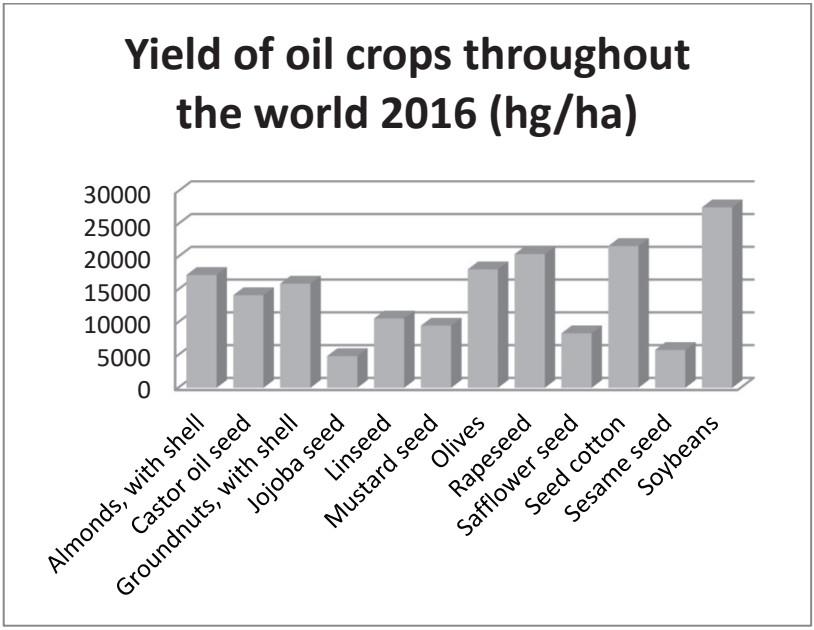
Figure 7.4. Graph showing yield (kg/ha) of oil crops worldwide from 2010-2016 (source FAO database).



7.2 Vegetable oil and seed oil

Seed oil is mainly stored in the plant as neutral lipids, such as triacylglycerols, which are highly significant, not only for nutrition but also in many industries. It is known that virtually all plant seeds contain storage lipids in the form of triacylglycerol (TAG). Considering the importance of these lipids many efforts have focused on improving seed composition and yield of oils. Plants are a rich source of energy. The need for plant biomass can only be fulfilled by attempts to improve oil production in vegetative tissues. Vegetable oils are not only produced by seed and fruit pericarps (e.g. oil palm, olive and avocado) but also by other parts of the plant such as leaves that also produce oil. In the leaf mesophyll cells contain lipid droplets in the cytosol, which shows that many other tissues can synthesize triacylglycerols (Lin and Oliver, 2008).

Figure 7.5 Graph showing the yield of main oil crops.



Soybean is the largest oil-producing crop. Due to the heart-friendly nature of the unsaturated fatty acids that are found in vegetable oils, consumers prefer food, snacks, margarine, shortenings and many other products which are prepared using vegetable oils. Among the vegetable oils, the

most preferred oils are soybean, cottonseed, sunflower, safflower, rapeseed (also known as canola oil), and corn (maize). The high acid content in rapeseed or canola means that it has stability at high temperature, durability and it can remain liquid at low temperatures. These qualities make canola oil more advantageous. Vegetable oil has one of the highest trade shares (42%) of the production of all agricultural commodities. This share is expected to remain stable throughout the outlook period, with global vegetable oil exports expected to be 91 Mt. by 2026 (OECD-FAO Agricultural outlook 2017-2026).

Vegetable oils are a major low-cost source of dietary fatty acids in modern diets. Considerable research has been undertaken in the public and private sectors into the feasibility of producing omega-3 LC-PUFAs in oilseed crops.

Plant oils mostly have C18 fatty acids, whereas essential fatty acids include linoleic acid and a-linolenic acid but are devoid of LC-PUFAs (Long Chain Polyunsaturated Fatty Acids), such as arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which typically only enter the human diet as oily fish. The health benefits of the omega-3 LC-PUFAs, EPA and DHA are now well known (Saravanan *et al.,* 2010). It is also reported that omega-6 ARA is important for infant nutrition (Janssen and Kiliaan, 2013). Considering the importance of LC-PUFA, efforts have been focused on enhancing the composition of vegetable oils to include essential LC-PUFAs. The omega-3 forms, specifically EPA and DHA, have been targeted, with the ultimate goal of producing a terrestrial plant-based source of these so-called fish oils. Although significant efforts have been made toward this goal (Abbadi *et.al.,* 2004, Wu *et.al.,* 2005, Hoffmann *et.al.,* 2008, Cheng *et.al.,* 2010 and Ruiz-López′, 2012), due to the complexity of adding these non-native fatty acids into the oilseed profile, until recently there has been limited success.

All vegetable oils have approximately 92-98% triacylglycerols, and the remaining are polar lipids, mono- and diacylglycerols, free fatty acids and polyisoprenoid lipids. Among the polar lipids usually are phospholipids and galactolipids (Åppelqvist, 1989). Physical, chemical, physiological and nutritional properties of vegetable oils depend on the relative amount of fatty acids and triacylglycerol molecular species (Padley *et al.,* 1986).

7.3 Genetic modification of quality of seed oil

The genetic improvement of crops is a crucial component of the efforts to address pressures on global food security and nutrition (Ronald, 2011). Due to environmental changes such as erratic rainfall patterns which lead to either drought or flood, ultimately food production and food prices are affected. The productivity of plants is reduced by a wide range of environmental induced biotic and abiotic stresses such as salinity, rising temperatures, drought, heat, pathogens and pest attacks (Ahmad *et al*., 2019; Naikoo *et al.,* 2019). Because of these challenges plant breeders will have to look for new innovative tools, such as genetic engineering, together with traditional breeding for sustainable food production to feed the world (Jain, 2010). GM (genetically modified) approaches to crop breeding can greatly facilitate the introgression of novel resistance traits into elite crop cultivars. This could increase the genetic diversity of pest resistance traits within the crops as well as many other qualitative and quantitative traits. Remarkably, by introducing resistance faster than evolving pests counter-resistance, GM food may offer a route to sustainable crop protection. However, the potency of this approach primarily relies on the discovery of novel resistance genes to incorporate using GM techniques. The opinion of the public seems variable regarding GM crops. The Americas, Asia, and South Africa show positive responses toward GM crops, while in Europe people think it is risky and something new which is more dangerous than something old. Media plays a very important role in our society; GM crops are sometimes portrayed in a stereotyped way by the media. Some groups even pose GM crops as harmful crops for both humans and the environment. Sections of the media previously saw 'the GM debate' as a chance to make sensationalist or even scare-mongering news stories with headlines about 'Frankenstein foods' and 'Mutant crops could kill you' (Durant and Lindsey, 2000).

Conventional breeding through backcrossing programs can improve elite varieties with desirable traits, but it takes several years. Different types of molecular markers (e.g., RFLP, AFLP, SSR, SNP) and high-throughput biochemical methods (e.g., gas chromatography, near-infrared spectrometry) have been proven to be efficient for plant breeding to improve seed quality. Soybean is used worldwide as a dual-use crop and its seeds are rich in both protein and oil. Now global demand for soybean has increased drastically for the oil content from the seeds. Oil content is approximately 18% to 20% of the weight of soybean seeds, which is lower than other oilseed crops. Seeds of peanut (*Arachis hypogaea*), another legume, for example, contain approximately 45% oil per seed weight.

Industrial applications, nutritional improvements and production of novel fatty acids in oils improved varieties can be used as cost-effective for sustainable food and feed (Kinney, 1998; Abbadi *et al.,* 2004; Cahoon *et al.,* 2007; Burgal *et al.,* 2008). Omega fatty acids are known to show beneficial and protective effects against diseases. Omega-3 fatty acids can prevent heart disease and may also reduce the risk of cancer. Linoleic acid is the parent fatty acid of omega-6 fatty acids, while the parent fatty acid of omega-3 fatty acids is α-linolenic acid. Sunflower, corn and soybean oils, the most consumed oils, are rich in linoleic acid (Simopoulus, 2008). The consumption of these oils increases the intake of omega-6 fatty acids and results in a high omega-6/omega-3 fatty acid ratio in the human diet. Numerous studies showed that the ratio of omega-6/omega-3 fatty acids in the human diet is very high (15:1). Therefore, the level of omega-3 fatty acids in comparison to omega-6/omega-3 fatty acids should be increased in the human diet (Gomez-Candela *et al.,* 2011). Various studies showed that accumulation of TAG (Triacylglycerol) can be increased by induced mutation in those genes which are involved in TAG and fatty acid turnover such as COMATOSE (CTS2), sugar dependent1 (SPD1) or comparative gene identification-58 (CGI58) (Kelly *et al.,* 2013, Slocombe *et al.,* 2009 and James *et al.,* 2010). Bhabha Atomic Research Centre, Bombay, India, released the peanut mutant variety 'TG-26', which yielded 9.4 tons/h nuts at the farm level. The release of flax cultivars with cooking quality oil 'linola', based on induced mutations (Green, 1986; Dribnenki, 1996), is the latest contribution for changing oil quality as had been done before in 'Canola' from rapeseed, and high oleic acid in sunflower.

7.4 Mutation breeding

The term 'mutation' was coined by Hugo de Vries to describe the sudden genetic change in higher plants which was stably inherited through many years (de Vries, 1901). Spontaneous mutants are valuable sources of diversity and variation, but intensive conventional breeding narrowed the genetic base of crop populations. There is a need to create variation by using induced mutation. Mutation is the heritable change to the genetic material; individuals that manifest modified characteristics on account of heritable changes are known as mutants (Bhat *et al.,* 2007b; Bhat *et al.,* 2005a; Bhat *et al.,* 2006a; Bhat *et al.,* 2006b; Raina *et al.,* 2018b; Ganai *et al.,* 2005; Bhat *et al.,* 2005b; Khursheed *et al.,* 2018a, 2018b, 2018c; Gulfishan *et al.,* 2013, 2012, 2010, 2011**;** Laskar *et al.*, 2015). DNA (deoxyribonucleic acid), is also known as the hereditary material, during replication of DNA, errors can occur which result in change or damage to

the hereditary material. Damage to DNA can also be caused by an individual's exposure to sunlight and ultra-violet (UV) radiation and sometimes diseases can lead to heritable changes to the genetic blueprint.

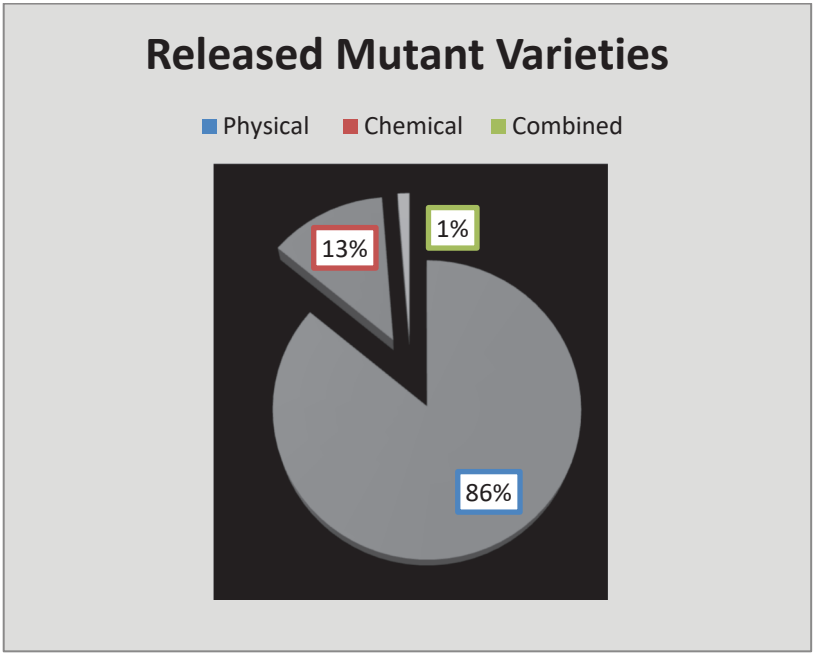
> "*A knowledge of the laws of mutation must sooner or later lead to the possibility of inducing mutations at will and so of originating perfectly new characters in animals and plants. And just as the process of selection has enabled us to produce improved races, greater in value and in beauty, so a control of the mutative process will, it is hoped, place in our hands the power of originating permanently improved species of animals and plants."* - De Vries, 1909.

The concept of utilizing induced mutations in breeding new forms was advocated by Hugo de Vries (1901). For the first time, a conclusive proof that ionizing radiations can induce mutations was reported by Muller (1927) when he succeeded in inducing certain variations in *Drosophila*. One year later Stadler (1928), worked on the effect of X-rays in maize, concluded that it was possible to obtain very high mutation rates through irradiation. Gustafsson (1947) reported effective and pioneering mutation work on the production of useful mutations in crop plants. Mutation breeding generally consists of the three-step process (a) inducing mutations, (b) screening for putative mutant candidates and (c) mutant testing and official release. Mutation induction has been widely used and highly successful in most species (Parveen *et al.,* 2006; Sharma *et al.,* 2009; Gulfishan *et al.,* 2015; Bhat and Wani, 2017a; Bhat and Wani, 2017b; Bhat *et al.,* 2007). Among these steps, the most difficult step is the second step, the screening of mutants and the selection of desired variants. Incredible advances have been made in the field of phenomics over the past five years. Still, the phenotypical selection is more laborious than genotypic selection (Fiorani and Schurr, 2013; Cobb *et al.,* 2013). Several workers supplemented comprehensive knowledge on the function and applicability of induced mutations for the improvement of genetic resources in several crop plants across the globe (Bhat and Wani, 2015; Bhat *et al.,* 2006; Amin *et al.,* 2016; Khursheed *et al;* 2015, 2016; Raina *et al*., 2018a; Wani *et al.,* 2017). Mutation breeding is considered as a coherent tool to create variability in a crop species in a very short period as compared to hybridizations. Different workers have employed wide ranges of physical and chemical mutagens for the improvement of traits in various crops (Bhat 2007; Laskar *et al.,* 2018a, 2018b in lentil; Tantray *et al.,* 2017 in black cumin; Bhat *et al.,* 2007a; Bhat *et al.,* 2007b Bhat *et al.,* 2006d; Raina *et al.,* 2017 in chickpea; Khursheed *et al.,* 2019 in faba bean; Hassan *et al.,* 2018 in fenugreek; Amin *et al*., 2019 in black cumin).

Significant progress can be seen by breeders in a number of crops. Breeders have developed soybean varieties with more protein and oil on a per hectare basis and improved overall yield. However, the selection of high yielding genotypes, with more protein and oil content, is not yet very successful (Mahmoud *et al.,* 2006).

The Joint FAO/IAEA Agriculture and Biotechnology Laboratories is established 35 km from its headquarters in Vienna, Austria. The FAO/IAEA Laboratories spearhead the use of "atoms for peace". The main goal is to combat the global food crisis and development by research as well as sustainable production by protecting plants and livestock, with an emphasis on the improvement of farmers' incomes and to ensure food safety for the future generations. Applications of these technologies have resulted in socio-economic impacts which are frequently measured in millions or billions of US dollars annually. Since 1964 the Joint FAO/IAEA Division has assisted with the safe use of nuclear techniques and related technologies in food and agriculture. It played a significant role in using radiation technologies for the betterment of global agricultural research in different divisions or areas such as animal production and health, food and environmental protection, insect pest control, plant breeding and genetics, and soil and water management and crop nutrition (www-naweb.iaea.org). Every year several mutant varieties are registered from countries worldwide into the mutant variety database. These varieties are mutated through chemical, physical, combined or somaclonal variation. Mutant varieties produced with ionizing radiation, specifically gamma rays, predominate in the database of registered mutant varieties. About 3275 mutated varieties have been released by using physical mutagen followed by 375 and 37 varieties released by using chemical and combined treatments respectively (Raina *et al.,* 2016) (Figure 7.6).

Figure 7.6 Distribution of mutant crop varieties released reported by Joint Division of the Food and Agriculture Organization of the United Nations and the International Atomic Energy Agency (joint IAEA/FAO) Mutant Varieties Database by using mutagenic agents up to 2017.



Top leading countries of mutant released varieties are China, Japan, India, Russian Federation, Netherlands, Germany, the United States of America and Bangladesh. The total number of mutant varieties released was 3,247 between 1950-2017. Among these, China is topmost having 826 mutant varieties followed by Japan having 481 and then India with 340 mutant varieties. This is the record of 67 years showing mutant released varieties (MVD 2018).

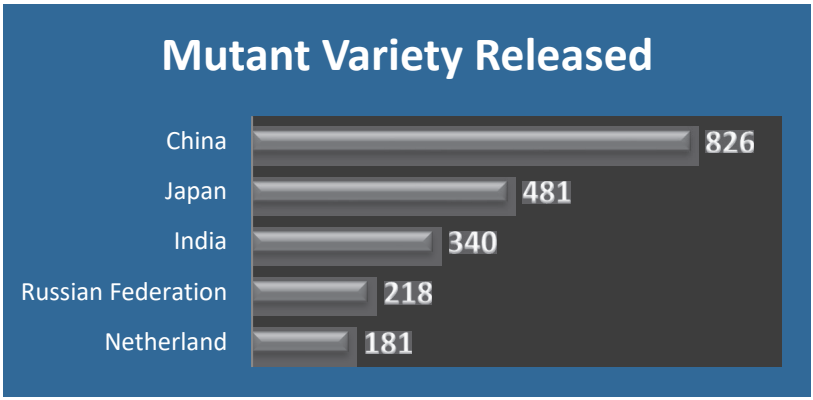


Figure 7.7 Distribution of mutant crop varieties by top five leading countries showing their number up to 2017.

A major hurdle in plant mutation breeding is raising a large mutant population where the mutant population is raised and evaluated to create variability and after that identification of desirable variants. Efforts are made to the dissociation of chimeras, also known as mosaics or sectoral differences. It is known that cells of different genotypes exist alongside the tissues of the same mutant plant. This happens in sexually produced crops because single cells in the form of gametes are the basis for the next generation, thus resolving any chimeras. While in vegetatively propagated crops, several cycles of regeneration are required to produce solid genotypically homogeneous material (Van Harten, 1998; Mba *et al.*, 2009). Plant breeders are also attracted to *in vitro* techniques such as micropropagation, embryo rescue, protoplasts, somatic embryogenesis, etc. as these in vitro techniques seem an efficient way to introduce variation and further multiplication is helpful in the selection of desired genotypes (Ahloowalia, 1998). Due to the growing world population breeders have no choice except to increase production and yield of crops. But this goal is really hard to achieve using traditional methods because of climate change. Hence, mutation-based plant breeding has been aimed at upgrading the well-adapted varieties by altering one or two major traits. Mutant varieties have specialized characters such as plant height, maturity, seed shattering, and disease resistance, which contribute to increased yield and quality traits. Quality traits include oil profile and content, malting quality, and size and quality of starch granules. Rice, wheat, barley and maize with short height genotypes are significant to increasing grain yield

because of their resistance to lodging and suitability for high planting density.

Every day human beings confront different types of problems for food production, food price and sustainability and due to climatic changes, environmental issues are very common with agriculture practices. Indeed, at the present time the demand is to avail food for everyone and to reach the goal of zero hunger by 2030 (FAO). We know that oilseed crops have a large trade share so modification of the fatty acid profile of the seed oil has been one of the main tasks faced by oilseed breeders over the past forty years. Success in this field has been of paramount importance for the worldwide expansion of some oilseed crops**.** About 104 varieties of oil crops have been released by mutation breeding up to 2017 throughout the world (FAO).

Table 7.1 Oil crop varieties with improved character are listed below.

Svalof's Primex	Sweden	1950	*Sinapis alba* L.	White mustard	Higher seed yield and oil percentage
Regina varraps elite A	Sweden	1953	*Brassica napus* L.	Rapeseed	Higher seed yield and oil percentage
Seco	Sweden	1961	*Sinapis alba* L.	White mustard	High yield, oil content, resistance to shattering, early flowering and stalk stiffness
Regina varraps elite F	Sweden	1962	*Brassica napus* L.	Rapeseed	Higher seed yield and oil percentage
Redwood 65	Canada	1965	*Linum usitatissimum* L.	Flax/linseed	High oil content
Trico	Sweden	1967	*Sinapis alba* L.	White mustard	Increased seed yield and oil content
Aruna	India	1969	*Ricinus communis* L.	Castor bean	Very early maturity (120 days instead of 270 days as original variety), yield slightly higher
Huyou 4	China	1970	*Brassica napus* L.	Rapeseed	Tolerance to low temperature, resistance to diseases, high and stable yield
TG 3	India	1973	*Arachis hypogaea* L.	Groundnut	Increased pod number, high yield and good adaptability
TG 1 (Vikram)	India	1973	*Arachis hypogaea* L.	Groundnut	Large seed size, maturity (135 days), oil content (47-48%) and TMV resistance
RLM 198	India	1975	*Brassica juncea* L.	Mustard	Improved oil content, early maturity (5-6 days) and high yield

Sowbhagya (157-B)	India	1976	*Ricinus communis* L.	Castor bean	Late maturity, dwarfness, resistance to shattering, suitable for intercropping
TG 4 (TG 14)	India	1976	*Arachis hypogaea* L.	Groundnut	Uniform maturity, high yield and good adaptability
Pervenetch	Russian Federation	1977	*Helianthus annuus* L.	Sunflower	High oil content (67.4-75.4%), altered fatty acid composition (oil is similar to olive oil)
RC8	India	1978	*Ricinus communis* L.	Castor bean	Late maturity, higher TGW, higher plant height, more internodes and higher yield
TM-2	India	1978	*Brassica juncea* L.	Oriental mustard	Altered pod morphology (black/brown seeds) and high yield (25%)
Dufferin	Canada	1979	*Linum usitatissimum* L.	Flax/linseed	High oil content and better resistance to Melampsora lini (Ehrenb.) Lev.
Xinyou 1	China	1979	*Brassica napus* L.	Rapeseed	Good seedling growth, tight stature, tolerance to low temperatures, resistance to drought, salinity and alkaline soils, good quality
Xiuyou 1	China	1979	*Brassica napus* L.	Rapeseed	Early maturity, suitable for rice-rice-rape multiple cropping, high yield, resistance to diseases and low temperatures
Huahuang 1	China	1980	*Brassica napus* L.	Rapeseed	Improved viability
RLM 514	India	1980	*Brassica juncea* L.	Oriental mustard	High oil content, less erucic acid (11%), early maturity, high grain yield (22 % more), large grain size and resistance to shattering

Kalika (BM 3-7)	India	1980	*Sesamum indicum* L.	Sesame	Semi-dwarfness, compact growth, number of seeds per capsule and increased yield (15%)
Khersonskaya 10	Russian Federation	1981	*Ricinus communis* L.	Castor bean	Improved oil content and high yield
Briscola	Italy	1981	*Olea europaea* L.	Olive	Reduced plant height by 50 %, easy harvest and male sterility
TG 17	India	1982	*Arachis hypogaea* L.	Groundnut	High yield, plant type (no secondary branches) and high harvest index
Zheyou 7	China	1983	*Brassica napus* L.	Rapeseed	Altered maturity and good quality
Co 2	India	1984	*Arachis hypogaea* L.	Groundnut	High yield and higher shelling percentage
Ganyou 5	China	1984	*Brassica napus* L.	Rapeseed	Tolerance to low temperature, reduced plant height, pod length increased, resistance to white rust and Sclerotinia, early maturity, higher and stable yield
Shambal (BAU-M/248)	Bangladesh	1984	*Brassica juncea* L.	Oriental mustard	Short stem and larger seed size
Ansanggae	Korea, Republic of	1984	*Sesamum indicum* L.	Sesame	Resistance to shattering, high yield and resistance to diseases (leaf blight, Phytophtora stemrot and fusarium wilt)
Stellar	Canada	1987	*Brassica napus* L.	Rapeseed	Linolenic acid (3%), linoleic acid (28%), low erucic acid and low glucosinolate
Jingkui 1	China	1987	*Helianthus annuus* L.	Sunflower	Improved plant structure and altered maturity

Xiangyou 11	China	1987	*Brassica napus* L.	Rapeseed	Tolerance to abiotic stresses and good quality
RL 1359	India	1987	*Brassica juncea* L.	Chinese mustard	Short duration, high yield, high TGW, oil content (43%), erect plant type, tolerance to aphids
Zarja 87	Russian Federation	1988	*Linum usitatissimum* L.	Flax/linseed	Late maturity, good quality and high yield
TGS 1 (Somnath)	India	1989	*Arachis hypogaea* L.	Groundnut	Early maturity, large seed size, high yield, sequential flowering and high oil content
Tismenitskii	Russian Federation	1989	*Brassica napus* L.	Rapeseed	Good quality, oil content, resistance to mildew and resistance to snow mold
Ivanna	Russian Federation	1990	B*rassica napus* L.	Rapeseed	White rhizocarp, resistance to mildew and resistance to insects
USHA	India	1990	*Sesamum indicum* L.	Sesame	High yield, uniform maturity and resistance to disease
UMA	India	1990	*Sesamum indicum* L.	Sesame	Early and uniform maturity, high oil content
TAG-24	India	1991	*Arachis hypogaea* L.	Groundnut	Earliness, semi-dwarf habit, yield stability, high harvest index, shorter internodes, dark green, small leaves and high water use efficiency
Safal	Bangladesh	1991	*Brassica juncea* L.	Oriental mustard	High seed yield, high oil yield (43-44%), higher biomass yield, tolerant to aphids in field conditions, plants are strong, erect and very tall (about 180 cm), tolerant to Alternaria disease
Agrani	Bangladesh	1991	*Brassica juncea* L.	Oriental mustard	Early maturity, bigger pod and seed size, tolerant to Alternaria disease, high yield and high oil content (44%)

TG-22	India	1992	*Arachis hypogaea* L.	Groundnut	High yield, medium-large seed size, seed dormancy, tolerance to acidic soils
Eshtar	Iraq	1992	*Sesamum indicum* L.	Sesame	Larger capsule size, reduction of branches and high oil content
Rafiden	Iraq	1992	*Sesamum indicum* L.	Sesame	Early maturity, high oil content and high yield
Babil	Iraq	1992	*Sesamum indicum* L.	Sesame	Early maturity, high oil content and high yield
Suwonkkae	Korea, Republic of	1992	*Sesamum indicum* L.	Sesame	Good quality, higher protein content, resistance to lodging, resistance to diseases and higher yield
TKG-19A	India	1993	*Arachis hypogaea* L.	Groundnut	High yield, large seed size, maturity (120 days), slightly reduced oil content with increased protein percentage, tolerant to acidic soils
Abasin-95	Pakistan	1995	*Brassica napus* L.	Rapeseed	High yield, early maturity and high adaptability
Yangbaeckkae	Korea, Republic of	1995	*Sesamum indicum* L.	Sesame	High oil quality and high yield
ANK-S2	Sri Lanka	1995	*Sesamum indicum* L.	Sesame	Resistance to diseases, high yield, vegetation (78-80 days), potential yield (1890 kg/ha) and resistance to Fusarium, Phytophthora and other diseases
Abasin-95	Pakistan	1995	*Brassica napus* L.	Rapeseed	High yield, early maturity and high adaptability
Yangbaeckkae	Korea, Republic of	1995	*Sesamum indicum* L.	Sesame	High oil quality and high yield
ANK-S2	Sri Lanka	1995	*Sesamum indicum* L.	Sesame	Resistance to diseases, high yield, vegetation (78-80 days), potential yield (1890 kg/ha) and resistance to Fusarium, Phytophthora and other diseases

Pungsankkae	Korea, Republic of	1996	*Sesamum indicum* L.	Sesame	Determinate growth, resistance to shattering of grains and high yield potential
Pungsankkae	Korea, Republic of	1996	*Sesamum indicum* L.	Sesame	Determinate growth, resistance to shattering of grains and high yield potential
TG-26	India	1996	*Arachis hypogaea* L.	Groundnut	High yield, semi-dwarfness, tolerance to peanut bud necrosis disease, fresh seed dormancy, compact pod setting, response to inputs
Linola 989	Canada	1996	*Linum usitatissimum* L.	Flax	Oil quality
Zlata	Czech Republic	1996	*Sinapis alba* L.	White mustard	Early flowering and vigor
Taka 1	Egypt	1996	*Sesamum indicum* L.	Sesame	Resistance to wilt
Taka 2	Egypt	1996	*Sesamum indicum* L.	Sesame	Resistance to wilt and root rot
Taka 3	Egypt	1996	*Sesamum indicum* L.	Sesame	High yield and resistance to diseases (insects)
Binasharisha-3	Bangladesh	1997	*Brassica napus* L.	Rapeseed	Early maturity (85-90 days), high yielding rapeseed variety, plant is erect, tolerance to Alternaria disease, maximum seed yield potential is 2.4 tons/ha (av. 1.85 tons/ha), seed contains 44% oil with low content of erucic acid (25%)
Binasharisha-4	Bangladesh	1997	*Brassica napus* L.	Rapeseed	Early maturity (80-85 days), high yielding rapeseed variety, more tolerance to Alternaria disease, maximum seed yield potential is 2.5 tons/ha (av. 1.9 tons/ha), seed contains 44% oil with low content of erucic acid (27%)

Seodunkkae	Korea, Republic of	1997	*Sesamum indicum* L.	Sesame	Resistance to diseases and high yield
Suwon 155	Korea, Republic of	1998	*Sesamum indicum* L.	Sesame	Improved oil quality and high yield
Zornitsa	Bulgaria	2000	*Lens culinaris* Medik.	Lentil	High yield, high protein content (28.7%), good culinary and organoleptic quality, resistance to anthracnose, viruses and ascochyta blight
Binasarisha-5	Bangladesh	2002	*Brassica napus* L.	Rapeseed	Salt tolerance
TPG-41	India	2002	*Arachis hypogaea* L.	Groundnut	High yield, large seed, maturity 120 days, 25 days seed dormancy, released for all India post-rainy situation under irrigation
NIFA-Raya	Pakistan	2003	*Brassica napus* L.	Rapeseed	High yield and higher oil content
NIFA-Mustard Canola	Pakistan	2003	*Brassica juncea* L.	Mustard	Reduction of plant height, early maturity, good quality, higher oil content, moderately resistant to Alternaria blight (*Alternaria brassicae*), Sclerotinia stem rot (*Sclerotinia sclerotiorum*) and downy mildew (*Peronspora parasitica*) diseases. Based on its quality characteristics, oil of MM-NIFA-Mustard Canola is suitable for human consumption and its meal is fit for animal use as part of their ration
Zhongzhi11	China	2003	*Sesamum indicum* L.	Sesame	High yield, good quality, vigor, resistance to stem blight and fusarium wilt, lodging resistance
Binatil-1	Bangladesh	2004	*Sesamum indicum* L.	Sesame	Tolerance to stem rot (*Macrophomina phaseolina* L.), high seed yield and high oil content

TG-37A	India	2004	*Arachis hypogaea* L.	Groundnut	Semi-dwarfness, compact pod setting, high yield and smooth pod surface
NUDB-38	India	2004	*Brassica napus* L.	Rapeseed	Early maturity and high yield potential
Durr-e-NIFA	Pakistan	2005	*Brassica napus* L.	Rapeseed	High yield, higher oil content, early maturity and broader adaptability to rainfed and irrigated environments
RADA	Bulgaria	2006	*Helianthus annuus* L.	Sunflower	Resistance to diseases
TAS-82	India	2007	*Helianthus annuus* L.	Sunflower	Tolerance to drought and black seed coat
TPM-1	India	2007	*Brassica juncea* L.	Mustard	Yellow seed coat and tolerance to powdery mildew
Mutant NUDB-26-11	India	2007	*Brassica napus* L.	Rapeseed	
Madan	Bulgaria	2008	*Helianthus annuus* L.	Sunflower	Large seeds, improved oil and protein content (>29% and >22% respectively)
Huayu 32	China	2009	*Arachis hypogaea* L.	Groundnut	High yield, good quality, medium drought and waterlogging tolerance
Yana	Bulgaria	2009	*Helianthus annuus* L.	Sunflower	High oil content and a large number of seeds per head
GPBD 5	India	2010	*Arachis hypogaea* L.	Groundnut	Large seed

Inshas 10	Egypt	2011	*Carthamus tinctorius* L.	Safflower	Spineless, high seed yield and yield component, high oleic fatty acid in oil, white flower, resistant for leaf spots and smut, resistant for tunnel insects and main pests
Inshas 11	Egypt	2011	*Carthamus tinctorius* L.	Safflower	Spineless, high seed yield and yield component (not mutation derived), high oleic fatty acid in oil, yellow flower, resistant for leaf spots and smut, resistant for tunnel insects and main pests
Binasarisha-7	Bangladesh	2011	*Brassica juncea* (L.) Czern & Coss	Indian mustard	Bold seed size and higher number of siliquae. Average seed yield is 2.0 t/ha. Maximum seed yield is 2.8 t/ha
Binasarisha-8	Bangladesh	2011	*Brassica juncea* (L.) Czern & Coss	Indian mustard	Average seed yield is 1.7 t/ha. Maximum seed yield is 2.4 t/ha
Binatil-2	Bangladesh	2011	*Sesamum indicum* L.	Sesame	Binatil-2 is characterized by the following improvements: higher yields, branched plant architecture, and tolerance to temporary waterlogged condition
Binasarisha-9	Bangladesh	2013	*Brassica napus* L.	Rapeseed	Average seed yield is 1.6 t/ha. Maximum seed yield is 1.95 t/ha. Other improved characters are shorter plant height and black seed coat color
Binatil-3	Bangladesh	2013	*Sesamum indicum* L.	Sesame	Binatil-3 is characterized by the following improvements: higher yields and branched plant architecture

Binatil-4	Bangladesh	2016	*Sesamum indicum* L.	Sesame	Binatil-4 is characterized by the following improvements: higher yield, less hairy stem, leaves and capsules, and tolerance to temporary waterlogged condition
NIAB-Sesame-2016	Pakistan	2016	*Sesamum indicum* L.	Sesame	Mutant with high-quality crystal-white seeds with 60.2% oil contents. The first mutant of sesame in Pakistan which is moderately resistant to bacterial blight, phyllody, and charcoal-rot diseases
NIAB-Pearl	Pakistan	2017	*Sesamum indicum* L.	Sesame	A higher number of fruiting branches and capsules per plant as compared to conventional varieties; About 10% higher oil content in seed as compared to conventional varieties; Thick stem and resistant to lodging as compared to the weak stem of conventional varieties; Best suited variety for new broad-line technology developed at NIAB for sesame cultivation

A number of varieties of oil crops with specific character improvements such as good quality, high yield, improved seed size, high harvest index, high and improved oil content and resistance to diseases have been released by mutation breeding across the world (FAO). Among the mutation-induced oil crop varieties in different countries, India developed 32 mutant varieties (most of the groundnut crop varieties) and ranked first, followed by China and Bangladesh with 15 and 13 mutant developed varieties respectively.

7.5 Linseed

Linseed is also known as flax and is one of the oldest crops cultivated for oil and fibre. The botanical name, *Linum usitatissimum,* was given by Linnaeus in his book "Species Plantarum" (Linnaeus, 1857) and it belongs to the family Linaceae. It is an annual herbaceous plant with a shallow root system. Linseed is commonly called flax and is used in North America and Asia. Flax is an erect annual plant growing to 1.0-1.25 m tall, stem: slender, leaves: green, narrow, alternate, lanceolate, flower: white, purple, blue, pink or red blossoms with five petals, fruit: round, dry capsule containing around ten seeds. The oil content of the seed varies from 33 to 47%.

Flaxseed cakes are very good manure and used as animal feed throughout the country. The whole plant of flax has great economic value. Many varieties of linseed have been specialized for oilseed and fibre by developing this species (Millam *et al.,* 2005).

Linseed oil is enriched with more than 50% linolenic acid; the 18-carbon fatty acid with three double bonds which is a unique feature among other vegetable oils (FAO). It has a total oil content of around 28-30%, thus it is grown for oil as well as fibre. Omega fatty acids are known to show beneficial and protective effects against diseases. Omega-3 fatty acids may reduce the risk of cancer and heart disease. Linseed oil has more than 50% linolenic acid which is suitable for its industrial application but for edible purposes, the linolenic acid content is very high so should be reduced.

By comparing the yields of linseed from 2004 to 2016 (Figure 7.8), it can be observed that linseed yield fluctuated in some years but since 2013, it continuously increased. This indicates that linseed is now a crop in demand due to the need for its valuable oil which has multiple benefits.

Figure 7.8 Graph showing linseed yield (world) 2004-2016 (source FAO).

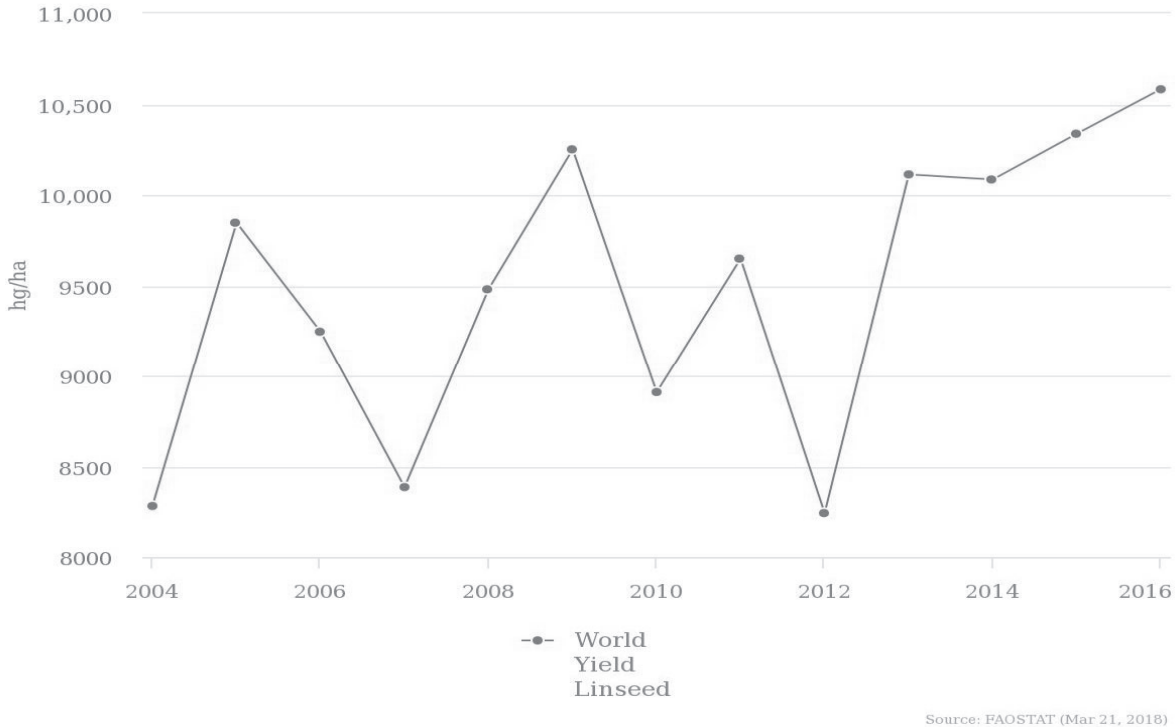
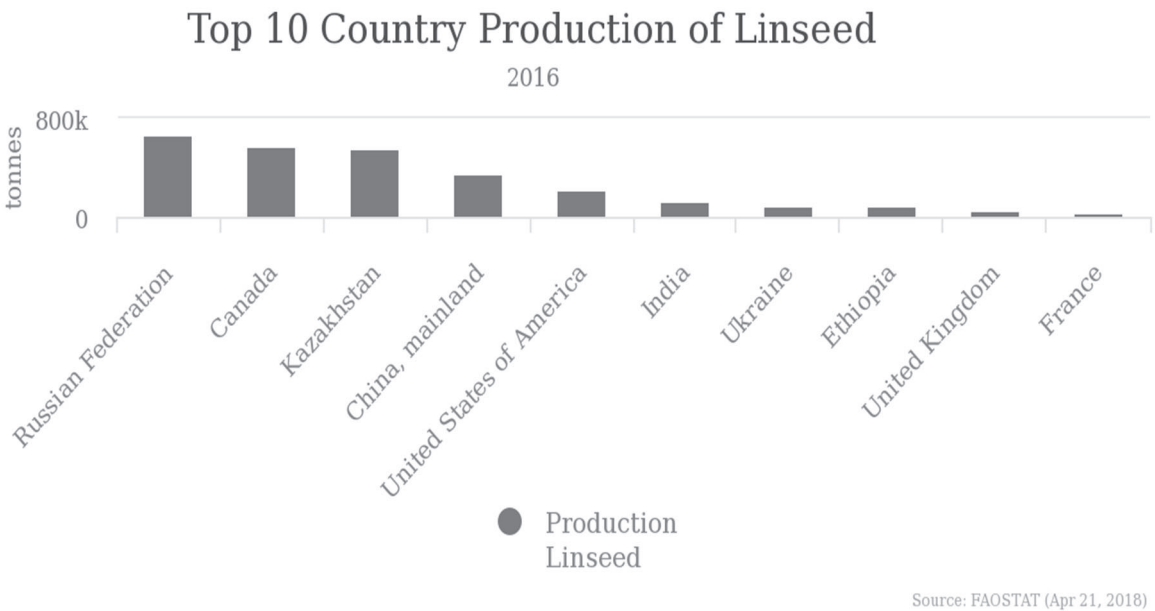
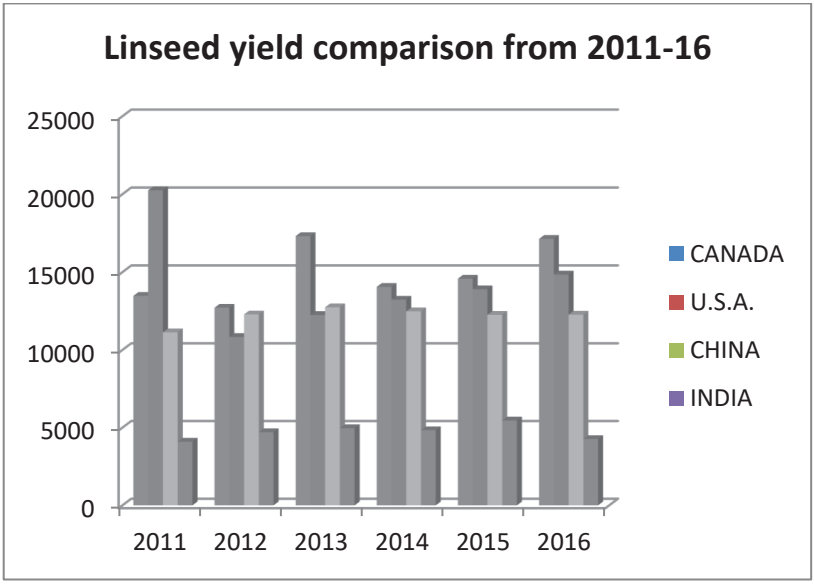


Figure 7.9 Graph showing the ten leading countries of linseed production.



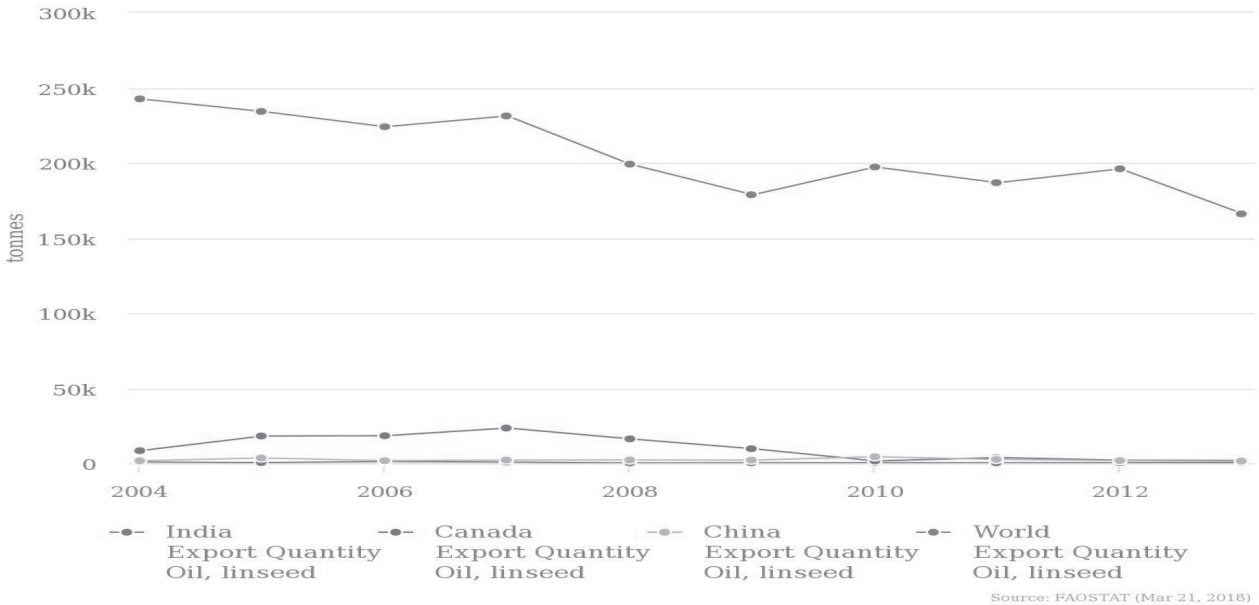
According to the FAO database, in 2016 the top five leading countries of linseed were the Russian Federation, Canada, Kazakhstan, China and the United States of America. Whereas India was ranked sixth among the top ten countries of linseed production (Figure 7.9).

Figure 7.10 Yield comparison of linseed.



If we compare the linseed yields for Canada, the United States of America, China and India from 2011 to 2016 then it is shown that yield decreased between 2011 and 2016 in the United States of America while other countries showed increased yields. In 2011 the United States of America was the largest producer with a yield of 20252 kg/ha, followed by Canada, China and India having yields of 13481, 11134 and 4095 kg/ha respectively. By 2016 Canada had become the largest producer of linseed followed by the United States of America, China and India (Figure 7.10).

Figure 7.11 Graph showing linseed oil export 2004-2013.



It is clear (Figure 7.11) that the export quantity of linseed oil decreased gradually during 2004-2009 and finally in 2013 export was lowest. We can assume that the decrease in export was due to increased consumption by the exporting country or due to low production. Either way, the demand for linseed oil is higher to meet the requirement. This is possible through the innovative method of oil production on a large scale.

Linseed varieties LINOLA in Australia in 1984 and SOLIN in Canada in 1990 with low linolenic acid have been successfully developed. In India, some genotypes with less than 1% linolenic acid were also developed by the national linseed program in collaboration with BARC, Mumbai. Linseed oil with low linolenic acid content can be widely used as cooking oil.

Conventional, as well as molecular approaches, are used to emulate the fatty acid profile of linseed oil and the same efforts are being used to modify soybean oil (*Glycine max*) and canola oil. These oils have ALA content which may replace fish oil in the diet and be beneficial for human health. Further modifications are necessary to enhance the alanine content in oils (Cahoon, 2003; Scarth and Tang, 2006).

Linseed varieties were also developed through mutation breeding but the number of improved varieties is remarkably low, being only seven. The mutant variety Dufferin was officially approved in 1979, being developed by hybridization and the variety Redwood 65 was obtained by irradiation with X-rays and only these two varieties show improved attributes of high oil content.

Table 7.2 Induced mutant varieties of linseed (source: mvd.iaea.org).

Variety Name	Country	Registration Year	Short Description
Redwood 65	Canada	1965	The mutant variety Redwood 65 was officially approved in 1965. It was developed by irradiation of seeds with X-rays. Main improved attribute of mutant variety is high oil content.
Heiya 4	China	1978	The mutant variety Heiya 4 was officially approved in 1978 and was developed by hybridization with mutant obtained by irradiation with gamma rays. Main improved attributes of mutant variety are early maturity, resistance to lodging, tolerance to moisture, salinity and alkalinity.
Dufferin	Canada	1979	The mutant variety Dufferin was officially approved in 1979. It was developed by hybridization with mutant variety Redwood 65 obtained by irradiation with X-rays. Main improved attributes of mutant variety are high oil content and better resistance to *Melampsora lini* (Ehrenb.) Lev.
Ningya 10	China	1982	The mutant variety Ningya 10 was officially approved in 1982. It was developed by irradiation of seeds with gamma rays (100 Gy). Main improved attributes of mutant variety are early maturity, branching, resistance to lodging and good quality.
Heiya 6	China	1985	The mutant variety Heiya 6 was officially approved in 1984. It was developed by hybridization with two mutants obtained by irradiation with gamma rays (one mutant irradiated with 200 Gy and the other mutant with 300 Gy). Main improved attributes of mutant variety are high yield, resistance to lodging, resistance to diseases, resistance to salinity and alkalinity.

Zarja 87	Russian Federation	1988	The mutant variety Zarja 87 was officially approved in 1988. The hybrid seeds (LD-147 x Complex) was treated with a water solution of EI. Main improved attributes of variety are late maturity, good quality and high yield.
Heiya 7	China	1989	The mutant variety Heiya 7 was officially approved in 1989 and developed by gamma rays (100 Gy). Main improved attributes of mutant variety are high yield and good quality.

Linseed mutated varieties Heiya 4, Ningya 10, Heiya 6, Heiya 7, Zarja 87, Dufferin, Redwood 65 were released from 1989 to 2017. This data showed that only three countries, Canada, China and the Russian Federation, developed mutant varieties from their three respective continents. It has been 29 years since the last induced variety and other countries are still waiting for a good result.

7.6 Fenugreek

Fenugreek is grown across the globe including in parts of North Africa, Mediterranean Europe, Russia, Middle East, China, India, Pakistan, Iran, Afghanistan, parts of the Far East and SE Asia, Australia, the USA, Canada and Argentina. It is recognized as a global spice crop in all the major continents depending on soil and climatic conditions. India was once the largest producer and still holds the largest area of fenugreek harvested in the world (Acharya *et al.,* 2006).

It is an annual forage legume and a traditional spice crop that has been grown for centuries across the Indian subcontinent. India, despite being the largest fenugreek producer in the world, does not have a major share of the global fenugreek trade due to high internal consumption (Basu 2006; Basu and Agoramoorthy, 2014). There are several research reports regarding the health benefits of fenugreek, making it more important economically, agronomically and environmentally throughout the world.

The fenugreek stem is thick, erect, hairy and green in color. The leaves are compound trifoliate, alternate and composed of three toothed ovate leaflets. The flowers, solitary or in groups of two, are borne on leaf axils and are white or pale yellow to light purple and present a triangular shape (hence the name of the genus, *Trigonella* which is extracted from the old Greek name, meaning ‘three-angled’ (Petropoulos, 2002)). The fruit is an

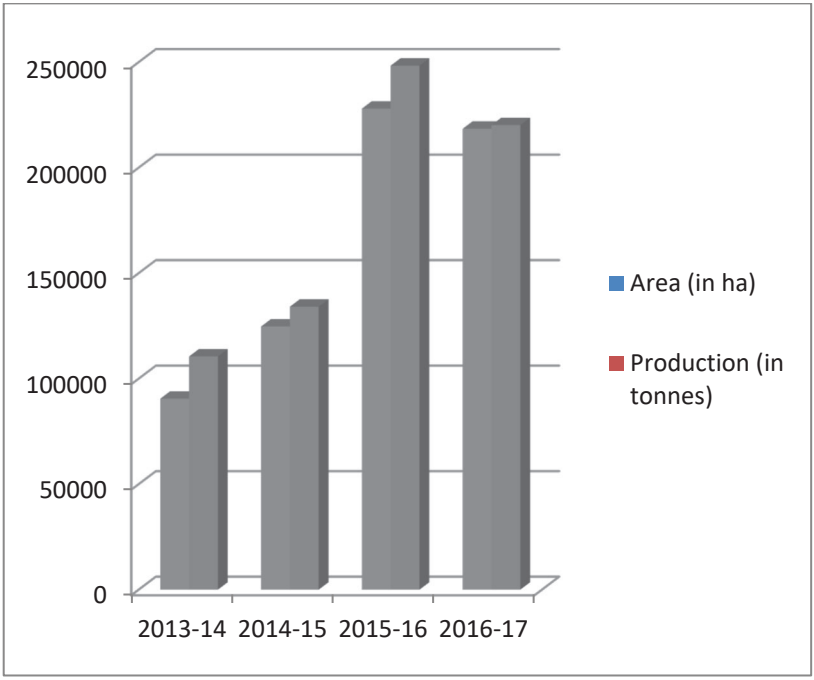
elongated arched pod and the seeds are located side by side, at maturity the seeds become very hard, flattened and their color changes from light brown to reddish-brown. The seeds are having a prominent groove that delineates two unequal parts (Acharya *et al.,* 2006). It possesses a strong celery odor and is used in butterscotch, cheese, licorice, pickle, rum, syrup and vanilla flavors and may be of interest to the perfume industry (Duke, 2012).

In fatty acid profiling, sixteen fatty acids were identified in fenugreek seed oil. Oil content in the fenugreek varieties ranged from 4.32 to 11.62%. Among sixteen fatty acids identified in fenugreek seed oil, linoleic acid (18:2 n-6) was the major contributor, the amount of which ranged from a minimum of 33.36 % to a maximum of 43.41 %. This was followed by α-Linolenic acid (18:3 n3), palmitic (16:0), stearic acids (18:0) and oleic acid (18:1 n-9).Other fatty acids viz., myristic acid (14:0), pentadecanoic acid (15:0), cis-10-heptadecenoic acid (17:1), heptadecanoic acid (17:0), cis-11,14-eicosadienoic acid (20:2 n-6), cis-11-eicosenoic acid (20:1 n-9), eicosanoic acid (20:0), heneicosanoic acid (21:0), 13-docosenoic acid (22:1 n-9), docosanoic acid (22:0) and tetracosanoic acid (24:0) were also identified in the range of 0.10% to 2.01 %. These indicated that fenugreek seed lipids are a good source of PUFA and the oil is rich in n-3 and n-6 content. The n-6: n-3 fatty acids ratio in fenugreek seed oil is very near to the ideal ratio for human consumption. PCA identified linolenic and oleic acid as the most important traits responsible for variation and can be used as a target trait for improving quality through breeding (Rathore *et al.,* 2016)

In recent years, due to the immense therapeutic and medicinal value of fenugreek and also broad adaptability with very few expectations of soil condition, fenugreek cultivation spread from America to India (Acharya *et al.,* 2006; Petropoulos, 2002). Even this plant is also known as a new species in Canada. Genotypes of fenugreek are adapted to the climatic conditions of western Canada. In such cases, mutation breeding can be used to generate genetic variability in the gene pool for various yield attributing traits (Fehr, 1991). Up to now, dramatic changes can be seen in various legume crops (Sigurbjornsson and Micke, 1974; Sigurbjornsson, 1983; Toker *et al.,* 2007). To increase diosgenin content in *T. corniculata* (a close relative of fenugreek), Mahna *et al.* (1994) used mutation breeding. In plants, generally, mutations are induced by a variety of mutagens either chemically or physically. RH 3112 cultivar with higher diosgenin content and seed yield and RH 3118 cultivar with higher protein content are two main cultivars which were made by induced mutations

(Laxmi *et al.,* 1980; Laxmi and Datta 1987). Chemical mutagens also play a very important role in the production of new improved varieties of fenugreek. Basu (2006) produced an improved variety of fenugreek by using the chemical mutagen Ethyl Methanesulfonate (EMS). Improved characters in a new population are higher height, seed yield, seed number per pod, biomass yield, the total number of pods and number of twin pods.

Figure 7.12 Fenugreek area wise production in India (source: Spice Board of India).



Fenugreek production along with cultivation area increased remarkably during 2015-2016 but decreased in 2016-2017 (Figure 7.12), which shows that there is a need to improve fenugreek production in available cultivation areas.

Improvement in fenugreek has been achieved by various methods based on genetics (Prajapati *et al.,* 2010; Harish *et al.,* 2011 and Fufa, 2013), salinity (Garg, 2012), drought tolerance (Ahari *et al*., 2009), fodder (Solorio-Sánchez *et al.,* 2014), symbiotic association (Ali *et al.,* 2012), and nutraceutical (Acharya *et al.,* 2006). For morphological screening by the

effect of magnets, UV and ethidium bromide (Gadge *et al.,* 2012), as well as for chlorophyll mutation induced by mutagen gamma rays, EMS and sodium azide (Bashir *et al.,* 2013) and improvement in the nutraceutical properties of fenugreek has been carried out by induced mutation screening by the chemical mutagen EMS (Acharya *et al.,* 2006). A combination of physical and chemical mutagen is also used to increase variability in fenugreek (Wani, 2009).

Mutant screening is a very crucial step in mutation breeding, Rajni *et al.* (2010) recommended 4.5 mM concentration of sodium azide to introduce variability and selection of useful genotypes of fenugreek with improved seed yield. Rajkumari, (2012) exposed fenugreek seeds to gamma rays (1-5 Kr) and treated seed with various concentrations of solutions of EMS, after harvesting M_2 generation was raised as progenies. It was shown that both mutagens created variability for yield attributing characters such as an increase in pods, seeds and seed weight. It was concluded that variation in fenugreek progenies could be useful for the selection of new genotypes.

In Kenya, a mutant variety, RH 3112, was developed from a local cultivar by treating with gamma ray irradiation. Mutant variety RH 3112 which had almost double the yield with 1.78% diosgenin, increasing up to 15% more than the mother plant. Another spontaneous mutant, RH 3129, is another example with twin pods and 1.35% diosgenin from the Moroccan cultivar (Petropoulos, 1973).

Fenugreek is a promising crop, with multiple uses of different parts of a single plant. Low field conditions and developing new cultivars with the implementation of mutation breeding for a wide range of adaptations in adverse climatic conditions could be a boon for elite farmers.

Fenugreek genotypes with variation in fatty acids composition may develop new cultivars for innovative end-uses solely or blended with other vegetable oils. Since fenugreek is not established as an oil crop, or it is neglected, significant genotypes may be utilized to introduce high-quality vegetable oil (Rathore *et al.,* 2016).

7.7 Future prospects

At the present time, as well as in the future due to climate change, human beings are confronting major challenges such as temperature rise, drought, cyclones, hailstorms and floods which ultimately, directly or indirectly,

adversely affects agriculture and food production (Report of the Intergovernmental Panel on Climate Change, 2007).

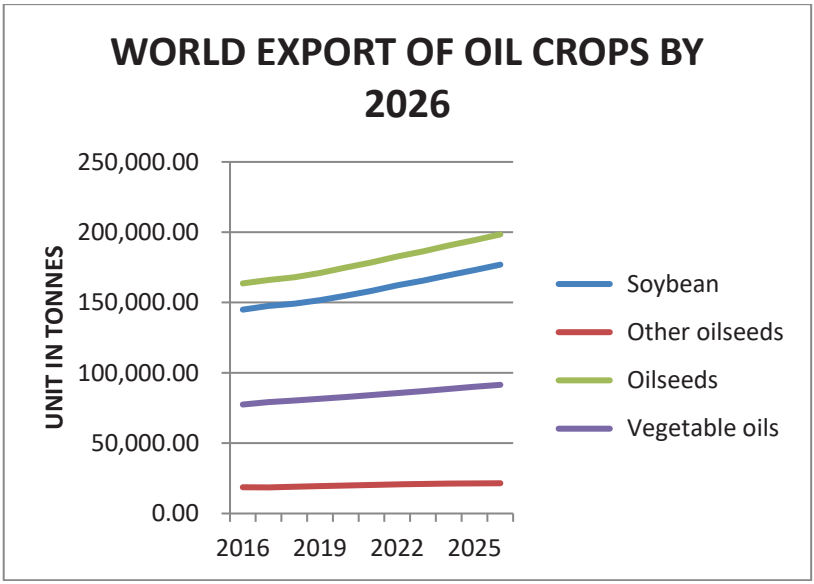
It is expected that by 2050 the human population will reach 9 billion, it has been estimated that 44% of the required additional calories will come from oil crops ("Prospects for world supply and demand of vegetable oils", Food and Agriculture Organization of the United Nations, www.fao.org). Traditional methods for increasing oil yield and improving quality are not sufficient. Oil crops are an essential ingredient for food supply with the growing world population. Novel technologies such as omics genetic marker selection are now being utilized in the plant as well as the animal system and may be highly significant for oil crop improvements in the future (Teh *et al.,* 2017).

The green revolution started in the late 1960s which resulted in genetic gains of crops with the development and massive distribution of high yielding and resilient cereal crop varieties around the world. The consequent marked increases in food production in many food-deficit countries were credited with saving billions of people from starvation, especially in Asia (Ejeta, 2009, Ejeta, 2010).

With dwindling or stagnant agricultural land and water resources, the sought-after increases will, therefore, be attained mainly through the enhancement of crop productivity under eco-efficient crop production systems. 'Smart' crop varieties that yield more with fewer inputs will be pivotal to success. Plant breeding must be re-oriented to generate these 'smart' crop varieties (Mba *et al.,* 2012).

According to the OECD-FAO Agricultural Outlook 2017-2026, world export of soybean, oilseeds and vegetable oils is expected to increase by 2026 (Figure 7.13). It is the time to increase the production of these crops by innovative techniques that hold great promises for crop improvement.

Figure 7.13 Graph showing world export of soybean, oilseeds and vegetable oils from 2016-2026.



Dataset: OECD-FAO Agricultural Outlook 2017-2026

The introduction of new genes and their variants into crops from novel sources will be critical to replicating the impacts of the green revolution as the current generational challenges demand. FAO (2011) recommended that 'a genetically diverse portfolio of improved crop varieties, suited to a range of agroecosystems and farming practices, and resilient to climate change' is key to sustainable production intensification.

7.8 Conclusion

Conclusively, this review points out the importance of oil crops with 42% global trade share of production of all the agricultural commodities and global risk of the high demand for oil and oil products due to population increase. Population increase is inevitable and all we have to fulfill the demand for all the agricultural commodities. Some major oilseed crops such as soybean, peanut, rapeseed mustard, sunflower, safflower, Sesamum, linseed, castor and cottonseed are grown worldwide and because of high consumer demand, genetic improvement of oilseed crops is the only option to meet the production demand.

Many innovative techniques have been employed to develop high-quality oilseed crops with improved qualitative and quantitative characters. One of

the most important, inexpensive and efficient methods is mutation breeding of oilseed crops. By using a mutagenic agent in any crop, the mutation is induced and plants with desirable changes are screened and finally improved varieties are released after testing. The Joint FAO/IAEA Division assists the safe use of nuclear techniques and related technologies in food and agriculture and every year several mutant varieties are registered from worldwide into the mutant variety database. Linseed, an oil plant having more than 50% linolenic acid and omega-3 fatty acids (known to reduce the risk of heart disease) is cultivated in cooler regions. About seven varieties of linseed have been officially released, among these varieties only two, named as Dufferin and Redwood 65, showed improved oil quality. Fenugreek is a multipurpose crop used as a vegetable, spice and oil from its seeds. India is the topmost producer of fenugreek. Induced mutagenesis has been applied to fenugreek, but success is limited.

Due to increases in the human population, we need ‘smart’ crop varieties that yield more, even in adverse climate conditions with low inputs that will be fundamental to success. Ultimately ‘a genetically diverse portfolio of improved crop varieties, suited to a range of agroecosystems and farming practices, and resilient to climate change’ is key to sustainable production intensification.

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CHAPTER 8

MUTATION BREEDING TECHNIQUE FOR THE IMPROVEMENT OF PULSE CROPS WITH SPECIAL REFERENCE TO FABA BEAN (*VICIA FABA* L.)

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Abstract: Genetic variability is an essential prerequisite for crop improvement programs. Lack of required genetic variability limits the scope of selection for better genotypes in crop plants. Induced mutations

can help to refurbish the variability which has been lost during evolution. Mutation breeding efforts are aimed at increasing the yield and economic value by incorporating desirable characters such as disease and insect resistance, better grain quality and shorter growth duration in crop plants. The mutation breeding programs across the globe have led to the official release of 3278 mutant crop varieties. A large number of these varieties have been released in developing countries including India, which has positively impacted their economy. By integrating induced mutagenesis with robust molecular techniques, tolerance to biotic and abiotic stresses, resistance to diseases, lodging and improvement in protein and mineral quality can be achieved in crop plants.

Keywords: Induced mutagenesis, pulse crops, faba bean, agronomic traits, genetic variability, mutagens.

8.1 Introduction

Green plants are the ultimate resources to human life. As the human population increased, greater and safer supplies of food had to be found and gradually production systems based on plant domestication were developed. The cultivation of crops historically has been influenced by ecological and agricultural conditions in addition to food gathering preferences. Genotypes that have adapted to a wide range of climatic and edaphic conditions have been selected for cultivation (Novak and Brunner, 1992).

Grain legumes, commonly known as pulses, belong to family Fabaceae - the third largest flowering plant family after Orchidaceae and Asteraceae. Different kinds of legumes provide us with food, medicines, timber and contain a high percentage of quality proteins, minerals and vitamins (Bhat *et al.,* 2006b; Bhat *et al.,* 2006a; Bhat *et al.,* 2005a; Bhat *et al.,* 2007b; Siag *et al.,* 2005). Pulses are wonderful gifts of nature with the unique ability of biological nitrogen fixation suitable for mixed and alternate cropping, they have deep root systems, mobilize insoluble soil nutrients and bring qualitative changes in the physical properties of soil which make them known as fertility restorers (Bromfeild *et al.,* 2001). Of all the crop plants of economic interest to humans, none probably has been selected for so many useful traits as pulse crops. These crops contain high amounts of proteins, amino acids, macro and micro-nutrients, vitamins, fibers and carbohydrates for balanced nutrition. Their by-products provide nutritious fodder for livestock. Pulses have been reported to reduce the levels of cholesterol and blood glucose in human beings. The newly emerging

health consciousness among people is a motivating force for adopting a nutritionally complete vegetarian diet involving legumes as a component.

The production of pulses in the country has been practically stagnant for the last two decades. This has been due to the intrusion of high yielding varieties of cereals in the areas traditionally cropped with pulses. Secondly, unlike cereals, pulses have been grown on marginal land conditions of moisture stress and low soil fertility. The situation is likely to worsen in the immediate future as pulse crops are unable to compete with high yielding varieties of cereals and other remunerative crops. As a result, pulses have neither been grown on good land nor have they been given due attention concerning fertilizers, pesticides and other practices of good management.

In India during 1950-51, the per cent share of pulses of the total food grains vis-à-vis area, production and productivity were 19.62, 16.55 and 84.48 percent respectively. This trend continued till 1960-61 and started declining from 1970-71 due to no breakthroughs in production technology. Presently, except area stabilization, the production during 2014-15 has gone down to 6.79 per cent due to the stagnation in productivity of pulses as compared to other food grains (Table 8.1).

Plants encounter a number of environmental stresses and in light of changing climatic conditions coupled with increasing pressure on global food productivity there is a staid demand for stress-tolerant crop varieties (Takeda and Matsuoka, 2008; Jain, 2010; Newton *et al*., 2011). Recent research has showcased a number of key genes, quantitative trait loci (QTLs) and molecular networks that mediate plant responses to drought, salinity, heat and other abiotic stresses (Krasensky and Jonak, 2012; Ahmad *et al*., 2019a,b; Naikoo *et al*., 2019). Climate change may well lead to significant losses of genetic diversity within a species (Suprasanna *et al*., 2014). It is, hence, desirable that additional genetic variability needs to be induced through mutagenesis or other contemporary biotechnological and molecular approaches (Bhat *et al.,* 2006d; Bhat *et al.,* 2005b; Jain, 2012).

Induced mutations have been used for improvement of crops by developing new plant types with increased genetic diversity (Bhat, 2007; Bhat *et al.,* 2007a; Datta *et al*., 1993; Ranalli, 2012; Auti, 2012; Datta, 2014; Bhat *et al.,* 2007 Raina *et al.,* 2018a). Micke *et al*. (1990) used induced mutations for obtaining desirable genetic changes for agronomic traits such as high yield, flower color, disease resistance and early maturity

in various crop, fruit and ornamental plants. Pulse crops are slightly poor in partitioning the photosynthates from their vegetative parts to grains. A lot of dry matter goes into the production of stalks which leads to a low harvest index. In such crops, the morphological framework must be reconstituted in such a way that the maximum dry matter produced by a plant is efficiently partitioned between grains and the vegetative parts.

8.2 Mutagenesis: An Overview

Mutation breeding, a much-heralded short cut breeding method, brings novel and high yielding genotypes (Bhat, *et al.,* 2006; Bhat and Wani, 2015; Singh *et al*., 2011; Raina *et al*., 2017; Khursheed *et al.,* 2018a; Tantray *et al.,* 2017; Hassan *et al.,* 2018; Laskar *et al*., 2019). Mutations have been employed to improve morphological, physiological and disease resistance in addition to quantitative and qualitative traits (Ganai *et al.,* 2005; Bhat and Wani, 2017; Bhat and Wani, 2017a; Toker *et al*., 2011; Nakagawa *et al*., 2011; Laskar *et al*., 2015; Wani, 2017; Wani *et al*., 2017; Patial *et al*., 2017; Ramachander *et al*., 2018; Laskar *et al*., 2018a; Raina *et al.,* 2018b; Verma *et al*., 2018; Wani, 2018; Laskar *et al.,* 2018b; Goyal *et al*., 2019; Raina *et al*., 2019; Gulfishan *et al.,* 2012; Gulfishan *et al.,* 2013). Practical mutation breeding procedures strictly for plant breeding purposes were developed after the publications of Freisleben and Lain (1942, 1943a, 1943b). The knowledge accumulated vis-à-vis optimal treatment doses, treatment conditions and mutation frequency/spectra after the systematic studies of Gustafsson (1947), Mackey (1956) and Hoffman (1959). The dawn of the "atomic age" following World War II, saw a boom of interest in utilizing ionizing radiation for peaceful purposes (Nilan *et al*., 1965). Developing countries began to play an increasing role in mutation breeding work and new varieties of crops soon appeared in the market (Sigurbjornsson and Micke, 1966; Sigurbjornsson and Micke, 1974; Parveen *et al.,* 2006; Raina *et al.,* 2016 Gulfishan *et al.,* 2011; Gulfishan *et al.,* 2010; Bhat *et al.,* 2007).

Regarding chemical mutagenesis, the first elaborated report was presented by Auerbach and Robson (1942) who showed that mustard gas (dichloro ethylsulphide) could induce chromosomal breaks in *Drosophila*. Since then, several chemical mutagens have been identified for their mutagenic properties (Roebbelen, 1959; Ehrenberg *et al*., 1961; Auerbach, 1961; Rapoport *et al*., 1966). In 1969, the joint FAO/IAEA, Vienna division started to organize courses for plant breeders on the induction and use of mutations. Thus, it may be justified to consider 1969 as the year that

marked the establishment of mutation breeding as a practical tool available to plant breeders in their endeavors to develop more productive cultivars with high yield and better resistance to stresses, diseases and improvement in the quality of proteins (Datta, 2014).

For the past 48 years, mutation breeding efforts have resulted in the official release of 3278 (http://mvgs.iaea.org) new crop varieties to farmers, either as direct mutants or from their progenies, for commercial cultivation. The major portion of these mutant varieties was developed in the continent of Asia (1993) followed by Europe (955), North America (200), Africa (69), Latin America (51) and Australia and Pacific (10).

8.3 Mutagenesis in Faba Bean

Faba bean (*Vicia faba* L.) has been cultivated since ancient times for both animal and human nourishment. Faba beans are eaten while still young and tender, enabling harvesting to begin as early as the middle of February, however, if left to mature fully they are usually harvested in late April. Moreover, the faba beans have the potential to treat Parkinson's disease, hypertension, renal failure and liver cirrhosis. Since the faba bean is homozygous there is a great scope for improvement of its genotype through mutation breeding.

Induced mutations can improve one or two traits without disrupting the rest of the genome. Being a self-fertilized crop, induced mutagenesis in the recent past has produced appreciable results in faba bean (Gulfishan *et al.,* 2015; Sharma *et al.,* 2009; Khursheed *et al.,* 2018b). Biological damage in an earlier generation is usually used to evaluate the mutagenic potency and the sensitivity of the biological system. Faba bean has been reported to be highly responsive to both physical and chemical mutagens (Khursheed *et al.,* 2018c). In light of this fact, Vandana and Dubey (1988), Kumar *et al.* (1993) and Laskar and Khan (2014) studied the mutagenic sensitivity of faba bean in the M_1 generation and reported that germination, seedling growth, pollen fertility, maturity time and survival rate were adversely affected by the application of mutagens. Also, plant height, branching, number of leaves, pods, seeds and total plant yield showed varying responses to different mutagenic concentrations. Frequency and spectrum of mutations in the M_2 generation were partitioned into those for chlorophyll, sterile and vital categories (Vandana, 1991; Vandana, 1992a, 1992b; Fatma, 2007). The sterile mutants were flowerless, fruitless, cleistogamous and underdeveloped. Vital mutations were classified based on characters involving mutations for cotyledonary leaf, plant height,

branching, leaf bristle, floral characters, maturity period, pod size and seed color and texture.

8.4 Chlorophyll Mutations and Chromosomal Aberrations

Chlorophyll mutation frequency is considered to be a dependable index for evaluating the effectiveness and efficiency of mutagens (Gustafsson, 1951; Monti, 1968; Auti, 2012; Wani, 2017). Significant differences between the relative proportions of different kinds of chlorophyll mutations induced by physical or chemical mutagens have been previously reported by Ehrenberg *et al.* (1961), Nilan and Konzak (1961), Siddiq and Swaminathan (1969), Vishnoi and Gupta (1980), Vandana (1991) and Auti (2012). Yasin (1996) studied the frequency and spectrum of chlorophyll and leaf mutation by gamma rays, EMS and nitrous oxide (N_2O) and reported the highest number of 'chlorina' type of mutations. Among the mutagens used, EMS was found to be more effective than gamma rays and nitrous oxide.

Meiotic abnormalities are reliable indices for estimating the mutagenic sensitivity of crop species (Amin *et al.,* 2019). Alterations in the form of chromosome breakage due to mutagens and later their reunion is of practical interest in mutagenesis programs (Amin *et al.,* 2016). Induced chromosomal alterations have been extensively investigated to understand the efficiency and mechanism of action of mutagens in faba bean (Khursheed *et al.,* 2015, 2016). Vandana and Dubey (1992), Bhat *et al.* (2005), Prashant and Verma (2005), Fatma (2007) and Laskar and Khan (2014) reported different types of anomalies in root tip cells such as fragmentation, clumping and stickiness of chromosomes, star metaphase, giant nuclei, binucleate cells, micronuclei, bridges and laggards, while meiotic abnormalities consisted of multivalent association such as rings or chains of bivalents, fragmentation of the nucleolus, precautious separation of bivalents at metaphase-I, single, double and multiple bridges and unequal distribution of chromosomes at two poles at anaphase I/II etc. The percentage of mitotic or meiotic anomalies at various stages was directly correlated to the dose of mutagen used. DES dosage induced a higher percentage of abnormal cells than EMS (Vandana and Dubey, 1992; Vandana *et al*., 1996; Parveen, 2006). Similar anomalies have been reported by Sjodin (1971).

Bhat *et al.* (2007) gave a comparative analysis of meiotic aberrations induced by diethyl sulfate (DES) and sodium azide (SA) and found that both the mutagens elicited various chromosomal aberrations in meiosis,

while Khan *et al.* (2007) investigated the clastogenic effects of 8-hydroxyl quinolone (8-HQ) and observed the dose-dependent decrease in chiasma frequency at prophase and metaphase-I. The other meiotic aberrations observed were stickiness, laggards, univalents, multivalents, bridges, fragments, precocious separation etc. The stickiness of the chromosomes was the most common aberration, followed by precocious separation and laggards. Chromosome stickiness may be caused by genetic or environmental factors. Fatma and Khan (2009) observed dose-dependent increases in various chromosomal abnormalities such as stickiness, bridges, non-synchronization, misorientation and cytomixis in mutagen treated populations of faba bean. Prashant and Verma (2005) induced a medium-strong asynaptic mutant in faba bean using EMS treatment. A significant decrease in the number of chiasmata and pollen fertility was reported in the mutant as compared to the control. The inheritance of this asynaptic mutant was monogenic and recessive. Ignacimuthu and Babu (1989) reported that chromosome breakage and reunion of broken ends could lead to the formation of bridges. Bridges may have been produced due to sub-chromatic exchanges or unequal exchange of dicentric chromosomes. The chromatin transmigration between pollen mother cells (PMCs) with cytoplasmic channels is considered as a source of aneuploid and polyploid gametes (Yen *et al.,* 1993).

8.5 Variability for Quantitative Traits

Few studies have been undertaken to compare the mutagenic agents for their ability to induce genetic variability for quantitative traits (Gregory, 1965; Sjodin, 1971; Abdalla and Hussein, 1977; Fillipetti and De Pace, 1983; Chapman, 1981; Chapman, 1986; Joshi and Verma, 2004; Yasin *et al.,* 2006; Goyal *et al*., 2011; Auti, 2012; Khursheed *et al.,* 2019). Enhanced variability for polygenic traits were induced by various mutagenic treatments which were reflected by a shift in mean values and increased inter and intra family variability in subsequent generations. The coefficient of inter-family variability was much higher than those for intrafamily variability, indicating the better scope of selection (Vandana, 1991). The highest phenotypic, genotypic and environmental coefficient of variability was recorded by Vandana (1992a) for seed yield and number of pods, whereas it was lowest for days to flowering and test weight. High heritability values for seed yield and traits such as test weight, seeds per plant, seeds per pod and pods per plant have also been reported by Bakheit and Mahaday (1988) and Nanda *et al.* (1988).

Globally, mutation breeding techniques have hitherto resulted in the development of twenty mutant varieties in faba bean with improved yield and qualitative characteristics. The hhighest number of such mutant varieties were released from Germany (6) followed by Poland (5) and Russian Federation (4) as depicted in Figure 8.1. In India, a single high yielding and wilt resistant variety 'Tuwaitha' was developed through gamma irradiation in the year 1994. The description and the year of release of all such mutant varieties are given in Table 8.2. These mutant varieties, besides commercial cultivation in the farmer's field, could also be exploited as suitable source material in breeding, genetic and functional genomic research.

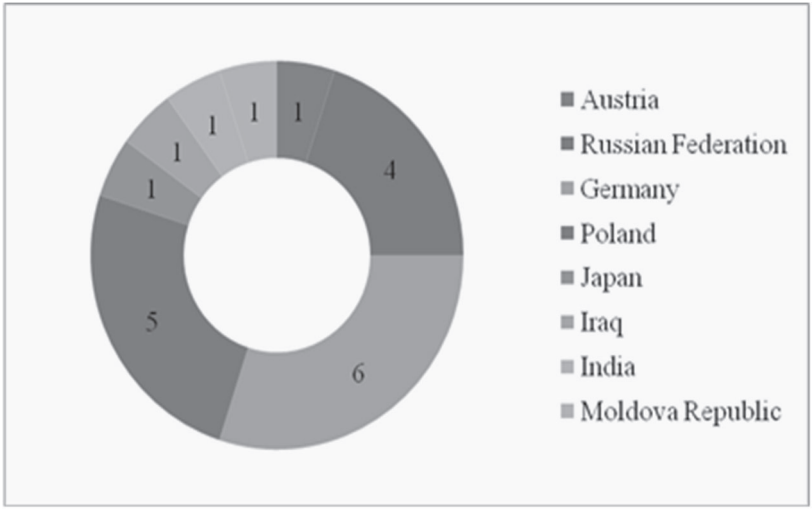

Figure 8.1 Country-wise mutant varieties of faba bean developed through induced mutagenesis (Source*:* Joint FAO/IAEA, Vienna Mutant Variety Database (MVD); http://mvgs.iaea.org).

8.6 Conclusion

Pulses are an important and economical source of vegetable proteins and occupy a pivotal place in the diet of people worldwide. India, despite having great agricultural potential, has long been facing a shortage of pulse production. Development of high yielding varieties is an important goal of mutation breeding programs. In recent years, induced mutations have been used as an important supplementary tool for developing several

improved varieties of pulse crops in different agro-ecological conditions which can significantly boost the agricultural production world over. In India, so far 84 improved varieties of pulses including one variety of faba bean have been developed. Besides a wealth of genetic variability has been generated for use in cross-breeding programs. However, the method is to be used selectively for crops where wider variability is desired or where conventional techniques pose problems. Further, there are certain characters more amenable to change; crops in which such characters are desired to be changed need to be chosen for improvement. In recent times, induced mutations have been gaining increasing importance in plant molecular biology as a tool to identify and isolate desirable genes of agronomic importance. These techniques, however deserve special attention in the days ahead to make cultivation of pulse crops a promising, remunerative and viable option for farmers across the world.

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Table 8.1. Share of pulse crops to total food grains in India (A- million ha, P- million metric tons, Y- kg/ha).

Year	Pulses			Food grains			Pulses % to Food grains		
	A	P	Y	A	P	Y	A	P	Y
1950-51	19.09	8.41	441	97.32	50.82	522	19.62	16.55	84.48
1960-61	23.56	12.70	539	115.58	82.02	710	20.38	15.48	75.92
1970-71	22.54	11.82	524	124.32	108.42	872	18.13	10.90	60.09
1980-81	22.46	10.63	473	126.67	129.59	1023	17.73	8.20	46.24
1990-91	24.66	14.26	578	127.84	176.39	1380	19.29	8.08	41.88
1995-96	22.28	12.31	552	121.01	180.42	1491	18.41	6.82	37.02
2000-01	20.35	11.08	544	121.05	196.81	1626	16.81	5.63	33.46
2001-02	22.01	13.37	607	122.78	212.85	1734	17.93	6.28	35.01
2002-03	20.50	11.13	543	113.86	174.77	1535	18.00	6.37	35.37
2003-04	23.46	14.91	635	123.45	213.19	1727	19.00	6.99	36.77
2004-05	22.76	13.13	577	120.00	198.36	1652	18.97	6.62	34.93
2005-06	23.39	13.39	598	121.60	208.60	1715	18.41	6.42	34.87
2006-07	23.76	14.11	594	124.07	211.78	1707	19.15	6.66	34.80
2007-08	23.63	14.76	625	124.07	230.78	1860	19.05	6.40	33.58
2008-09	22.09	14.57	660	122.83	234.47	1909	17.98	6.21	34.55
2009-10	23.28	14.66	630	121.33	218.11	1798	19.19	6.72	35.03
2010-11	26.40	18.24	691	126.67	244.49	1930	20.84	7.46	35.80
2011-12	24.46	17.09	699	124.76	259.32	2079	19.61	6.59	33.61
2012-13	23.25	18.34	789	120.77	257.12	2129	19.25	7.13	37.06
2013-14	25.21	19.25	764	125.04	265.04	2120	20.16	7.26	36.03
2014-15	23.10	17.16	743	122.07	252.67	2069	18.92	6.79	35.91

Source: Pulses in India: retrospect and prospects (2016), Government of India Ministry of Agriculture and Farmers Welfare, http://dpd.dacnet.nic.in.

Table 8.2. Details of Faba bean (*Vicia faba* L.) varieties developed through mutation breeding worldwide.

S. No.	Mutant Variety Name	Country	Year of registration	Developed by	Main improved attributes
1	Karna	Austria	1983	Gamma rays on seeds	High yield.
2	Chabanskii	Russian Federation	1985	NEU	Early maturity, grain yield 5 t/ha, green mass 47 t/ha, dry matter 10.3 t/ha.
3	Ti-Nova	Germany	1986	60 Gy X-rays	Improved resistance to lodging, earlier and uniform maturity, reduced plant height, suitable for combine harvest.
4	Prikarpatskie 4	Russian Federation	1986	NEU+NMU+DES +DMS+EI on seeds	Resistance to bacterial blight.
5	Dino	Poland	1987	70 Gy gamma rays on seeds	Short stem, early maturity (4 days), but smaller TGW.
6	Stego	Poland	1987	70 Gy gamma rays	Short stem, early maturity (4 days), but smaller TGW.
7	KYU-82	Russian Federation	1987	0.01% NMU on seeds	Early maturity, resistance to diseases, high yield.
8	Fritel	Germany	1988	X-rays	Improved plant architecture.
9	Tigo	Germany	1988	X-rays	Improved plant architecture.
10	Tina	Germany	1988	EMS	Improved plant architecture.

11	Bronto	Poland	1989	60 Gy gamma rays	High yield.
12	Tifabo	Germany	1990	X-rays	Improved plant architecture.
13	Tisesta	Germany	1991	EMS	Improved plant architecture.
14	Rin-rei	Japan	1991	gamma and X-rays recurrent	Dwarfness.
15	Severinovskie 1	Russian Federation	1992	0.01% NMU on seeds	High yield, high protein content.
16	Tinos	Poland	1992	Crossing with ti mutant	Terminal inflorescence plants, dwarfness, determinate growth, early maturity.
17	Martin	Poland	1994	Crossing with one mutant TJ3177/77×3177/77	Resistance to *Ascochyta* and *Fusarium*, high yield.
18	Babylon	Iraq	1994	30 Gy gamma rays	Resistance to diseases, high yield, high protein content.
19	Tuwaitha	India	1994	40 Gy gamma rays	Resistance to wilt, high yield and protein content.
20	Geca 5	Moldova, Republic	2008	250 Gy gamma rays on seeds	Good quality, early maturity.

Source: Joint FAO/IAEA, Vienna Mutant Variety Database (**MVD**); http://mvgs.iaea.org.

CHAPTER 9

INDUCED CHROMOSOMAL ABERRATIONS IN GRAIN LEGUMES: *LENS CULINARIS* MEDIK.

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Abstract: Mutation is an evolutionary force that occurs at random in nature and creates a necessary contrast to the wild-type condition. Although mutations occur spontaneously in every organism the induced mutagenesis technique offers a way to accelerate the rate of mutations in desirable directions for controlled selection. Induced mutagenesis has been a powerful technique to generate desirable mutations in the trait(s) of interest for genetic improvement of economically important crops. Optimization of the mutagen doses/concentrations employed is a key prerequisite to achieve the maximum frequency of mutation with minimum hazards, as mutagenic potency and genotypic sensitivity are directly correlated, along with other treatment conditions such as temperature, pH, etc. In this context, cytological study (mitotic and

meiotic aberrations) offers the most reliable index for determining the potency of mutagen(s) dose(s)/concentration(s) against the specific plant genotype(s) and to select the optimum mutagen(s) dose(s)/concentration(s) in any successful mutation breeding experiments. This chapter provides an overview of induced mutagenesis, cytological aberrations and mutation breeding with specific references to lentil genetic improvement for practical implementation.

Keywords: Induced mutagenesis; Grain legumes; Lentil; Chromosome aberrations; Mutagen potency; Genotype sensitivity.

9.1 Introduction

Grain legumes, also called "pulses," are an annual leguminous crop belonging to the family Fabaceae (Leguminosae) which is the third largest family of dicotyledons, with approximately 800 genera and 20,000 species (Bhat *et al.,* 2006a; Bhat *et al.,* 2005a; Lewis *et. al.,* 2005). Pulses and cereals are believed to have evolved simultaneously in a symbiotic way and both are considered as complementary components of agricultural systems worldwide. Pulses represent an important component of agricultural food crops widely consumed in developing countries, hence are a vital crop for achieving food and nutritional security for both poor producers and consumers. As a matter of fact, in dietary terms, food legumes complement cereal crops as a source of protein and minerals while agronomically they serve as a rotation crop with cereals, reducing soil pathogens and supplying nitrogen to the cereal crops. Pulses also serve as a feed crop in many farming systems and fetch higher prices compared to cereals and are increasingly grown to supplement farmers' incomes. The important and diverse roles played by pulses in the farming systems and diets of poor people makes them ideal crops for achieving global goals of reducing poverty and hunger, improving human health and nutrition, and enhancing ecosystem resilience.

Crop production primarily depends on the climatic conditions, soil health, water resources and, most importantly, agricultural land and human resource availability, which are dynamic factors and therefore, plant breeding for creating new genotypes with improved yield and adaptability, needs to evolve continuously to achieve the food demands of a rapidly increasing global population. Pulses, being self-pollinating crops, exhibit a very narrow genetic base which represents a limitation for breeding programs, as the availability of genetic variations within the crop gene pool is a key prerequisite to executing any improvement experiment.

Induced mutagenesis is an extremely powerful technique to create genetic variability that either does not exist in nature or is not available to the breeders. Mutation breeding techniques have been proved to be the best approach for broadening the genetic base and breaking the genetic bottleneck, particularly in grain legumes. Although the concept of mutation was first developed by Hugo de Vries (1901), its practical significance was subsequently demonstrated by Stadler (1928) in barley and Goodspeed (1929) in *Datura* and *Nicotiana.* Since the time required is relatively short to breed improved varieties and the whole genome is not altered, only a small portion of it, these discoveries initiated crop improvement programs through induced mutation. Later, induced mutation techniques became highly convenient tools for the plant breeders to generate the much needed genetic variation in different crop species, thereby obtaining access to the hidden genetic possibilities within the crop genome for various crop improvement programs around the world.

Khan *et al*. (2015) pointed out that prior knowledge of the mutagenic mode of action and an accurate estimation of the mutagenic potential of different doses of a mutagen to be employed toward the target crop genotype are crucial for the success of any mutation breeding experiment. *In vitro* germination tests of mutagen-treated seeds for gaining an initial idea of mutagenic lethality is a useful criterion to determine the employable range of mutagen doses/concentrations. However, the study of chromosomal aberrations has been proved to be one of the most dependable indexes for estimating the mutagenic potency of specific doses/concentrations of mutagens on the crop genotype. Chromosomal alterations induced by chemical or physical mutagens directly affect cell division (mitosis or meiosis) and can lead to the induction of a wide range of sterility and mutations at different stages of plant growth and development. Therefore, evaluation of optimal doses/concentrations of mutagen through chromosomal aberration studies is an integral part of induced mutagenesis experiments. The rate of induced mutation depends on genotype sensitivity and the degree of exposure. Mutation breeding initially requires a large mutagenized population with a broad range of mutations to implement a selection of desirable trait(s). Hence, the primary objective of induced mutagenesis is to select only the mutagen and their doses/concentrations that can generate a maximum rate of mutations with minimum seedling lethality.

9.2 Research Species: *Lens culinaris* Medik.

Grain legumes play an important and diverse role in the farming system, thus are an ideal crop for achieving the goal of global food security (Amin *et al*., 2015). Lentil (*Lens culinaris* Medik.) is an old-world grain legume food crop that was domesticated in the Near East arc along with other pulses such as pea, chickpea, and faba bean in early Neolithic times (Helbaek, 1959; Ladizinsky, 1979). Lentil cultivars and their wild relatives are self-fertilizing diploids having chromosome number 2n = 2x = 14. Cytogenetic analysis of interspecific hybrids has accentuated that *Lens orientalis* is the progenitor of the cultivated lentil *Lens culinaris* (Cubero *et al.,* 2009). Sindhu *et al*. (1983) presented the karyotype of lentil that includes three sub-metacentric (one of them with a secondary constriction), one metacentric and three acrocentric chromosome pairs.

9.3 Mutagenic potency and genotypic sensitivity

Mutagens used to create genetic variations are typically divided into physical mutagens (ionizing radiation, e.g., particulate - α-rays, β-rays, thermal neutrons; non-particulate - X-rays and ɣ rays and non-ionizing radiation, e.g., UV ray) and chemical mutagens (EMS, dES, NMG, MMS, EO, hydroxylamine, nitrous acid, 5-bromouracil, 2-aminopurine and ethidium bromide, among others). The correct selection of variety, mutagen and dose is the key to achieving success in mutation breeding. At least two varieties from the best varieties released recently should be selected for mutagenesis. Since the potency of a mutagen varies from variety to variety, it will allow comparative analysis and, since the recommended varieties have best-adapted characters, it will be useful to improve one or two specific deficient characters. The dose of a chemical mutagen mainly depends on (i) concentration (ii) duration of treatment and (iii) temperature during treatment (Bhat *et al.,* 2007b; Toker *et al*., 2007). Modifying factors are: (i) pre-soaking, (ii) pH of the solution, (iii) metallic ions, (iv) carrier agents, (v) subsequent washing of seeds (post-washing), (vi) post drying and (vii) storage of treated seeds.

The mutagenic potency and genotypic sensitivity are both interlinked and interdependent. Acquaah (2007) stated that the optimum dose rate of a mutagen can be determined with careful experimentation. The optimum dose of mutagen is considered to be the lethal dose-50 (LD50) i.e., the dose at which at least 50% of the treated plants survive up to maturity. Determining the optimum doses of mutagen for a particular genotype is

the primary criterion to start a mutation breeding program, because an over dose may result in a very small population for applying selection, whereas an under dose tends to induce very few mutations. The chromosomal aberrations induced by mutagens have been reported by many researchers in several plants such as in *Pisum sativum* (Kallo, 1972), *Lens culinaris* (Reddy and Annadurai, 1992), *Cicer arietinum* (Ganai *et al*., 2005), *Vigna* spp. (Goyal and Khan, 2009), and *Vicia* spp. (Khursheed *et al*., 2015; Khan *et al*., 2015 Bhat *et al.,* 2006d; Bhat *et al.,* 2005b; Bhat *et al.,* 2006b).

In the present study, Hydrazine hydrate (HZ) and gamma rays (γ rays) were employed alone and in combination treatments to assess the mutagenic potency in microsperma and macrosperma cultivars of lentil based on induced chromosomal aberrations.

9.4 Cytological observations

Chromosomal aberrations were studied at different stages of microsporogenesis. Lentil contains 7 bivalents (2n = 2x = 14) of small size with a relatively large genome of 4063 Mbp. No such abnormalities were observed in pollen mother cells (PMCs) of control plants which showed a formation of seven perfect bivalents (2n=14) at diakinesis and metaphase followed by normal separation (7:7) at anaphase I. However, meiotic study of the plants raised from treated seeds of both the cultivars DPL 62 and Pant L 406 of lentil showed broad-spectrum and frequency of different chromosomal aberrations such as stray bivalents, precocious separation, stickiness and clumping of chromosomes, fragments, bridge and fragments of broken bridge, laggard, micronuclei, cytoplasmic channel, and disturbed polarity at different stages of meiosis. Both the cultivars had similar spectra of meiotic aberrations with different frequencies. The data showing the frequency and spectrum of cytological aberrations in single and combination treatments of gamma rays and HZ and in controls have been presented in Table 9.1 and Figure 9.1. The metaphase stage of the meiotic cycle was found to be the stage most affected by the mutagenic treatments in both the cultivars. A dose-dependent increase in meiotic aberrations was observed with all mutagen treatments of gamma rays, HZ, and their combination in both the cultivars. The cytological study revealed that the microsperma cultivar Pant L 406 was more sensitive toward the mutagen treatments compared to the macrosperma cultivar DPL 62. Combination treatments of gamma rays and HZ gave the highest total frequency of meiotic abnormalities in both the cultivars, followed by gamma rays and

HZ. Stray bivalents at metaphase-I were more frequent in combination treatments than with gamma rays and HZ. Stickiness observed at metaphase-I was mostly compact chromatin mass type in all the mutagen treatments and their frequency was higher in higher treatments. Precocious separation of chromosomes to the poles was mostly observed in higher treatments of mutagens. Stickiness was the most prominent abnormality found at metaphase-I/II followed by stray bivalents. Bridges at anaphase I were mostly single type. However, multiple bridges were also observed, but at low frequency. At anaphase I/II, laggards were noted at high frequency followed by bridges in DPL 62, whereas, in Pant L 406 bridges occurred at higher frequencies than laggards. The cytoplasmic connections between two PMCs, termed cytomixis, was another abnormality observed in the present study, while no visible transfer of chromosome was detected. At telophase I/II, cytomixis was high, followed by micronuclei.

Similar results were also reported by many workers in different plants after treatments with physical and chemical mutagens, viz. Anis and Wani (1997*)* in *Trigonella foenum-graecum*; Dhamyanthi and Reddi (2000) and Gulfishan *et al*. (2013) in *Capsicum annuum*; Singh (2003) in *Vigna radiata*; Singh and Chaudhary (2005) in chili*;* Khursheed *et al*. (2015), Bhat 2007, Bhat *et al.,* 2007, Bhat *et al.,* 2007a; in *Vicia faba*; Kumar and Singh (2003) in *Hordium vulgare*; Kumar and Dubey (1998) in *Lathyrus sativus*; Alka *et al.* (2012) in *Linum usitatissimum* L.; and Aslam *et al*. (2012) in *Cichorium intybus* L.

According to Zeerak (1992), induced structural changes in chromosomes and gene mutations by mutagens prevent the homologous chromosomes pairing that results in the occurrence of univalents during cell division. Asynapsis (lack of chromosome pairing during the late prophase I) and/or desynapsis (inability to retain chiasmata in synapsed homologous chromosomes) which cause either no pairing or premature separation of bivalents respectively are considered to be the basis of occurrences of univalents at metaphase-I. Koduru and Rao (1981) opined that asynaptic or desynaptic genes in prophase I cause univalents. Gottschalk and Kleine (1976) reported that two groups of genes, namely *As* and *Ds,* control the chromosome pairing and chromosome pairing failure when genes are present in the recessive state. Therefore, it can be inferred that the genes responsible for homologous chromosomes pairing during prophase I might be mutated due to mutagen treatment that resulted in the occurrences of univalents in the present study. It was also believed that precocious chiasma terminalization could also be the cause of univalents (Bhat *et al.,* 2007; Bhat *et al.,* 2006; Bhat and Wani, 2015; Gottschalk and Kaul,

1980a; Sidhu, 2008). Hence, reduction in chiasma frequencies with increased heterology might be the reason for a high number of univalents with increasing concentration of mutagens (Gottschalk and Kaul, 1980b; Jabee and Ansari, 2005). The laggards at anaphase and telophase stages also arise from some of these univalents. In the subsequent generations, the mutagenic effect ceases, and the repair mechanism comes into action that reduces the occurrence of univalents, and thereby, the normal pairing of bivalents. Thus, gamma rays, MMS and DES might have induced genic disturbances due to mutagenic activity and hence the disturbances in homology and pairing of homologous chromosomes.

Multivalents such as trivalents, tetravalents, hexavalents, heptavalents and octavalents were observed at metaphase-I in the treated populations. The mutagen causes breakage in chromosomes and thereafter structural changes as translocations occur during the reunion of broken parts which give rise to the multivalent formation. Chughtai and Hasan (1979) observed multivalents in *Lens esculenta* with EMS, MES and MMS treatments and suggested the terminal affinities among broken chromosomes results in chromosomal translocation. Many researchers attributed the multivalent formation to irregular pairing and breakage followed by translocation and inversion (Zeerak, 1992; Vandana and Kumar, 1996; Kumar and Sinha, 1991; Anis and Wani, 1997; Kumar and Gupta, 2009; Gulfishan *et al.,* 2012; Gulfishan *et al.,* 2013; Ganai *et al.,* 2005; Bhat and Wani, 2017; Bhat and Wani, 2017a). According to Lea (1955), the random fusion of broken chromosomal ends generates unequal alteration in chromosome structure producing multivalents.

Stickiness or clumping of chromosomes in one or different groups was the most common meiotic aberration observed at metaphase-I/II. Stickiness could be attributed to the induced depolymerization of nucleic acid by mutagens (Kumar *et al*., 2003; Kumar and Tripathi, 2003; Jabee *et al*., 2008) or the changes in structural arrangement and partial dissociation of nucleoproteins (Katiyar, 1978; Myers *et al*., 1992; Kumar *et al*., 2003; Kumar and Rai, 2007c). Chromosomal breakage caused by mutagenic treatments and random orientation may produce stickiness among the chromosomes at metaphase (Jabee and Ansari, 2005). It may also be due to genetic and environmental factors (Gulfishan *et al.,* 2012; Gulfishan *et al.,* 2013; Ganai *et al.,* 2005; Bhat and Wani, 2017; Bhat and Wani, 2017a; Rao *et al*., 1990; Nirmala and Rao, 1996; Baptista-Giacomelli *et al*., 2000a). Hence, in the present case, the mutagen treatments were responsible for the observed stickiness by disturbing the structure of the chromosomes during their condensation from prophase I to metaphase II.

Precocious separation at metaphase-I/II was also one of the most common cytological aberrations. The precocious movement of chromosomes may be due to chromosome stickiness or early terminalization of chromosomes and/or uneven movement of chromosomes during anaphase (Premjit and Grover, 1985). Agarwal and Ansari (2001) pointed out that the disturbing homologous pairing of chromosomes and abnormal spindle activity might be the reason for the precocious movement of chromosomes. Kumar and Gupta (2009) reported abnormal spindle activity; other researchers also raised the issue of chromatids reunion during prophase as possible reasons (Rees and Thompson, 1955; Lewis and John, 1966; Newmann, 1966). Precocious separation of univalents causes desynapsis or asynapsis during meiosis (Gulfishan *et al.,* 2015; Sharma *et al.,* 2009; Parveen *et al.,* 2006; Gulfishan *et al.,* 2010; Bose and Saha, 1970; Kaul and Nirmala, 1993; Kumar and Rai, 2007a; Roy *et al*., 1971). Furthermore, dissimilarity in chromosomes of homologous pairs followed by abnormal spindle activity could be the reason for random chromosome movement, of which some were destined to be precocious.

The occurrence of chromosome lagging (laggards) as univalents or as whole bivalents at anaphase I/II are the result of abnormal spindle formation and chromosomal breakage. It may be due to delayed terminalization or stickiness of chromosome ends that fails chromosomal movement (Permjit and Grover, 1985; Jayabalan and Rao, 1987b; Soheir *et al*., 1989). Das and Roy (1989) opined that mutagen affects the spindle fiber's activity of directing chromosomes to the polar regions that results in the appearance of laggards at anaphase I. Pagliarini (1990) reported delayed chiasma terminalization as another possible reason for laggards formation. Kumar and Rai (2007a) also support the opinion that laggards might have appeared due to improper spindle functioning. Kumar and Gupta (2009) reported that laggards may also be formed from fragments of broken bridges cause by spindle pull toward the poles.

The development of chromatin bridges might be due to the failure of chiasmata in a bivalent to terminalize, which leads to stretching of chromosomes between the poles. Bhattacherjee (1953) attributed bridge formation to the interlocking of bivalent chromosomes, while Sinha and Godward (1972) attributed it to paracentric inversions. The dicentric chromosomes arise from chromosome breakage and then lateral reunion also forms chromosomal bridges pulled equally to both the poles by spindle fiber (Gulfishan *et al.,* 2011; Anis *et al.,* 1998). Bridges can also be attributed to the general stickiness of chromosomes at metaphase which further led to their inability to separate or to the breakage and reunion of

chromosomes (Ahmad, 1993; Anis and Wani ,1997; Kumar and Gupta, 2009).

Unequal separation of chromosomes toward poles at anaphase due to non-disjunction of homologous chromosomes at metaphase may be attributed to the stickiness of chromosomes that could lead to unequal distribution of chromosomes in the daughter nuclei (Anis and Wani, 1997). Mitra and Bhowmik (1996) related unequal separation of chromosomes to spindle irregularities. The unequal separation of chromosomes may also appear from the random movement of univalents to either pole (Kumar and Singh, 2003). Kumar and Rai (2007c) believe that unequal separation of chromosomes in meiosis I and II might be the outcome of non-oriented bivalent formation due to spindle dysfunction or due to the formation of univalents at diakinesis or metaphase. Disturbed polarity at telophase stages was recorded in a certain percentage of the PMCs which could be due to spindle disturbance.

Korniche (1901) was first to report cytomixis in pollen mother cells (PMCs) of *Crocus sativus,* followed by Gates (1911) in PMCs of *Oenothera gigas*. Cytomixis was defined as a process of transmigration of chromatin between adjoining cells. Since then, various factors have been proposed that cause cytomixis, such as the influence of genes (Kaul and Nirmala, 1993), abnormal formation of the cell wall during premeiotic divisions (Kamra, 1960), action of agents such as colchicines (Sinha, 1988), action of chemical mutagens (Bhat *et al*., 2006), rotenone (Amer and Mikhael, 1986), changes in the biochemical process that involve microsporogenesis modifying the micro-environment of affected anthers (Kaul, 1990), effect of gamma irradiation resulting in an unbalanced and sterile genetic system (Amma *et al*., 1990), the presence of a male-sterile mutant gene with altered frequency by environmental factors (Nirmala and Kaul, 1994) and environmental stress and pollution (Haroun *et al*., 2004). The cytological consequences of cytomixis are the production of aneuploid plants with certain morphological peculiarities (Sheidai *et al*., 1993) or production gametes with no reduction in chromosomal number as reported in *Aegilops* (Sheidai *et al*., 2002), which leads to polyploidy with significant evolutionary importance. The frequency and intensity of cytomictic flow depend on the nature of the connection between adjacent cells. There are two possibilities of such connections between PMCs, i.e., direct fusion and cytoplasmic channels. The connection through channels were generally single, however multiple cytoplasmic channels were also observed, though rarely. In some instances, donor cells became empty after passing the entire chromatin material to the recipient cells, while

partial migration of the chromatin material results in the formation of aneuploid cells.

Micronuclei are the product of chromosome fragments and laggards which fall short of reaching the poles (Kumar and Duby, 1998b). Laxmi *et al*. (1975) suggested that irregular distribution of acentric fragments or laggards results in the formation of micronuclei at telophase which affects the number and size of pollen grains formed from abnormal PMCs. Micronuclei at dyad or tetrad stage of PMCs in a mutagen-treated population might have resulted due to non-orientation of chromosomes and laggards since they were of frequent occurrence. Micronuclei cause loss of genetic material resulting in daughter cells deficient in one or more chromosome. This usually brings about pollen grains sterility.

9.5 Conclusion

Cytological analysis for the meiotic behaviors of M_1 plants revealed various types of meiotic abnormalities. The frequency of meiotic abnormalities increased with the increase in the dose of the mutagens. The microsperma cultivar Pant L 406 was found to be more sensitive toward the mutagen treatments compared to the macrosperma cultivar DPL 62. Combination treatments of gamma rays and HZ gave the highest total frequency of meiotic abnormalities in both the cultivars, followed by gamma rays and HZ.

Mutagen treatment induced a wide range of chromosomal aberrations at different stages of meiotic/mitotic cell division which, if the cell repair mechanism failed, could have significant genetic consequences. Although, most of the large chromosomal aberrations are detected easily by control mechanisms of cell division, smaller chromosomal aberrations mostly remain undetected and result in mutated daughter cells. Meiosis results in the production of haploid gametes for the next generation, therefore, any unrepaired aberrations could affect the inheritance of traits that are observable in the next generation as mutant phenotypes. Another major genetic consequence of meiotic aberration is the altered ploidy level in the daughter cells which could give rise to plants with observable morphological mutation due to imbalance in the expression of the gene(s). Since chromosomal aberration provides an actual picture of the interaction between mutagen and genotype, the study of chromosomal aberration through a pilot experiment must be performed before initiating a large-scale induced mutagenesis for mutation breeding programs. This helps to determine the type of mutagen and strength of that mutagen which is the

best fit for the particular crop genotype, which is also an economical and time-saving technique for the breeders.

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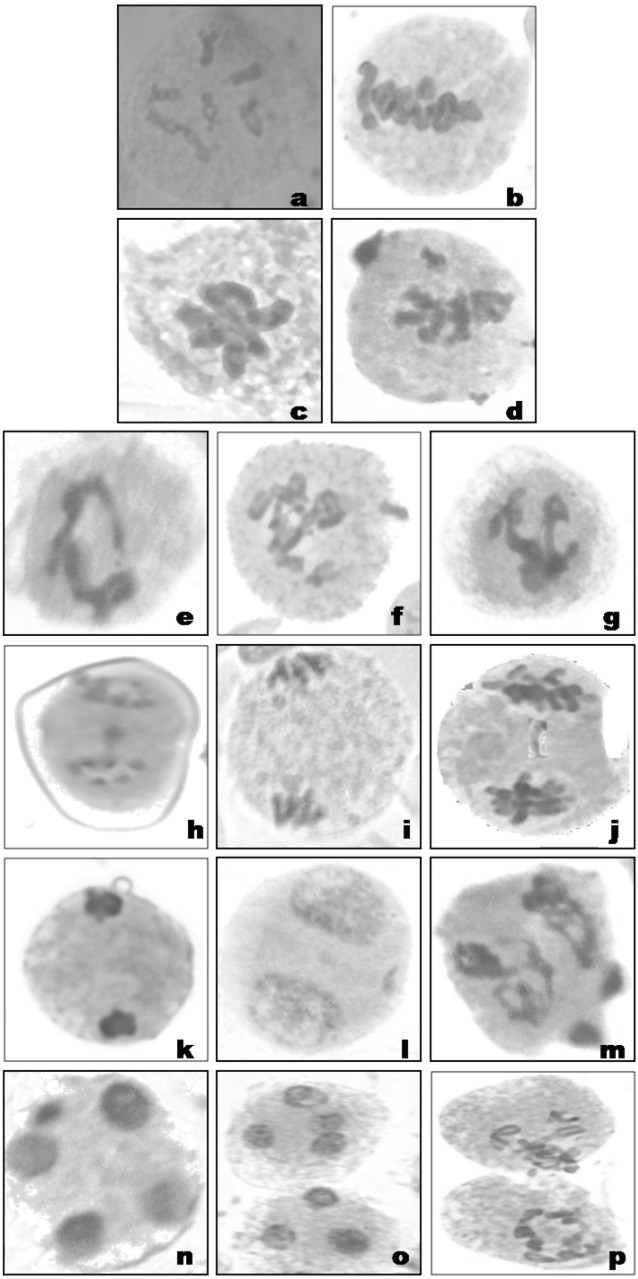
Table 9.1: Frequency of meiotic abnormalities in various mutagenic treatments in lentil.

Treatment	cv. DPL 62 (macrosperma)			cv. Pant L 406 (microsperma)		
	Total No. of PMCs observed	**Total No. of abnormal PMCs**	**Total frequency (%)**	**Total No. of PMCs observed**	**Total No. of abnormal PMCs**	**Total frequency (%)**
Control	**297**	-	-	**315**	**-**	**-**
0.1% HZ	312	9	2.88	355	18	5.07
0.2% HZ	283	20	7.07	282	24	8.51
0.3% HZ	299	25	8.36	324	32	9.88
0.4% HZ	289	29	10.03	299	33	11.04
TOTAL	**1183**	**83**	**7.02**	**1260**	**107**	**8.49**
100 Gy γ rays	307	17	5.54	325	21	6.46
200 Gy γ rays	309	21	6.80	331	27	8.16
300 Gy γ rays	294	28	9.52	279	30	10.75
400 Gy γ rays	298	31	10.40	311	35	11.25
TOTAL	**1208**	**97**	**8.03**	**1246**	**113**	**9.07**

100Gy γ rays+0.1% HZ	279	17	6.09	264	22	8.33
200Gy γ rays+0.2% HZ	300	26	8.67	303	34	11.22
300Gy γ rays+0.3% HZ	285	25	8.77	301	33	10.96
400Gy γ rays+0.4% HZ	266	33	12.41	243	31	12.76
TOTAL	**1130**	**101**	**8.94**	**1111**	**120**	**10.80**
GRAND TOTAL	**3521**	**281**	**7.98**	**3617**	**336**	**9.29**

Figure 9.1 (next page): PMCs showing meiotic abnormalities in the treated population of lentil. (a) 7 bivalents at Diakinesis-I (Control); (b) Chromosomes arranged in the equator at Metaphase- I (Control); (c) stickiness and clumping at Metaphase- I; (d) stray bivalent at Metaphase- I; (e) chromatin bridge at sticky Anaphase; (f) bridge and fragments of broken bridge at Anaphase; (g) chromatin bridges at Anaphase; (h) precocious separation of chromosome at early Anaphase; (i) chromosome on opposite pole at Anaphase- I (Control); (j) laggard at Anaphase; (k) Telophase- I (Control); (l) micronuclei at Telophase- I; (m) multiple chromatin bridges at Anaphase- II; (n) micronuclei at Telophase- II; (o) linear arrangement of three nuclei due to disturbed polarity at Telophase- II; (p) cytoplasmic channel and chromosome movement.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CHAPTER 10

EXPLORATION, APPLICABILITY AND CONCLUSIONS OF INDUCED MUTAGENESIS ON BROAD BEAN

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Abstract: The present investigation was carried out to study the mutagenic effects of ethyl methanesulfonate (EMS), gamma rays, methyl methanesulfonate (MMS) and their combination treatments in two varieties of broad bean *(Vicia faba* L.) var. minor and var. major. The seeds of the two varieties of *Vicia faba* L. were exposed to different concentrations and treatments of EMS, MMS, gamma rays and their combinations to study their effects on biological and cytological parameters. Biological, cytological and agronomic parameters were studied. The effect was studied up to three generations named as M_1, M_2, M_3, negative mutations were discarded, and positive mutations were encouraged, harvested and mutant lines with a positive impact on agronomic characters were established having better yield content.

10.1 Objectives

The main objective of the study was to induce the additional genetic variability for quantitative traits and increase the yield potential of the crop by isolating promising mutants. Various other aspects of the study were:

10.1.1 Biological damage in M_1 and M_2 generations.

10.1.2 Effectiveness and efficiency of the mutagens.

10.1.3 Spectrum and frequency of chlorophyll and viable macromutations.

10.1.4 Estimation of genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), heritability (broad sense) and genetic advance (per cent of the mean).

10.1.5 Cytological investigation of M_1, M_2 and M_3 generations.

10.1.6 Cytological investigation of the isolated mutants.

10.1.7 Calculation of chiasmata frequency in M_1, M_2 and M_3 generations.

10.1.8 Isolation of desired mutant lines.

10.1.9 Evaluation of protein content in the isolated mutant lines.

10.1.10 Determination of the variation in seed protein patterns in controls and the isolated mutants of *Vicia faba* L. through SDS-PAGE gel scanning.

10.1.11 The data obtained in M_1, M_2 and M_3 populations were statistically analyzed to ascertain the extent of variation and improvement in genetic characters in treated plants in comparison to control.

The significant findings are summarized below:

10.2 Sensitivity studies

The mutagenic effect was studied on some M_1 parameters such as seed germination, seedling height, plant survival, pollen fertility and various quantitative characters such as days to flowering, number of flowers per plant, days to maturity, plant height (cm), number of fertile branches per plant, number of pods per plant, number of seeds per pod, mean pod length (cm), mean pod girth (cm), 100-seed weight, total yield per plant and

number of leaves per plant. Besides, the cytological investigation was carried out to assess the comparative effect of physical and chemical mutagens, separately as well as in combination. The extent and nature of injury in the M_1 generation varied with the plant varieties, mutagens and their doses/concentrations.

a) Seed germination, seedling growth, plant survival and pollen fertility decreased with an increase in mutagenic treatment as studied by Bhat (2006a).

b) Chromosomal aberrations showed a dose-dependent increase with mutagenic treatments as reported by Bhat, *et al.,* (2005b, 2006b, 2007a, 2007b) in *Vicia faba* L.

c) The chromosomal aberrations induced by EMS, gamma rays, MMS and their combination treatments were univalents, multivalents, stickiness, precocious separation of bivalents, disturbed metaphase and fragments at metaphase-I/II. The induced chromosomal aberrations at anaphase-I/II included bridges, with or without fragments, laggards, disturbed anaphase, unequal separation and non-disjunction. The induced chromosomal aberrations at telophase-1/II included disturbed polarity, micronuclei, multinucleate condition and cytomixis. The end products of meiosis were triads, pentads and hexads, along with tetrads as reported by Bhat, *et al.,* (2005a, 2005b, 2006a, 2006d, 2017, 2017b) in *Vicia faba* L. and Gulfishan *et al.,* (2010, 2011, 2012, 2013) in *Capsicum*.

d) In general, the frequency of chromosomal aberrations was highest at metaphase stages, followed by anaphase and telophase stages. Chromosomal aberrations were the same in M_1, M_2 and M_3 generations, but their frequencies were lesser in M_2 than M_1 and in M_3 lesser than M_2 as reported by Bhat, (2007) in *Vicia faba* L.

e) In general, the variety minor was more sensitive as compared to the var. major as reported by Bhat, *et al.,* (2006, 2007) in *Vicia faba* L.

f) Studies on various quantitative parameters revealed the general effectiveness of higher treatments and the stimulatory effect of lower and intermediate treatments in the M_1 generation as reported by Ganai *et al.*, (2005) in chick pea.

g) The coefficient of variation was high among the treated populations as compared to controls in the M_1 generation.

h) Chromosomal aberrations and pollen sterility were dose-dependent and increased with dose/concentration of mutagens.

i) The chiasma frequency showed more reduction in combination treatments than the individual mutagenic treatments. The order of potentiality of mutagens to cause a reduction in chiasma frequency was gamma rays+MMS>gamma rays+EMS>MMS>gamma rays>EMS in both the varieties.

10.3 Qualitative mutations

10.3.1 A wide spectrum of chlorophyll and viable morphological mutants were isolated in the M_2 generation. Lower and intermediate doses/concentrations of EMS, gamma rays, MMS and their combination mutagenic treatments induced the maximum frequency of mutations as compared to higher doses/concentrations treatments.

10.3.2 Frequency of chlorina mutants was highest, followed by xantha and albina in both the varieties of the broad bean as reported by Bhat *et al*,.(2005, 2007a) in *Vicia faba* L. The closed flower mutants with chlorophyll mutants were also reported by Bhat *et al.*, (2005, 2007a) in *Vicia faba* L.

10.3.3 The var. minor showed more chlorophyll mutations than var. major.

10.3.4 The most promising and striking mutants were tall, dwarf, and semi-dwarf, mutants with broad leaves, big flowers, number of flowers, elongated pods, broader pods, giant seeds, bold seeds, black bold seeds, brown small seeds and small seeds with normal gray color. The morphological mutants with agronomically desirable features could be utilized for further improvement of this crop.

10.3.5 No dose-dependent increase was observed for chlorophyll and viable mutation frequency.

10.3.6 The mutagenic effectiveness measured based on the frequency of chlorophyll mutations divided by a dose of the mutagen revealed MMS to be most effective, followed by gamma rays in combination with MMS and EMS in causing mutations. Gamma rays in combination with EMS were least effective.

10.3.7 The mutagenic efficiency was calculated based on inhibition in seed germination (MF/R), seedling injury (MF/I), lethality (MF/L), sterility

(MF/S) and meiotic abnormalities (MF/M). Lethality (MF/L) was generally high followed by meiotic abnormalities (MF/M) and sterility (MF/S).

10.3.8 Intermediate and lower doses of EMS, gamma rays and MMS treatments were most efficient based on all criteria used.

10.3.9 Some fascinating mutants such as closed flower mutants and desynaptic mutants were isolated. Their detailed cytomorphological investigations were carried out and the causes and consequences of mutations were discussed.

10.3.10 The interaction coefficient (K) of combination treatments for various biological parameters in M_1 and chlorophyll mutation frequency in M_2 revealed less than additive effects. However, synergistic or additive effects were also obtained in some combinations.

10.3.11 The maximum frequency of morphological mutants exhibiting altered morphological features were isolated in combination treatments of gamma rays and EMS in both the varieties.

10.4 INDUCED POLYGENIC VARIATIONS

10.4.1 The mean values of different quantitative traits showed positive and negative shifts in the M_2 generation, however, the positive shift in the mean was more pronounced in the M_3 generation, especially for yield and yield contributing traits.

10.4.2 The mean flowering time was reduced by 6-8 days (EMS), 4-7 days (gamma rays),4-5 days (MMS), 4 days (gamma rays+EMS) and 3 days (gamma rays+MMS) in var. minor, while the flowering time was reduced by 8-9 days (EMS and gamma rays), 7-8 days (MMS), 6-7 days (gammarays+EMS) and 2-4 days (gammarays+MMS) in var. major in the M_3 generation.

10.4.3 The mean duration of maturity time was reduced by 7-10 days (EMS), 6 days (gamma rays), 5-6 days (MMS), 4-5 days (gamma rays+EMS) and 2-3 days (gamma rays+MMS) in var. minor, whereas maturity time was reduced by 8-10 days (EMS), 7 days (gamma rays), 5-6 days (MMS), 4-5 days (gamma rays+EMS) and 3-5 days (gamma rays+MMS) in M_3 generation of var. major.

10.4.4 The mean number of flowers per plant increased by 13-14 flowers (EMS), 10-12 flowers (gamma rays), 8-10 flowers (MMS), 5-6 flowers (gamma rays+EMS) and 2-5 flowers (gamma rays+MMS) in var. minor, whereas the mean number of flowers per plant increased by 11-13 flowers (EMS), 9-11 flowers (gamma rays), 8 flowers (MMS), 6-7 flowers (gamma rays+EMS) and 4-5 flowers (gamma rays+MMS) in var. major in the M_3 generation.

10.4.5 The average plant height increased by 5-6 cm (EMS), 4-5 cm (gamma rays), 4 cm (MMS), 2-3 cm (gamma rays+EMS) and 1-3 cm (gamma rays+MMS) in var. minor, whereas mean plant height increased by 4-5 cm (EMS), 3 cm (gamma rays and MMS), 1-2 cm (gamma rays+EMS) and 1-2 cm (gamma rays+MMS) in var. major in M_3 generation.

10.4.6 The mean number of fertile branches per plant increased by 4-5 (EMS), 3-4 (gamma rays and MMS), 2-3 (gamma rays+EMS) and 1-2 (gamma rays+MMS) in var. major in the M_3 generation.

10.4.7 The mean number of pods per plant showed significant increase in var. minor, the mean values of pods per plant showed 14-16 pods (EMS), 12-13 pods (gamma rays and MMS), 11-12 pods (gamma rays+EMS) and 3-11 pods (y-rays+MMS), while in var. major it was 10-12 (EMS), 7-9 (gamma rays), 5-7 (MMS), 4-5 (gamma rays+EMS) and 1-4 (gamma rays+MMS) in var. minor in the M_3 generation.

10.4.8 The mean number of seeds per pod also showed a significant increase in the M_3 generation, particularly 0.1, 0.2 and 0.3% of EMS and 10kR, 20kR and 30kR doses of gamma rays in var. minor and 0.1% and 0.2% of EMS in var. major.

10.4.9 A significant increase in mean values of pod length was obtained at 0.1% and 0.2% of EMS in var. minor, whereas a significant increase in mean values of pod length was achieved in 0.1% and 0.3% EMS in var. major in the M_3 generation.

10.4.10 The pod girth also showed a considerable increase. The maximum increase in pod girth was recorded in var. major in M_3 generations (0.1% EMS).

10.4.11 The mean values for 100-seed weight showed considerable increase *i.e.,* 5-6 g (EMS), 3-4 g (gamma rays and MMS) and 2-3 g (gamma rays+EMS and gamma rays+MMS) in var. minor, whereas in var.

major 100-seed weight (g) increased by 4-5 g (EMS), 2-3 g (gamma rays+MMS) and 1-4 g (gamma rays+EMS and gamma rays+MMS) in the M_3 generation.

10.4.12 The total yield per plant also showed considerable increase *i.e.,* 3-4 g (EMS and gamma rays), 2-3 g (MMS), 2-3 g (gamma rays+EMS and gamma rays+MMS) in var. minor, whereas in var. major the increase was 2-4 g (EMS, gamma rays and MMS) and 2-3 g (gamma rays+EMS and gamma rays+MMS) in the M_3 generation) The mean number of leaves per plant also showed considerable increase *i.e.,* 4-7 leaves in var. major and 3-7 leaves in var. minor in the M_3 generation.

10.4.13 The increase in mean values was accompanied by an increase in genetic parameters.

10.4.14 The increase in the number of pods per plant, pod length, pod girth, seeds per pod and 100-seed weight played a significant role in boosting the total yield per plant in treated populations.

10.4.15 The estimates of genotypic coefficient of variation (GCV), heritability (broad sense) and genetic advance (GA) was high in treated populations as compared to controls in the M_2 generation. All these genetic parameters increased further in M_3 for yield and yield contributing traits. This indicated that these characters could be transmitted to future generations and will be of much importance for the improvement of *Vicia faba* L. varieties.

10.4.16 The protein content increased significantly in the isolated mutant lines, whereas a significant decrease was also noticed in others.

10.5 Performance of isolated mutants

10.5.1 Isolated strains of var. minor.

10.5.1.1 Minor-A: This was isolated from 0.1% of EMS treated populations and was round bold seeded. Its shift in mean was 12.33 in several fertile branches per plant, 27.70 in several pods per plant, 1.47 seeds per pod, 2.26 cm pod length, 2.80 cm pod girth, 100-seed weight 9.37 g and average yield per plant was 13.07 g. This mutant was high yielding.

10.5.1.2 Minor-B: This was isolated from 0.2% of EMS treated populations and was round bold seeded. The seed coat bore peculiar

sculpturing, the shift in mean was 12.00 in many fertile branches per plant, 27.04 in pods per plant, 1.23 in seeds per pod, 2.33 cm in pod length, 2.70 cm in pod girth, 14.53 g in 100-seed weight and 13.07 g in yield per plant. It was a high yielding mutant.

10.5.1.3 Minor-C: This was isolated at 10kR gamma rays and had round bold seeds. The peculiar design was found on the seed coat. Its shift in mean was 11.33 in fertile branches per plant, 27.87 in pods per plant, 1.27 in seeds per pod, 2.63 cm in pod length, 2.53 cm in pod girth, 15.53 in 100-seed weight and 12.23 in yield per plant. This strain was high yielding.

10.5.1.4 Minor-D: This was isolated at 20kR gamma rays. It was round bold black seeded. Its shift in mean was 10.00 in fertile branches per plant, 26.87 in pods per plant, 1.33 in seeds per pod, 2.43 cm in pod length, 2.40 cm in pod girth, 15.36 in 100-seed weight and 11.90 in yield per plant. This strain was high yielding.

10.5.1.5 Strain-E: This was isolated in 0.03% MMS. The seed coat bore peculiar sculptures. Its shift in mean was 10.66 in fertile branches per plant, 26.37 in pods per plant, 1.73 in seeds per pod, 1.70 cm in pod length, 2.30 cm in pod girth, 18.36 g in 100-seed weight and 11.07 g in yield per plant. It was high yielding.

10.5.1.6 Minor-F: This was isolated at 0.01% MMS and the seeds were green with peculiar sculptures on the seed coat. The striking feature of this strain was seven flowers on a common peduncle. The shift in mean was 10.50 in fertile branches per plant, 25.70 in pods per plant, 1.73 in seeds per pod, 1.23 cm in pod length, 1.83 cm in pod girth, 17.70 g in 100-seed weight and 11.23 g in total yield per plant. It was high yielding.

10.5.1.7 Minor-G: This was isolated at 30kR gamma rays. It was a tall mutant with brown seeds. Its shift in mean was 10.33 in fertile branches per plant, 24.70 in pods per plant, 1.50 in seeds per pod, 1.l0 cm in pod length, 1.60 cm in pod girth, 16.70 g in 100-seed weight, 11.70 g in yield per plant. It was high yielding.

10.5.1.8 Minor-H: This was isolated at 0.02% MMS. It was semi-dwarf and bore peculiar sculptures on the seed coat. Its shift in mean was 10.16 in fertile branches per plant, 24.04 in pods per plant, 1.47 in seeds per pod, 1.70 cm in pod length, 1.80 cm in pod girth, 16.20 g in 100-seed weight and 9.90 g in yield per plant. It was high yielding.

10.5.1.9 Minor-I: This was isolated in 0.3% EMS. It was round bold seeded. Its shift in mean was 10.00 in fertile branches per plant, 23.04 in pods per plant, 1.63 in seeds per pod, 1.80 cm in pod length, 2.00 cm in pod girth, 15.20 g in 100-seed weight and 9.23 g in yield per plant. It was high yielding.

10.5.1.10 Minor-J: This was isolated at 10kR+0.1 % EMS. Seeds were bold green. Its peculiar feature was elongated pods. Its shift in mean was 9.16 in fertile branches per plant, 22.54 in pods per plant, 1.90 in seeds per pod, 1.90 cm in pod length, 1.93 cm in pod girth, 16.53 g in 100-seed weight and 9.06 g in total yield per plant. It was high yielding.

10.5.1.11 Minor-K: This was isolated at 20kR+0.3% EMS. Its shift in mean was 8.86 fertile branches per plant, 29.80 in pods per plant, 1.67 in seeds per pod, 1.83 cm in pod length, 1.77 cm in pod girth, 6.49 g in 100-seed weight and 9.05 g in yield per plant. It was high yielding.

10.5.1.12 Minor-L: This was isolated at l0kR+0.02% MMS. It was a tall mutant with bold seeds. Its shift in mean was 8.86 in fertile branches per plant, 32.90 in pods per plant, 1.87 in seeds per pod, 1.83 cm in pod length, 1.67 cm in pod girth, 6.07 g in 100-seed weight and 9.67 g in yield per plant. It was high yielding.

10.5.1.13 Minor-M: This was isolated at 10kR+0.01% MMS. It was round-flat bold seeded with a sculptured seed coat. Its shift in mean was 10.56 in fertile branches per plant, 32.50 in pods per plant, 2.27 in seeds per pod, 1.26 cm in pod length, 2.07 cm in pod girth, 7.23 g in 100-seed weight and 10.63 g in yield per plant. It was high yielding.

10.5.1.14 Minor-N: This was isolated at 20kR+0.2% EMS and had six flowers on a common inflorescence. Its shift in mean was 10.49 in fertile branches per plant, 31.90 in pods per plant, 2.56 in seeds per pod, 1.60 cm in pod length, 2.13 cm in pod girth, 7.46 g in 100-seed weight and 9.60 g in yield per plant. It was high yielding.

10.5.1.15 Minor-O: This was isolated at 0.3% EMS. Its shift in mean was 10.59 in fertile branches per plant, 29.77 in pods per plant, 1.88 in seeds per pod, 1.60 cm in pod length, 1.50 cm in pod girth, 6.43 g in 100-seed weight and 9.62 g in yield per plant. It was high yielding.

10.5.2 Isolated mutants of var. major

10.5.2.1 Major-A: This was isolated in 0.1% EMS and was giant bold seeded. Its shift in mean was 5.97 in fertile branches per plant, 23.40 in pods per plant, 1.60 in seeds per plant, 2.31 cm in pod length, 2.23 cm in pod girth, 2.03 g in 100-seed weight and 12.17 g in yield per plant. It was a high yielding mutant.

10.5.2.2 Major-B: This was isolated in 0.3% EMS and was round giant bold seeded. Its shift in mean was 6.97 in fertile branches per plant, 21.40 in pods per plant, 1.50 in seeds per pod, 2.47 cm in pod length, 2.l3 cm in pod girth, l3.36 g in 100-seed weight and 13.84 g in yield per plant. It was high yielding.

10.5.2.3 Major-C: This was isolated at 10kR gamma rays. It was round seeded. The seed coat showed peculiar markings. Its maximum shift in mean was 7.97 in fertile branches per plant, 19.04 in pods per plant, 1.50 in seeds per pod, 2.14 cm in pod length, 2.10 cm in pod girth, 12.70 g in 100-seed weight and 12.84 g in yield per plant. This strain was high yielding.

10.5.2.4 Major-D: This was isolated at 30kR gamma rays. It was round bold seeded with sculptures on the seed coat. Its shift in mean was 7.80 in fertile branches per plant, 17.90 in pods per plant, 1.70 in seeds per pod, 2.44 cm in pod length, 1.37 cm in pod girth, 11.53 g in 100-seed weight and 12.34 g in yield per plant. This strain was high yielding.

10.5.2.5 Major-E: This was isolated in 20kR gamma rays. The seed coat was sculptured. It shift in mean was 11.84 fertile branches per plant, 22.40 in pods per plant, 1.77 in seeds per pod, 2.44 cm in pod length, 2.10 cm in pod girth, 11.20 g in 100-seed weight and 11.84 g in yield per plant. It was high yielding.

10.5.2.6 Major-F: This was isolated at 0.01% MMS. The seed coat was sculptured. The shift in mean was 10.64 in fertile branches per plant, 20.57 in pods per plant, 1.60 in seeds per pod, 1.31cm in pod length, 1.77 cm in pod girth, 10.36 g in100-seed weight and 10.84 g in total yield per plant. It was high yielding.

10.5.2.7 Major-G: This was isolated at 0.02% MMS. It was round bold seeded. Its shift in mean was 11.97 in fertile branches per plant, 19.90 in pods per plant, 1.70 in seeds per pod, 1.80 cm in pod length, 1.77 cm in pod girth, 10.60 g in 100-seed weight, 11.84 g in yield per plant. It was high yielding.

10.5.2.8 Major-H: This was isolated at 10kR+0.1% EMS. It was round bold seeded with sculptures in the seed coat. Its shift in mean was 10.80 in fertile branches per plant, 18.90 in pods per plant, 1.57 in seeds per pod, 1.31 cm in pod length, 1.97 cm in pod girth, 12.63 g in 100-seed weight and 14.84 g in yield per plant. It was high yielding.

10.5.2.9 Major-I: This was isolated in 10kR+0.2% EMS. It was round bold seeded with peculiar structures. Its shift in mean was 9.97 in fertile branches per plant, 17.40 in pods per plant, 1.73 in seeds per pod, 1.54 cm in pod length, 1.83 cm in pod girth, 10.53 g in 100-seed weight and 13.84 g in yield per plant. It was high yielding.

10.5.2.10 Major-J: This was isolated at 20kR+0.02% EMS. It was round bold seeded with peculiar sculptures in the seed coat. Its shift in mean was 9.97 in fertile branches per plant, 15.57 in pods per plant, 1.84 in seeds per pod, 1.47 cm in pod length, 1.73 cm in pod girth, 10.86 g in 100-seed weight and 12.84 g in total yield per plant. It was high yielding.

10.5.2.11 Major-K: This was isolated at 20kR+0.03% MMS. It was round bold seeded with sculptures on the seed coat. Its shift in mean was 10.77 in fertile branches per plant, 17.87 in pods per plant, 1.60 in seeds per pod, 1.58 cm in pod length, 1.90 cm in pod girth, 11.63 g in 100-seed weight and 10.83 g in yield per plant. It was high yielding.

10.5.2.12 Major-L: This was isolated at 10kR+0.01% MMS. It was mutant with bold seeds. Its shift in mean was 10.97 in fertile branches per plant, 19.73 in pods per plant, 1.50 in seeds per pod, 1.69 cm in pod length, 1.80 cm in pod girth, l1.13 g in 100-seed weight and 11.61 g in yield per plant. It was high yielding.

10.5.2.13 Major-M: This was isolated at 20kR+0.3% EMS. It was tall bold seeded with sculptures on the seed coat. Its shift in mean was 7.80 in fertile branches per plant, 20.57 in pods per plant, 1.64 in seeds per pod, 1.36 cm in pod length, 1.70 cm in pod girth, 10.83 g in 100-seed weight and 11.39 g in yield per plant. It was high yielding.

10.5.2.14 Major-N: This was isolated in 0.2% EMS. It was round bold seeded with sculptures on the seed coat. The shift in mean was 19.04 in pods per plant, 1.60 in seeds per pod, 2.14 cm in pod length, 1.88 cm in pod girth, 10.23 g in 100-seed weight and 10.5 g in yield per plant. It was high yielding.

10.5.2.15 Major-O: This was isolated in 0.03% MMS. It was round bold seeded. Its shift in mean was 10.23 in fertile branches per plant, 19.29 in pods per plant, 1.60 in seeds per pod, 1.80 cm in pod length, 1.84 cm in pod girth, 10.36 g in 100-seed weight and 11.50 g in yield per plant. It was high yielding.

10.6 Conclusions In general, lower and intermediate treatments of EMS, gamma rays, MMS and their combination treatments induced greater variability and proved to be more effective and efficient than the higher treatments. The present findings lead to the following conclusions:

10.6.1 The two varieties of broad bean viz., var. minor and var. major required low and intermediate treatments of EMS (below 0.3%), MMS (below 0.03%) and gamma rays (below 30kR) and their combination to induce maximum variability and more viable mutants.

10.6.2 Appropriate doses of gamma rays (10kR and 20kR) were used in combination with EMS (0.1%-0.4%) and MMS (0.01%-0.04%) to induce maximum mutation frequency.

10.6.3 The increase in mean values coupled with an increase in genetic variability from the M_2 to M_3 generation, especially for yield contributing traits, suggested further possibilities of selecting more promising lines with high yield and high heritability in both of the varieties of broad bean.

10.6.4 The number of fertile branches per plant, pods per plant, seeds per pod, mean pod length, mean pod girth, and 100-seed weight were positively and strongly correlated with yield in the broad bean, whereas, seed size showed a low positive correlation with protein content.

10.6.5 The efficiency of micro mutations could be increased by selecting normal-looking plants of M_1 segregating families as well as non-segregating families with high variance and desired shift in mean, whereas the efficiency of macromutations could be increased by selecting M_1 plants with maximum damage as well as normal-looking plants of segregating M_1 families.

10.6.6 The meiotic aberrations indicate the mutational load. The frequency of meiotic aberrations decreased from M_1 to M_2 and M_2 to M_3 and further decreased in isolated strains which represent the occurrence of stability in mutated genotype from the M_1 to M_3 generation and the isolated mutants of both the varieties of *Vicia faba* L.

Therefore, based on the present findings, it is suggested and concluded that the mutants isolated in *Vicia faba* L. will be very important in its improvement. The overall performance of the isolated mutants in general, and alterations for yield contributing characters in particular, is worth maintaining. The mutant lines are under evaluation for the stability of yield and yield contributing parameters and after their test and positive performance they may be recommended for multiplication and further use.

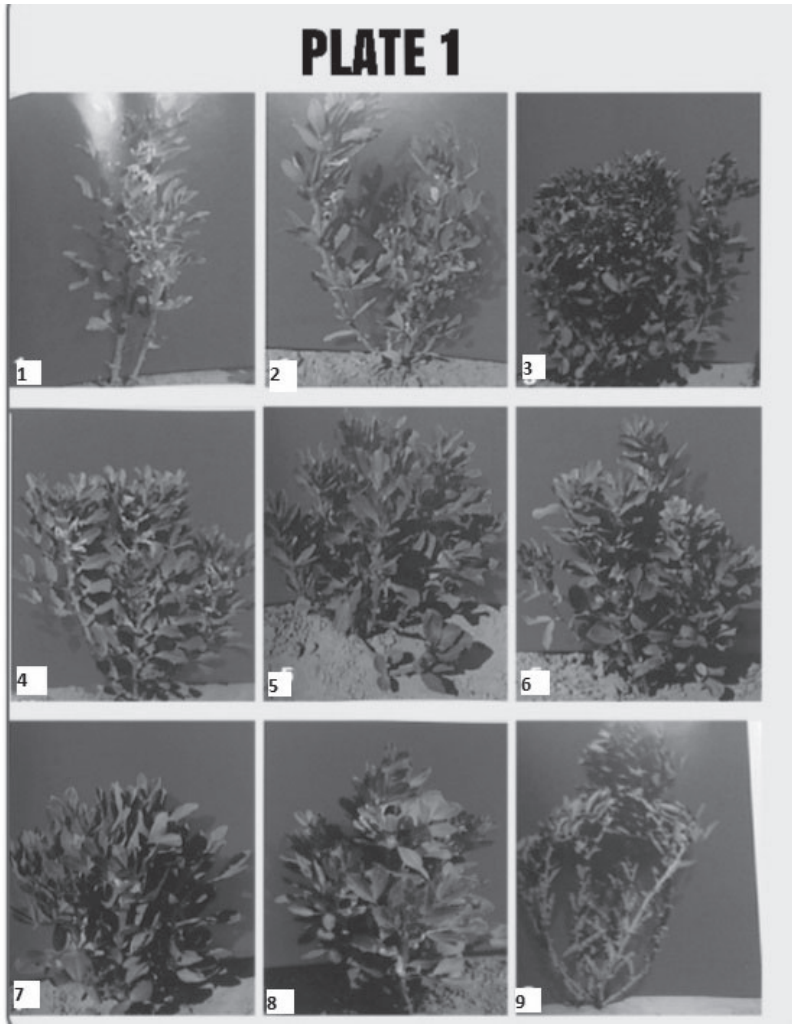
PLATE 1
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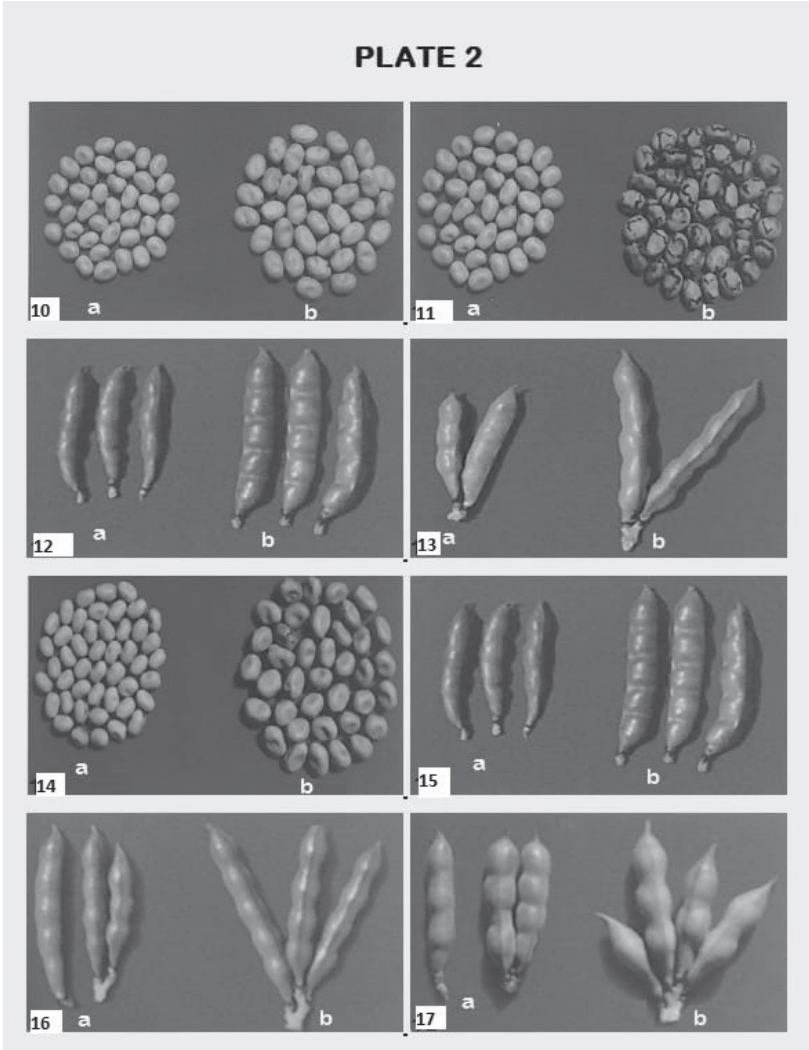
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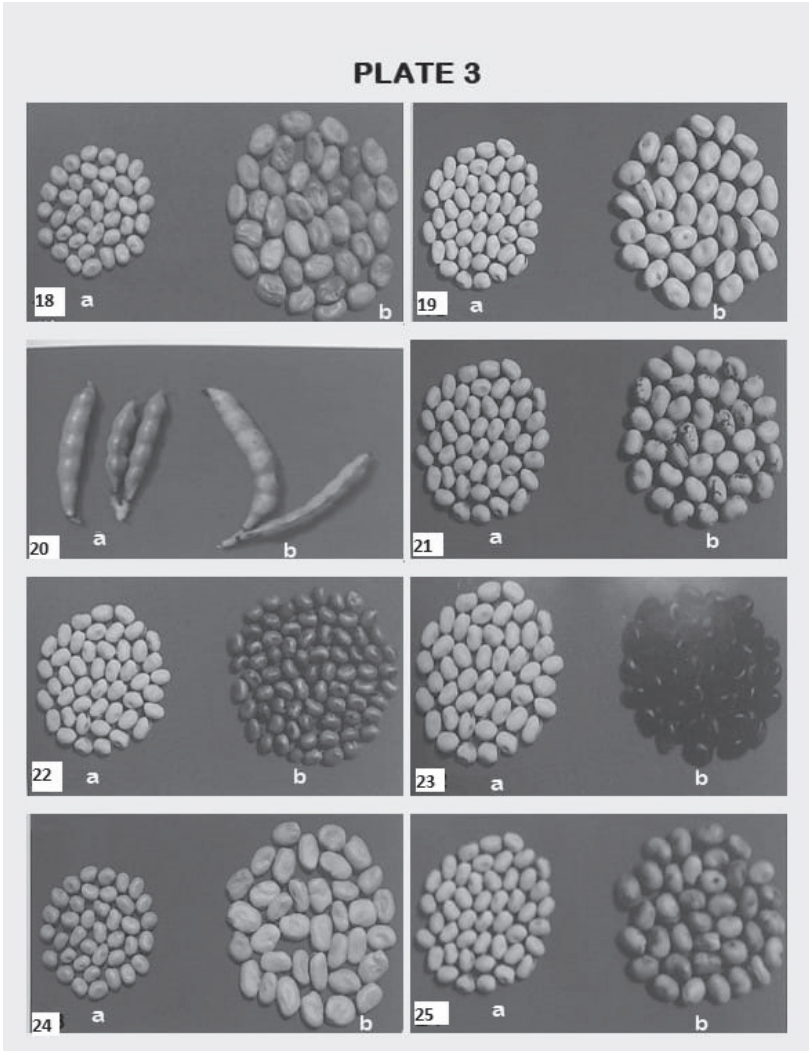
11 a b
12 a b
13 a b
14 a b
15 a b
16 a b
17 a b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19 a b
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21 a b
22 a b
23 a b
24 a b
25 a b

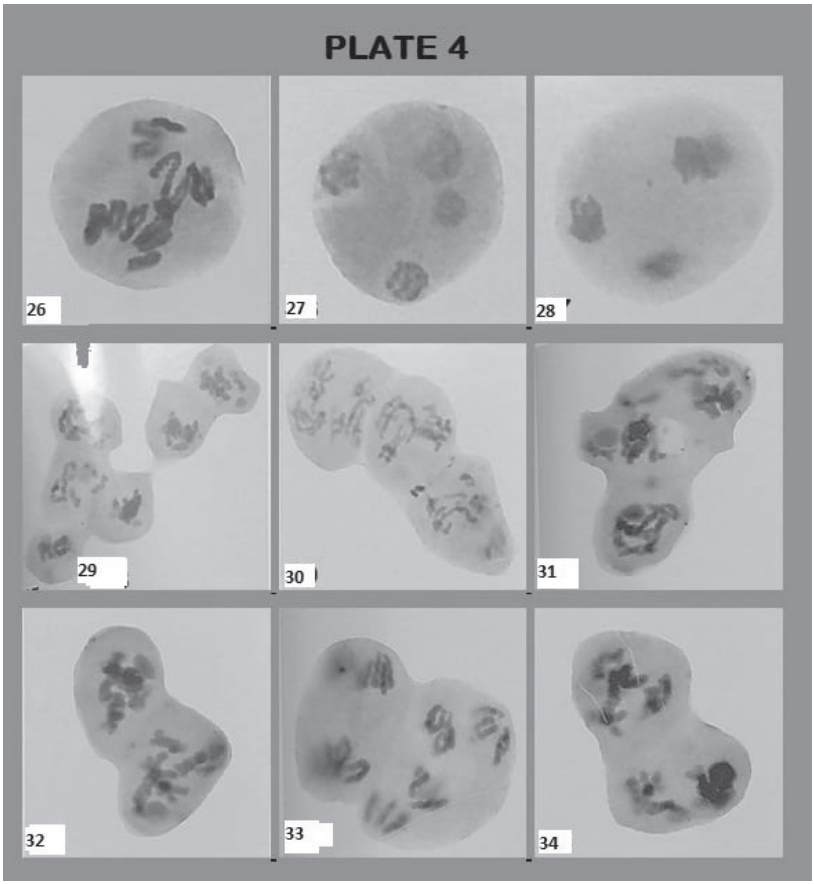
PLATE 4
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Plate 10.1

Fig. 1. Tall mutant (10kR+0.1%EMS).

Fig. 2. Tall mutant with altered leaf shape (20kR+0.2%EMS)

Fig. 3. Tall bushy mutant (10kR+0.2%EMS).

Fig. 4. Broad leaf mutant (20kR+0.3%EMS).

Fig. 5. Bushy mutant with more flowers per plant (10kR+0.1%EMS).

Fig. 6. Bushy semi-dwarf mutant (10kR+0.2%EMS).

Fig. 7. Bushy dwarf mutant with broad leaves (20kR+0.2%EMS).

Fig. 8. Bushy mutant with more flowers and leaves (20Kr+0.03%MMS).

Fig. 9. High yielding mutant (10Kr+0.02%MMS)

Plate 10.2

Fig. 10 a) Control seeds, b) Giant bold seeds of Major –B, mutant seeds were very bold with a normal seed coat.

Fig 11 a) Control seeds, b) Bold seeds of Major –C, mutant seeds were bold and showed peculiar structures on the seed coat.

Fig 12 a) Control pods, b) Mutant pods of Major –D.

Fig 13 a) Control pods, b) Mutant pods of Major –E.

Fig 14 a) Control seeds, b) Mutant seeds of Minor –C, mutant seeds were bold with a green seed coat.

Fig 15 a) Control pods, b) Mutant pods of Major –D.

Fig 16 a) Control pods, b) Mutant pods of Major –B.

Fig 17 a) Control pods, b) Mutant pods of Minor –B.

Plate 10.3

Fig 18 a) Control seeds, b) Giant bold seeds of Major –A, mutant seeds were very bold with a brownish seed coat.

Fig 19 a) Control seeds, b) Mutant seeds of Minor –L, mutant seeds were bold.

Fig 20 a) Control pods, b) Mutant pods of Minor –C.

Fig 21 a) Control seeds, b) Mutant seeds of Minor –I, mutant seeds were bold.

Fig 22 a) Control seeds, b) Mutant seeds of Minor –G, mutant seeds were brownish in appearance.

Fig 23 a) Control seeds, b) Mutant seeds of Minor –D, mutant seeds were bold with a black seed coat.

Fig 24 a) Control seeds, b) Giant bold seeds of Major –B, mutant seeds were very bold with a normal seed coat.

Fig 25 a) Control seeds, b) Mutant seeds of Minor –A, mutant seeds were bold with a darkish seed coat.

Plate 10.4

Fig 26. PMC showing 2 bridges at anaphase-I (var. major, 20 kR+0.04% MMS).

Fig 27. PMC showing non-synchronization at telophase –II (var. minor, 0.2% EMS).

Fig 28. PMC showing non-synchronization at telophase –II (var. major, 0.4% EMS).

Fig 29. Six PMCs showing cytomixis through direct fusion and by the formation of tubes (var. minor, 20kR gamma rays).

Fig 30. Three PMCs showing cytomixis by direct fusion (var. major, 40 kR gamma rays).

Fig 31. Three PMCs showing cytomixis by direct fusion (var. minor, 10 kR+ 0.1% EMS).

Fig 32. Two PMCs showing cytomixis by direct fusion (var. major, 20 kR+ 0.02% MMS).

Fig 33. Two PMCs showing cytomixis by direct fusion among the two, one PMC showing disturbed anaphase-II (var. minor, 0.1% EMS).

Fig 34. Two PMCs showing cytomixis by direct fusion (var. major, 0.03% MMS).

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CHAPTER 11

GENETIC IMPROVEMENT OF GRAIN LEGUMES IN DEVELOPING COUNTRIES THROUGH INDUCED MUTAGENESIS

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Abstract: Today the major challenge around the world is to ensure food availability along with nutritional security to every person. Protein-energy malnutrition is a serious menace faced by human beings all over the world, despite the numerous efforts to curb it. The rapidly burgeoning population, urbanization, changing climatic conditions, loss of acreage for legume crops and other abiotic and biotic stresses are the key factors responsible for the lack of legume crop productivity. The lower production of food legumes further exacerbates the situation for poor people, making protein-rich diets inaccessible to them, especially in developing countries. It is therefore essential to adopt new methods, apart from conventional breeding techniques, through which the qualitative and quantitative characters of legumes can be enhanced which would ultimately lead to an increase in the per capita production of legumes. Induced mutagenesis offered possibilities to improve various qualitative and quantitative traits. It is now being widely used as an alternative tool to create variability and improve the yield of legumes which is the need of the hour. Collaborative efforts between governments, scientists, breeders and farmers are required to resolve the situation of grain legume crisis.

Keywords: Protein-energy malnutrition, induced mutagenesis, mutagen, qualitative and quantitative traits, mutant, genomics.

11.1 Introduction

The growth and development of any country are often evaluated by its nutritional index. It is a matter of global concern to provide food security, along with nutritional security, to all sections of society. In the present scenario, the adequate production and financial accessibility to food are the essential factors needed to eradicate hunger and malnutrition. Across the globe, protein-energy malnutrition (PEM) is a common health problem but is generally more prevalent in developing countries. In developing countries, basically more emphasis is laid down on the cultivation of cereals for socio-economic reasons. To uplift the production of pulses and resolve the problem of protein-energy malnutrition, a paradigm shift in research approaches is needed, with more focus on the cultivation of grain legumes besides cereal crops. Grain legumes are important crop plants due to their high protein and essential amino acids content. Leguminous crops are consumed worldwide for their leaves, edible pods and dry seeds. They complement the cereal crops in providing a perfectly balanced diet and are the main source of dietary protein for the vegetarians all over the world. They are an integral part of the diet of the poor people who cannot afford or access the protein-rich diets obtained from animal products due to the soaring prices. So, they are often regarded as "Poor Man's Meat" (Akibode and Maredia, 2011). Nowadays, vegetable proteins are highly preferred as an alternative for animal proteins such as meat and poultry products due to social, health and religious preferences. The presence of tremendous health-promoting factors and nutritional components makes grain legumes a valuable part of the diet. Besides containing rich amounts of proteins and micronutrients, grain legumes such as the soybean, kidney bean, and faba bean also contain bioactive phytochemical compounds such as polyphenols, phytohemagglutinins (lectins), flavonoids, fiber, oligosaccharides, saponins, etc. These compounds possess antioxidant activities which offer several health benefits such as prevention of cancer, reducing the risk of cardiovascular diseases, diabetes, inflammation and lowering of blood cholesterol level, etc. (Malika Bouchenak and Myriem Lamri-Senhadj, 2013, Ganesan and Xu, 2017).

Legumes have also been used as high protein feed supplements for livestock. In developing countries, forage legumes have played a crucial role in dairy and meat production for centuries (Russelle, 2001). Apart from the traditional food and forage uses, grain legumes can be used in liquid form to produce milk, yogurt, and infant formula (Garcia *et. al*., 1998). Besides this, grain legumes have been used in oils, gums, dyes, and inks (Morris, 1997) as well as in the manufacture of biodegradable plastics

((Paetau *et. al*., 1994). Due to their nitrogen fixation ability grain legumes play a vital role in stabilizing disturbed ecosystems, including those that are fire prone (Arianoutsou and Thanos, 1996). All these features make grain legumes a multipurpose crop. The enhanced productivity rate of legumes will help in alleviating the problem of protein-energy malnutrition which is also the breeding ground of several infectious diseases all around the world, especially in developing countries, ensuring nutritional security and thus laying a strong foundation stone for the future.

11.2 Botanical description and Taxonomical classification

Legumes, or pulses, are flowering plants belonging to the Leguminosae or Fabaceae family. The word legume is derived from the Latin verb legere which means to gather (Allaire and Brady, 2010). This family possesses some 690 genera and 18,000 species. The Leguminosae family is further classified into three sub-families: Papilionoideae, Caesalpinioideae, and Mimosoideae. Each sub-family is identified by its flowers. The sub-family Papilionoideae contains mainly edible legume crops which include the soybean, chickpea, bean, and pea, among others (Morris, 2003). The flowers of leguminous plants are hermaphrodite, containing both the stamen and pistil and have a short hypanthium, usually cup-shaped. As the plants are self-fertile, they have limited genetic diversity. However, hybridization occurs frequently in nature as any plant can pollinate another due to the hermaphroditic properties therein (Weaver, 2003). The flowers are usually pentamerous, possessing five sepals and petals. The stamens are ten in number and are mostly diadelphous, while the pistil comprises of a single long style and stigma, and a superior ovary with one locule containing two or more marginal ovules. The flower arrangement is of papilionaceous type. The posterior large petal called the 'banner' or 'standard' folds over the rest of petals for protection. In front of the posterior petal are two lateral petals called 'wings,' between which the two innermost anterior petals unite to form a boat-shaped structure called the ‘keel’ (Morris, 2003). The stamens and pistil are enclosed within the keel (Earle, 1971). After pollination, the growing ovary develops into the pod. The pod contains the seeds of the plant (Allaire and Brady, 2010).

The taxonomical description of the leguminous plant is as follows:

Kingdom: Plantae—Plants
Subkindom: Tracheobionta—Vascular plants
Superdivision: Spermatophyta—Seed plants
Division: Magnoliophyta—Flowering plants
Class: Magnoliopsida—Dicotyledons
Sub-class: Rosidae
Order: Fabales
Family: Fabaceae

11.3 Scenario of Pulse Production and trade

The pulses have a long history of cultivation like cereals and have been an important constituent in human diets since pre-historic times (Fuller *et. al.,* 2001; Caracuta *et. al.,* 2015). Across the world, in the wake of the "Green Revolution," more importance was given to the cultivation of cereals, causing a significant progression in their production. The focus was shifted from the cultivation of pulses, despite them being a rich source of proteins and providing multifaceted agronomic benefits. The pulse crops received less attention in contrast to cereals. Poor policy, diversification of land toward cereal cultivation and less emphasis on research and developmental programs on pulses has led to a reduction in their supply, effectively driving prices higher and decreasing per capita consumption (Kennedy and Bouis, 1993; Kataki, 2002; Akibode and Maredia, 2011). But gradually in recent years, a considerable change has been witnessed in the global production and trade of pulses due to increasing awareness toward their nutraceutical value. Nowadays, there have been a significant increase in the acreage for pulses.

In developing countries, the majority of the population relies mainly on legumes for their protein need because of cultural, religious and social reasons. The consumption demand increases each year as against the production rate, so to ensure adequate availability to the people it is essential to maintain a balance between the domestic production and consumption rate. India, despite being the largest producer in the world, imports a surplus quantity of pulses annually to meet its domestic consumption need. The steadily increasing population and stagnant production rate are the main factors that resulted in the severe decline of the per capita availability of pulses. In 2011–2013, on average, pulses occupied 80.3 million hectares of the global crop area, producing 72.3 million metric tons of grain. The global pulse production recorded during 2014 was 77. 6 million tons with an average yield of 910 kg/ha (Figure 11.1).

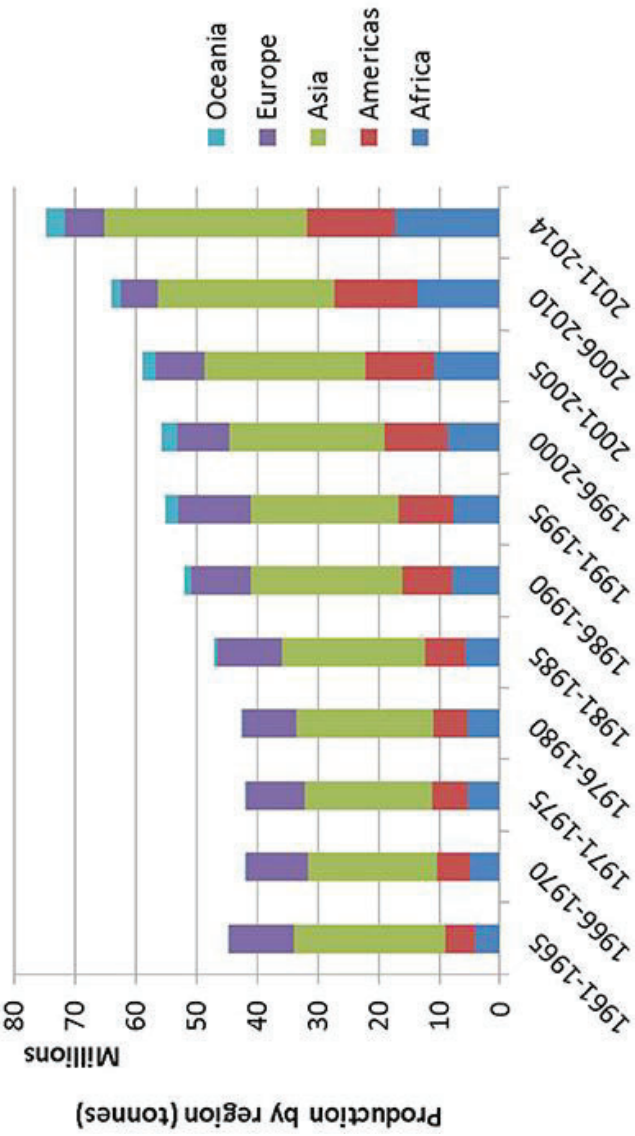

Figure-11.1 Average pulse production by region (FAO, 2016a). (Source: Tan *et.al.,* 2017).

Asia is the largest producer of pulses and among the countries, India is the largest producer and consumer of pulses. In terms of pulse production, India ranks first in the world, occupying about 29% of the world area and 19% of the world's production (Singh *et.al.,* 2015). The legume production recorded in India during 2012-2013, and 2013-2014 was 18.3 and 19.25 million tons respectively, while in 2014-2015, it was reported to be 17.2 million tons, with a total consumption demand of 26.8 million tons. In the consecutive year, 2015-2016, the production of pulses was found to be 17 million tons, with a total consumption demand of 27.1 million tons. Due to subsequent droughts, the production from a peak of 19.25 million tons recorded during 2013-14 in India dipped to 17.2 million tons in 2014-15. But gradually as a result of a considerable increase in the area coverage and productivity, the total production of pulses increased during 2016-17 and was estimated to be about 22.95 million tons, greater by 3.70 million tons (19.22%) than the previous year's production of 19.25 million tons recorded during 2013-14. The production of pulses in India during 2016-2017 was also higher than their five-year average production by 5.32 million tons (30.16%) (Ministry of Agriculture, Government of India).

India produces about 35% of the world acreage and nearly 20% of the world pulses production. More than two-thirds of the global chick pea and pigeon pea production are in India. India reportedly holds almost 70% of chickpea acreage, about 75% of pigeon pea acreage, and over 40% of the lentil area in the world (FAOSTAT, 2014). India is immediately followed by Canada, with an average pulses production of 5.8 million tons in 2014. The world's third-largest producer of pulses is Myanmar, with a productivity of 5 million tons (Table 11.1). As per the data released by FAO, in Asia, Myanmar increased its legume production about 20-fold during the examined period and becomes the world's third-largest producer. In China, due to a shift in consumption towards animal-based protein, the total pulse production dropped by more than half over the past fifty years. Dry beans top the list of pulse crops grown, followed by dry peas, chickpea, cowpea, lentils, pigeon pea and broad beans (FAOSTAT 2016).

Country	Production (mmt)				Principal pulse crops
	1961	1981	2001	2014	
India	12.9	10.8	12.2	19.98	Chickpeas, beans, pigeon peas
Myanmar	0.2	0.4	2.0	5.0	Beans, pigeon peas, chickpeas
Canada	0.1	0.2	3.4	5.8	Peas, lentils
China	8.5	6.4	5.1	4.5	Beans, broad beans, peas
Brazil	1.8	2.4	2.5	3.3	Beans
Nigeria	5	0.6	2.3	2.2	Cowpeas
Ethiopia	0.6	0.9	1.2	2.6	Broad beans, beans, chickpeas, peas
Australia	0	0.3	2.7	3.0	Lupines, lentils, chickpeas
USA	1.1	1.7	1.3	2.4	Beans, peas
Tanzania, U. Rep.	0.1	0.3	0.8	1.8	Beans
Rest of the world	15.0	17.5	22.6	27.02	
Total	40.8	41.6	55.9	77.6	

Table 11.1 Major pulse producer countries in the world (FAO, 2014, 2016b) (Source: Tan *et.al.*, 2017).

In India and other developing countries, the shortfall in the availability of pulses is currently being met through imports. But on a long perspective, excessive import will affect the country's economy and nutritional security. Thus, it is important to become self-reliant and reduce the yield gap to improve the productivity for delinking the dependence on foreign countries. In recent years, the popularity of legumes has also gone up due to the awareness campaign started by the organization called People for the Ethical Treatment of Animals (PETA) which educates people about the benefits of consuming vegetable proteins. These days vegetable proteins are highly preferred over animal proteins such as meat and poultry products due to health issues.

11.4 Economic importance

Grain legumes includes all the pulse crops such as chickpea, pigeon pea, green gram, lentil, peas, beans etc. and also some major oilseed crops such as groundnut and soybeans (Reddy, 2013). The grain legumes or pulses are particularly rich in lysine protein, thereby complementing the conventional cereal-based carbohydrate-rich diets, which are otherwise deficient in lysine and tryptophan (Broughton *et. al.,* 2003; Ufaz and Galili, 2008). Additionally, secondary metabolites such as isoflavonoids and several essential minerals and vitamins are also found in pulses (Cannon *et. al.,* 2009). Pulses are high in complex carbohydrates and fiber, while being low in calories (260-360 kcal/100g dried pulses), due to which they are digested slowly and gives a feeling of satiety. The iron content in pulses help in the transportation of oxygen throughout the body, which boosts the energy production and metabolism. The fiber present in pulses binds to toxins and cholesterol in the gut which helps in their removal as these are not generally absorbed by the body. This improves heart health and lowers the blood cholesterol. Pulses are also rich in complex carbohydrates, micronutrients, protein and B-vitamins, which are essential constituents of a healthy diet. Being low in fat and rich in fiber, pulses are excellent for digestive health, managing cholesterol, and regulating energy levels. Simultaneously the pulses are also rich in foliate, iron, calcium, magnesium, zinc and potassium (Pulse Australia, 2008).

The fat composition in the legume seeds is 2%–21% with the beneficial composition of exogenic unsaturated fatty acids such as linoleic (18:2) acid (21%–53%). Legume lipids are rich in α-linolenic (18:3) acid (4%–22%), which is an essential fatty acid for the biosynthesis of omega-3 polyunsaturated fatty acids (PUFA) ranged 2.5%–41.7% of fatty acid (Campos *et.al.,* 2010). Along with the macro and micro nutritional needs, several health benefits have been linked with the inclusion of edible pulses in the diet. Their low glycaemic index (GI) has been associated with the management of diabetes and diabetes-related diseases (Rizkalla *et. al.*, 2002; Sievenpiper *et. al.*, 2009) while the bioactive components have been scrutinized for their effective properties on human health. For example lectins have an immunomodulatory effect, protease inhibitors have an anti-inflammatory effect, and angiotensin I-converting enzyme (ACE) inhibitory peptides have anti-hypertensive properties (Rochfort and Panozzo, 2007; Roy *et. al*., 2010). The contribution of pulses in reducing colon cancer, cardiovascular disease, increased satiety, lowered body mass index and obesity risk have also been reported in recent years (Boye *et.al.,* 2010; McCrory *et. al.,* 2010; Jukanti *et. al.,* 2012). Pulses may have a

significant anti-cancerous effect since the phytochemicals, saponins, and tannins found in pulses possess antioxidant and anti-carcinogenic properties (Mudryj *et.al.,* 2014). The phytoestrogens present in grain legumes can offer an alternative therapy for hormone replacement therapy with beneficial effects on the cardiovascular system, and might even improve menopausal symptoms (Prakash and Gupta, 2011).

Soil nutrient depletion is a particular problem of small landholders in developing countries. Grain legumes play a major role not only in food security but also contribute to the improvement of soil health in terms of increasing organic manure content in the soil as well as by fixing atmospheric nitrogen to enrich soil fertility, thereby increasing the productivity of soil in terms of yield of the subsequent crop or cropping system as a whole (Reddy, 2005a). Legumes play an important role in natural ecosystems, agriculture, and agroforestry. The ability of legumes to fix N_2 in symbiosis makes them excellent colonizers of low-nitrogen environments, and an economic and environmentally friendly crop. Due to the nitrogen-fixing properties, pulses are often grown as an intercrop or as a mixed crop to replenish soil nitrogen levels, thereby reducing the need for fertilizers. The uptake of nitrogen, sulfur, and phosphorus by cereals have been increased when cultivated with pulse crops, resulting in an enhanced yield and grain quality (Li *et. al.,* 2003, 2004b; Agegnehu *et. al.,* 2006; Banik *et. al.,* 2006; Gooding *et. al.,* 2007).

The by-product of the leguminous plant, i.e. the straw, is used as an important source of fodder for livestock. The legume straw is generally favored over cereal straw because of its better nutritional quality owing to the higher nitrogen and lower fiber contents (Lopez *et. al.,* 2005). Although grain crops are the major feed sources in intensive animal and milk production, forage legumes are required to maintain animal health (Wattiaux and Howard, 2001). In developing countries, meat and dairy production are almost solely dependent upon forage legumes and grasses. The prevalent forage legume used in temperate climates is alfalfa (*Medicago sativa*) (Russelle, 2001). It is noteworthy that in addition to being a food and fodder source, the grain legumes play an important contribution toward climate change mitigation. The emission of greenhouse gases (GHG) such as carbon dioxide (CO_2) and nitrous oxide (N_2O) compared with agricultural systems based on mineral nitrogen fertilization are lower with grain legumes. The grain legumes also have an important role in the sequestration of carbon in soils and reduce the overall fossil energy inputs in the system (Lemke *et. al.,* 2007, Jeuffroy *et.al.,* 2013, Stagnari *et.al.,* 2017). The diverse and important roles played by

pulses in farming systems and the diets of people make them ideal crops for achieving the sustainable development goals of reducing poverty and hunger, improving human health and nutrition and enhancing ecosystem resilience (Kabata *et.al.,* 2017). Keeping in view the diversified significance of grain legumes to humans and the environment, the UN General Assembly declared the year 2016 as the International Year of Pulses.

11.5 Constraints for pulses production

The grain legumes are adversely affected by different kinds of biotic and abiotic stresses. The richness of legumes in Nitrogen and Phosphorus makes them attractive for insects, pests and diseases (Sinclair and Vadez, 2012). A large quantity of legume crops are damaged by pests such as pod borers, aphids, termites and caterpillars. The biotic stresses that affect the production of pulses include Fusarium wilt (chickpea, pigeon pea, lentil), Ascochyta blight (lentil, chickpea), rust (pea and lentil), powdery mildew (pea), root rot (chickpea, moong and lentil) and anthracnose (kidney bean, urad) etc. (Johansen C. *et. al.,* 1994; Muehlbauer *et.al*., 2006). Similarly, the different abiotic stresses encountered by grain legumes include drought (Kashiwagi *et. al.,* 2013), high temperature (Kumar and van Rheenen, 2000), cold stress (Sandhu *et. al.,* 2007), salinity (Flowers *et. al*., 2010), water logging (Sultana *et. al*., 2014), and Al toxicity (Butare *et. al*., 2011; Yang *et. al*., 2013). Besides these stresses, lack of knowledge, non-availability of improved seeds, poor technical guidance, lack of subsidy for inputs, lack of knowledge about seed rate, seed treatment, weed management dosage, seed storage, poor irrigation and poor marketing etc. were the socio-economic factors responsible for low pulse production (Subbarao *et. al*., 2001, Pande *et. al.,* 2003, Kumar and Bourai, 2011).

The lack of high yielding varieties, loss of acreage for legumes, post-harvest damage, absence of mechanized harvesting, inappropriate technologies and different types of biotic and abiotic stresses are the major constraints responsible for the inefficient production of legumes in developing countries. To increase the yield plateau in legumes, efforts are required to develop high yielding varieties by creating variations in the existing varieties through breeding programs. The conventional plant breeding methods such as mass selection, pedigree selection and somatic hybridization have been used in agricultural practices for many decades to produce genetic variations and enhance productivity. But these methods have certain limitations and drawbacks also, such as the transfer of undesirable traits along with the traits of interest. Artificial induction of

mutation with the help of mutagenic agents offered the possibilities of creating genetic variation and improving various qualitative and quantitative traits in the crop plants which is a pre-requisite of any successful breeding program. These days the nature and function of genes which are the building blocks and basis of plant growth and development are thoroughly understood by mutagenic studies (Adamu and Aliyu, 2007).

11.6 Induced Mutagenesis

Induced mutagenesis is the process whereby sudden heritable changes occur in the genetic information of an organism, not caused by genetic segregation or genetic recombination, but induced by chemical, physical or biological agents (Bhat *et al.,* 2006b; Bhat *et al.,* 2006a; Bhat *et al.,* 2005a; Bhat *et al.,* 2007b; Roychowdhury and Tah, 2013). Hugo de Vries (1901) is credited for the introduction of the term mutation while working on *Oenothera lamarckiana* (evening primrose). The discovery and genetic analysis of white-eyed mutants of *Drosophila* in 1910 by Morgan led to the beginning of systematic studies on mutation. Although mutations are spontaneous, the frequency of such mutations is too low to be considered effective for plant breeding. However, the efficacy of spontaneous mutations in genetic studies prompted the geneticists to search for alternate ways of inducing mutations. This work resulted in the discovery of the mutagenic potential of X-rays in *Drosophila* by H. J. Muller (1927) and barley by Stadler (1928). The first commercial mutant cultivar, chlorina was obtained by Goodspeed (1929) in the genus *Nicotiana* after X-ray irradiation. Later on, the mutagenic effects of mustard gas and some other chemical compounds were discovered by Auerbach and Robson in 1946. In recent years, induced mutagenesis has become a key area of research for genetical studies. It has been profitably utilized in raising a large number of economically superior and desirable genotypes of crop plants.

Induced mutagenesis has gained momentum since the beginning of the last decade and has been considered as one of the driving forces for evolution (Toker, 2009). It has played a pivotal role in creating genetic variations in crops having a narrow genetic base, which was impossible via conventional breeding programs. The usefulness of mutagens in mutation breeding depends not only on its mutagenic effectiveness (mutations per unit dose of mutagen) but also on its mutagenic efficiency about undesirable changes such as sterility, lethality, injury etc. (Bhat *et*

al., 2007; Bhat *et al.,* 2007a; Bhat *et al.,* 2006d; Bhat *et al.,* 2005b; Girija and Dhanavel, 2009). The mutants derived through induced mutagenesis show superior traits over the cultivars, such as synergistic higher yields due to enhanced resistance to biotic and abiotic factors, improved nutrient content and other quality traits. The mutant crop varieties are more adaptable to the environment, require less agricultural input, and thus are more economical to grow and contribute to more environment-friendly agriculture (Mba, 2013). Mutagenesis is a well-functioning branch of plant breeding and supplements the conventional methods in a favorable manner. It confers several advantages in plant improvement by upgrading a specific character without altering the original genetic make-up of the cultivar (Bhat *et al.,* 2007; Bhat *et al.,* 2006; Bhat and Wani, 2015; Bhat, 2007; Gottschalk, 1986; Toker *et. al.,* 2007). However, T-DNA insertional mutagenesis is likely to result in the complete disruption of gene function rather than in generating allelic series of mutants with partial loss of function and thus will not produce the range of mutation strengths necessary for crop improvement (Parry *et. al.,* 2009).

With the advent of induced mutagenesis, the prospects to boost up the agricultural productivity have been made possible which was affected by various factors such as rapid industrialization, burgeoning population and climate change etc. Both qualitative and quantitative variations could be induced by induced mutagenesis in a comparatively shorter time by altering alleles at known loci as well as at previously unknown loci, besides altering linkage groups (Konzak *et. al.,* 1977). During the last few years, research on mutagenesis has expanded rapidly to understand its impact on the crops. The spectrum of the effect induced by the mutagens varies with the varying mutagens and doses in different plant species. Thus, it is essential to select a particular mutagen and its optimum dose according to plant species for the successful running of the mutation breeding program and also to minimize the adverse effects. Mutation breeding efforts continue around the world today. Mutagenesis, when combined with bio-molecular technologies such as plant tissue culture and molecular markers, plays a very important role in crop improvement (Lagoda, 2009).

Up until 2014, 3,218 mutant varieties have been officially cataloged on the FAO/IAEA mutant database of Mutant Variety and Genetic Stock (http://mvgs.iaea.org). According to the FAO/IAEA database, Asia is credited for the development of more than half of the mutant derived varieties. About 60% of the mutant crop varieties have been released in Asia. China, alone holds more than 25% of the share of the officially

released mutant varieties globally. Interestingly, China along with two other Asian countries, Japan and India, are the top three countries with the most officially released mutant crop varieties (Kharkwal and Shu, 2009). The distribution of mutant crop varieties by continents has been shown in Figure 11.2. The global impact of mutation-derived varieties developed and released in major crops all over the world has been reviewed and published by Ahloowalia *et. al.* (2004).

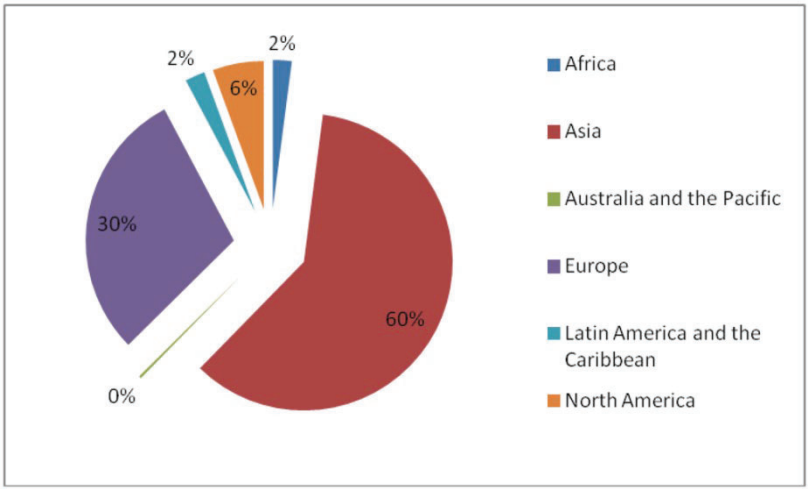

Fig-11.2 Distribution of mutant varieties developed in different continents (Joint FAO/IAEA Mutant Variety Database http://mvgs.iaea.org/).

Induced mutagenesis is used to create genetic variability in the crop species which can further be exploited for germplasm enhancement and could also be utilized for developing new cultivars. Through induced mutagenesis, a new variety is developed in a comparatively shorter time compared to hybridization. Generally, 11-12 years are needed to develop a new variety through hybridization, whereas it usually takes only 8-9 years in the case of induced mutagenesis (Brock, 1977). Many desirable traits such as resistance against biotic and abiotic stresses, enhanced nutritional value, improved yield etc. which were normally restricted to a limit in hybridization have been induced through mutations in the Leguminosae family (Toker and Cagirgam, 2004). Interestingly, the mutant varieties developed through induced mutagenesis possess both improved agronomic traits and nutritional quality, hence their popularity has gone up among the consumers. Simultaneously, basic studies such as the biochemical and

plant developmental pathways have been elucidated by induced mutation studies. For example, identification of key genes involved in floral organ development, which ultimately led to the construction of the ABC model of flower development, was made possible through the isolation and molecular characterization of floral mutants of *Arabidopsis* and *Antirrhinum* (Coen and Meyerowitz, 1991). At present induced mutagenesis is randomly being used for the identification of a gene by knock down of the phenotypic expression. The mapping of genes in a crop is possible via induced mutagenesis (Roychowdhury and Tah, 2013).

11.7 Types of mutation

Mutations can be broadly divided into intragenic or non-structural mutations (occurring within a gene in the DNA sequence), intergenic or structural mutations within chromosomes (inversions, translocations) and mutations leading to changes in the chromosome number (polyploidy, aneuploidy and haploidy) (Auerbach, 1976). Besides this, it is important to distinguish between nuclear and extranuclear or plasmon (mainly chloroplast and mitochondrial) mutations, which are of considerable interest to agriculture (Pathirana, 2011).

11.7.1 Intragenic or non-structural mutations

The DNA sequence is made up of triplets of bases known as codons which eventually translate into amino acids. The mutations which cause a change in the base pair sequence of genes are known as intragenic mutations or non-structural mutations. They include -

11.7.1.1 Point mutations - These involve a single-base pair change and may result in missense codons (codes for a different amino acid), silent codons (no change in amino acid coding) and nonsense codons (amino acid codon is changed to a translation stop codon). Point mutations are also known as single-base substitutions, which contribute to the build-up of single-nucleotide polymorphisms (SNPs).

11.7.1.2 Insertions and deletions - Insertions and deletions of one or more nucleotides, collectively known as indels may result in changes to codons and frame shifts in codon reading frames with qualitative and quantitative effects on the proteins produced.

11.7.1.3 Mutations in the specific gene regions - Mutations in the specific gene regions such as promoter, coding, stop, intron and 3' untranslated sequences.

11.7.1.4 Transposon insertion - Transposon insertion is also known as insertional mutagenesis. The transposon refers to DNA sequences that can transpose, move in and out of chromosomes. They are often activated by environmental stress and their activity can knock out or activate genes by inserting into or exiting a gene.

11.7.2 Intergenic or structural mutations

Intergenic or structural mutations cause variations in the chromosome structure of an organism. They arise as a result of either chromosomal breakage or rearrangements. As a result of chromosome breakage, the sequence of bases may be inverted, in which case the linear order of genes is opposite to the wild type orientation while in the case of chromosomal rearrangements a piece of a chromosome is relocated on the same chromosome (intrachromosomal translocation) or transferred to a different location (inter-chromosomal translocation).

11.7.3. Changes in the chromosome number

These include either a reduction in the genome number or an increase in the genome number, either by genome duplication (autoploids) or genome addition (alloploids) (Lundqvist *et.al.,* 2012).

11.8 Kinds of mutagens

11.8.1 Physical mutagens

The physical mutagens comprise high energy radiation such as gamma rays, UV-rays, X-rays, alpha particles, beta particles, and neutrons etc. They affect the genetic material because of their high penetration ability inside the living cells. The effect of radiation on living cells and tissues is directly proportional to the degree of penetration of the radiation. The discoveries of X-rays by Roentgen in 1895, radioactivity by Becquerel in 1896, and radioactive elements by Marie and Pierre Curie in 1898 opened up the new channels for humans to induce mutations deliberately in plants using physical mutagens (Mba, 2013). Electromagnetic radiation is classified into two types i.e. ionizing and non-ionizing radiation based on

the source, the amount of energy that is involved, and the frequency etc. (Mba *et.al.,* 2010). The ionizing radiations are further distinguished into two classes according to differences in linear energy transfer (LET). Alpha particles, neutrons, and heavy-ion beams have high LET, while gamma rays, X-rays and electron beams have low LET (Morita *et.al.,* 2009). Neutrons which possess high LET cause deletions of about 300bp to 12kbp in length in plant genomes (Li *et. al.,* 2001). Recent research has shown that heavy-ion beams, also classified as high LET radiation, possess the capability to induce structural changes in chromosomes, such as inversions and translocations (Shikazono *et. al.,* 2005).

Among the physical mutagens, gamma rays are the most widely used mutagen by plant breeders because of their ability to penetrate deeper into biological matter due to their short wavelength (Amano, 2006). Gamma rays cause damage to DNA in two ways, either it will cause DNA double-strand breaks (DSBs) by the direct deposition of ionizing energy, or generate reactive oxygen species (e.g. H_2O_2, $^{\bullet}OH$, $O_2^{\bullet -}$) produced by ionization-induced water radiolysis which causes damage at the nucleotide level and thus affects the genome indirectly (Lee *et. al.,* 2009; Lagoda, 2012). It has been found that gamma rays induced both insertion and deletion of nucleotides and point mutations (Nawaz and Shu, 2014; Li *et al.,* 2016). The frequency of mutation is estimated to be one mutation/6.2 Mb (Sato *et. al.,* 2006). The non-ionizing radiations such as UV-rays are known to cause heritable mutations by forming cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine photoproducts (Ikehata and Ono, 2011). The different kinds of physical mutagens used in induced mutation breeding programs and their properties are given in (Table 11.2).

11.8.2 Chemical mutagens

Chemical mutagens have attained popularity since they are easy to use, do not require any specialized equipment, and can provide a very high mutation frequency. Chemical mutagens cause single-base-pair (bp) changes, or single-nucleotide polymorphisms (SNPs) (Sikora *et. al.,* 2011). Based on their specific reaction with DNA, the chemical mutagens are classified into four major groups such as deaminating agents (nitrous acid), alkylating agents (ethyl methane sulphonate and methyl methane sulphonate), base analogs (5 -Bromouracil), and intercalating agents (acridine orange) (Table 11.3). The alkylating agent Ethyl methanesulfonate (EMS) is a widely used chemical mutagen in mutation breeding because it creates a high frequency of non-lethal point mutations

(Talebi *et. al.,* 2012). Along with its effectiveness, EMS is also preferred because it is easy to handle and it can be disposed of by its detoxification through hydrolysis (Pathirana, 2011). Worthwhile EMS causes the alkylation of guanine and forms O-6-ethyl guanine which often misspairs with thymine instead of cytosine in the complementary strand. This misplacement produces base pair substitutions resulting in G/C to A/T transition (Greene *et al*., 2003). With the advances in genetic mapping and molecular markers, mutant phenotypes caused by EMS mutation can be linked to specific gene sequences using forward genetics (Peters *et.al.,* 2003). Also, EMS mutagenesis can induce changes in the gene of interest and allow for the study of gene function using reverse genetics (Cooper *et. al.,* 2008).

11.9 Methodology of induced mutagenesis

The objective in induced mutation breeding is to induce maximum genomic variation with a minimum decrease in viability (Gulfishan *et al.,* 2013; Ganai *et al.,* 2005; Bhat and Wani, 2017; Bhat and Wani, 2017a; Sikora *et.al.,* 2011) and the success of any induced mutagenesis program is dependent on several criteria and on the accuracy of the method adopted. The critical factors determining the spectrum and rate of induced mutations include the type of mutagen used, the administered dose and dose rate, and the method of treatment including choice of materials, pre- and post-treatment handling etc. The use of the correct procedure for mutagenic treatment, the development and management of the mutant population, and selection of mutant are the key elements in mutant exploitation (Mba *et.al.,* 2013).

The procedure involved in the induced mutation breeding approach has been displayed in Figure 11.3. The first step in induced mutagenesis is to select a mutagen of particular interest. After this, before proceeding toward mutagenic treatment, the effective dose of the particular mutagen is estimated by determining the LD_{50}. Thereafter the selected plant part (seeds, buds, flower, etc.) are treated with the particular mutagen. Once the treatment is over, the treated parts are grown to raise the M_1 generation. The seeds harvested from the M_1 generation are again sown in the next consecutive season to raise the M_2 generation. Simultaneously M_3 and M_4 generations are raised. The appropriate selection and screening of the mutant through various identification markers is the most important step in any induced mutation breeding exercise. Most mutations are usually not expressed in the first generation as they are recessive. The mutant plants

could be selected through visual screening, biochemical attributes and cytological detection etc. In mutation breeding, an efficient screening approach is essential since a high mutation induction rate, followed by a poor screening approach could result in inefficiency and ultimately project failure (Ukaia and Nakagawab, 2012). The seeds of the selected mutant plant progenies with the desired trait are then evaluated further in the field, along with the normal ones to check their homogeneity and stability. The stabilized homozygous population are then tested at multi-location sites for two to three years after which it could be released as a new variety. The mutant progenies obtained could also be used in hybridization experiments to create genetic variations.

11.10 Significance of induced mutagenesis in grain legume improvement

The main endeavor of mutation breeding programs is to create genetic variability with mutagenic agents and complement the existing germplasm resources (Parveen *et al.,* 2006; Gulfishan *et al.,* 2011; Gulfishan *et al.,* 2010; Gulfishan *et al.,* 2012; Konzak *et.al.,* 1977). The mutagens induced genomic variation in several desired quantitative and qualitative traits of the crop. The spectrum of the effect induced by the mutagens differs with the varying mutagens and doses in different plant species. The desirable mutations of high frequency in the crops can be recovered through the study of mutagen effectiveness and efficiency (Gulfishan *et al.,* 2015; Sharma *et al.,* 2009; Badere and Choudhry, 2007). During the last few years, a significant amount of work has been undertaken on induced mutagenesis in grain legumes through the incorporation of physical and chemical mutagens to understand its effect on productivity and how it would help to improve the malnutrition situations. The moderate concentrations of mutagens were found to be the most effective and efficient for inducing mutations (Wani *et.al.,* 2014). According to Kaul (1989), the mutagens that cause the least damage and yield high useful mutants are the most desirable ones.

Many cultivars in pulse crops have been derived through induced mutation breeding directly or indirectly by involving mutants as parents to derive new cultivars (Ahloowalia *et. al*., 2004). Around the globe so far, approximately 432 legume varieties have been released and are in commercial cultivation. The number of officially released mutant varieties of legumes through mutation breeding has been given in table 11.4. These include soybean (170), groundnut (72), mungbean (36), chickpea (21),

lentil (13), cowpea (12), urdbean (9), pigeon pea (7) and others (92) (http://mvgs.iaea.org). In *Cicer arietinum* L. the glabrous mutants were observed in EMS mutated populations (Pundir and Reddy, 1989) while the mutants with increased seed size and shape by the treatment of both EMS and gamma rays have been reported by Wani and Anis (2008). In chickpea, gamma rays were most effective in generating a disease-resistant variety (CM 98) against Ascochyta blight and wilt (Haq *et.al.,* 1999). In Bangladesh Shamsuzzaman and Islam (2005) were successful in isolating an early-maturing chickpea mutant L-84 through mutation breeding. The mutant strain matured significantly earlier by about two weeks compared with its parent variety Binasola-2 and had the highest seed yield. Along with the quality parameters in chick pea, mutagenic treatment with gamma rays has also been found to increase the degree of softness of seed, thus improving the cooking quality (Graham *et. al*., 2002).

Legumes (Botanical Name)	**Number of mutants released**
Arachis hypogea (Groundnut)	72
Cajanus cajanus (Pigeon pea)	7
Cicer arietinum (Chickpea)	21
Dolichus lablab (Hyacinth bean)	1
Lathyrus sativus (Grass pea)	3
Lens culinaris (Lentil)	13
Glycine max (Soybean)	170
Phaseolus vulgaris (French bean)	59
Pisum sativum (Pea)	34
Trifolium alexadrinum (Egyptian clover)	1
*T. incarnatum (*Crimson clover)	1
T. pratenus (Red clover)	1
T. subterraneum (Subterranean clover)	1
Vicia faba (Faba bean)	20
Vigna unguicularis (Azuki bean)	3
V. mungo (Black gram)	9
V. radiata (Mungbean)	36
V. unguiculata (Cowpea)	12
Total	**462**

Table 11.4 Number of officially released mutant varieties of legumes through mutation breeding. Data source: FAO/IAEA Mutant variety database, 2015.

Generally, the grain legumes show low productivity rates due to their high susceptibility toward abiotic stresses (Freyer *et. al.,* 2005; LópezBellido *et. al.,* 2005; Voisin *et. al*., 2014). Biotic and abiotic factors hinder legume productivity to a large extent. This issue can be resolved by manipulating and modifying the genetic architecture of legumes through induced mutagenesis which nullifies the effect of biotic and abiotic stresses. Induced mutagenesis helps the grain legumes to combat the different types of stresses. The stress-tolerant plants in grain legumes via conventional and molecular breeding approaches have been developed utilizing the genomic resources and genes associated with plant abiotic stress responses (Araujo *et.al.,* 2014). A drought-tolerant mutant of lentil was isolated by Lal and Tomar (2009) through induced mutagenesis. The nitrate reductase activity, wax content and grain yield were found to be higher in the mutant line. The mutant crop varieties contributed significantly toward food and nutritional security with their enhanced resistance toward biotic and abiotic factors, higher yield, improved nutrient content and requirement for less agricultural inputs (Kharkwal and Shu, 2009).

Since induced mutagenesis facilitates the isolation, identification and cloning of genes it is possible to design crops with improved yield, increased stress tolerance and longer life span as well as reduced agronomic inputs usage (Ahloowalia and Maluszynski, 2001). The morphological mutants induced through gamma rays and EMS in chickpea showed improved agronomic traits. The yield pattern of the mutants was high compared to the untreated plants (Wani, 2011). Auti (2005) reported that in mungbean both chemical mutagens (Sodium Azide and EMS) in lower concentrations showed a stimulatory effect, while gamma radiation and the higher concentrations of chemical mutagens had an inhibitory effect on the morphological traits. Singh *et. al.* (1988) reported a pentaphyllous mutant in *Vigna mungo* at 20kR of gamma rays with improved nutraceutical values. The bold seeded mutants induced by gamma rays in *Vigna mungo* has been documented by Singh (1996). The positive effects of gamma rays on productivity and seed protein content in soybean in M_2, M_3 and M_4 generations have been corroborated by Pavadai *et. al.* (2010). Kharkwal (1998) studied total protein content of 44 chickpea micromutants and 35 macromutants induced as a result of treatment with physical (gamma rays and fast neutrons) and chemical mutagens (EMS and NMU). He reported that all mutant genotypes showed significantly higher levels of protein content than the parental varieties. According to him, it was possible to concurrently increase the protein content in the chickpea mutant with an increase or without a loss in grain weight and yield. The effectiveness and efficiency of mutagens are higher

at lower and moderate doses and have been reported in *Lathyrus sativus* (Nerkar, 1977), *Vigna mungo* (Kumar *et. al.,* 2007), *Glycine max* (Khan and Tyagi, 2010a) and *Vigna radiata* (Lavanya *et. al.,* 2011).

Even though the legumes are an integral part of the human diet for protein and starch, their widespread use has been restricted owing to the presence of some anti-nutritional substances such as saponins, lectins, protease inhibitors, non-protein amino acids (NPAAs), alkaloids, cyanogenic glycosides, pyrimidine glycosides, tannins, isoflavones, oligosaccharides, erucic acid, and phytates (Mikic *et.al.,* 2009). A reduction in saponin content through gamma radiation at a radiation dose level of between 4 and 6 kGy in *Vigna radiata* L. has been reported by Siddduraju *et.al.* (2002a). A low TI content induced by gamma rays in two mutants (early maturing and flat pod) of winged bean has been found by Dhadke and Kothekar (2005). The mutant legume plants show a considerable (40–60%) reduction in the antitryptic activity which indicates the significance of induced mutagenesis in enhancing the nutritional quality of grain legumes.

The mutants developed through induced mutagenesis can have a huge impact on the food security and economy of a nation. The release of 'TG' (Trombay groundnut) cultivars of groundnut in India has boosted the Indian economy by millions of dollars (D'Souza, 2008). Out of nine mutant varieties of blackgram (urdbean) released in India, four have been developed at the Bhabha Atomic Research Centre (BARC) in Mumbai. One of the mutant varieties of blackgram, TAU-1, has become the most popular variety cultivated in Maharashtra State in India, occupying an area of about 500,000 hectares. The additional production contributed an amount of Rs. 300 crores (about 60 million US dollars) annually to the national income in India (Kharkwal *et.al.,* 2004). Similarly, the mungbean mutant variety Binamoog-5, in Bangladesh, has substantially contributed toward food security (Ali Azam, personal communication, April 2008). Likewise, the first high yielding chickpea mutant cultivar 'CM-72' resistant to Ascochyta blight released in Pakistan in 1983 has brought a revolutionary change in the economy of farmers. The first example of mutant legume crop cultivated in the world was the chickpea developed at I.A.R.I., New Delhi and released by the Government of India for agricultural commercialization. The four mutant varieties of chickpea, Pusa – 408 (Ajay), Pusa – 413 (Atul), Pusa – 417 (Girnar), and Pusa – 547 possess high yield and Ascochyta blight and wilt disease-resistant properties. Another chickpea mutant variety, Pusa – 547, released in 2006 for commercial cultivation, has attractive bold seeds, thin testa, and good cooking quality besides a high yielding trait (Kharkwal *et.al.,* 1998, 2004, 2005 and 2008).

11.11 Conclusion and prospects

Induced mutagenesis has emerged as an effective technique to induce genetic variability in staple crops, especially in grain legumes which have a narrowed genetic base due to their self-pollinating nature. The genetic diversity could be broadened through the use of physical and chemical mutagens. Mutation breeding offers a chance to improve the germplasm of the existing varieties and develop superior varieties, which is the basic aim of any breeding program. This technique has an edge over conventional breeding methods in enhancing the qualitative and quantitative traits of the crop. With a rapidly burgeoning population and shrinking land resources, it is mandatory to maintain a balance between production rate and consumption demand of staple crops to avert a shortage crisis and ensure food availability to every person. The mutant varieties raised through mutation breeding show better levels of survivability as well as improved agronomic traits which would play a pivotal role in addressing food security at the global level. The nutraceutical value of food is also enhanced through this methodology. It is worth mentioning that the mutant cultivars can be sustained under the varied agro-climatic conditions of different regions, thus maintaining a sustainable agricultural cropping system.

In developing countries, malnutrition and hunger are the silent killers of humanity. With the introduction of induced mutation breeding methodology, improved varieties of cereals and grain legumes have been developed to combat these issues. Although in recent years the pioneering work on grain legumes has been improved, it is still proceeding far behind the cereals in terms of mutant varieties released. More research work on grain legumes has to be thoroughly done to improve the scenario in developing countries. This methodology had a tremendous role in boosting the economy of developing nations. The livelihood of many people relying on agriculture has been improved through the cultivation of mutant cultivars. In the future, the induced mutants could be used as ideal study material for functional genomic studies. The structure and function of a gene can be easily understood through the deployment of mutational events. In spite of the aforementioned advantages, challenges still lie ahead as it is a major challenge to incorporate the induced mutagenesis into mainstream agricultural practices. A collaborative effort between breeders, farmers, and research institutes is needed to resolve this issue. Increasing awareness and provision of more funds will help in popularizing this technique for crop improvement.

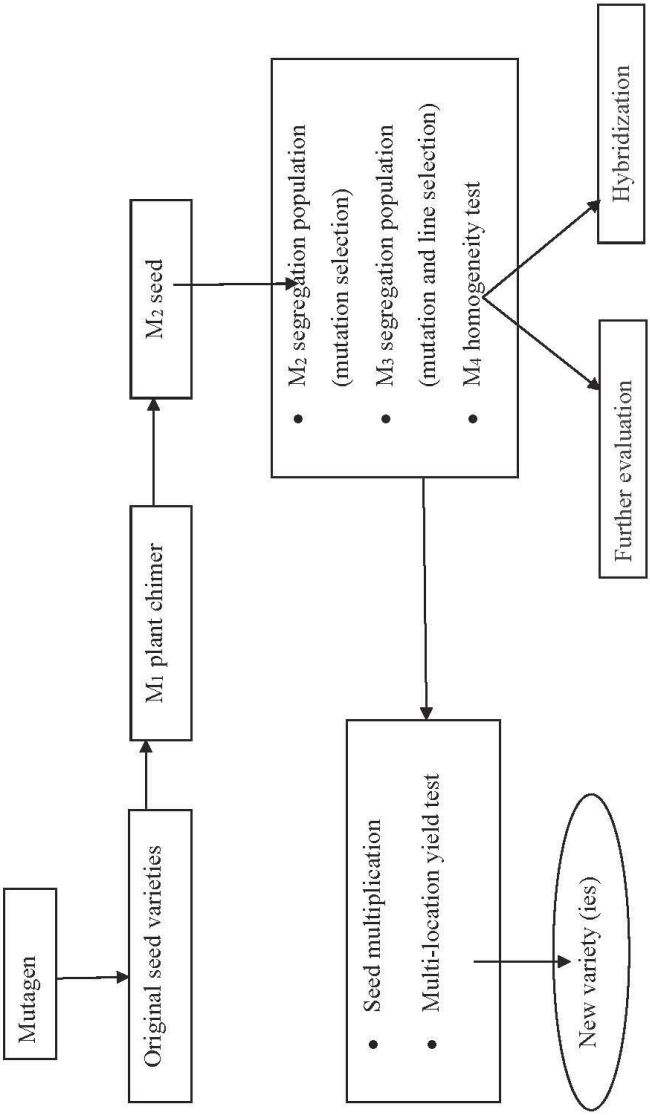

Figure 11.3 Methodology adopted in induced mutagenesis breeding program *(Source:* Oladosu *et. al.,* 2016).

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Table-11.2 Various types of physical mutagens and their properties (Source: Mba *et.al.* 2013).

Mutagen	Source	Energy	Characteristics	Hazard
X-rays	X-ray machine	Commonly 50-300 keV	Electromagnetic radiation, penetrates tissues from a few millimeters to many centimeters.	Dangerous, penetrating
Gamma rays	Radioisotopes and nuclear reaction	Up to several MeV	Electromagnetic radiation, penetrates through whole part in plants.	Dangerous, very penetrating
Neutrons (fast, slow and thermal)	Nuclear reactors or accelerators	From less than 1eV to several MeV	Uncharged particles, slightly heavier than proton, observable only through interaction with nuclei, penetrate tissues to many centimeters.	Very hazardous
Beta particles, fast electrons or cathode rays	Radioactive isotopes or accelerators	Up to several MeV	An electron, ionizing much less densely than alpha particles, penetrates plant tissues up to several several cm.	May be dangerous
Alpha particles	Radioisotopes	2-9 MeV	A helium nucleus, heavily ionized, penetrates up to a small fraction of a mm into the tissues.	Very dangerous
Protons or deuterons	Nuclear reactors or	Up to several GeV	Nucleus of hydrogen, penetrates tissues up to several	Very dangerous

	accelerators		centimeters.	
Low energy ion beam	Particle accelerators	Dozens of keV	Ionized nucleus of various elements, penetrates a fraction of mm into plant tissues.	Dangerous
High energy ion beams	Particle accelerator	Up to GeV	Ionized nucleus of various elements, penetrates up to a few cm into plant tissues.	Dangerous

Table-11.3 Various types of chemical mutagens and their mode of action (Source: Mba *et.al.* 2013).

Chemical mutagen group	Example	Mode of action
Deaminating agent	Nitrous acid	Acts through deamination, the replacement of cytosine by uracil which can pair with adenine and thus through subsequent cycles of replication lead to transitions.
Alkylating agents	Ethyl methanesulfonate (EMS),methyl methanesulfonate (MMS), dimethyl sulfate (DMS), diethyl sulfate (DES), 1-methyl-2-nitro-1-nitrosoguanidine (MNNG),1-ethyl-2-nitro-1-nitrosoguanidine (ENNG), N,N-dimethylnitrous amide (NDMA), N,N diethylnitrous amide (NDEA), 1-methyl-1-nitrosourea (MNU), and 1-ethyl-1-nitrosourea (ENU)	They react with bases and add methyl or ethyl groups and, depending on the affected atom, the alkylated base may then degrade to yield a baseless site, which is mutagenic and recombinogenic or mispair to result in mutations upon DNA replication.
Base analogs	5-bromouracil (BU), 5 bromodeoxyuridine, 2-aminopurine (2AP)	Incorporates into DNA in place of the normal bases during DNA replication thereby causing transitions (purine to purine or pyrimidine to pyrimidine).
Intercalating agents	Acridine orange, proflavin, ethidium bromide	They intercalate DNA bases thereby causing a distortion of the DNA duplex and the DNA polymerase in turn recognizes this stretch as an additional base and inserts an extra base opposite this stretched (intercalated) molecule. This results in frameshifts i.e. an alteration of the reading frame.

CHAPTER 12

PHYSICAL AND CHEMICAL AGENTS INDUCED STRUCTURAL AND NUMERICAL CHANGES IN CHROMOSOMES

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Abstract: Industrial development has widely affected the environment in recent decades. Since the mid-1940s synthetic chemicals have become an integral part of life. These synthetic chemicals possess potential health hazards to humans and other organisms. The usage of such chemicals has enormously increased in day to day life. Among these, some of the synthetic chemicals have become mutagens which have a higher risk for malignancy. These mutagenic chemicals alter the DNA in somatic cells that are one of the key events in the process of carcinogenesis. There are different types of mutagen, such as base analogs (5-Bromouracil, 2-Aminopurine); chemical mutagens (Ethyl methanesulfonate, Methyl methanesulfonate, Nitrous acid, Sodium azide); physical agents (UV light, Radiation) and Intercalating agents (Acridines). Mutagens cause single-strand and double-strand breaks in DNA. Mutagen induced chromosomal aberrations can be divided into two main classes - i) chromatid type aberrations involving both single and double chromatid type aberrations; ii) chromosome type aberrations such as dicentric, inversion and ring chromosome in the G_0 or G_1 stage of cell cycle; whereas chromatid type aberrations such as breaks and gaps are produced during S or G_2 stage. In general, most chemical mutagens are S-dependent and therefore produce chromatid type aberrations. On the basis of scoring endpoints, the DNA materials are classified as (1) pynotic symptoms or cytotoxicity, (2) chromosomal disintegrity or clastogenicity, (3) segregational inaccuracy or aneugenicity, (4) genic changes or mutagenicity. Most of the studies deal

with clastogenic aberrations only. The breakage is caused in the DNA at the subchromatid, chromatid and chromosomal levels, while some of the breakages are repaired, others manifest themselves in the successive cell cycle in various forms, such as gaps, fragments, bridges and exchanges. The term polyploidy is used to refer to the state in which there are extra sets of chromosomes in the cell. Aneuploidy is the condition in which there is either an extra chromosome or a missing chromosome. Aneuploidy is the most common type of chromosomal disorder, occurring in approximately 4% of live births. Many physical and chemical agents are useful in polyploidization for crop improvement in plant breeding. Numerical and structural alterations in chromosomes are a defining characteristic of the cancer cell genome. Structural chromosomal rearrangements have received considerable attention for their role in tumorigenesis, whereas the role of numerical chromosomal changes in cancer is less clearly understood.

Keywords: Mutation; Chromosome; Mutagen; cell cycle; clastogens; aberrations; Aneuploidy; polyploidy

12.1. Introduction

Chromosomal aberrations (CA) are one of the significant biological consequences of human exposure to genotoxic agents and ionizing radiation. In epidemiological studies, it has been shown that people with elevated frequencies of CA in their peripheral blood lymphocytes have a significantly elevated risk of developing cancer (Bonassi *et al*., 1995, 2000; Hagmar *et al*., 1994,1 998a, 1998b). Chemical mutagens act in one of two ways; they are either radio mimetic in that they actually produce chromosome structural changes in different stages of the cell cycle or they induce only chromatid aberrations and require an intervening round of chromosomal DNA replication for clastogenic action (Kihlman *et al*., 1971). Most chemical agents produce only the chromatid type of aberrations via replication of damaged chromosomal DNA (Brewen and Payne, 1976, 1978). Metaphase chromosomes have a highly coiled and condensed structure that is microscopically visible. Part of a wide spectrum of DNA changes is generated by the action of mutagens. CA breakpoints occur preferentially in the active chromatid (Obe *et al.*, 2002). CA are a small fraction of a huge amount of changes in chromosomal DNA and reflect the enormous plasticity of the genome, which has far-reaching consequences for evolution (Caporale 1999). CA are induced by

agents that damage chromosomal DNA (Natarajan, 1976; Roberts, 1978; Singer and Grunberger, 1983).

12.2. Mutagens

H. J. Muller (1927) first reported that X-rays could induce mutations in *Drosophila.* This was followed by C. J. Stadlers who found that the same was true in the plant *Hordeum vulgare*. A large number of agents or factors have since become available to induce mutations. Any agent which can alter the basic structure or sequence of DNA is called a mutagen. Mutagens can be found in any part of the environment. The word 'mutagen' comes from the word part 'gen,' which is found in many scientific terms. 'Gen' means the 'origin' or 'creation' of something. All mutations are changes in the nucleotide sequence of DNA. Chromosome mutations involve large portions of DNA. UV radiation is one of the mutagens that exists in our environment. Other mutagens include X-rays, gamma rays, extreme heat, or chemicals that react with DNA molecules. These agents can change the way molecules bond and react with one another, which increases the likelihood that a mistake will be made in the nucleotide sequence. The mutagenic agents may be classified into the following two broad groups: physical mutagens and chemical mutagens.

12.2.1 Physical mutagens

Different types of radiation which have mutagenic properties fall under the physical mutagens group. These consist of high energy radiation that could easily penetrate living cells and affect the genetic material. The effect of radiation on living cells and tissues is directly proportional to the degree of penetration of the radiation. The radiation is a part of the electromagnetic spectrum having a shorter wavelength and higher energy than visible light, such as UV rays, X-rays, gamma rays and cosmic rays or particulate radiation produced by the decay of radioisotopes.

When someone watches a black and white television for an hour, he/she will be hit by 1 mrem radiation. If they watch a color television, the effect is doubled, If someone is diagnosed by X-ray, they will be hit by 150 mrem radiation, and every time someone gets their teeth portrayed, they get hit by 20 mrem radiation, Note that 1 to 2 dosages of mrem is already able to induce mutation.

12.2.1.1 Radiation

Radiation was the first mutagenic agent discovered and its effects on DNA were first reported in the 1920s. Roentgen discovered X-rays in 1895, Becquerel discovered radioactivity in 1896, and Marie and Pierre Curie discovered the radioactive elements in 1898 (Evans *et al*., 1978; Takahashi *et al*., 1982). The most significant forms of radiation are light, heat, and ionizing radiation. Electromagnetic radiation such as X-rays and ultraviolet rays have more penetration into cells and tissues. The penetration power of electromagnetic radiation is inversely proportional to the wavelength. Radiation is grouped into two classes on the basis of its effects. (i) Ionizing radiation causes ionization in the atoms present in its path. The types of ionizing radiation are: (1) particulate and (2) non-particulate radiation. Particulate radiation consists of high energy, atomic particles generated due to radioactive decay. The non-particulate ionizing radiation is represented by X-rays and gamma rays. (ii) Non-ionizing radiation: Ultraviolet rays are the only non-ionizing radiation with mutagenic properties. The wavelength ranges from 100–3900A^{o} and they are specifically absorbed by purines and pyrimidines in DNA. The maximum absorption of UV rays is by DNA as well as by pyrimidines and particularly thymine which occurs at the wavelength of 254 nm (Tedesco *et al*., 1997).

The genetic effects of radiation are either direct or indirect. The direct effect of radiation is produced due to ionization directly in the DNA molecule, while their indirect effect is produced through ionization of molecules other than DNA and is believed to be mediated by free radical formation. Natural sources of radiation include cosmic rays from the sun and outer space, radioactive elements in soil and terrestrial products (wood, stone) and in the atmosphere (radon). Humans have created artificial sources of radiation such as X-rays, nuclear testing and power plants.

X-rays and particulate radiation provide high energy radiation when they pass through cells and tissues and they collide with molecules such as water and cause the expulsion of electrons, creating a positively charged ion. The ejected electron cannot remain in the free state and therefore is picked up by another ion, creating a negative ion. These are highly reactive chemicals that may act on genes, chromosomes and other parts of the cells produced by these ions when combining with oxygen. The splitting of water in the presence of oxygen results in mutagenic peroxides.

12.2.1.2 Chemical Mutagens

Charlotte Auerbach in 1942 showed that nitrogen mustard could cause mutations in cells. This poisonous mustard gas was used in World War I and II. Since that time, many other mutagenic chemicals have been identified in food additives, industrial chemicals, etc. (Hollaender, 1971). A large number of chemicals are known to cause mutation, employing different pathways. On the basis of their specific reaction with DNA, these chemical mutagens are classified into four major groups.

12.2.1.2.1 Base analogs

During DNA replication, base analogs structurally resemble purines and pyrimidines and may be incorporated into DNA in place of the normal bases, leading to substitution mutations. They also exist in two alternative forms i.e., keto or enol form of amino and imino form, and change spontaneously from one to another form as the normal bases. For example, 5′ Bromouracil (BU), an artificially created compound extensively used in research, resembles thymine because it has Br in the 5^{th} position, instead of the methyl group and has the same effect on its base-pairing behavior, 5 BU behaves like thymine and usually pairs with adenine. 5′aminopurine is an adenine analog which can pair with T or with C, causing A:T to G:C or G:C to A:T transitions.

Because of their constant likelihood of mispairing, each of these chemicals will continue to mutagenize with time. It requires subsequent rounds of replication for any mutation to be generated since this requires "mispairing" during replication. Before both strands of DNA have the "mutant information," it takes another round of replication before the mutation is stabilized. The mismatch repair system can still recognize and remove the inappropriate base until that occurs. This is termed "mutation fixation."

12.2.1.2.2 Chemicals alter the structure and pairing properties of bases

Nitrous acid is formed by the digestion of nitrites (preservatives) in foods. It causes C to U, C to T, and A to hypoxanthine deaminations. These chemicals react directly with certain bases and thus do not require active DNA synthesis in order to act, but still do require DNA synthesis in order to be "fixed". They are powerful mutagens in nearly every biological system since they are very commonly used. Examples of alkylators include ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS),

diethylsulfate (DES), and nitrosoguanidine (NTG, NG, MNNG). These mutagens tend to prefer G-rich regions, reacting to form a variety of modified G residues, the result often being depurination (Lawley and Thatcher, 1970). Some of these modified G residues have the property of inducing error-prone repair, although, mispairing of the altered base might also be possible. It also appears that alkylated bases can mispair during replication. The relative contribution of all of these mechanisms to actual mutagenesis is unclear.

12.2.1.2.3 Intercalating agents

Like insertion mutations, intercalating agents are compounds that can insert themselves between nucleotides in a DNA molecule. They may result in a frameshift, which can alter the codon reading frame and result in aberrant DNA transcription and replication, where this occurs, DNA polymerase may add an additional base opposite the intercalating agent. If this occurs in a gene, it induces a frameshift mutation (that means it alters the reading of the gene transcript, changing the amino acid composition to the encoded protein). Ethidium bromide is one such agent, widely used in gel electrophoresis, for instance, to find the DNA bands that have been separated on a gel. Molecular biologists are trained to handle this chemical carefully because of the mutagenic effect.

This intercalation distorts the shape of the DNA helix, which can cause the wrong bases to be added to a growing DNA strand during DNA synthesis. Intercalating agents tend to be flat, planar molecules such as benzo[a]pyrene, a component of wood and tobacco smoke. Thousands of birth defects occurred because the intercalating agent was in some preparations of thalidomide, an anti-nausea drug given to some pregnant women. The following agents interact with bases of DNA and insert between them: acridine orange, proflavin and ethidium bromide which are flat, multiple ring molecules.

12.2.1.2.4 Chemicals altering DNA structure

A large number of molecules that bind to bases in DNA and cause them to be noncoding are referred to as "bulky" lesions. Agents, such as psoralens for example, cause intra- and inter-strand crosslinks. Chemicals such as peroxides cause DNA strand breaks. There are a number of chemicals present in the environment which are potentially mutagenic. Parasitic fungi of field crops, mushrooms, certain vegetables and medicinal herbs are major sources of natural mutagenic agents. Environmental mutagens

include air and water pollutants, food additives and preservatives, agricultural chemicals, cosmetics, drugs, pesticides, cigarettes and industrial products such as benzidine, vinyl chloride, and asbestos.

12.2.2 Chromosomal aberrations are due to DNA damage and repair

DNA double-strand breaks (DSB) are the principal lesions in the process of CA formation (Bryant, 1998; Natarajan, 1978; Obe *et al.,* 1992). DSBs arise spontaneously at quite significant frequencies through a variety of cellular processes and can be directly induced by ionizing radiation, certain antibiotics, or endonucleases (Obe *et al*., 1992, Pfeiffer, 2000). Sources of spontaneously induced DSBs are DNA replication and DNA excision repair by accumulating single-strand breaks (Caldecott, 2001; Dianov and Neill, 2001), transposition (Kooistra *et al*., 1999), VDJ-recombination (Errami *et al*., 1996; Grawunder *et al*., 1998; Kienker *et al*., 2000), antibody class switching (Manis *et al.*, 1998), mitotic recombination (Bergerat *et al*., 1997; Keeney and Kleckner, 1995; Keeney *et al*., 1997) and oxidative damage (Newcomb and Loeb, 1998).

UV radiation and a majority of chemical mutagens are not able to induce DSB directly but lead to other lesions in chromosomal DNA, either during repair or DNA synthesis, which may give rise to DSB and eventually to CA. If left unrepaired, the DSB may lead to broken chromosomes. Unrepaired DSB but also CA may be cell lethal in interphase or mitosis, respectively. If repaired improperly, the DSB may lead to mutations, chromosome rearrangements, and oncogenic transformation. DSB is repaired by at least three different mechanisms in eukaryotic cells. (i) Homologous recombination repair (HRR), precisely restores the original sequence at the break and is a highly accurate process; (ii) single-strand annealing (SSA), which leads to the formation of mainly interstitial deletions; (iii) nonhomologous DNA end joining (NHEJ) which joins two broken ends directly and usually generates small scale alterations such as base-pair substitutions, insertions and deletions at the break site. HRR is generally initiated by one single DSB to generate both correct intrachromosomal repair products, and incorrect exchange type CA that is the two DNA ends of a single DSB interact with the two strands of a homologous unbroken DNA duplex. DSB between two direct repeat sequences can be repaired by SSA which leads to the deletion of one repeat unit and the intervening sequence. The repair products usually contain small scale changes NHEJ and deletions SSA in contrast to the

restitution generated by HRR. To generate an exchange type CA, both NHEJ and SSA require at least two initial DSBs that means the four ends of two DSBs interact cross-wise with each other. The repair pathways mentioned before compete actively for the repair of a DSB. In the regulation of these pathways what circumstances determine the pathway which is used is still mysterious but appears to occur at different levels during development and cell cycle progression (Haber, 2000). Likewise, it is not known to what extent a given DSB repair process contributes to the formation of a certain type of CA. A single DSB is sufficient for homologous recombination (HR) while at least two initial DSBs are required for NHEJ and SSA. In most cases, at least as long as the number of initial breaks remains small, DSB is likely to be repaired correctly or lead to small scale DNA alterations in the range of a few base pairs or kilobases. In some cases, DSB may lead to large scale alterations visible as CA under the light microscope.

12.2.3 Kinds of chromosomal aberrations

Abnormalities in the structure are formed by either chromosomal breakage or unequal crossing over, resulting in deletions, ring chromosomes, duplications, translocations, insertions and inversions (Fig. 12.1). A single break in one of the chromatids will produce a terminal deletion, whereas two breaks in two chromatids can result in an interstitial deletion or an inversion. When a chromatid is lost or gained in the process, the rearrangement is unbalanced (Jacobs, 1977; Borgaonkar, 1994; Schinzel, 1994; Moore and Best, 2001). The unbalanced nature results in developmental delay, poor growth and birth defects (Savage, 1976). Structural abnormalities acquired after birth may cause tumors or leukemia by altering the cell cycle (Arlett and Lehmann, 1978). These structural aberrations make up a significant portion of chromosomal genetic diseases (Savage, 1976, 1991; Obe *et al*., 2002).

Balanced or unbalanced chromosomal aberrations may be inherited by offspring from parents as de novo rearrangements, to be formed in a single gamete or zygote. There is a low risk of physical or mental impairment resulting from balanced chromosomal aberrations. Sometimes when the abnormality occurs as a de novo event, the risk for genetic disease or phenotypic effects is increased, even though the parents have a normal karyotype. This is due to either submicroscopic deletion or duplication at the breakpoints or from functional genes occurring near or on the break points (Evens and O'Riordan, 1975; Ishidate *et al*., 1998; Kirkland, 1998; Van Gent *et al*., 2001; Moore and Best, 2001).

12.2.3.1 Deletions

A portion of chromatin from a single chromosome that is lost is called a deletion. A deletion results in partial monosomy and unbalanced rearrangements (Neal *et al*., 2006). Single breaks cause terminal deletions with a subsequent loss of the single chromatid. This is called a terminal deletion (Jablonicka *et al*., 1989). When two breaks occur in the same arm of a chromosome, interstitial deletions are formed by a loss of the chromatin between the breaks and a rejoining of the remaining segments. Deletions can be scored by using light microscopy to represent the loss of many genes that are physically located in the same band or region of the chromosome and result in monosomy (Chen *et al*., 2004). Deletions that survive to birth are associated with a very high risk of birth defects and intellectual impairment. If the deletion occurs in tumor suppressor genes or oncogenes a high risk of cancer and/or leukemia is conferred (Palanikumar and Panneerselvam, 2008; Moore and Best, 2001).

When a break occurs in a single chromatid, it is called a chromatid break. If the break occurs in both chromatid arms it is called an isochromatic break. When the break is smaller than the chromatid width, then this is called a chromatid gap or isochromatic gap. Normally the gap is too small and can be repairable (Jablonicka *et al*., 1989).

12 2.3.2 Terminal deletions

In human chromosomes, many terminal deletions cause well-described syndromes (Griffith, 2000). If a single break occurs capping of the broken end with a telomere results (Fig.12.2). One of the earliest and best-delineated syndromes due to a terminal deletion is the cri-du-chat syndrome with loss of part of the short arm of chromosome 5. This is due to a very small deletion involving a break at band 5p15.2 (Tyagi *et al.*, 2010). The infant has a round face with wide-set eyes, but the older child and adult develop an elongated asymmetrical face. There is severe intellectual impairment. Individual case reports of terminal deletions have been reported for most of the human chromosomes (Rooney, 2001). The most common terminal deletion is the end of the short arm of chromosome 4, which results in intellectual impairment or developmental delay, microcephaly, large, simply folded ears, clefting of the lip and palate, external genital abnormalities, and characteristic facial features (Jefferson *et al*., 1986).

12.2.3.3 Interstitial Deletions

The banding technique led to the discovery of many syndromes that are due to small interstitial deletions (Fig.12.3). Interstitial deletions cause two breaks with loss of the interstitial segments (Klein *et al*., 2007). Partial monosomies caused by interstitial deletions can produce severe abnormalities and death of the embryo (Leelavathy *et al.,* 2011). Only embryos with small deletions are likely to survive. It is difficult to analyze conventional cytogenetic techniques, and many small interstitial deletions probably go undetected. In chromosome 15 one interstitial deletion that has been studied extensively is a deletion just below the centromere. This deletion is found in two distinct and clinically very different syndromes, Prader–Willi (PWS) and Angelman syndromes (AS) (Buiting, 2010). PWS is characterized by intense hyperphagia, obesity, poor muscle tone, hypoplastic genitalia and moderate intellectual impairment, while AS is associated with ataxia, severe intellectual impairment, delayed or absent speech, spontaneous outbursts of laughter and characteristic facial features.

12.2.3.4 Ring Chromosomes

A ring chromosome is formed from two terminal deletions (Fig.12.4). There is a break in both the short arm and the long arm, with the fusion of the ends of the centromeric segment and loss of the two terminal segments (Jablonicka *et al*., 1989; Sessink *et al*., 1994; Demily *et al*., 2014). Ring chromosomes represent a type of terminal deletion and are mitotically unstable during replication. Three forms of ring chromosome are common: (i) large rings with minimal loss from the terminal segments of the short and long arms; (ii) very small rings as extra chromosomes in the karyotype; (iii) rings formed from the X chromosome, which is generally found in females with features of Turner syndrome (Sybert and McCauley, 2004).

12.2.3.5 Microdeletions (Contiguous Gene Syndromes)

Microdeletion is a special category of interstitial deletions because of their small size which often escapes from conventional cytogenetic methods. Microdeletions are referred to as contiguous gene syndromes because they involve the loss of a series of closely linked genes having similar characters (Kodama *et al.,* 1997). There may be variations in the size of the deletions in different patients, but there are considerable similarities in the physical features of patients, related to the overlap of deleted

chromosomal segments (Schwartz and Graf, 2002). Contiguous gene syndromes may also be the result of small duplications. A list of some common contiguous gene syndromes due to microdeletions or duplications is given in Table 12.1. These deletions may involve only 1–2Mb of deoxyribonucleic acid (DNA) or less, in the same chromosomal band, and are rarely visible at the microscopic level. The fluorescence in situ hybridization technique could be useful to detect the microdeletion.

An example of a common interstitial deletion is the deletion within band 22q11.2 that is related to conotruncal heart malformations, hearing loss, calcium metabolism defects, dysmorphic facial features, and developmental delay or intellectual impairment (Adeyinka, 2004). Both the DiGeorge sequence and Velocardiofacial syndrome are associated with microdeletions of this region and are thought to be different manifestations of the same genetic deficiency. It is important to recognize that these deletions may be carried in the heterozygous state in an unaffected or very mildly affected parent as well as in the more severely affected offspring. Thus they present a significant risk for recurrence in future offspring.

12.2.3.6 Duplications

Duplications are unbalanced rearrangements which result in partial trisomy (Patel *et al*., 2006). Duplications are primarily from unequal crossing over (Fig.12.5), especially in regions of the genome where repeat DNA sequences are found. Direct duplications retain the same order of gene loci and chromosome bands in relation to the centromere as the parent chromosome, but inverted duplications show a complete reversal of loci and bands contained in the duplication. Duplications on one chromosome produce partial trisomies when paired with a normal chromosome in a diploid cell. One example is an inverted duplication of a segment of the long arm of chromosome 15, which is generally observed as an extra dicentric chromosome (Roback *et al*., 1991). The second example is a duplication of the proximal long arm of chromosome 22 as an extra dicentric chromosome (cat eye syndrome) is also relatively common and is associated with coloboma of the eye, intellectual impairment and anal atresia (Zannotti *et al.,* 1980).

12.2.3.7 Microduplications

Microduplications are very much rarer than microdeletions and represent another type of contiguous gene syndrome. The best known of the microduplication syndromes occurs on the short arm of chromosome 11

(within band p15.5) which results in Beckwith–Wiedemann syndrome with high birth weight, omphalocele and overgrowth of the tongue (Heyningen *et al*., 1985).

Microduplications, or submicroscopic duplications, are chromosomal duplications that are too small to be detected by using conventional cytogenetics methods. Specialized testing is needed to identify these duplications. Microduplications are typically one to three megabases (Mb) long and involve several contiguous genes. The exact size and location of a microduplication that causes a syndrome may vary, but a specific "critical region" is consistently involved. The phenotype of microduplication syndromes is due to changes in a few critical dose-sensitive genes, or in some cases, a single gene, often less clear and less well defined. In addition, some microduplication syndromes may be inherited from apparently normal parents raising important issues regarding incomplete penetrance and ascertainment bias in these newly described clinical entities.

12.2.3.8 Translocations

Translocations involve breaks in two different chromosomes with an exchange of segments (Jablonicka *et al*., 1989; Sessink *et al*., 1994). There are many structurally different types of translocations, as with inversions, there is no loss of genetic material, although the breakpoint can cause disruption of a critical gene or juxtapose pieces of two genes to create a fusion gene that induces cancer. In general, however, the problem with translocations occurs during meiosis and manifests as reductions in fertility. In humans, there are two major types of translocation: reciprocal translocations in which there is no visual loss of chromatin, and Robertsonian translocations in which the long arms of two acrocentric chromosomes are joined with loss of the two short arms (Brown *et al*., 1993; Savage, 1993; Simpson and Savage, 1994).

12.2.3.9 Reciprocal Translocations

Reciprocal translocations are characterized by an exchange of chromatin between different chromosomes. A single break occurs in each chromosome, and the noncentric segments are exchanged without the visible loss of any chromatin (Fig.12.6). However, the two new derivative chromosomes may have very different morphologies depending on the breakpoints. The carrier of a reciprocal translocation generally has no phenotypic effects due to the rearrangement except for possible

reproductive abnormalities including infertility, spontaneous abortions and abnormal offspring (Jalbert *et al*., 1980; Brown and Kovacs, 1993).

The pairing of homologous pairs at meiosis is altered in translocation carriers. The two derivative chromosomes and their two normal homologous pairs form a cross-shaped quadrivalent at pachytene with each homologous segment pairing with its counterpart. Each chromosome consists of two chromatids and, thus, at each point, the quadrivalent consists of four chromatids as pairing and segregation take place after DNA replication. There are four basic segregation patterns from a reciprocal translocation quadrivalent. In most cases, two chromosomes move to one daughter cell and two to the other; in rare situations, three chromosomes segregate together, leaving one to move alone (Jacobs, 1977; Jalbert *et al*., 1980).

12.2.3.10 Adjacent I Segregation

Adjacent nonhomologous centromeres move to the same pole. A zygote with partial trisomy of one chromosome and partial monosomy of the other is because of an unbalanced chromosomal complement. The amount of alternate segregation as always equal to the amount of adjacent I segregation. Since to the cell they both involve separating homologous centromeres. If the breakpoint is very far from the centromere, the cell does not become confused and only alternate and adjacent I segregation will be observed.

12.2.3.11 Adjacent II Segregation

Adjacent homologous centromeres moving to the same pole will result in large amounts of unbalanced chromatin. The amount of adjacent II segregation depends upon how far the breakpoint is located from the centromere. If the breakpoint of translocation is located very near the centromere it is sending to which pole, and a significant amount of adjacent II segregation will occur.

12.2.3.12 Robertsonian Translocations

W.R.B. Robertson, a cytogeneticist studied numerical chromosome changes in several orthopteran populations (Robertson, 1916). The formation of a Robertsonian translocation actually results from breaks in the short arm, in the long arm or within the centromere of the two chromosomes that form the ‘fusion’ product (Fig.12.7). The resulting derivative chromosome may be either monocentric or dicentric, depending

on the position of the breaks and exchange of chromatin segments. During meiosis, Down syndrome, or trisomy 21 is usually the result of a nondisjunction event. Because of a translocation between chromosome 14 and 21, or between 22 and 21, approximately 4% of Down individuals actually are affected. For every Down child born, five normal and five balanced translocation heterozygotes are seen. Since 80% of trisomy 21 fetuses are spontaneously aborted, the theoretical risk of a translocation heterozygote producing a Down offspring is 1/3.

12.2.3.13 Inversions

Inversions are formed by two breaks in the same chromosome with the exchange of the two ends. Inversion is thus essentially formed in the same manner as translocations, except that the breaks and exchange occur in the same chromosome. Two types of inversion are found. One break occurs in each arm of the chromosome and, thus, the centromere is included in the inverted segment of the pericentric chromosome (Fig.12.8). Due to the movement of the centromere, the banding patterns change and also the shape of the chromosome may change. Alternatively, a paracentric chromosome is formed when both breaks occur in the same arm and, therefore, the centromere is not included in the inverted segment (Fig.12.9). This alters the banding patterns, but not the shape of the chromosome.

The major difference between pericentric and paracentric inversions involves the position of the centromere in the recombinant products. Since the region within the inversion loop remains balanced, the recombination products of the pericentric inversion each retain a single copy of the centromere and disjoin normally during mitosis. In contrast, because the region outside the inversion loop is either duplicated or deleted, the recombination products from the paracentric inversion receive either two copies or no copies of the centromere.

12.3 Numerical changes in chromosome

Among chromosomal variations, the changes in number can be easily observed. There are two types, one is euploid variation, which involves changes in the number of entire sets of chromosomes and the other type are aneuploid variations, which involves changes in a single chromosome within a set. Most sexually reproducing organisms in eukaryotes are diploids (2n), but various algae and fungi exist as haploids throughout their life cycle. Bridges in 1916 discovered *Drosophila* lacking an X

chromosome (XXY females) which gave rise to a serious of study of aneuploid organisms.

12.3.1 Monoploids

The monoploid organisms have one genome (n) in their body cells and when it occurs in gametes (sperms and eggs) it is termed haploidy. Most micro-organisms such as bacteria, fungi and algae; the gametophytic generation of bryophytes; the sporophytic generation of some angiospermic plants such as *Sorghum*, *Triticum*, *Hordeum*, *Datura*, etc and certain hymenopteran male insects for example wasps, bees, etc have one genome in their body cells. Compared to their diploid prototypes monoploids are usually smaller and less vigorous. Characteristically, monoploid plants are sterile, subsequently, the chromosomes have no regular pairing partners (homologous chromosomes) during meiosis, and meiotic products are deficient in one or more chromosomes. Monoploids may originate in several ways:

(1) Male bees, wasps, and ants are monoploid. In the normal life cycles of these insects, males develop from unfertilized eggs by the process of parthenogenesis.

(2) The anther technique for producing monoploids does not work in all organisms or in all genotypes of an organism. Barley, an important crop plant, has developed a useful technique. Diploid barley, *Hordeum vulgare,* can be fertilized by pollen from a diploid wild relative called *Hordeum bulbosum. Z*ygotes result with one chromosome set from each parental species with this fertilization. In the ensuing somatic cell divisions, however, the chromosomes of *H. bulbosum* are eliminated from the zygote, whereas all the chromosomes of *H. vulgare* are retained, resulting in a haploid embryo.

(3) Development of more than one embryo in the seed is known as polyembryony. Development of haploids through polyembryony has been reported in *Linum*, pepper and several horticultural crops.

(4) Semigamy is a form of apomixis in which the sperm nucleus enters the egg cell but fails to fuse with the egg nucleus. In semigamy, the sperm and egg nuclei develop independently resulting in the formation of seeds with maternal and paternal chimeras of haploid tissues, for example, Egyptian cotton (*Gossypium borbadense*).

12.3.2 Aneuploidy

Aneuploidy is another major category of chromosome mutations in which the chromosome number is abnormal. An individual organism whose chromosome number differs from the wild type by part of a chromosome set is called aneuploid.

Each time a cell divides it must duplicate the entire genome and distribute one copy of each chromosome into each daughter nucleus. Millions of cell divisions occur every minute in the adult human. Therefore the maintenance of a diploid chromosome content requires that each chromosome is segregated with high fidelity during every division. Aneuploidy occurs as a result of errors in chromosome partitioning during mitosis.

There are two types of aneuploidy. One is hypoploidy and the other one is hyperploidy.

Hypoploidy: Loss of one or two chromosomes from a diploid set.

(a) **Monosomic**: Loss of one chromosome from one pair ($2n - 1$) or from two different pairs ($2n - 1 - 1$).
(b) **Nullisomic**: Loss of one chromosome pair ($2n - 2$)

Hyperploidy: Addition of one or two chromosomes to one pair or two different pairs.

(a) **Trisomic**: Addition of one chromosome to one pair ($2n + 1$) or two different pairs ($2n + 2 + 2$)

Aneuploids are useful tools for locating genes on a specific chromosome in wheat, cotton, tobacco, oats and other crops. Aneuploids are used for developing alien additions and alien desirable genes from one species to another.

12.3.2.1 Consequences of Aneuploidy

Aneuploidy in the germline presents a significant barrier toward successful organismal development. In humans, aneuploidy is the leading cause of miscarriage and mental retardation (Hassold *et al*., 2007; Brown, 2008). Most congenital aneuploidies arise from errors in chromosome segregation in maternal meiosis I (Hassold *et al*., 2001). Only three autosomal trisomies are viable and other human autosomal monosomies are lethal. Trisomy of chromosome 13, 18 and 21, are the smallest human

chromosomes regarding the number of genes they encode. Of these viable trisomies only Down syndrome patients—who are trisomic for chromosome 21—survive until adulthood. Many studies have shown that aneuploidy is deleterious for cellular growth *in vitro*. An array of aneuploid yeast strains carrying one or more additional chromosomes proliferates more slowly than euploid strains under normal growth conditions, showing a G_1 cell cycle delay and increased uptake of glucose (Torres *et al*., 2007; Pavelka *et al*., 2010; Niwa *et al*., 2006). Fibroblasts derived from human patients with Down syndrome proliferate more slowly than non-isogenic diploid control cells (Segal and McCoy, 1974). Gains and losses of whole chromosomes concurrently alter the copy number of hundreds of genes. As most genes on aneuploid chromosomes are transcribed and translated, the production of additional proteins is responsible for the inhibitory effect of aneuploidy on cellular growth *in vitro* (Torres *et al*., 2007, 2010; Pavelka *et al*., 2010; Williams *et al*., 2008). Aneuploidy is caused by chromosome segregation errors in mitosis, whereas structural chromosomal alterations are produced by inappropriate repair of DNA double-strand breaks (Obe and Durante, 2010).

12.3.3 Diploidy

Two genomes (2n) in each somatic cell of the diploid organisms is the characterization of diploidy. Most animals and plants are diploids. The diploidy is related to fertility, balanced growth, great vigorosity, adaptability and survivability of the diploid organisms.

12.3.4 Polyploids

Polyploids contain more than two genomes in the organisms. Among plants and animals, polyploidy occurs as triploidy (3n), tetraploidy (4n), pentaploidy (5n), hexaploidy (6n), heptaploidy (7n), and octaploidy (8n), respectively. In natural populations, it is not common to see ploidy levels higher than tetraploid. But most of our important crops and ornamental flowers are polyploid, e.g., wheat (hexaploid, 6n), strawberries (octaploid, 8n), many commercial fruit and ornamental plants.

Polyploidy is most common among angiosperms and some of the economically important polyploid angiospermic plants are peanuts, oats, coffee, strawberry, cotton, barely, sweet potato, apple, alfa-alfa, banana, tobacco, plum, sugar cane, potato, sorghum, clover and wheat.

12.3.4.1 Origin of Polyploidy

(1) Irregularities in a new generation of plants because somatic doubling-cells sometimes undergo irregularities at mitosis and give rise to meristematic cells.

(2) Reproductive cells may have an irregular reduction or equation division in which the sets of chromosomes fail to separate completely to the poles at anaphase and thus become incorporated in the same nucleus resulting in the doubling of chromosome number in the gamete.

(3) By exposure to chemicals such as colchicine, etc., or heat or cold the somatic doubling of the genome is accomplished either spontaneously or it can be induced in high frequency.

12.3.4.2 Incidence of Polyploidization

Polyploidization is the increase in genome size caused by the inheritance of an additional set of chromosomes (Figure 12.1). The duplicated sets of chromosomes which may originate from the same or a closely related individual is called "**autopolyploid**" or from the hybridization of two different species it is known as "**allopolyploidy**". With duplicated sets of chromosomes that share homology but are adequately distinct due to their separate originsthese pairs of chromosomes are known as homeologs when polyploidization is involved (Figure 12.1). Polyploidy is specifically widespread among hybrid taxa, an association thought to be driven by problems with meiotic pairing in diploid hybrids, which are solved if each homeologous chromosome has its own pairing partner. Moreover, diploid hybrids form unreduced gametes contain the same number of chromosomes as somatic cells at unusually high rates (Ramsey and Schemske, 2002), increasing the rate of formation of polyploids from hybrid lineages. By joining traits from two parental species and ensuring fair segregation of these traits, allopolyploids potentially benefit from "hybrid vigor", where hybrids have characteristics that make them superior to both parental species and an altered ecological niche without the problems associated with segregation and breakdown at the F_2 generation that occurs among diploid hybrids.

Mutations affecting ploidy occur relatively frequently in both plants and animals. Plants produce unreduced gametes, a common route to polyploidization, at an average rate of ~0.5% per gamete (Ramsey and Schemske, 1998). The production of polyploid animals contributes by both unreduced gametes and polyspermy. Among chicken embryos, 0.9% are

triploid or tetraploid (Bloom, 1972), and among spontaneous human abortions, 5.3% are triploid or tetraploid (Creasy *et al*., 1976). Gene duplication events are much rarer, with a roughly 10−8 chance of occurring per gene copy per generation (Lynch, 2007). Aneuploids were four times more common than polyploids among the aborted fetuses examined by Creasy *et al.* (1976). In various plant genera, the rate at which polyploids arise and persist is on the order of 0.01 per lineage per million years, roughly 1/10th the rate of speciation (Meyers and Levin, 2006). One would expect a large fraction of plant species to have undergone polyploidization at some point in their past evolution with such a high rate of polyploidization per speciation. Previous studies had suggested that polyploidy occurred sometime in the past in 57% (Grant, 1963) to 70% (Goldblatt, 1980; Masterson, 1994) of flowering plants, based solely on chromosome numbers. Recent genomic analyses indicate that an early polyploidization event may predate the radiation of flowering plants (Bowers *et al.*, 2003), suggesting that 100% of angiosperms are paleopolyploid. Although polyploidization is less prevalent in animals, nearly 200 independent examples of polyploidy have been reported in insects and vertebrates, with many more cases known among other invertebrate groups (Gregory and Mable, 2005).

12.3.4.3 Immediate Effects of Polyploidization

(a) Generally, genome size increase will result in an increase in cell volume (Cavalier-Smith, 1978; Gregory, 2001), while the particular relationship between ploidy and cell volume varies among environments and taxa. (b) Increasing adult body size in plants and invertebrates more than in vertebrates is caused by polyploidization (Gregory and Mable, 2005; Otto and Whitton, 2000). (c) A surprising feature of many newly formed polyploids is that their genomes are unstable and undergo rapid repatterning (Wendel, 2000). For example, Song *et al*. (1995) in newly created polyploid *Brassica* hybrids, observed extensive genomic rearrangements and fragment loss within five generations. (d) In addition to structural changes, polyploids often exhibit changes in gene expression (Liu *et al.,* 1998). This is especially true of allopolyploids, which exhibit changes in methylation (Salmon *et al*., 2005), alterations in imprinting (Josefsson *et al*., 2006), and biased expression of homeologs (Udall *et al*., 2006).

12.3.4.4 Induction of Polyploidy

Polyploidy can be induced in common diploid organisms by the following methods:

A. Cell generation – In mosses (Bryophytes), polyploidy has been induced by cutting their diploid sporophytes and keeping the sporophytes in moist conditions. The cells of the cut end regenerated threads which were true protonema and produced diploid gametophytic generation instead of monoploidic generation.

B. Physical agents –

(i) Temperature shocks - Extreme temperature changes sometimes result in a higher frequency of polyploid cells.

(ii) Centrifugation - Centrifugation can cause polyploidy in the cells of plant seedlings, eg. *Nicotiana*.

(iii) X-rays - X-rays have been found to induce polyploidy in normal diploid plant cells.

C. Chemicals – Chemicals such as colchicine, chloral hydrate, acenaphthene, veratrine, sulfanilamide, ethyl, mercury chloride. Polyploidy has been induced in plants by hexachlorocyclohexane. These chemical substances disturb the mitotic spindle and cause non-segregation of previously duplicated chromosomes and therefore, convert the diploid cells into tetraploid cells.

12.3.4.5 Kinds of Polyploidy

There are two kinds of polyploidy

(i) Allopolyploids

(ii) Autopolyploids

(i). Allopolyploidy- The prefix "allo" indicates that nonhomologous sets of chromosomes are involved. A polyploid organism that originates by combining complete chromosome sets for two or more species is known as allopolyploid or alloploid and such a condition is referred to as allopolyploidy. Hybrid polyploids or bi-species or multi-species polyploids are also known as alloploids. An allopolyploid which arises by combining genomes of two diploid species is termed **allotetraploid** or

amphidiploid. Alloployploidy can be developed by interspecific crosses and fertility is restored by chromosome doubling with colchicine treatment. Alloploidy has played a greater role in crop evolution then autopolyploidy.

P_1: Species X × Species Y

(AA) ↓ (BB)

(Diploid) (Diploid)

F_1: AB

Diploid sterile hybrid

↓ Colchicine

AABB

Fertile amphidiploid tetraploid

Like a new species in appearance and behavior, produced by the union of unreduced or diploid or polyploid gametes from different diploid or polyploid species could produce in one step an amphipolyploid or allopolyploid. Let A represent a set of the genome in species X, and let B represent another genome in a species Y. The F_1 hybrids of these species then would have one A genome and another B genome. By treating them with colchicine the F_1 diploid but sterile hybrids can be converted into fertile allotetraploids.

Uses of Alloploidy

1. Alloploidy plays an important role in tracing the origin of natural allopolyploids. The study of chromosome pairing in a cross between an allopolyploid and a diploid species enables tracing of the origin of the polyploidy species.

2. Alloploidy sometimes leads to the creation of new crop species. Triticale is the best example, which is allopolyploid between wheat and rye. It combines desirable characters of both the species, *i.e.*, the grain quality of wheat and the hardiness of rye. Triticales are of two types, viz. primary triticales and secondary triticales. Primary triticales are derivatives of a cross involving either tetraploid wheat or hexaploid wheat with rye. Secondary triticales are derivatives of the cross either between two primary triticales or between primary triticale and wheat.

3. The interspecific gene transfer can be carried out in two ways, viz. by alien addition and alien substitution. In the case of alien addition, one chromosome of the wild species is added to the normal complement of the cultivated species. In the case of alien substitution, one pair of the chromosome is substituted in the cultivated species with those of the wild donor species. Such types of gene transfer are generally made for disease resistance. This type of gene transfer has been achieved in crops such as wheat, tobacco, cotton and oats.

B. Autopolyploids

The ploidy that involves only homologous chromosome sets is indicated by the prefix "auto". Four sets of genomes of a tetraploid produced by the somatic doubling of a diploid and likewise, eight sets of genomes of an octaploid produced by the somatic doubling of a tetraploid. The combination of unreduced diploid or tetraploid gametes from the same species would accomplish the same result.

Seedless varieties of economically important plants have been produced by autotriploid, therefore it has great commercial value. For example, in Japan, H. Kihara produced seedless watermelons, which were autotriploids.

Origin of Autopolyploids

Autopolyploids naturally occur at a low frequency and can be induced artificially using various techniques, such as heat and chemical treatments, decapitation, and selection from twin seedlings.

Triploids

Triploids are usually autopolyploids. They are constructed by geneticists from the cross of a 4*x* (tetraploid) and a 2*x* (diploid) or they arise spontaneously in nature. The 2*x* and the *x* gametes unite to form a 3*x* triploid.

Triploids are characteristically sterile. The problem, like that of monoploids, lies in pairing at meiosis. Synapsis, or true pairing, can take place only between two chromosomes, but one chromosome can pair with one partner along part of its length and with another along with the remainder, which gives rise to an association of three chromosomes. Paired chromosomes of the type found in diploids are called bivalents. Associations of three chromosomes are called trivalents, and unpaired

chromosomes are called univalents. Hence in triploids, there are two pairing possibilities, resulting in a trivalent or in a bivalent plus a univalent. Paired centromeres segregate to opposite poles, but unpaired centromeres pass to either pole randomly. Cultivated varieties of banana, apple, sugar beet and watermelon are triploids and seedless. They have higher sugar contents, are larger in size and are generally resistant to molds.

Autotetraploids

Autotetraploids arise naturally by the spontaneous accidental doubling of a 2*x* genome to a 4*x* genome, and autotetraploidy can be induced artificially by colchicine.

12.4 Conclusion

In this review, a realistic understanding of different kinds of chromosome aberration formation is presented. The different endpoints presented here are indicators of mutagenic and carcinogenic activity. The formation of chromosomal aberrations is neither the molecular mechanism nor the biological significance of the phenomenon that has been clarified. The recent development of molecular cytogenetics namely FISH has facilitated analysis of a specific CA (Zhang *et al*., 2002). In conclusion, the chromosomal aberrations are microscopically observed visible changes in the chromosomes and chromatids.

Genetic changes when they arise have been called mutations. The term usually refers to any change in the amount or organization of genetic material. To make things easier, the genetic changes are either cytologically visible in the chromosomal level or invisible in a gene or point mutation. In general aneuploids and polyploids are useful in crop improvement in many ways. Aneuploids are useful tools for locating genes on a specific chromosome in wheat, cotton, tobacco and other crops. Monosomics are used in transferring chromosomes with desirable genes from one species to another. In polyploids, allopolyploidy has wider applications than autopolyploidy. Both triploids and tetraploids have been used to trace the origin of natural species in creating new species, and in interspecific gene transfer.

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Table 12.1 Contiguous gene syndromes (Moore and Best 2001).

Syndrome	Duplication or deletion	Critical chromosomal region
Saethre–Chotzen	Deletion	7p21-p22
Grieg cephalopolysyndactyly	Deletion	7p13
Williams	Deletion	7q11.2
Wolf-Hirschhon	Deletion	7q11.23
Langer–Giedion	Deletion	8q24.1
DiGeorge 2	Deletion	10p13
WAGR[a]	Deletion	11p13
Beckwith–Wiedemann	Duplication	11p15
Prader–Willi/Angelman	Deletion	15q11-13
Angelman	Deletion	15q11.2-q13
Rubenstein–Taybi	Deletion	16p13.3
Miller–Dieker	Deletion	17p13.3
Smith–Magenis	Deletion	17p11.2
Charcot–Marie–Tooth, type 1A	Duplication	17p11.2-p12
Diamond-Blacfan anemia	Deletion	19q13.2
Alagille	Deletion	20p11.2-20p12
DiGeorge 1/velocardiofacial	Deletion	22q11.2
Cat eye	Duplication	22q11
Microdeletion 22q13	Deletion	22q13.3
Mental retardation, nonspecific		Xp21.3-p22.1
Kallmann/contiguous genes	Deletion	Xp22.3
Duchenne muscular dystrophy/contiguous genes	Deletion	Xp21

[a]WAGR; Wilms tumor, Aniridia, Genitourinary anomalies, mental Retardation

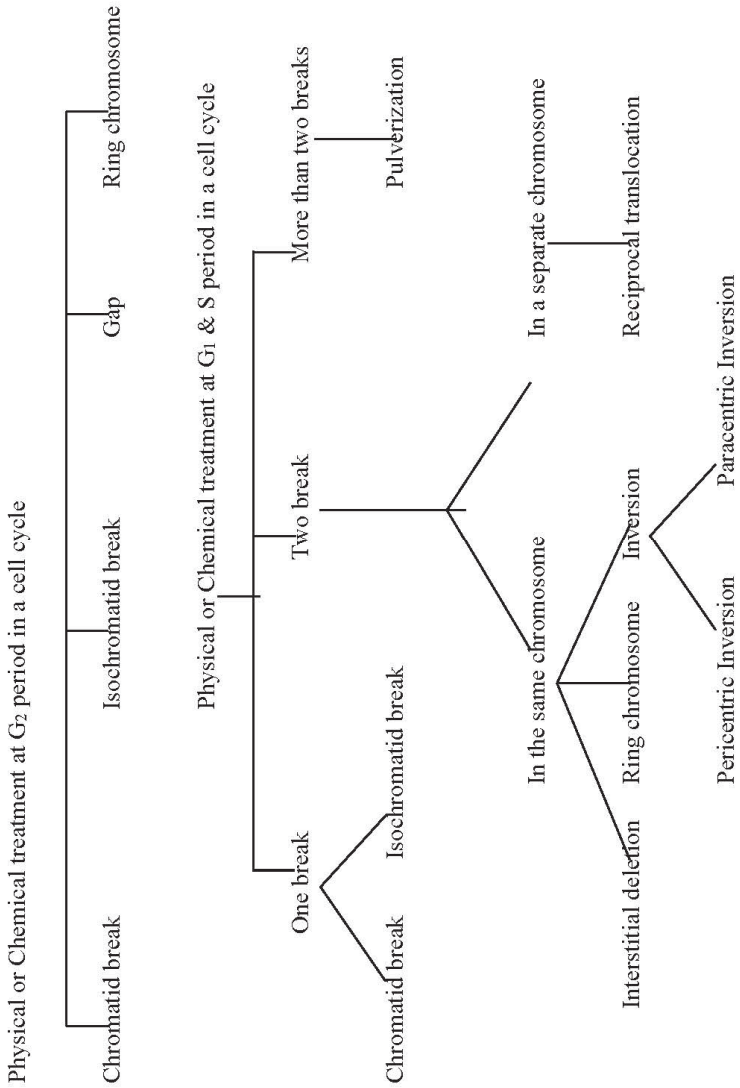


Fig. 12.1 Kinds of chromosomal aberrations.

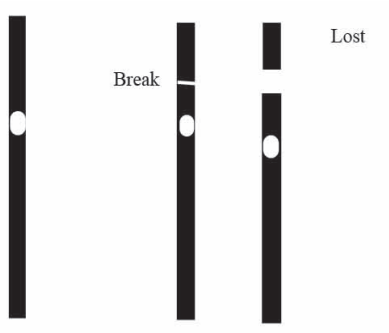


Fig. 12.2 Terminal deletion.

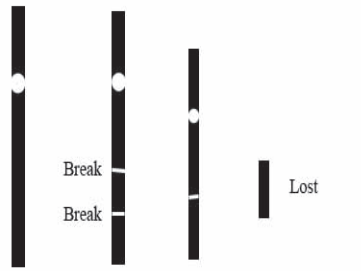


Fig. 12.3 Interstitial deletions.

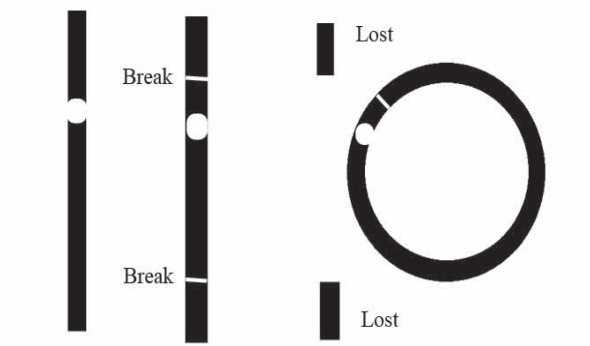


Fig. 12.4 Ring chromosomes.

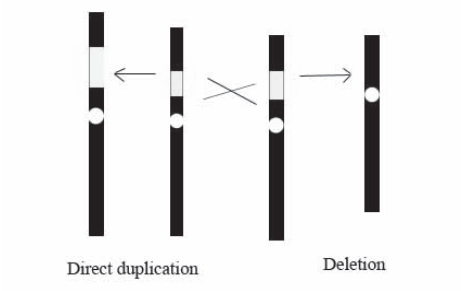


Fig.12.5 Duplication and Deletion.

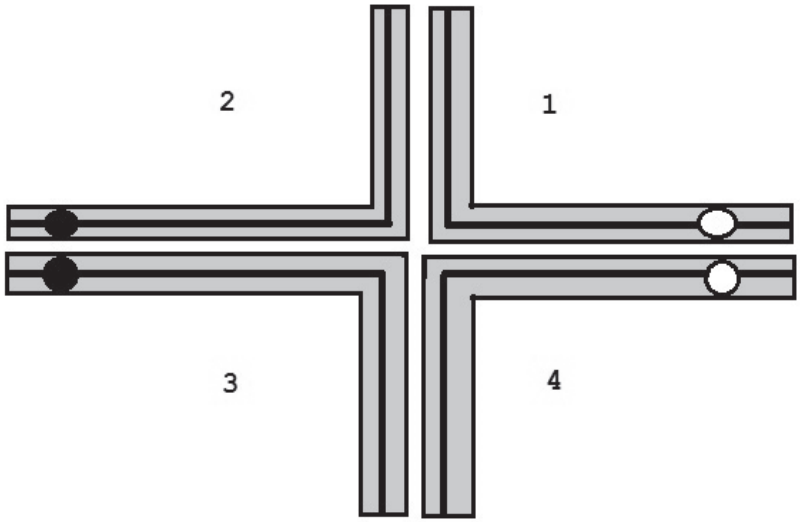


Fig.12.6 Reciprocal translocation in meiosis I – A quadrivalent.

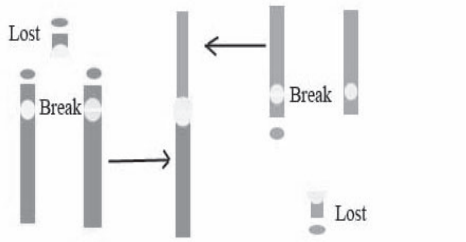
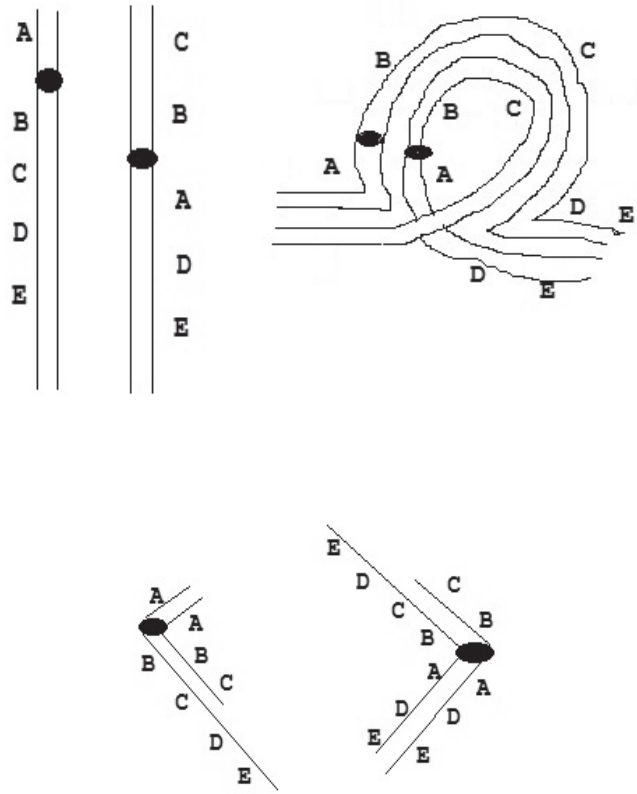


Fig.12.7 Robertsonian translocations.

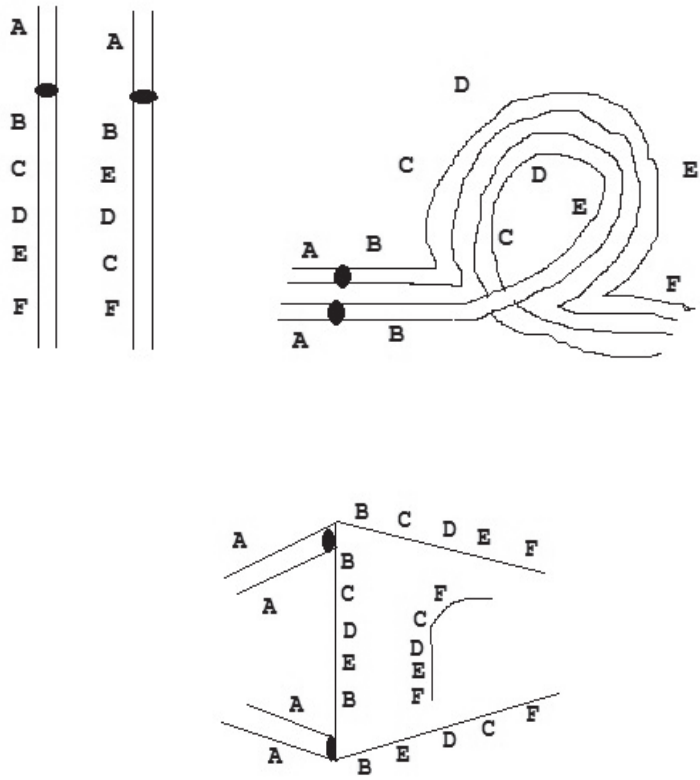
Metaphase Meiosis I Anaphase Meiosis I



Crossover is between B and C

Fig.12.8 Pericentric inversion.

Metaphase Meiosis I Anaphase Meiosis I



Crossover between C and D

Fig.12.9 Paracentric inversion.

CHAPTER 13

CORRELATION ANALYSIS FOR BIOCHEMICAL ASPECTS OF ISOLATED MUTANTS OF FABA BEAN

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Abstract: The present investigation was carried out to determine the correlation of some important biochemical aspects of mutants isolated in the M_3 generation of faba bean (*Vicia faba* L.) using both physical and chemical mutagenesis. The correlation analysis was obtained among total seed protein, iron, manganese and zinc contents. Significant and positive correlation was observed between protein and mineral elements, whereas both significant and non-significant correlations were observed between mineral elements. Non-significant and positive correlation was observed between iron-zinc and manganese-zinc, whereas significant and positive correlation was observed between manganese and iron. A correlation study for different character pairs is an important step in mutation breeding programs. The correlation studies help to find out the relation between different characters under study.

Key words: Faba bean, seed protein, iron, manganese, zinc, mutation breeding.

13.1 Introduction

The productivity of pulses plays a vital role in achieving food and nutrition security in developing countries such as India, Pakistan and Bangladesh. The decline in per capita availability of food due to an unparalleled rate of population growth gravely demands the attainment of enhanced productivities, especially in developing countries. As per the Food and Agriculture Organization of the United Nations (FAO), the human population will increase to 9 billion by the end of 2050 and the feeding of such a huge population will be a challenging task. With the increasing population, dwindling arable lands, depleting water resources, increased urbanization and industrialization, hunger ghosts would haunt the millions of poor people across the globe (FAO, 2009). The population explosion, fast urbanization and severe climate changes represent the challenges which affect the overall crop productivity and are considered major causes of food insecurity in India. As per the report of FAO (2013), developing and under-developed countries are worst hit by the problems of food insecurity and increasing malnutrition, and this can only be remedied by implementing policies on agricultural intensification. In addition to the man-made activities, the productivity of plants is reduced by a wide range of environmental biotic and abiotic stresses, such as salinity and rising temperatures, drought and heat, pathogens and pest attacks (Ahmad *et al*., 2019a, b; Naikoo *et al*., 2019). Man has applied different approaches to reducing food insecurity from time to time. Out of such approaches, mutation breeding has played a key role in increasing the yield of different crop plants. Mutation breeding is considered as a coherent tool to create variability in a crop species in a very short space of time as compared to hybridizations. Several workers have supplemented comprehensive knowledge on the function and applicability of induced mutations for the improvement of genetic resources in several crop plants across the globe (Bhat *et al.,* 2006b; Bhat *et al.,* 2006a; Bhat *et al.,* 2007b; Amin *et al.,* 2016; Laskar *et al*., 2015; Khursheed et al; 2015; Bhat *et al.,* 2005a; Raina *et al*., 2016; Raina *et al*., 2018a, 2019; Wani *et al.,* 2017; Laskar *et al*., 2019). The main merits of induced mutagenesis are the scope it gives of improving a single trait in a crop species without significantly altering the entire genome governing desirable agronomic characters. Induced mutagenesis also plays a vital role in the enhancement of genetic variability which forms the basis of each and every crop improvement

program (Bhat *et al.,* 2007a; Bhat *et al.,* 2006d; Bhat *et al.,* 2005b; Raina *et al*., 2018b). Several workers have employed wide ranges of physical and chemical mutagens for the improvement of traits in various crops (Laskar *et al.,* 2018a,b in lentil; Tantray *et al.,* 2017 in black cumin; Bhat *et al.,* 2007; Bhat *et al.,* 2006; Bhat and Wani 2015; Bhat 2007; Bhat *et al.,* 2007 and Khursheed *et al.,* 2019 in faba bean; Hassan *et al.,* 2018 in fenugreek; Amin *et al*., 2019 in black cumin; Ganai *et al.,* 2005 in *Cicer*; Parveen *et al.,* 2006 in *Trigonella*). Against this backdrop, the present investigation was carried out to determine the correlation analysis of some important biochemical aspects of mutants isolated in the M_3 generation of faba bean var. Vikrant and var. PRT-12 using induced mutagenesis.

Case Study of Induced mutation breeding in Faba bean

13.2 Materials and Methods

13.2.1. Raising M_1 generation

Seeds of both of the varieties of faba bean were treated with gamma rays, Ethyl methanesulfonate (EMS) and their combination treatments as detailed below:

13.2.1.2. Physical mutagen: Gamma rays (γ rays)

Dry and dormant seeds of each variety were irradiated with 100, 200, 300 and 400 Gy of gamma rays with a radioisotope ^{60}CO source at the National Botanical Research Institute, Lucknow, Uttar Pradesh, India.

13.2.1.3 Chemical mutagen: Ethyl methanesulfonate (EMS)

Healthy seeds of uniform size of each variety were presoaked for 9 hours in distilled water and treated with 0.01, 0.02, 0.03 and 0.04 % of EMS for 6 hours with intermittent shaking at room temperature of 22±1ºC. The solution of EMS was prepared in phosphate buffer of pH 7. Only freshly prepared solutions were used for all the treatments. The pH of the solution was maintained by using buffer tablets manufactured by MERCK manufactures, Mumbai, India. After treatment, the seeds were thoroughly washed in running tap water to remove excess mutagen.

13.2.1.4 Combination treatment: Gamma rays + EMS

For combination treatments, dry seeds of each variety were first irradiated with gamma rays at 100, 200, 300 and 400 Gy doses and then treated with

0.01, 0.02, 0.03 and 0.04 % EMS. The procedure adopted was similar to that for the individual treatment.

13.2.1.5 Sample size

In both the varieties 350 seeds were used for each treatment and control.

13.2.1.6 Controls

For each variety, presoaked seeds were again soaked in phosphate buffer for 6 hours to serve as controls.

13.2.2. Raising M_2 generation

Twenty-seven healthy seeds from each normal looking M_1 plant of all different treatments with their respective controls in both the varieties were planted in plant progeny rows in M_2 generation during the rabi season of 2013. Different treatments and controls comprised of 50 progenies. The distance between seeds in a row and between the rows was kept at 30×60 cm, respectively. Three replications were maintained in each treatment in a completely randomized block design (CRBD).

Observations were made on ten normal looking plants of each progeny for each treatment with their controls. Data were collected on an individual plant basis on different quantitative traits. Plants showing morphological, chlorophyll and other variations were discarded from each progeny. Seeds were collected only from the normal looking M_2 plants.

13.2.2.1 Raising M_3 generation

For raising the M_3 generation, two treatments of gamma rays (100 and 200 Gy) and EMS (0.01% and 0.02%) alone and their combinations (100 Gy gamma rays+0.01% EMS and 200 Gy gamma rays+0.02% EMS) for each variety were selected which gave the maximum seed yield in the M_2 generation. 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 the controls, particularly for yield components under study in the M_2 generation.

13.2.2.2 Correlation coefficient (r) and Regression (R^2)

13.2.2.3 Correlation coefficient (r)

Correlation coefficient, a statistical measure which indicates association between two or more than two traits generally denoted by the symbol 'r' was calculated through SPSS 17.0 software (Chicago: SPSS Inc.).

13.2.2.4 Regression analysis (R^2)

The estimation of relationships among variables were analyzed by regression and calculated through excel spreadsheet using Microsoft office excel 2007.

13.3 Experimental Results

Mutants were isolated in the M_3 generation from both the varieties (Table 13.1).

Table 13.1: Brief description of the mutants isolated in the M_3 generation of *Vicia faba* L.

Strain Number	Origin/Treatment
Var. Vikrant	**Vikrant (Control)**
Strain-A	100 Gy γ rays
Strain-B	0.01% EMS
Strain-C	0.02% EMS
Strain-D	100 Gy γ rays+0.01% EMS
Var. PRT-12	PRT-12 (Control)
Strain-E	100 Gy γ rays
Strain-F	100 Gy γ rays+0.01% EMS

13.4 Correlation Analysis of Isolated Mutants

Significant and positive correlation was observed between protein and mineral elements. The regression line and square of correlation for protein and zinc (R^2=0.686), protein and iron (R^2=0.815) and protein and manganese (R^2=0.698) indicates a significant and positive relationship. Both significant and non-significant correlations were observed between mineral elements. Non-significant and positive correlations were observed between iron-zinc and manganese-zinc. The regression line and square of correlation for iron and zinc (R^2=0.307) and manganese and zinc

(R^2=0.285) designates a non-significant and positive relationship. A significant and positive correlation was observed between manganese and iron. The regression line and square of correlation for manganese and iron (R^2=0.774) indicates a significant and positive relationship (Table 13.3; Fig. 13.1).

Table 13.2: Mean for total seed protein (%), iron, manganese and zinc contents (µg. g^{-1}DW) of the high yielding mutants isolated in M_3 generation of *Vicia faba* L.

Strain No.	Protein $\bar{x} \pm$ S.E	Iron (Fe) $\bar{x} \pm$ S.E	Manganese (Mn) $\bar{x} \pm$ S.E	Zinc (Zn) $\bar{x} \pm$ S.E
Control (Vikrant)	26.06^{c}±0.01	67.17^{d}±0.03	16.37^{d}±0.04	31.44^{d}±0.09
Strain- A	26.24^{b}±0.03	67.38^{cd}±0.06	17.22^{c}±0.03	32.22^{b}±0.02
Strain –B	27.31^{a}±0.02	68.42^{b}±0.06	18.22^{b}±0.02	33.23^{a}±0.02
Strain- C	27.35^{a}±0.02	69.75^{a}±0.12	19.35^{a}±0.22	32.31^{b}±0.08
Strain- D	26.32^{b}±0.03	67.45^{c}±0.07	18.26^{b}±0.05	31.67^{c}±0.09
Control (PRT-12)	26.05^{c}±0.01	67.13^{c}±0.01	16.39^{c}±0.09	31.72^{c}±0.03
Strain-E	27.29^{a}±0.01	68.43^{a}±0.06	18.28^{a}±0.04	33.37^{a}±0.05
Strain-F	26.28^{b}±0.03	67.43^{b}±0.07	17.27^{b}±0.05	32.21^{b}±0.03

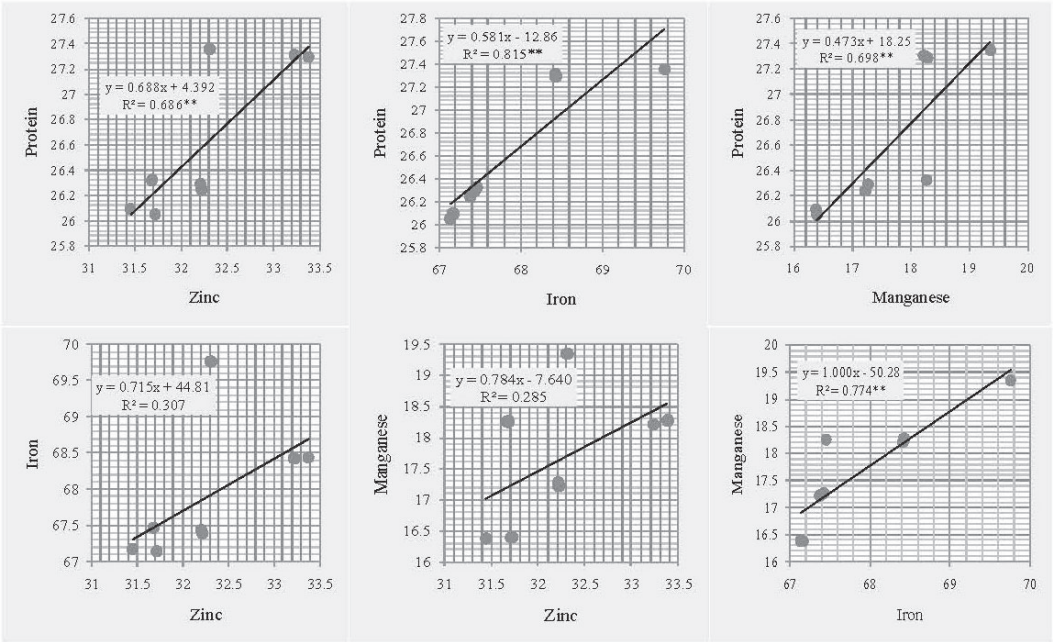
Different letters show significant difference at $p \leq 0.05$. Means with the same letter are not statistically different.

Table 13.3: Correlation coefficient matrix for yield and its component traits, seed protein and mineral elements in the high yielding mutants of faba bean.

	Protein	zinc	iron	manganese
protein	-	.828*	.904**	.837**
	-	.011	.002	.010
	-	8	8	8
zinc	-	-	.557	.535
	-	-	.152	.171
	-	-	8	8
iron	-	-	-	.880**
	-	-	-	.004
	-	-	-	8

** Correlation is significant at 0.01 level
* Correlation is significant at 0.05 level

Fig. 13.1: Regression analysis between different character pairs of high yielding mutants of faba bean. (R-Values with asterisks are significant).



13.5 Discussion

Induced mutagenesis in faba bean could be a novel strategy for increasing food security and reducing malnutrition problems, especially in India, by inducing genetic variability in protein and mineral elements (Bhat and Wani, 2017; Bhat and Wani, 2017a; Bhat, 2007; Khursheed *et al*., 2018a,b,c). Correlation studies for different character pairs, especially between yield and its component traits, is an important and necessary step in mutation breeding programs (Kaul and Grag, 1982; Raina *et al*., 2017). The correlation studies help to determine the relationship between different characters under study. Both significant and non-significant correlations were observed among the isolated mutants in the M_3 generation. Correlation studies using induced mutations have been reported by Khursheed *et al*. (2016). A significant and positive correlation was observed for yield and its component traits. This might be the pliotropic effects of mutated genes. The correlation for yield with other traits has been reported in mung bean (Canci and Toker, 2014) and lablab bean (Asaduzzaman *et al*., 2014). Non-significant but positive correlation was observed between yield and protein content in the mutants, indicating the difficulty of improving yield and protein simultaneously. Similar findings have been reported by Asaduzzaman *et al*. (2014).

Correlation studies between protein and mineral elements and between mineral elements were also recorded in the present experimentation. Significant and positive correlation was observed between protein and mineral elements. However, both significant and non-significant correlations were observed between mineral elements. Significant and positive correlation among mineral elements was observed between iron and manganese, whereas non-significant and positive correlation was recorded between zinc with iron and manganese. This differential response may be attributed to agonistic and antagonistic metabolic pathways as has been reported by Kabata-Pendias (2011).

13.6 Future Prospectus

The main aims in a breeding program are recognizing the desired genetic variability for agro-economical traits, utilizing that variability, correlation between agro-economical traits and propagating the desired populations to future generations. Even though traditional breeding methods are helpful to utilize the available genetic variability in the cultivated germplasm, resulting in the development of several high yielding and better adaptable

varieties of faba bean, the limitation is the huge time required for such procedures. The correlation studies have shown a significant and positive correlation between protein and mineral elements and between manganese and iron. Non-significant and positive correlation was observed between iron-zinc and manganese-zinc. Correlation studies for different character pairs, especially between yield and its component traits, is an important and necessary step in mutation breeding programs.

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CHAPTER 14

ISOLATION OF PROMISING MUTANTS OF M_3 GENERATION IN BROAD BEAN AND THEIR PROTEIN ELECTROPHORETIC PATTERNS THROUGH SDS-PAGE

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Abstract: Mutation breeding is one of the most promising tools used to induce genetic variability and the creation of new plant varieties having promising agronomic characters. The mutagens employed in plant breeding are physical and chemical mutagens. In the present study, both physical and chemical mutagens and their combination treatments were utilized to induce genetic variability and isolate promising mutants from two varieties of broad bean having a comparatively narrow genetic base. Subsequently, M_1, M_2 and M_3 generations were raised and promising mutants were isolated from both variety Minor and variety Major and were named as Minor A, Minor B, Minor C, Minor D, Minor E, Minor F, Minor G, Minor H, Minor I, Minor J, Minor K, Minor L, Minor M, Minor N, Minor O and Major A, Major B, Major C, Major D, Major E, Major F, Major G, Major H, Major I, Major J, Major K, Major L, Major M, Major N, Major O. Their agronomic characters were thoroughly studied and analyzed through statistical analysis which reveals their increased productivity. The cytological examinations reveal certain chromosomal

aberrations, but their frequency was very low. Protein electrophoretic patterns were determined by SDS-PAGE gel scanning.

Keywords: chromosomal aberrations, SDS-PAGE, agronomic characters, Mutagens, Mutants, genetic variability.

14.1 Mutagens and mutations

Plants are facing a wide range of environmental induced stresses and to overcome the deleterious effects of such stresses requires an alteration in the secondary metabolites such as phenolics (Ahmad *et al*., 2019a, b, c; Niakoo *et al*., 2019). Induced mutations can play a critical role in the development of plants that reveal improved tolerance to a range of biotic and abiotic stresses. Mutagens are the agents that have the potential to change the genotype of an organism. The genotype is a genetic structure of cells and organisms. Mutagens are of two types, that is physical mutagens and chemical mutagens. Physical mutagens are radiation such as gamma-rays, X-rays, beta rays, UV rays and fast-moving neutrons. Some physical radiation is ionizing and some non-ionizing. Chemical mutagens are chemicals that are widely used to induce alterations in the genetic architecture of an organism. In plants among the physical mutagens, gamma-rays are widely used in gamma gardens in different doses to improve agronomic characters. In chemical mutagens different doses in different concentrations are prepared to induce mutations. Most preferred mutations are induced at seed level. Mutations are sudden inheritable induced changes in genotype for improving the phenotype and consequently agronomic characters (Bhat *et. al.,* 2006a; Raina *et al*., 2017; 2018a; Laskar *et al*., 2018a; 2019; Wani *et al*., 2017; Tantray *et al*., 2017). Physical as well as chemical mutagens are used to give impetus for change to the genetic architecture of organisms, particularly to plants. Change in genotype brings about a change in phenotype in plants, particularly in agronomic characters. This change is harvested to improve the agronomic characters to bring positive change in productivity. Mutations are harvested by geneticists and plant breeders across the globe to produce new improved varieties with improved agronomic characters and productivity (Khursheed *et al*., 2018a; 2019). Mutations occur both at chromosome and gene level, however the desirable mutations are gene mutations (Amin *et al*., 2016; Goyal *et al*., 2019).

14.2 Results

14.2.1 High yielding mutants

The details of mutants isolated based on their yield performance in M_3 as compared to their parents are given in Tables 14.1 and 14.2. The phenotypic correlation among different quantitative characters in the varieties minor and major are given in Tables 14.3 and 14.4. Since yield per plant is the most dependable character, certain mutants which were distinctly much superior as compared to others for seed yield per plant were selected in the M_2 generation. Some of these mutants were morphologically quite distinct especially in seed size, seed shape, seed coat color and pod size, whereas others were more or less similar to their respective controls. All these mutants were raised in progeny rows in the M_3 generation and were evaluated not only for the seed yield but also for their quantitative characters. Representative photographs of mutants are given from Figs. 14.13-14.20.

14.2.2 Cytological studies in isolated strains

The cytological study of isolated strains of var. minor and var. major showed almost all the chromosomal aberrations but were found in low frequency. The cytological aberrations in mutant generations and subsequent mutants have also been reported by Gulfishan *et. al.,* (2012), Gulfishan *et. al.,* (2013), Parveen *et. al.,* (2006), Sharma *et. al*., (2009). Bhat *et. al*., (2007), Bhat *et. al*., (2007a), Bhat and Wani, (2017), Khursheed *et al.,* (2015) Amin *et al.,* (2019). Representative cytological features of isolated strains of both the varieties are shown in Plate 14.1 (Figs. 14.1-14.12).

14.2.3 Meiotic studies in strains isolated from variety minor

Most of the chromosomal aberrations observed in the treated population of *Vicia faba* L. in M_I, M_2 and M_3 generations were also observed in the strains isolated from var. minor but the frequency of these chromosomal aberrations was comparatively very much less than the frequency of chromosomal aberrations observed in the treated population of M_I, M_2 and M_3 generations as reported by Bhat and Wani, (2015), Bhat *et al.,* (2007), Bhat and Wani, (2017) in *Vicia Faba* L.

14.2.4 High yielding mutants in var. minor

All the mutants selected in the M_2 generation showed a very significant increase in average plant yield in M_3 in comparison to their parents.

14.2.4.1 Number of fertile branches per plant: A significant increase was recorded for the number of fertile branches per plant in isolated strains of var. minor. The maximum shift in mean was 12.33 in Minor A, followed by 12.00 in Minor B and 11.33 in Minor C. The maximum GCV was 27.78 in Minor M, 27.34 in Minor N and 27.04 in Minor K. The heritability and genetic advance showed a significant increase. The maximum heritability was 93.06 in Minor E, followed by 91.99 in Minor B and 90.75 in Minor A, while the lowest heritability was 64.69 in Minor D. The maximum GA was 63.25% in Minor K, followed by 62.19% in Minor E and 61.77% in Minor M.

14.2.4.2 Number of pods per plant: The number of pods per plant showed a significant increase over the parental variety. The maximum shift in mean was 32.90 in Minor L, followed by 32.50 and 31.90 in Minor M and Minor N respectively and the lowest was 22.54 in Minor J. The genetic parameters showed a significant increase in the mutant strains. The highest GCV was 19.84% in Minor I, followed by 18.59% and 18.14% in Minor J and Minor M respectively and the lowest was 13.69% in Minor L. The heritability and genetic advance estimates showed an exceptionally significant increase. The maximum heritability was 93.84% in Minor A, followed by 93.68% in Minor C and 92.51% in Minor I and the lowest was 77.13% in Minor K. The maximum GA was 50.08% in Minor I followed by 46.23% in Minor J and 45.22% in Minor H, whereas the lowest was 31.86% in Minor K.

14.2.4.3 Number of seeds per pod: A significant increase was recorded for seeds per pod in isolated strains of var. minor. The maximum increase was 2.56 in Minor N, followed by 2.27 in Minor M and 1.90 in Minor J and the minimum was 1.23 in Minor B. The genetic parameters continued to show an increase for this parameter also which is an important contributing character for total yield per plant. The maximum GCV was 38.12% in Minor G, followed by 35.85% in Minor D and 35.16% in Minor E, while the lowest was 27.28% in Minor J. The heritability and genetic advance showed a considerable increase. The maximum heritability was 93.45% in Minor A, followed by 92.33% in Minor E and 84.77% in Minor K and minimum 69.60% in Minor H. The maximum genetic advance was

89.00% in Minor E, followed by 85.19% in Minor D and 80.71% in Minor K, while the lowest value of GA was 62.46% in Minor 1.

14.2.4.4 Pod length (cm): A considerable and significant increase in pod length was obtained in different strains of var. minor. The maximum increase in the mean shift was 2.63 in Minor C, followed by 2.43 in Minor D and 2.33 in Minor B, whereas the lowest was 1.23 in Minor F. The genetic parameters also showed a considerable increase. The maximum GCV was 28.84% in Minor F, followed by 27.18% in Minor I and 26.42% in Minor J, while the minimum was 20.51% in Minor D. The heritability and genetic advance also showed a significant increase in the selected strains. The maximum heritability was 89.50% in Minor C, followed by 85.89% in Minor F and 85.71% in Minor M, while the minimum was 66.40% in Minor B. The maximum GA was 70.71% in Minor F, followed by 64.11 in Minor G and 63.89 in Minor I and minimum was 46.23% in Minor A.

14.2.4.5 Pod girth (cm): There was a significant increase in the mean shift in different strains isolated from var. minor. The maximum shift in mean was 2.80 in Minor A, followed by 2.70 in Minor B and 2.53 in Minor C, while the minimum was 1.50 in Minor O. The genetic parameters showed a significant increase over the parent. The maximum GCV was 33.15% in Minor I, followed by 30.99% in Minor H and 28.55% in Minor K, while the minimum was 19.24% in Minor C. The heritability and genetic advance showed a significant increase for this parameter. The maximum heritability was 93.22% in Minor A, followed by 91.32% in Minor B and 90.43% in Minor D, whereas the minimum was 76.33% in Minor L. The maximum GA was 83.53% in Minor I, 78.72% in Minor H and 70.77% in Minor K, while the minimum was 46.37% in Minor C.

14.2.4.6 Seed weight (g): It is an important contributing character to assess the total yield per plant. The shift in mean showed an exceptionally better increase for seed weight in the isolated strains of var. minor. The maximum shift in mean was 18.36 in Minor E, followed by 17.70 in Minor F and 16.70 in Minor G, whereas the minimum was 6.06 in Minor L. The genetic parameters showed a significant increase over the parent variety. The maximum GCV was 28.19% in Minor L, followed by 27.47% in Minor K and 25.38% in Minor M, while the minimum was 13.78% in Minor A. The heritability and genetic advance also showed a significant increase. The maximum heritability was 89.88% in Minor K, followed by 88.81% in Minor L and 88.58% in Minor M, while the minimum was 70.33% in Minor A. The maximum GA was 70.07% in Minor L, 68.79%

in Minor K and 62.96% in Minor M and minimum was 12.32% in Minor G.

14.2.4.7 Yield per plant (g): It is the character by which total improvement of the variety is assessed but is contributed to by many other characters such as pods per plant, seeds per pod, pod length (cm), pod girth (cm), seed weight (g), fertile branches per plant, etc. A significant increase was recorded in the mean shift in all the isolated strains. The maximum shift was 13.07% in Minor B, followed by 13.07% in Minor A and 12.23% in Minor C, whereas the minimum was 9.06% in Minor 1. The genetic parameters showed a considerable increase. The maximum GCV was 32.50% in Minor D, followed by 25.47% in Minor K and 25.35% in Minor J and the minimum was 12.71% in Minor C. The estimates of heritability and the genetic advance were very high. The maximum heritability was 94.93% in Minor A, followed by 90.55% in Minor M, 90.01% in Minor K and the minimum heritability was 66.91% in Minor C. The maximum GA was 63.78% in Minor K, 62.66% in Minor J, followed by 60.37% in Minor L, whereas the minimum GA was 27.48% in Minor C.

14.3 High yielding mutants in var. major

All the mutants selected in the M_2 generation showed a very significant increase in average plant yield as compared to their parents in M_3.

14.3.1 Number of fertile branches per plant

A significant increase was recorded for a number of fertile branches per plant in isolated strains of var. major. The maximum shift in mean was 11.97 in Major G, followed by 11.64 in Major E and 10.64 in Major F. The maximum GCV was 33.10% in Major J, 32.89% in Major I followed by 32.55% in Major D, minimum GCV was 26.33% in Major F. The heritability and genetic advance showed a significant increase. The maximum heritability was 88.94% in Major D, followed by 87.75% in Major C and 87.56% in Major E, whereas the lowest heritability was 70.91% in Major A. The maximum GA was 81.03% in Major D, followed by 78.99% in Major C and 76.61% in Major I.

14.3.2 Number of pods per plant

The number of pods per plant showed a significant increase over the parental variety. The maximum shift in mean was 23.40 in Major A, followed by 22.40 and 21.40 in Major E and Major B respectively and the lowest was 15.57 in Major 1. The genetic parameters showed a significant increase in the number of pods per plant. The highest GCV was 20.03% in Major I, followed by 19.60% and 19.15% in Major G and Major C respectively and the lowest was 15.50% in Major D. The increase in heritability and genetic advance was exceptionally significant over the parent. The maximum heritability was 90.63% in Major C, followed by 87.26% in Major A and 86.45% in Major D. The lowest heritability was 74.03% in Major H. The maximum GA was 48.33% in Major I, followed by 47.83% in Major C and 47.77% in Major G, while the lowest GA was 37.54% in Major F.

14.3.3 Number of seeds per pod

A significant increase was recorded in the number of seeds per pod in isolated strains of var. major. The maximum increase was 1.77 in Major E, followed by 1.70 in Major D and Major G and the minimum shift in mean was in Major L, Major B and Major C. The genetic parameters continued to show an increase in this character which is an important contributing factor for yield per plant. The maximum GCV was 28.31% in Major A, followed by 26.00% in Major E and 26.22% in Major I, whereas the lowest was 23.82% in Major H. The heritability and genetic advance showed a considerable increase over the parent. The maximum heritability was 91.56% in Major A, followed by 91.56% in Major E and 89.71% in Major C and minimum was 79.60% in Major G and D. The maximum genetic advance was 76.15% in Major A, followed by 66.53% in Major I and 65.32% in Major E, whereas the lowest was 56.37% in Major G.

14.3.4 Pod length (cm)

A considerable and significant increase in pod length was obtained in different strains of var. major. The maximum increase was 2.47 in Major B, followed by 2.44 in Major D and Major E, while the lowest was 1.31 in Major F and Major H. The genetic parameters also showed a considerable increase. The maximum GCV was 35.83% in Major J, followed by 35.68% in Major E and 34.81% in Major H, whereas heritability and

genetic advance increase in the selected strains. The minimum was 22.51% in Major A.

The maximum heritability was 89.82% in Major J, followed by 84.54% in Major I and 83.75% in Major H, while the minimum was 64.5.6% in Major D. The maximum GA was 89.84% in Major J, followed by 84.14% in Major H and 83.16% in Major I and minimum was 50.00% in Major D.

14.3.5 Pod girth (cm)

A significant increase in the mean shift was found in different strains isolated from var. major. The maximum shift in mean was 2.23 in Major A, followed by 2.13 in Major B and 2.10 in Major C and Major E, while the minimum shift in mean was 1.37 in Major D. The genetic parameters showed a significant increase over the parent variety. The maximum GCV was 27.66% in Major C, followed by 26.93% in Major B and 26.74% in Major I, while the minimum was 21.17% in Major E. The heritability and genetic advance showed a significant increase over the parent in this parameter. The maximum heritability was 94.97% in Major A, followed by 82.45% in Major I and 82.32% in Major J, whereas the minimum was 67.67% in Major H. The maximum GA was 65.41% in Major C, 63.72% in Major I and 61.81% in Major J, while the minimum was 46.86% in Major E.

14.3.6 Seed weight (g)

It is also an important yield contributing character to assess the total yield per plant. The shift in mean showed an exceptionally better increase for seed weight in the isolated strains of var. major. The maximum shift in mean was 13.36 in Major B, followed by 12.70 in Major C and 12.03 in Major A, while the minimum was 10.36 in Major F. The genetic parameters showed a significant increase over the parent variety. The maximum GCV was 19.12% in Major C, followed by 18.77% in Major E, whereas the minimum was 12.44% in Major A. The heritability and genetic advance also showed a significant increase. The maximum heritability was 95.33% in Major C, followed by 93.92% in Major I and 87.61% in Major A, while the minimum was 72.37% in Major F. The maximum GA was 49.09% in Major C, 44.82% in Major E and 42.90% in Major D and minimum was 30.84% in Major A.

14.3.7 Yield per plant (g)

It is the character by which total improvement of the variety is assessed but is contributed to by many other characters such as pods per plant, seeds per pod, pod length (cm), pod girth (cm), seed weight (g), fertile branches per plant, etc. A significant increase was recorded in the shift in mean in all the isolated strains. The maximum shift was 14.84 in Major H, followed by 13.84 in Major B and 12.84 in Major C, while the minimum was 10.84 in Major F. The genetic parameters showed a considerable increase over the parent. The maximum GCV was 18.32% in Major C, followed by 16.51% in Major F and 16.41% in Major J and the minimum was 13.54% in Major A. The estimates of heritability and genetic advance showed exceptionally high values. The maximum heritability was 92.69% in Major A, followed by 90.42% in Major C, 84.32% in Major J and minimum was 78.33% in Major B. The maximum GA was 45.79% in Major C, followed by 39.64% in Major J, 39.38% in Major F, while the minimum was 34.15% in Major A.

14.4 Seed protein content (%)

Since broad bean is important for its high protein content, attempts were made to evaluate the crude protein content and to assess the extent of induced variability in different treatments and mutant lines of two broad bean varieties along with controls in the M_3 generation. A wide range of variability of protein content was induced in most of the treatments and mutant lines. The maximum increase in protein content was 30.39%, 29.41% and 26.47% at 10kR+0.1% EMS, 10kR+0.2% EMS and 0.02% MMS respectively in var. minor, while in var. major the maximum increase was 33.89%, 28.03%, 25.52% and 23.01% at l0kR+0.2% EMS, 0.1% EMS, 0.2% EMS and 20kR+0.3% EMS respectively. The maximum decrease in protein content was 20.59%, 22.55% and 17.16% at 20kR+0.03% MMS, 10kR+0.02% and 0.03% MMS in var. minor, while in var. major the maximum decrease in protein content was 30.54%, 18.83% and 15.48% at 20kR+0.03%,10kR+0.02% and 0.01% MMS respectively. Among different mutant lines, the maximum increase in protein content was 33.82%, 38.72%,45.58% and 38.23% as compared to control in Minor A, Minor C, Minor D and Minor F, respectively and 28.03%, 28.87% and 31.38% in Major A, Major B and Major M respectively (Graph 1 and 2). In general individual mutagenic treatments were most effective in increasing the seed protein content. Varietal sensitivity was noted by the fact that the maximum increase in protein content was observed in the var.

minor rather than var. major. Besides, similar treatments increased the protein content in one variety and decreased it in the other variety during the investigation.

14.5 Protein electrophoretic patterns

The electrophoretic analysis was performed to determine the variation in seed protein patterns in controls and the isolated mutants of *Vicia faba* L. varieties. The estimates of the mean coefficient of variability and percentage change over the control for protein content of varieties minor and major is given in Tables 14.5 and14.6. The data obtained from the SDS-PAGE gel scanning are presented in Tables 14.7 and 14.8 and the corresponding gel is presented in Figs. 14.21 and 14.22. The results in Tables 14.7 and 14.8 showed that the number of recorded protein bands ranged from 11-19 in mutants, while 11 protein bands were found in controls. They also indicated that there are differences in protein patterns between control and treated plants. In general, the electrophoretic protein pattern comprised 26 recognizable migration distances, each representing a protein fraction. It was also observed that there was an occurrence of common protein bands that were consistent in the control as well as mutants. The mutants i.e., Minor D, Major A and Major B showed the maximum number of protein bands followed by Major D and Major L, Major K and Major J.

14.6 Discussion

14.6.1 Breeding Potential of created variability

The breeding potential of a crop plant is to exploit the existing genetic variability through selection or created variability. Mutation breeding techniques are the best method to enlarge the genetically conditioned variability of a species within a short period and has played a significant role in the development of many new crop varieties (Raina *et al*., 2016; 2018b; Laskar *et al*., 2018b; Khursheed *et al.,* 2018b, c; Bhat *et. al,.*2006c). Induced mutagenesis plays a very important role in enhancing genetic variability for crop improvement by inducing micromutations in addition to visible macromutations and is the simplest and fastest way to isolate mutants of agronomic and economic significance. The primary strategy in mutation-based plant breeding has been to upgrade the well-adapted varieties by altering one or two major traits. These include mainly the yield contributing traits. Besides, induced mutagenesis offers the

possibility of the induction of desirable attributes, perhaps those that either cannot be found naturally or have been lost. Induced mutagenesis finds a prominent place in the augmentation and recreation of genetic variability which was lost by a rigid selection or narrow base of germ plasm of a crop plant under improvement. The potentiality of mutations for this purpose, however, depends upon the efficiency of the induction of mutation (Siddqui and Yousufzai, 1988; Laskar *et al.*, 2015; Raina *et al*., 2019; Khursheed *et al*., 2016). The enhancement of mutation frequency and the alteration of the mutation spectrum in a predictable manner remain at all times important aspects of mutation research. Increasing the number of mutated genes over a certain threshold essentially needs extensive research for refined methods and treatment conditions. It is often suggested that the manipulation of sieves in the mutation process would seem to be one of the means of obtaining a certain degree of phenotypic specificity. To ensure a speedy generation of variability for a specific trait to be improved, a mutation breeder has to go through all basic events met in the methodology to ensure reliable information about the mutagenic sensitivity of biological material and the extent of effectiveness and efficiency of the mutagen in question. Mutagens vary in their mode of action, effectiveness, efficiency and the spectrum of mutations induced. Similarly, genotypes show differential sensitivity toward mutagens even at the varietal level.

The basic information on mutagenic sensitivity, the efficiency of mutagens, methods of handling the material and treatment methods required to maximize mutation induction is essential for any mutation breeding program. Although ionizing radiation remains the most employed tool of inducing mutations, several chemicals are equally and even more potent in their mutagenic effects. Combinations of different mutagens, if their mutation induction process is independent and capable of interaction, should increase mutation frequency and alter the mutation spectrum. Numerous mutant varieties through induced mutation have developed significant economic impact, sustaining crop production and greatly contributing to the increase in food production. According to IAEA mutant varieties data base (2004), more than 2300 new crop varieties, all carrying novel induced variations, have been officially registered.

14.6.2 Assessment of superiority of mutagens

Generally, the criteria such as germination, injury, lethality, sterility, chromosomal aberrations in the M_1 generation, chlorophyll and viable mutation frequency in the M_2 generation are used to assess the superiority

of mutagens (Thakur and Sethi, 1995; Kumar and Dubey, 1998c; Khan, 1999, Bhat *et al.,*2006, Bhat *et al.,*2005, Bhat *et al.,*2007b, Bhat 2007). Broad beans *(Vicia faba* L.) possess low genetic variability due to frequent self-pollination. Crossing produces too limited variability therefore it was chosen as an experimental material for induction of variations through mutagenesis (Bhat *et al.,*2005a).

14.6.3 Estimation of induced genetic variability in various traits

The present investigation was planned to estimate the extent of induced genetic variability in various traits i.e., days to flowering, number of flowers per plant, plant height, days to maturity, number of pods per plant, pod length (cm), pod girth (cm), number of seeds per pod, 100-seed weight, total yield per plant and number of leaves per plant in separate and combined applications of ethyl methanesulphonate (EMS), gamma-rays, methyl methanesulfonate (MMS) and their combination treatments.

14.6.4 Heritability

The heritability is a property not only of a character but also of the population and the environmental circumstances which the individuals are subjected to. Since the value of heritability depends on the magnitude of all the components of variance, a change in any one of these may affect it. Heritability in the parental populations was low for all the polygenic traits. However, a wide range of heritability was observed in the treated populations. Heritability estimates were comparatively low in M_2 but increased considerably in the M_3 generation. This could be due to the increased homozygosis of the genes involved among treated populations. In general, heritability was medium to high for almost all quantitative traits. Different workers have different opinions regarding the range of heritability for various quantitative traits. Nevertheless, a wide range of heritability induced by physical and chemical mutagens has been reported by different workers (Khan, 1984; Ignacimuthu and Babu, 1993; Srivastava and Singh, 1993; Sharma and Sharma, 1982). The disparity in results could be because heritability is a property, not only of a character, but also of the population, environment and the circumstances which the genotypes are subjected to (Falconer, 1960). Kaul and Bhan (1974) suggested that all the genetic components are influenced by gene frequencies (which differ from population to population according to the history of the population) and by the environmental variance, since more variable conditions reduce heritability and more uniform conditions

increase it. The high heritability estimates in the quantitative characters are useful for selecting suitable types based on their phenotypic performance. The increase in heritability was associated with an increase in variability for most of the yield traits in M_3 providing further chances for selecting plants with better yield and high heritability. Increased variability in the form of high heritability for different quantitative characters has also been reported by earlier workers (Nayeem and Ghasim, 1990; Sharma *et al.,* 1990, Bhat, 2007). A rational approach toward the improvement of any crop plant involves selection. A selection within the base population and utilization of selected material would produce germplasm that would result in the isolation of desired mutants.

Johnson *et al.* (1955) advocated that heritability estimates along with genetic advance are usually more helpful than heritability value alone in predicting the resultant effects of selection. This is probably because heritability estimates are subject to genotype-environment interactions (Lin *et al.,* 1979, Bhat, 2007).

14.6.5 Genetic advance

Genetic advance is indicative of the expected genetic progress for a particular trait under suitable selection procedures and consequently carries much significance in self-pollinated crops. The estimated values of genetic advance (percentage of mean) differed in different mutagenic treatments and also from trait to trait. Like heritability, genetic advance also increased in M_3 as compared to the M_2 generation for yield contributing traits. This is due to an increase in genetic variability component *i.e.,* the induced genetic changes for quantitative characters. From the plant breeding point of view, this should mean a higher response to selection (Sharma and Sharma, 1982). The higher values of heritability and genetic advance also suggest that mutations have mostly occurred at the loci having additive effects (Lawrence, 1965). An increase in heritability coupled with an increase in genetic advance has also been reported earlier (Khan, 1984; Ignacimuthu and Babu, 1992; Lokesha and Veeresh, 1993). In general, results relating to yield and yield components are quite encouraging since they possess sufficiently high values of heritability and genetic advance.

14.6.6 Agronomic characters of Isolated Mutants

The high yielding mutants isolated in M_3 generations revealed that a considerable increase in yield and yield contributing traits could be achieved in these mutants. The increase in 100-seed weight and number of pods per plant, number of seeds per pod, pod length and pod girth were probably the main reasons for boosting the plant yield to such a great extent. In other words, these traits appeared to be highly correlated with yield. The general selection was effective for fertile branches per plant, pods per plant, 100-seed weight, pod length, pod girth and total yield per plant in almost all the mutants as was evident from the increased values of genetic parameters. All the twelve yield contributing traits in the high yielding mutants showed an increase in the genotypic coefficient of variation, heritability and genetic advance, indicating that these characters could be transmitted to future generations and further improvement of these polygenic traits was possible in the subsequent generations. Burton (1951) suggested that the genotypic coefficient of variation, together with high heritability estimates, would give a better picture of the extent of genetic advance to be expected by selection. Because of the above facts, all yield-related traits showed high genotypic variability coupled with high heritability and genetic advance indicating that their heritability was largely due to additive genetic effects.

In the present investigation, many fasciated mutants were isolated in the M_3 generation. The fasciated character involved seed size, seed shape, seed coat color, flower shape, etc. Some mutants among them were agronomically important while some mutants showed inferior yield. Fasciated mutants are a valuable genetic resource and may benefit plant breeding programs of diverse taxa aimed at improving yield and resistance to certain diseases/lodging in them. Fasciated mutants so far have been reported in several leguminous crops viz., pigeon pea (Bhatnagar *et al.,* 1967), mungbean (Singh, 1981), soyabean (Albertsen *et al.,* 1983), chickpea (Knights,1993; Gaur and Gaur, 1999), broad bean (Bhat *et al.,* 2006c), pea (Gottschalk,1977), lentil (Tyagi and Gupta, 1991), *Capsicum annuum* (Kumar *et al.,* 2006).

Among these fasciated mutants, some were superior, while some were inferior agronomically. The agronomically inferior mutants were late maturing with reduced numbers of fruits per plant, fewer seeds per fruit, poor harvest index resulting in an adverse effect on yield, while the agronomically superior yields were possibly due to an increase in the number of branches, flowers and pods per plant, higher yield, higher

harvest index and higher seed yield per plant. So far studies made on several fasciated mutants indicate that the gene for fasciation had both positive and negative effects from the viewpoints of plant breeding. Kumar *et. al.* (2006) studied the inheritance of fasciation in F_2 families of five crosses involving a fasciated mutant as one of the parents and suggested that a single recessive gene controls the stem fasciation. Stem fasciation was also reported to be recessive and monogenetically controlled in pea (Scheibe, 1954), pigeon pea (Sinha *et al.,* 1967) and chickpea (Knights, 1993). Gottschalk (1977) found that three genes control the stem fasciation in induced *Pisum* mutants. In general, those fasciated mutants which had a higher number of branches, flowers per plant and more seeds per fruit were found to be agronomically superior. Further, the gene/genes for fasciation seems to exert a pleiotropic effect in a majority of the mutants reported so far. The bold seeded mutants isolated in the present investigation are of special interest since these mutants showed considerable improvement in the yield besides an increase in pod size.

14.6.7 Cytological observation

Cytological observation of these mutants revealed 6 bivalents (2n=12) at diakinesis/metaphase-I, although some meiotic aberrations such as stickiness, precocious separation, bridges and disturbed telophase were also obtained, but in very low frequency. The normal cytological behavior of these mutants may indicate their genetic nature, however, cryptic structural changes in the chromosome could not be denied, which is in agreement with the findings of Bhat *et al.,*2005b; Bhat *et al.,*2006b; Bhat *et al.,*2006d; Gulfishan *et, al.,* 2010; Gulfishan *et, al.,* 2010 in *Vicia faba* L. ; Ganie *et, al.,* 2005 in chickpea; Gulfishan *et, al.,* 2010 in *Capsicum annuum.*

14.6.8 Future Breeding potential of Isolated mutants

The morphological mutants isolated in the present study included mutants with agronomically desirable features that could be utilized in future breeding programs. A wide range of variability was observed for the fertile branches, pods per plant, pod length, pod girth, seed and yield per plant of the mutants evaluated in M_3 generation. The mutant progenies displayed a tremendous increase in mean values of these traits in comparison to control. Selection for fertile branches, number of pods per plant, pod length, pod girth, number of seeds per pod and yield per plant in the M_3

generation was found to be effective for all the isolated mutants, as is evident from the manifold increase in the values of their genetic parameters in comparison to the control and the rest of the M_3 population. All the seven traits showed increased values of heritability and genetic advance, possibly due to a close resemblance between the corresponding values of PCV and GCV, indicating that these traits can be transmitted to future generations and further improvement of these traits is possible in subsequent generations. The degree of association of plant characters has been helpful as a basis of selection. Several significant changes toward the desirable side were induced in the correlation coefficients of various pairs of traits in the mutants isolated in the M_3 generation. The correlation among yield contributing traits in a population is a composite of the effects of selection, gene linkage and pleiotropy (Sehrawat *et al.,* 1996). The usefulness of mutations in weakening, strengthening or altering character association has been demonstrated in lentil (Sharma and Sharma, 1981a). If the nature of selection practiced in the control and treated population is the same, any difference in the correlation coefficients in the two populations will be due to the effect of mutagens on gene linkage and altered pleiotropic effects of newly mutated genes. However, according to Gottschalk (1987), climatic factors can also influence the pleiotropic pattern positively or negatively. Such alteration in correlation among various traits may be utilized to enhance the rate of selection response in a primary trait. In the present investigation positive and significant correlation between fertile branches per plant and yield per plant, fertile branches per plant and pods per plant and pods per plant vs. yield per plant was observed in almost all the mutants as against controls. This reflects a desirable change in plant type after mutagenic treatment, which may have resulted in increased plant yield. Since the number of pods per plant has shown a significant relationship with yield, it is desirable to make a direct selection for these traits. The protein content was evaluated in the M_3 generation for all the selected treatments and mutant lines. It was evident from the results that variability, though not very high, was induced among various treated populations and mutant lines. Such mutant genotypes can be expected to be of practical value in a protein improvement program and would offer a greater potentiality for cross-breeding since their increased protein content is not expected to be at the expense of grain yield, grain density or grain weight. The protein content increased significantly in several treatments. The mutant lines showed either significant or insignificant increase or decrease in protein content. In general, a wide range of variability was induced for protein content in treatments as well as mutant lines of the M_3 generation, suggesting the possibilities of

isolating high yielding-high protein mutant lines in future generations. One important fact observed during the present investigation was that increase in seed weight was not always associated with a significant increase in protein content as is evident from the bold seeded mutants viz., where the protein content decreased slightly despite a considerable increase in the seed size. The electrophoretic analysis showed variation in seed protein patterns of isolated mutants of *Vicia faba* L. varieties.

14.6.9 SDS-PAGE

The data obtained from the SDS-PAGE gel scanning showed an increase in a number of protein bands in mutants. The controls showed 11 protein bands, while in mutants protein bands ranged from 11-19. In general, the electrophoretic protein pattern comprised 26 recognizable migration distances, each representing a protein fraction. The mutants *i.e.,* Minor D, Major A and Major B showed the maximum number of protein bands followed by Major D, Major L, Major K and Major L. Kharkwal (1998c) reported a very low positive correlation between seed weight and protein content in chickpea, but a positive correlation was observed in the case of grain density and protein content. He further reported a high variability associated with a wide range among different mutant lines and isolated different high protein mutants in chickpea. He concluded that useful variability for quality characters such as protein content could be successfully induced, isolated and significantly improved through mutagenesis in grain legume crops. A number of induced mutant varieties with improved protein content, particularly in the case of grain legumes have been developed and reported by several workers (Kharkwal *et al.,* 1988, Micke, 1988; Shaikh *et al.,* 1982; Gottschalk *et al.,* 1975; Bhat *et al.,* 2007).

In brief, the results have revealed that the lower and moderate treatments of physical and chemical mutagens used in the present investigation proved to be efficient in increasing the genetic variability for yield-oriented selection in broad bean. The isolated mutants possess the more desirable plant architecture associated with high yield and higher seed protein content than their respective controls. They can be evaluated in future generations and after multi-locational trials released as new varieties. The meiotic aberrations indicate the mutational load. The frequency of meiotic aberrations decreased from M_1 to M_2 and M_2 to M_3 and further decreased in isolated strains which represent the occurrence of stability in mutated genotype from the M_1 to M_3 generation and in the isolated mutants of both the varieties of *Vicia faba* L. Thus, the genetic

variability induced by EMS, gamma-rays, MMS and their combination treatments may effectively be exploited for the improvement of broad bean and other self-pollinated crops.

14.7 Seed protein content (%)

Since the broad bean is important for its high protein content, attempts were made to evaluate the crude protein content and to assess the extent of induced variability in different treatments and mutant lines of two broad bean varieties along with controls in the M_3 generation.

A wide range of variability for protein content was induced in most of the treatments and mutant lines. The maximum increase in protein content was 30.39%, 29.41% and 26.47% at l0kR+0.1% EMS, 10kR+0.2% EMS and 0.02% MMS respectively in var. minor, while in var. major the maximum increase was 33.89%, 28.03%, 25.52% and 23.01% at l0kR+0.2% EMS, 0.1% EMS, 0.2% EMS and 20kR+0.3% EMS respectively. The maximum decrease in protein content was 20.59%, 22.55% and 17.16% at 20kR+0.03% MMS, 10kR+0.02% and 0.03% MMS in var. minor, while in var. major maximum decrease in protein content was 30.54%, 18.83% and 15.48% at 20kR+0.03%, l0kR+0.02% and 0.01% MMS respectively (Graph 14.1 and 14.2).

Among different mutant lines, the maximum increase in protein content was 33.82%, 38.72%, 45.58% and 38.23% as compared to control in Minor A, Minor C, Minor D and Minor F, respectively and 28.03%, 28.87% and 31.38% in Major A, Major B and Major M respectively (Graph 14.3 and 14.4). In general, individual mutagenic treatments were most effective in increasing the seed protein content. Varietal sensitivity was noted by the fact that the maximum increase in protein content was observed in the var. minor than var. major. Besides, similar treatment increased protein content in one variety and decreased it in other variety during the investigation.

14.8 Protein electrophoretic patterns

The electrophoretic analysis was performed to determine the variation in seed protein patterns in controls and the isolated mutants of *Vicia faba* L. varieties. The data obtained from the SDS-PAGE gel scanning are presented in Tables 14.7 and 14.8 and the corresponding gel is presented in Figs. 14.21 and 14.22. The results in Tables 14.7 and 14.8 showed that

the number of recorded protein bands ranged from 11-19 in mutants, while 11 protein bands were found in controls. They also indicated that there are differences in protein patterns between control and treated plants. In general, the electrophoretic protein pattern comprised 26 recognizable migration distances, each representing a protein fraction. It was also observed that there was an occurrence of common protein bands that were consistent in the control as well as in the mutants.

The mutants i.e., Minor D, Major A and Major B showed the maximum number of protein bands followed by Major D and Major L, Major K and Major J.

Table 14.1: Brief description of the mutants isolated in M_3 generation of *Vicia faba* L var. minor.

Mutants	Treatment	Salient features
Variety minor	Control	Round seeds.
Minor-A	0.1% EMS	Round bold seeds, high yielding.
Minor-B	0.2% EMS	Round bold seeds, seed coat sculptured, high yielding.
Minor-C	10kR gamma-rays	Round bold green seeds, seed coat colored, high yielding.
Minor-D	20kR gamma-rays	Round bold black seeds, high yielding.
Minor-E	0.03%MMS	Sculptured seed coat, high yielding.
Minor-F	0.01% MMS	Greenish sculptured seed coat, high yielding.
Minor-G	30kR gamma-rays	Tal I mutant, round brown seeds, high yielding
Minor-H	0.02%MMS	Semi dwarf, sculptured seed coat, high yielding.
Minor-I	0.3% EMS	Round bold seeded, high yielding.
Minor-J	10kR+0.1 % EMS	Bold green seeds, high yielding.
Minor-K	20kR+0.3% EMS	Bold seeds, high yielding.
Minor-L	10kR+0.02% MMS	Tall mutant, bold round seeds, high yielding.
Minor-M	20kR+0.2% EMS	Flat bold seeds with sculptured seed coat, high yielding.
Minor-N	0.2% EMS	Hexa flowered, high yielding.
Minor-O	0.3% EMS	High yielding.

Table 14.2: Brief description of the mutants isolated in M_3 generation of *Vicia faba* L var. major.

Mutants	Treatment	Salient features
Variety minor	Control	Round seeds,
Major-A	0.1% EMS	Round bold seeds, high yielding.
Major-B	0.3% EMS	Round bold seeds, seed coat sculptured, high yielding.
Major-C	10kR gamma-rays	Round bold green seeds, seed coat colored, high yielding.
Major-D	30kR gamma-rays	Round bold black seeds, high yielding.
Major-En	20kR gamma-rays	Sculptured seed coat, high yielding.
Major-F	0.01% EMS	Greenish sculptured seed coat, high yielding.
Major-G	0.02%MMS	Tal I mutant, round brown seeds, high yielding.
Major-H	10kR+0.I% EMS	Semi dwarf, sculptured seed coat, high yielding.
Major-I	10kR+0.2% EMS	Round bold seeded, high yielding.
Major-J	20kR+0.02% MMS	Bold green seeds, high yielding.
Major-K	20kR+0.03% MMS	Bold seeds, high yielding, Tal I mutant, bold round seeds, high yielding.
Major-L	10kR+0.0I%MMS	Flat bold seeds with sculptured seed coat, yigh yielding.
Major-M	20kR+0.3% EMS	Hexa flowered, high yielding.
Major-N	0.3% EMS	High yielding.
Major-O	0.03%MMS	Round bold seeds, high yielding.

Table 14.3: Phenotypic correlation between various pairs of quantitative traits of M_3 mutants of broad bean *(Vicia faba* L.) var. minor.

Mutants	Treatment	Fertile branches / plant Vs. Pods/plant	Fertile branches /plant Vs. Yield/plant (g)	Pods/plant Vs. Yield/plant (g)
Minor(parent)	Control	0.422	0.388	0.400
Minor-A	0.1% EMS	0.995**	0.995	0.991**
Minor-B	0.2% EMS	0.996**	0.998	0.993**
Minor-C	10kR gamma-rays	0.994**	0.985	0.992**
Minor-D	20kR gamma-rays	0.994**	0.977	0.991**
Minor-E	0.03% MMS	0.994**	0.973	0.992**
Minor-F	0.01% MMS	0.991*	0.979	0.990*
Minor-G	30kR gamma-rays	0.990 NS	0.977	0.987*
Minor-H	0.02%MMS	0.999 NS	0.971	0.986*
Minor-I	0.3% EMS	0.988 NS	0.969	0.981NS
Minor-J	10kR+0.1% EMS	0.993**	0.977*	0.982NS
Minor-K	20kR+0.3% EMS	0.996**	0.985**	0.995**
Minor-L	10kR+0.02% MMS	0.993*	0.990**	0.986*
Minor-M	10kR+0.01% MMS	0.993*	0.985**	0.984NS
Minor-N	20kR+0.2% EMS	0.995**	0.981*	0.992**
Minor-O	0.3% EMS	0.996*	0.971 *	0.987**

** = Significant at 1%
* = Significant at 5%
NS = Non-significant

Table 14.4: Phenotypic correlation between various pairs of quantitative traits of M_3 mutants of broad bean *(Vicia faba* L.) var. major.

Mutants	Treatment	Fertile branches / plant Vs. Pods/plant	Fertile branches /plant Vs. Yield/plant (g)	Pods/plant Vs. Yield/plant (g)
Major(parent)	Control	0.400	0.422	0.388
Major-A	0.1% EMS	0.994**	0.990**	0.814**
Major-B	0.3% EMS	0.990**	0.987**	0.800**
Major-C	10kR gamma-rays	0.991**	0.987**	0.773**
Major-D	30kR gamma-rays	0.992**	0.985**	0.724**
Major-E	20kR gamma-rays	0.982**	0.979NS	0.672*
Major-F	0.0I%MMS	0.991 **	0.980*	0.537*
Major-G	0.02%MMS	0.987*	0.979NS	0.811 **
Major-H	10kR+0.l% EMS	0.985NS	0.983**	0.741 *
Major-I	10kR+0.2% EMS	0.983NS	0.982*	0.530NS
Major-J	20kR+0.02% MMS	0.983NS	0.980*	0.502NS
Major-K	20kR+0.03% MMS	0.982NS	0.986**	0.792**
Major-L	10kR+0.0l% MMS	0.986*	0.981*	0.569*
Major-M	20kR+0.3% EMS	0.990**	0.983**	0.689*
Major-N	0.2% EMS	0.983NS	0.981 *	0.782**
Major-O	0.03% MMS	0.989*	0.984**	0.769**

** = Significant at 1%
* = Significant at 5%
NS = Non-significant

Table 14.5: Estimates of Mean (X), coefficient of variability (CV) and percentage change over control for protein content in high yielding mutant lines of broad bean *(Vicia/faba* L.) var. minor.

Mutants	Treatment	Mean±S.E.	CV(%)	% change over control
Minor(parent)	Control	20.40±0.45	7.45	-
Minor-A	0.1% EMS	27.30±0.83	8.72	+33.82
Minor-B	0.2% EMS	26.60±0.73	9.58	+30.39
Minor-C	10kR gamma-rays	28.30±0.86	8.26	+38.72
Minor-D	20kR gamma-rays	29.70±0.77	9.27	+45.58
Minor-E	0.03% MMS	26.80±0.78	10.91	+31.37
Minor-F	0.01% MMS	26.30±0.90	6.21	+38.23
Minor-G	30kR gamma-rays	28.20±0.55	10.14	+8.82
Minor-H	0.02% MMS	22.20±1.13	16.72	+20.59
Minor-I	0.3% EMS	24.60±1.30	12.47	-8.82
Minor-J	10kR+0.l% EMS	18.60±0.73	12.62	-13.72
Minor-K	20kR+0.3% EMS	17.60±0.70	14.40	+20.59
Minor-L	10kR+0.02% MMS	24.60±1.l9	1.19	-1.96
Minor-M	10kR+0.01% MMS	20.00±0.88	13.94	-12.25
Minor-N	20kR+0.2% EMS	17.90±1.07	18.89	-21.94
Minor-O	0.3% EMS	21.00±0.86	12.89	

Table 14.6: Estimates of Mean (X), coefficient of variability (CV) and percentage change over control for protein content in high yielding mutant lines of broad bean *(Vicia faba* L.) var. major.

Mutants	Treatment	Mean±S.E.	CV(%)	% change over control
Major(parent)	Control	23.90±0.28	3.66	-
Major-A	0.1% EMS	30.60±0.73	7.58	+28.03
Major-B	0.3% EMS	30.80±0.94	9.65	+28.87
Major-C	10kR gamma-rays	21.60±1.15	16.79	-9.62
Major-D	30kR gamma-rays	27.10±1.15	13.41	+13.39
Major-E	20kR gamma-rays	23.80±1.05	13.98	-0.42
Major-F	0.01%MMS	28.30±0.86	9.58	+18.83
Major-G	0.02%MMS	20.40±I.11	17.17	-14.64
Major-H	10kR+0.l% EMS	22.10±1.00	14.38	-7.53
Major-I	10kR+0.2% EMS	20.40±0.83	12.91	-14.64
Major-J	20kR+0.02% MMS	28.30±0.91	10.14	+18.41
Major-K	20kR+0.03% MMS	28.60±0.73	8.11	+19.66
Major-L	10kR+0.0l% MMS	31.40±0.79	7.97	+31.38
Major-M	20kR+0.3% EMS	29.40±0.63	6.84	+23.01
Major-N	0.2% EMS	20AO±1.10	17.01	-14.64
Major-O	0.03% MMS	22.10±1.15	14.88	-7.53

Table 14.7: Comparative analysis of molecular weights (KDa) of the different protein bands of the seeds of *Vicia faba* L. mutants (var. minor) using SDS-PAGE technique.

Band Number	Lane 1 marker	Lane 2 Minor A	Lane 3 Minor C	Lane 4 Minor F	Lane 5 Minor D	Control
1		-	-	-	-	-
2		75.2	-	74.0	74.0	-
3		72.0	72.0	72.0	72.0	-
4	-	-	-	-	-	-
5	67.0	66.2	-	66.0	66.6	-
6			64.0	63.0	63.0	-
7		59.0	-	-	-	-
8			54.0	54.2	54.2	-
9		51.0	-	-	50.2	-
10	48.0	47.0	46.2	-	48.0	48.0
11			-	45.2	-	-
12			-	-	42.0	42.0
13		38.4	37.4	37.4	38.4	-
14			-	-	35.4	35.4
15		33.0	33.0	32.2	32.0	32.0
16		30.2	29.2	-	-	-
17			-	28.2	28.4	-
18	24.0	23.3	-	23.0	23.4	24.0
19			23.0	-	-	-
20		20.2	20.0	20.4	20.2	20.2
21		18.0	17.0	17.2	17.0	17.2
22			-	-	-	-
23			-	14.2	14.0	14.0
24	12.4	12.0	12.0	12.0	12.0	12.0
25		11.4	11.2	11.2	11.2	11.0
26		10.4	10.4	10.4	10.9	10.2
Total bands		**15**	**13**	**16**	**19**	**11**

Table 14.8: Comparative analysis of molecular weights (KDa) of the different protein bands of the seeds of *Vicia faba* L. mutants (var. major) using SDS-PAGE technique.

Band Number	Lane 1 Marker	Lane 2 control	Lane 3 Major M	Lane 4 Major L	Lane 5 Major K	Lane 6 Major J	Lane 7 Major F	Lane 8 Major B	Lane 8 Major A	Lane 8 Major D
1		-	-	-	75.20	75.20	-	76.20	75.20	-
2		72.00	-	-	-	-	-	74.20	73.20	73.20
3		-	72.20	70.00	-	69.20	71.20	72.20	72.00	-
4	67.00	67.00	-	67.20	66.20	-	-	-	-	66.20
5		63.00	63.20	62.20	62.40	62.40	66.20	64.40	64.40	63.40
6		-	58.20	58.00	58.00	57.30	62.40	62.40	62.40	-
7		55.00	54.00	54.00	54.20	55.20	-	56.20	56.30	55.30
8		-	50.20	-	-	-	56.30	-	52.00	51.20
9	48.00	47.00	-	49.00	49.20	47.00	48.00	47.00	46.20	-
10		-	46.20	-	-	-	-	-	-	46.00
11		-	-	45.00	44.00	45.20	44.20	44.20	44.20	44.00
12		-	43.40	43.00	43.00	42.00	41.20	41.20	40.00	40.20
13		38.20	-	-	-	-	-	38.00	38.20	38.20
14		-	35.40	35.40	-	38.20	38.20	-	-	-
15		-	-	-	34.30	34.20	-	23.00	32.00	32.00
16		-	-	30.00	-	-	-	-	-	30.20
17		27.00	27.20	26.40	26.00	26.00	26.20	25.00	24.00	26.00
18	24.00	-	-	-	24.00	-	-	-	-	24.00
19		23.00	23.00	22.00	-	22.20	-	21.20	22.20	22.20
20		-	19.20	19.00	21.00	20.40	19.20	18.20	17.00	-

21		18.00	18.20	-	18.20	-	-	-	-	16.20
22		-	16.00	16.30	15.00	14.40	14.40	14.20	14.20	14.20
23		14.00	14.30	13.20	12.30	12.00	-	12.00	12.00	-
24	18.40	-	11.60	11.20	11.20	11.00	11.00	11.00	11.00	12.20
25		11.00	10.20	10.20	10.20	10.20	-	10.20	10.00	11.20
26		-	-	-	-	-	-	90.20	-	-
Total bands		**11**	**16**	**17**	**17**	**17**	**12**	**19**	**19**	**18**

Description of Figures – See Colour Centrefold

Fig. 14.1 PMC showing stickiness of five bivalents and one free bivalent at metaphase-1 (Minor-A).

Fig.14.2 PMC showing the stickiness of three bivalents and three separate bivalents at metaphase-1 (Minor-B).

Fig.14.3 PMC showing 1^{S1} and 3^{II} at metaphase-1 (Minor-D).

Fig. 14.4 PMC showing stickiness of five bivalents and one stray bivalents at metaphase-1 (Minor-C).

Fig.14.5 PMC showing one extra (6+1) bivalents (Minor-J).

Fig.14.6 A polyploid PMC showing 12 bivalents (Minor-E).

Fig.14.7 PMC showing stickiness at metaphase-1 (Minor-F).

Fig.14.8 PMC showing stickiness at metaphase-1 (Minor-M).

Fig.14.9 PMC showing 1^{IV} and 4^{n} at metaphase-1 (Minor-N).

Fig.14.10 PMC showing stickiness at metaphase-1 (Minor-O).

Fig.14.11 PMC showing stickiness of three bivalents, one tetravalent and one separate bivalent at metaphase-1 (Minor-Ci).

Fig.14.12 PMC showing the stickiness of three bivalents and three separate bivalents (Minor-H).

Fig.14.13 Control Plants (Variety Minor).

Fig.14.14 High yielding tall bushy mutant (Minor A, 0.1% EMS).

Fig.14.15 High yielding tall mutant (Minor B, 0.2% EMS).

Fig.14.16 High yielding tall bushy mutant (Minor C,10KR Gamma-rays).

Fig.14.17 High yielding tall mutant (Major H, 0.02% MMS).

Fig.14.18 High yielding tall bushy mutant (Major I, 0.3% EMS).

Fig.14.19 High yielding tall bushy mutant (Major J, 10KR + 0.2% EMS Gamma-rays).

Fig.14.20 High yielding tall bushy mutant (Major K, 10KR +0.03% MMS Gamma-rays).

Fig.14.21 SDS PAGE pattern of proteins of Mutants of variety minor along with control.

Fig.14.22 SDS PAGE pattern of proteins of Mutants of variety minor along with control.

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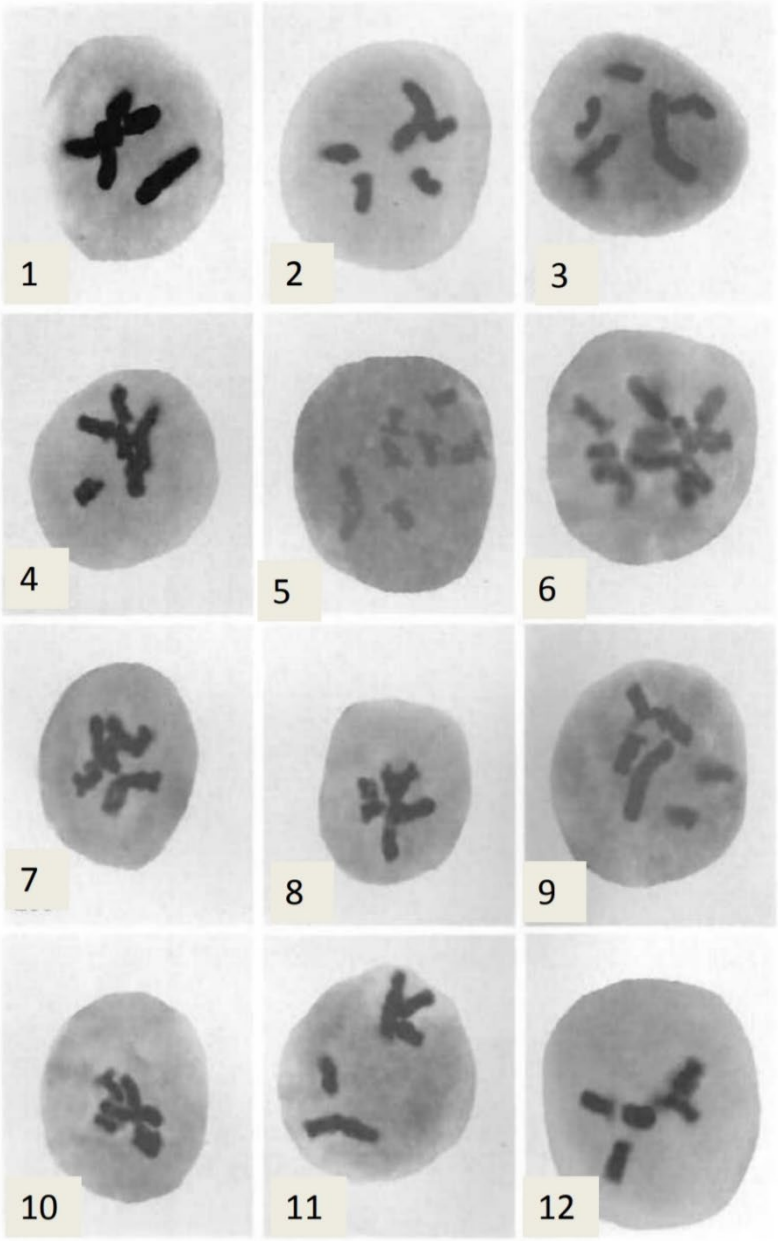
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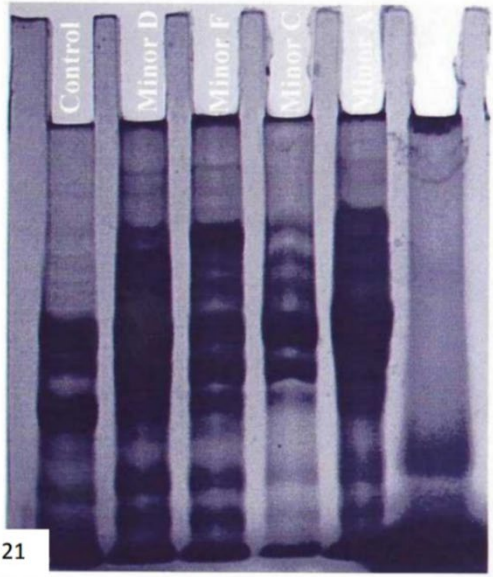
Control
Minor D
Minor F
Minor C
Minor A

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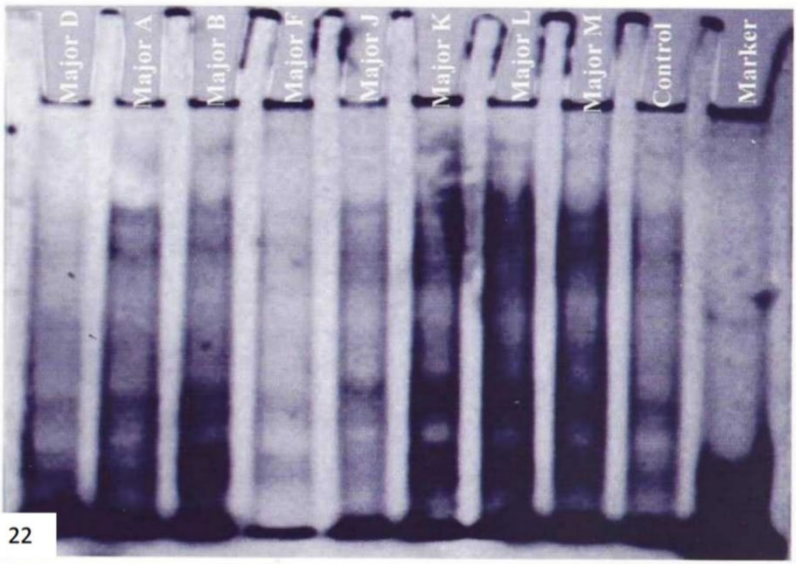
Major D
Major A
Major B
Major F
Major J
Major K
Major L
Major M
Control
Marker

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CHAPTER 15

SITE-DIRECTED MUTAGENESIS IN PLANTS

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Abstract: Genetic variations serve as a genetic resource to incorporate the desired traits in plants. The basic purpose of genetic variations is the improvement of crop yield and enhancement of quality for the betterment of human beings. Instead of natural mutations, researchers induced mutations in plants by different physical and chemical methods but these methods introduce mutations randomly in the genome. Random mutations cause changes in non-targeted genes along with the targeted genes of plant genomes which can disturb the vital plant processes. To overcome this problem targeted mutagenesis was introduced. Powerful tools have been designed for targeted mutagenesis to insert the mutations at specific locations in the genome to get the desired results. Nucleases such as zinc finger nucleases and transcription activator-like effector nucleases have been designed to insert the mutation at the predetermined position. Recently, by using the prokaryote adaptive immune system, the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system has been designed for targeted mutagenesis. Many benefits have been obtained by using targeted mutagenesis, for example, disease-resistant plants and herbicide-resistant plants have been produced. Moreover, the yield and quality of the crops have been improved.

Keywords: Mutation, mutagen, homologous recombination, non-homologous end joining, mutagenesis, CRISPR/Cas, TALEN, biotechnology.

Abbreviations: Chloroplastos alterados (*CLA*), Vacuolar h+-pyrophosphatase (*VP*), 5'-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*), Phytoene desaturase (*PDS*), Mildew-resistance locus (*MLO*), Acetolactate synthase

(*ALS*), Granule-bound starch synthase (*GBSS*), Auxin response factor 1 (*ARF1*), Abscisic acid insensitive 4 (*ABI4*), Fatty acid desaturase (*FDA*), Flower locus t (*FT*), Shriveled seed gene (*SHR*), Acetohydroxyacid synthase (*AHAS*), b-ketoacyl synthetase (*KAS11),* Defective chloroplasts and leaves (*DCL*), Inositol-pentakisphosphate 2-kinase (*IPK1*), Phosphinothricin acetyltransferase (*PAT*), Aryloxyalkanoate dioxygenase 1 (*AAD-1*), Sulfonylurea receptors (*SUR*), Chitinase (*CHN*), Abscisic acid (*AB*), Alcohol dehydrogenases (*ADH*), Transparent testa (*TT*), Mitogen-activated protein kinase (*MAPK*), Recognition of peronospora parasitica 4 (*RPP4*), Polyphenol oxidase (*PPO*), Radiation sensitive 23 (*RAD23*), Dominant suppressor of kar 2 (*DSK2*), N-terminal acetyltransferases (*NAT),* Glabrous 1 (*GLL*), Clavata3 (*CLV3*), Cruciferin (*CRU*), Betaine aldehyde dehydrogenase (*BADH*), Nodulin3 (*N3*), Dense and erect panicle 1 (*DEP1*), Caffeic acid o-methyltransferase (*COMT).*

15.1 Introduction

Induction of mutations in the genomes of plants is the most fascinating phenomenon for scientists to introduce new traits, especially in crop plants for the well-being of humans and other creatures (Bhat *et al.,* 2006b; Bhat *et al.,* 2006a; Bhat *et al.,* 2005a; Bhat *et al.,* 2007b). Today's focus for scientists is to produce plants that have resistance against herbicides, pathogens and adverse climate changes. The history of plant mutagenesis can be sketched back to 300BC when it was reported in two mutant crops in China. In 1934 the first mutant variety of tobacco was produced commercially. 77 cultivars were developed via mutagenesis before 1995 and varieties increased to 484 in 1995 and now this number is very large (Rafii *et al.*, 2016). As a breeding tool, mutagenesis became very popular from the 1950s onwards when a large range of crops and ornamental plant species were largely treated by irradiation to increase trait variation (Shu *et al.*, 2012).

Genetic variations are very important to maintain sustainable agriculture. In the past genetic variations were introduced by breeding plants possessing different traits to attain desirable traits in crops. With the development of the new scientific techniques, scientists developed different methods to introduce genetic variation in the genomes to cope with the increasing demand of crops with increases in the world population and changing climate (Gulfishan *et al.,* 2015; Sharma *et al.,* 2009; Parveen *et al.,* 2006; Gulfishan *et al.,* 2011; Gulfishan *et al.,* 2010; Bhat, 2007; Bhat *et al.,* 2007; Bhat *et al.,* 2007a; Bhat *et al.,* 2006d; Bhat *et al.,* 2005b; Svitashev *et al.,*

2015). To produce the genetic variation in plants, mutations are introduced in the genome by using different mutagenic techniques. In plants, the mutation can be introduced experimentally by induced mutagenesis, insertion mutagenesis and site-directed/targeted mutagenesis (Gulfishan *et al.,* 2012; Gulfishan *et al.,* 2013; Ganai *et al.,* 2005; Bhat and Wani, 2017; Bhat and Wani, 2017a; Bhat *et al.,* 2007; Bhat *et al.,* 2006; Bhat and Wani, 2015).

15.2 Induced Mutagenesis

Agents that can induce mutation in DNA are called mutagens. The nature of mutagens can be classified as physical and chemical. The choice of mutagen either physical or chemical depends on the type of mutation required to be introduced in genome. Normally chemical mutagens are used to introduce point mutations and physical mutagens to introduce gross lesions (Mba *et al.*, 2010). More than 70% of mutant varities are produced by physical mutagens, mostly ionizing radiation has been used in the last 80 years (Kodym and Afza, 2003).

Chemical mutagens are easy to use as compared to physical mutagens because they do not require any special equipment to apply. Simply, the material is soaked in the solution of chemical to induce mutation. More than 80% of mutant plant varities have been produced by using chemical mutagens as reported in the International Atomic Energy Association (IAEA) database. The efficiency of the mutagen depends upon the concentration of mutagen, length of treatment and the temperature during the experiment. Chemical mutagens should always be prepared fresh because they are highly reactive. (Oldach *et al.*, 2008). One of the main disadvantages of chemical and physical mutagens is that they are biohazardous and require skills to handle them.

15.3 Insertional Mutagenesis

Insertional mutagenesis has been used to generate mutation in plants for many years to study the gene and chromosomal behavior. The integrated gene can be T-DNA and endogenous or exogenous transposable elements. T-DNA is integrated into the genome by the co-cultivation of *Agrobacterium tumefaciens*, gene bombardment, or direct gene transfer via electroporation, microinjection or polyethylene-glycol treatment of protoplasts. The foreign gene can be integrated at any coding sequence which interferes with the normal expression of a gene. Several thousand

lines of *Arabidopsis thaliana* created through insertional mutagenesis are now available (www.arabidopsis.org/index.jsp). The maize Ac/Ds and Arabidopsis En/Spm transposon systems, T-DNA are considered the most efficient ways of insertional mutagenesis in plants, because well-organized transformation methods for them are available (Guiderdoni *et al.*, 2005).

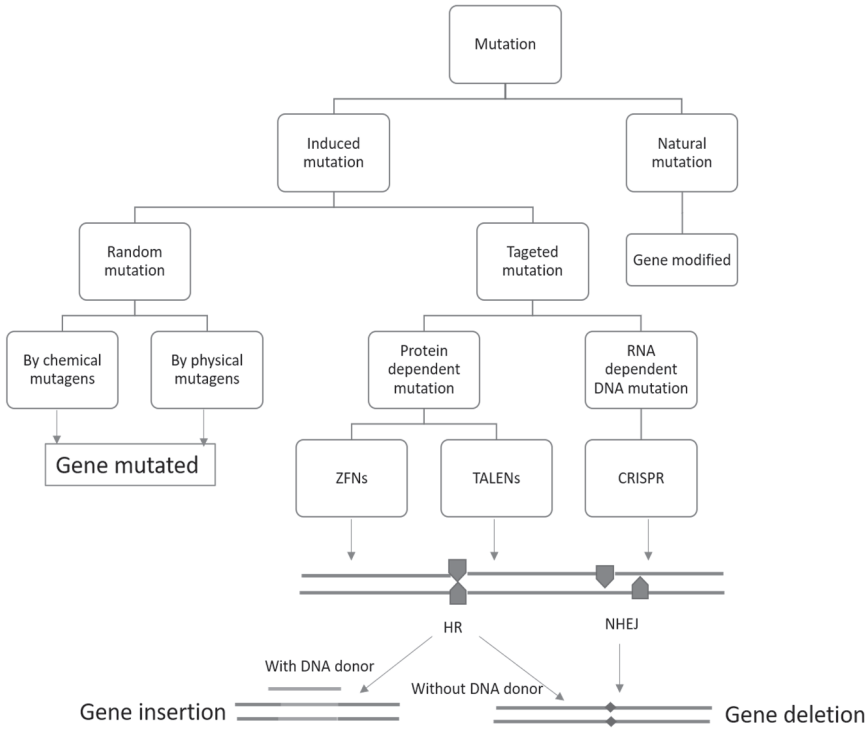

Figure 15.1 Types of mutations. There are two major causes of mutation, one is natural mutation and other is induced mutation. Induced mutation is further divided into random mutation (by a physical or chemical mutagen) and targeted mutation (by ZFNs, TALENs and CRISPR).

The purpose of mutagenesis is to introduce new traits for the improvement of crop quality and quantity and to deal with the forthcoming challenges of fluctuating climate conditions (reduction in water supply, increased temperature, pest attack and pollution etc). By using random mutagenesis, many mutant plants with new traits have been produced, but the main flaw of random mutagenesis was that it also affects the active genes of the plants

that are not under study and are required for the normal growth of plants under given conditions. Both chemical and physical mutagens reduce the viability of the plants by causing the changes in the promoter region of the genes which can lead to either suppression or overexpression of the non-targeted genes. In short, random mutagenesis does not help us to study specific gene expression and suppression in plants.

15.4 Site-directed mutagenesis/targeted mutagenesis

Integration of a foreign gene at the predetermined position is called site-directed/targeted mutagenesis. Gene targeting is mostly achieved by homologous recombination or non-homologous end joining. For more than twenty years gene targeting has been used efficiently in bacteria, yeast and mice (Rothstein, 1991). In this method DNA fragments are introduced into the cultured cells by homologous recombination by which means the DNA fragment is incorporated into the homologous locus. Numerous attempts have been made to attain gene targeting in higher plants over the last seventeen years. The gene is introduced into the cultured cells by direct or *Agrobacterium tumefacien* method and the gene is incorporated by non-homologous end joining. The efficiency of non-homologous end joining is better than homologous recombination for gene targeting. (Lloyd *et al.*, 2005).

Targeted mutagenesis is based on the DNA repair system by homologous recombination and non-homologous end joining. To repair a double-strand break in DNA (caused by UV, or a chemical or physical mutagen) requires an undamaged homologous sister chromatid to use as a template. During the targeted mutagenesis, foreign DNA having the homologous sites is used as a template for recombination. In most organisms, foreign DNA is inserted into the genome by homologous recombination. However, in plants, it is achieved by non-homologous end joining and results in the integration of foreign DNA in plant genome. Some strategies have been developed to enhance the homologous recombination by overexpression of RAD54, recA and ruvA proteins which are involved in homologous recombination (Weinthal *et al.*, 2013).

Targeted mutagenesis has turned out to be an effective technique, used to edit the genome of plants by using the engineered nucleases and synthetic oligonucleotides at a specific location. Nucleases induce the double-strand break at the target site which is then repaired by homologous recombination or non-homologous end joining, depending on the type of mutation to be

introduced (insertion or deletion). There are four types of targeted mutagenesis techniques:

1. Zinc finger nucleases (ZFNs).
2. Transcription activator-like effector nucleases (TALENs).
3. Oligonucleotide-directed mutations (ODM).
4. CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated) 9.

Table 15.1: comparison between different targeted mutagenesis techniques.

Properties	ZFNs	TALEN	CRISPR	ODM
Mode of recognition	Protein-DNA	Protein-DNA	RNA-DNA	RNA-DNA
DNA binding entity	Zinc finger protein	Transcription activator-like protein	RNA	Oligonucleotide
Nuclease	Fok1	Fok1	Cas9	No
Target	12bp	16bp	20bp	60-88nt
Construct	Zinc finger sequence linked with fok1	TAL sequence linked with fok1	A crRNA linked with tracer RNA and cas9	Chimeric RNA/DNA
Cost	Very expensive	Less expensive	Low cost	
Time consumption	Very time consuming	Less time consuming	Minimum time consuming	Moderate
Multiplexing	No	No	Yes	No
Success rate	Low	High	High	Middle
Mod of action	Double-strand break	Double-strand break	Double or single-strand break	Information strand direct conversion in target DNA

15.4.1 Zinc finger nucleases (ZFNs)

Zinc finger nucleases are well characterized engineered nucleases, in which non-specific DNA cleavage enzyme Fok1 is attached with the DNA binding zinc finger motif. ZFN is composed of two monomers each having three or four zinc fingers. Each zinc finger recognizes 3 nucleotide and 18 nucleotides by a whole zinc finger nuclease. Zinc finger domains are engineered to bind with specific DNA sequences and could be designed to identify every sequence in the plant genome. Hence, they have the potential to mutate every gene of the plant genome. ZFNs could be designed according to need with the unique combination of 18 nucleotides (Mani *et al.*, 2005).

ZFNs can be used as an effective vehicle for targeted mutagenesis in plant species. This tool has been successfully used for targeted mutagenesis in *Arabidopsis thaliana* and soybean by non-homologous end joining, as well as targeted mutagenesis by homologous recombination in *Arabidopsis* (Zhang *et al.*, 2010), tobacco (Townsend *et al.*, 2009) and maize (Shukla *et al.*, 2009). Targeted mutagenesis using ZFNs is also applicable the perennial trees such as apple and fig (Peer *et al.*, 2015).

15.4.1.1 Mechanism of action

Zinc finger nuclease is composed of a DNA binding domain fused with nuclease for DNA cleavage. Zinc finger nucleases bind with the DNA and then *fok1* nucleases dimerize. Zinc finger nucleases cleave the DNA by the double-strand break and induce repair system. In the absence of donor template, error-prone non-homologous end join repairs the break. In the presence of a homologous template, homologous recombination occurs, and the gene is edited. ZFNs have been designed to induce mutagenesis in plants by homologous recombination instead of non-homologous recombination. ZFNs have good efficiencies at introducing targeted mutagenesis in plants (Urnov *et al.*, 2010).

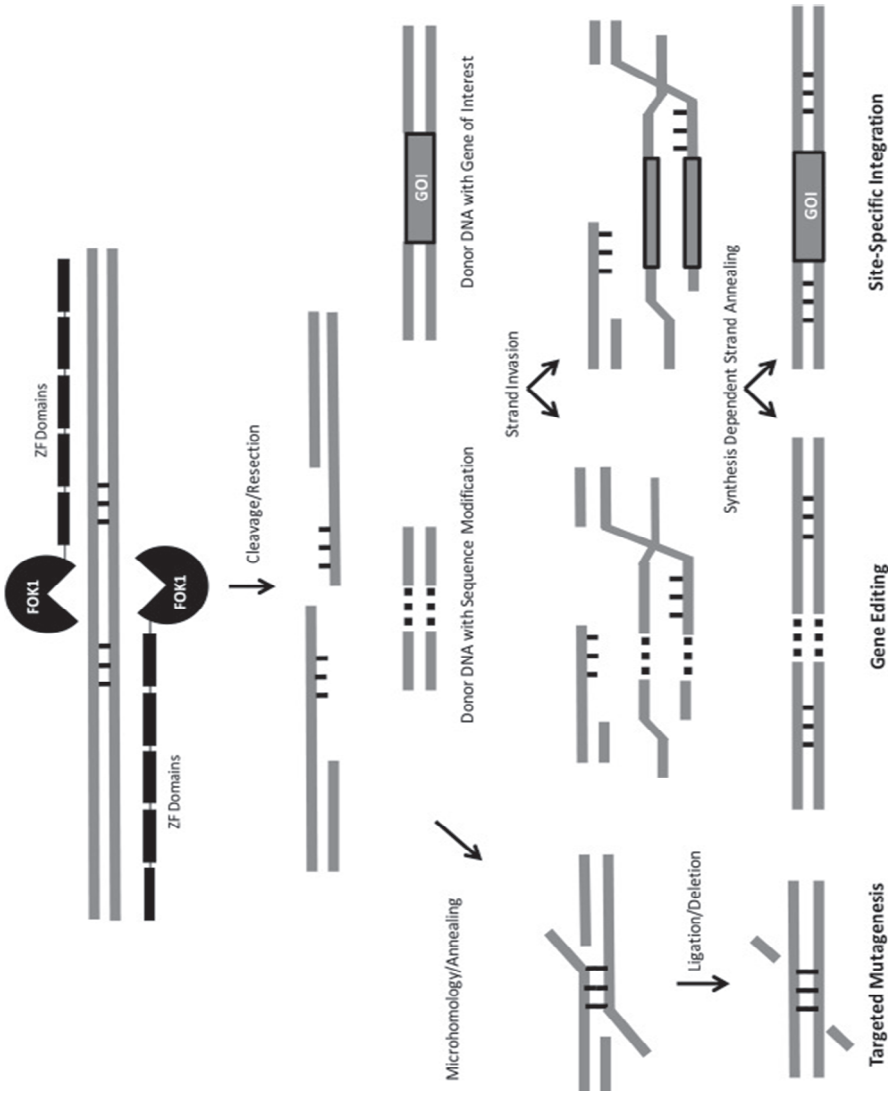

Figure 15.2 Gene editing by using zinc finger nucleases. Zinc finger nuclease binds to the DNA and nucleases fok1 dimerized and introduces double-strand break at the cleavage site. Without the templet double-strand break repair by the non-homologous end joining and deletion occurred (left). In the presence of templet homologous DNA double-strand break is repaired by homologous recombination either nucleotide substitution or deletion occur (center) or gene is integrated into the genome (left), depending upon the type of templet DNA (Petolino, 2015).

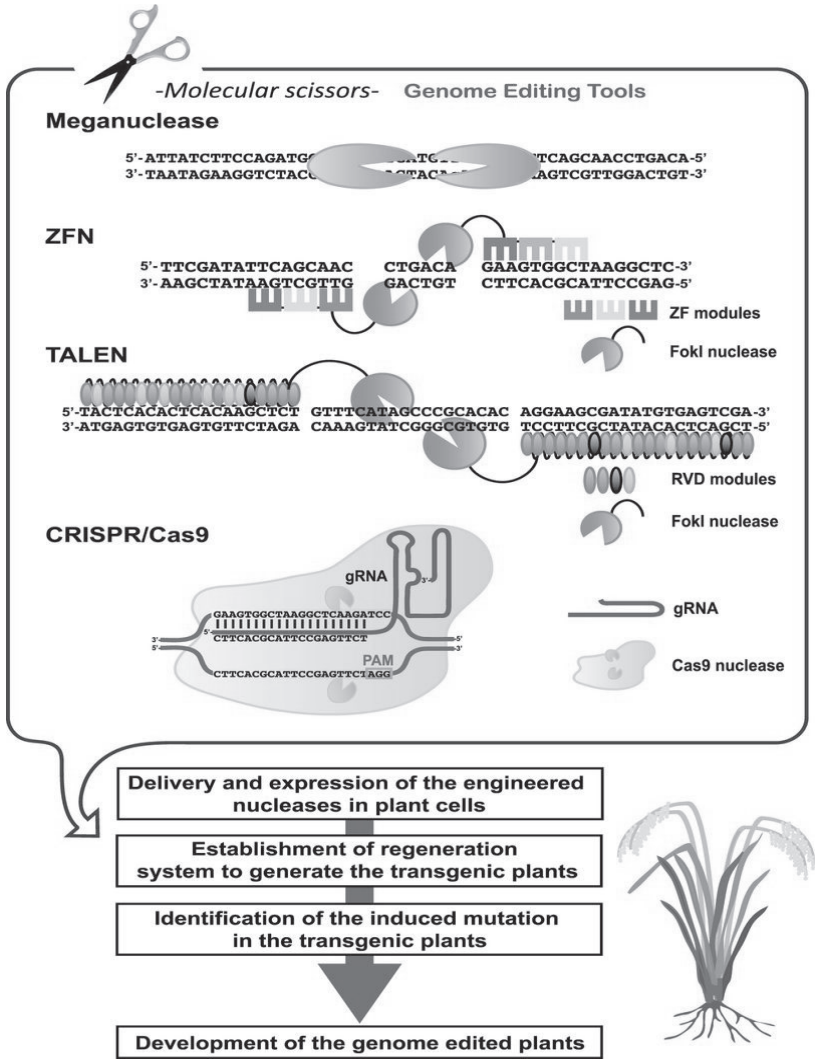

Figure 15.3 Flow diagram of genome editing using engineered nucleases (Osakabe and Osakabe, 2014).

Table 15.2: Plant species mutated by using ZFNs.

Species	Target gene	Reference
Arabidopsis thaliana	*ABI4, ADH1, TT4, MPK, RPP4 gene cluster, PPO*	(Osakabe *et al.*, 2010, Zhang *et al.*, 2010, Qi *et al.*, 2013, De Pater *et al.*, 2013)
Nicotiana benthamiana	*SurA/B, CHN50,*	(Townsend *et al.*, 2009, Wright *et al.*, 2005, Maeder *et al.*, 2008)
Zea mays	*IPK1, Pat, aad1*	(Shukla *et al.*, 2009, Ainley *et al.*, 2013)
Brassica napus	*KasII*	(Gupta *et al.*, 2012)
Glycine max	*DCL*	(Curtin *et al.*, 2011)

15.4.2 Transcription activator-like effector nucleases (TALEN)

TALEN is a newly developed technique for targeted mutagenesis. Transcription activator-like effector (TALE) is a DNA binding protein derived from the various plant-pathogen bacteria belonging to genus *Xanthomonas*. When bacteria infect the plant, they secrete TALE protein that moves into the nucleus of plant cells and binds with the DNA at a specific recognition site (Boch and Bonas, 2010). This property of the TALE protein has been used for genome editing. TALEN is composed of DNA binding TALE protein and Fok1 endonuclease. Several TALENs have been engineered for target binding. The activity of the TALEN protein is affected by the flanking region of DNA binding domain in eukaryotes including plant species (Voytas, 2013). In rice and *Arabidopsis*, the effect of N and C-terminal flanking regions of the DNA binding domain on the targeted mutagenesis has been studied. Truncated flanking regions enhance the TALEN activity (Cermak *et al.*, 2011). Several successful studies have been carried out for targeted mutagenesis by using TALEN in plants, listed in Table 15.3.

Hax3, is a DNA binding protein derived from the Brassicaceae pathogen *Xanthomonas campestris pv. armoraciae* strain 5, recognizing 12 base pairs. It has been used for new TALEN induced targeted mutagenesis in tobacco (Mahfouz *et al.*, 2011). Targeted mutagenesis has been induced in the *ADH1* gene of *Arabidopsis thaliana* by the custom TALE array commonly known as the Golden Gate System (Cermak *et al.*, 2011). Other than the model

plants, this technique has been used to mutate genes in crop plants such as rice, barley and maize. In rice, the disease susceptible gene and sucrose efflux reporter gene *OsSWEET14* were mutated by using TALEN. Moreover, by the genetic segregation, rice plants that do not have the selection marker and TALEN gene can be obtained (Li *et al.*, 2012). On a large scale, it was applied in rice and *Brachypodium* to knock out eight genes, as well as large genomic deletion by simultaneously expressing the two pairs of TALEN (Shan *et al.*, 2013a). In barley, it has been used to disrupt the gene in embryonic pollen culture having haploid cells (Gurushidze *et al.*, 2014).

15.4.2.1 Advantages of using TALEN

TALEN is given preference on the ZFNs for two reasons. First, it is less toxic as compared to ZFNs and second, it is driven by the strong constitutive promotor in contrast to conditional inducible promotors in ZFNs. It has been found that using the strong constitutive promotor is more efficient at inducing site-directed mutagenesis as compared to conditional inducible promotors (Christian *et al.*, 2013).

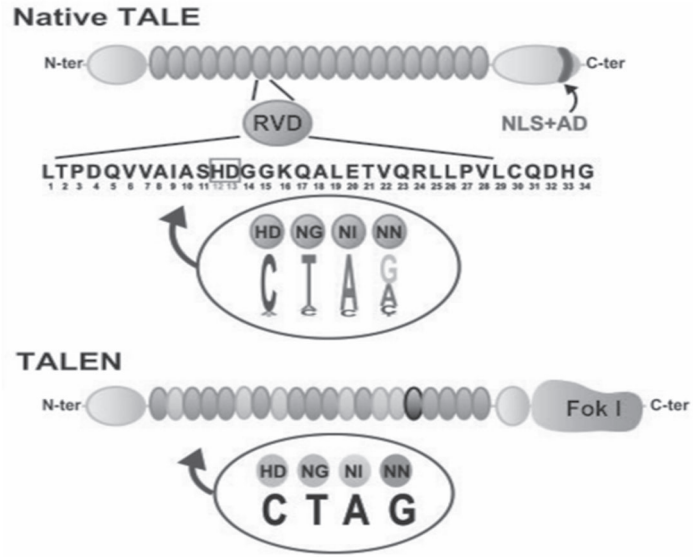

Figure 15.4: Inborn TALE of Xanthomonas is composed of tandem repeat domains of 33–35 amino acids. TALENs can identify the target sites that are separated by a 12–20bp spacer region (Osakabe and Osakabe, 2014).

Table 15.3: plant species mutated by TALEN

Plant name	Genes	Reference
Arabidopsis thaliana (Arabidopsis)	*ADH1, TT4, DSK2B, NATA2, GLL22a, MAPKKK1, GLL22b, CLV3, CRU3,*	(Christian *et al.*, 2013, Cermak *et al.*, 2011, Forner *et al.*, 2015, Johnson *et al.*, 2013)
Nicotiana benthamiana (Tobacco)	*ALS*	(Zhang *et al.*, 2013)
Oryza sativa (Rice)	*OsBADH2, 11N3, DEP1, BADH2, CKX2, SD1, EPSPS, MST7, MST8, PMS3, CSA, DERF1, LOX3, ALS, SWEET14, WAXY,*	(Zhang *et al.*, 2016a, Li *et al.*, 2012, Shan *et al.*, 2013a, Shan *et al.*, 2015, Wang *et al.*, 2015b, Ma *et al.*, 2015a, Li *et al.*, 2016b, Blanvillain-Baufumé *et al.*, 2017, Nishizawa-Yokoi *et al.*, 2016)
Hordeum Vulgare (Barley)	*HvPAPhy_a,*	(Wendt *et al.*, 2013)
Zea mays (Maize)	*GL2, IPK1A, IPK, MRP4*	(Char *et al.*, 2015, Liang *et al.*, 2014)
Brachypodium spp.	*ABA1, CKX2, SMC6, SPL, SBP, COII, RHT, HTA1*	*(Shan et al., 2013a)*
Solanum tuberosum (Potato)	*ALS, Vlnv*	*(Clasen et al.,* 2016*, Nicolia et al.,* 2015*, Butler et al.,* 2016*)*
Lycopersicum esculentum (Tomato)	*PROCERA, ANT1*	(Lor *et al.*, 2014)
Triticum aestivum (Wheat)	*MLO*	(Čermák *et al.*, 2015)
Glycine max (Soybean)	*FAD2-1A, FAD2-1B, FAD3A, PDS11, PDS18*	(Haun *et al.*, 2014, Demorest *et al.*, 2016, Du *et al.*, 2016)
Saccharum officinarum (Sugar cane)	*COMT*	(Jung and Altpeter, 2016)

15.4.3 Oligonucleotide-directed gene targeting

In oligonucleotide-directed mutagenesis, chimera (DNA/RNA) and modified DNA nucleotide are used for the site-specific mutagenesis. Targeted mutagenesis by oligonucleotide direct mutagenesis has been successfully carried out in bacteria, yeast, mammals and plant systems.

Different mutated plants were produced by using this technique, but it is very efficient in mammals as compared to plants. Oligonucleotide-directed gene targeting was first used on maize and tobacco. In both plants the acetolactate synthase (*ALS*) gene mutated by base changing and it was observed that it follows the Mendelian segregation rules and remains stable in generations. Chimaeric oligonucleotide is composed of 68 nucleotides which have a mutator region composed of five nucleotides flanked with 2'-O-methyl RNA bridge. Mutator DNA is complementary to the target site (Sauer *et al.*, 2016).

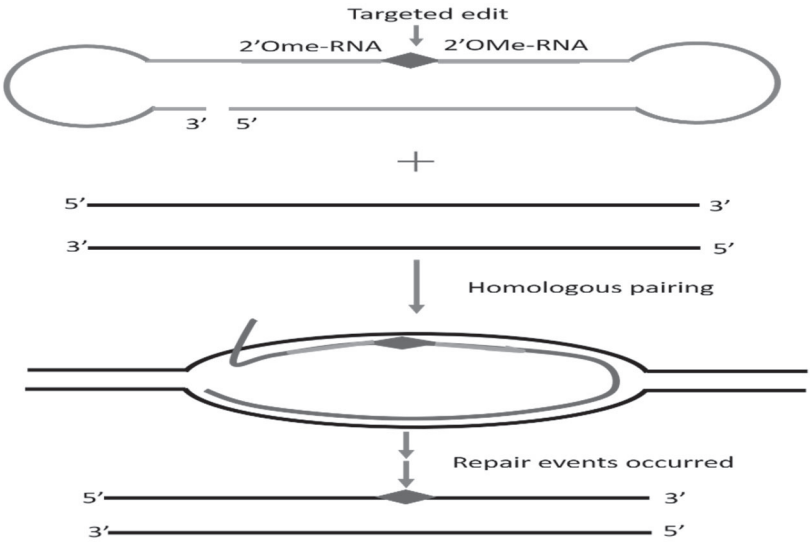

Figure 15.5: Mechanism of oligonucleotide-directed mutagenesis.

Oligonucleotide-directed mutagenesis is achieved by homologues pairing between the target gene to be corrected and the construct that has a mutated gene and then the mismatch is removed by the mismatch repair machinery of the plants. Correct homologous pairing is the rate limiting step in the repair frequency of this technique.

To begin with, this technique *ALS* in maize (Zhu *et al*., 1999) and tobacco was mutated and it was later applied on *Brassica napus* (Sauer *et al.*, 2016). In tobacco *ALS* gene, proline-196 was converted into threonine by oligonucleotide-directed mutagenesis. This was achieved by bombarding the chimera RNA/DNA having a mutation at proline-196, on the cultured

cells of tobacco and then it was observed that transformed cells have chlorsulfuron resistance. Similarly, tobacco mesophyll cells were transformed with the chimeric RNA/DNA constructs having the acetohydroxyacid synthase (*AHAS*) gene mutated at proline-196-alanine, threonine, glutamine and serine substitution and tryptophane-573 with leucine and it was observed that all plants were resistant to chlorsulfuron, except proline-196-leucine. The same approach was used to mutate the *AHAS* gene by oligonucleotide-directed mutagenesis in which serine-621-asparagine and proline-165-alanine with a single construct were used to induce resistance against both sulfonylurea and imidazoline herbicides.

Table 15.4: Plant species mutated by using oligonucleotide-directed mutagenesis.

Species	Target gene	Reference
Arabidopsis thaliana	*BFP*	(Sauer *et al.*, 2016)
Zea mays	*ALS*, kanamycin, tetracycline	(Zhu *et al.*, 1999)
Oryza sativa	*ALS*	(Okuzaki and Toriyama, 2004)
Triticum aestivum	*GFP*	(Dong *et al.*, 2006)
Brassica napus	*GFP*	(Sauer *et al.*, 2016)
Nicotiana benthamiana	*ALS*	(Kochevenko and Willmitzer, 2003)
Musa acuminata	Kanamycin	(Gamper *et al.*, 2000)
Spinacia oleracea	Kanamycin, tetracycline	(Kmiec *et al.*, 2001)

15.4.4 Clustered regularly interspaced short palindromic repeats/Cas 9 (CRISPR-associated)

This technique for genome editing was developed a few years ago by using the bacterial CRISPR type II prokaryotes adaptive immune system. This system uses Cas9 nuclease and single-guide RNA engineered to target a specific DNA sequence. It is considered easier to use as compared to ZFN and TALEN as it based only on one protein and single-guide RNA (Takahashi *et al.*, 2018).

Currently, this technique is now also used in plants for targeted mutagenesis and was reported for the first time in 2013 in rice and wheat, *A. thaliana* and *Nicotiana benthamiana*. Different reports have been given in which

CRISPR/Cas has been used for genome editing via protoplast transformation and in a plant using *Agrobacterium tumefaciens*. The mutation is carried out by both HR and NHEJ (Belhaj *et al.*, 2013).

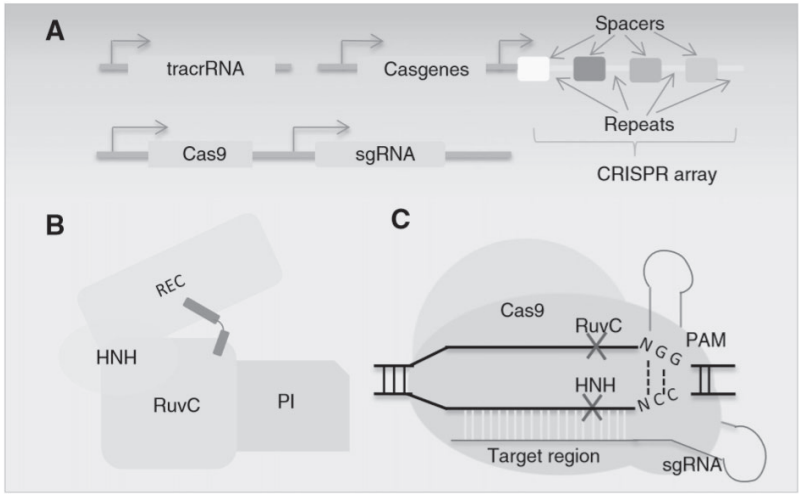

Figure 15. 6. Schematic diagram of CRISPR/Cas9. A) The top diagram is a display of CRISPR array in the prokaryote and the lower one is a representation of CRISPR construct for genome editing. B) general mechanism of how CRISPE/Cas9 work (Song et al., 2016).

15.5 How to use genome editing for plants

For mutagenesis in plants, Cas9 is modified to optimize the bacterial gene codons for plant expression. In plants, Cas9 is expressed under the 35S promoter of cauliflower mosaic virus or ubiquitin promotor of maize and single-guide RNA is expressed under RNA Polymerase III U3 or U6 promoter. Single-guide RNA is kept under the strong promoter to achieve high expression because the low expression of guide RNA can be a limiting factor of mutation efficiency. There are different methods used to introduce Cas9 and guide RNA into the plant sample using protoplast for transient expression assay, particle bombardment to the callus or *Agrobacterium* mediated transformation (Murai, 2017).

The efficiency of any system depends upon the target specificity of that system, therefore, the CRISPR system's efficiency depends on the target specificity of the single-guide RNA. An efficient CRISPR has good

specificity at the 3' end but the deviation at the 5' end is tolerable. So, to avoid the off-target effects of the CRISPR, the target recognition ability should be very high. There are two strategies to reduce the off-target effects and enhance target recognition ability. One of them is to design such type of cas9 nuclease that produces a nick in the DNA and design the paired cas9 monomer in such a way that they have the co-dependent nick production activity to avoid insertion and deletion mutation. The other strategy is to utilize the specialized single-guide RNA. For that 5' truncated guide RNA (17-18 nucleotide) is used to avoid off-target effects (Sander and Joung, 2014). Several plants have been targeted for mutagenesis by using CRISPR/cas9 as shown in Table 5.

Table 15. 5: plant species mutated by using CRISPR/Cas9.

Plant name	Gene	Reference
Camelina	*FAD2*	(Morineau *et al.*, 2017, Jiang *et al.*, 2017)
Glycine max	*FT2a, 07g14530, 01g38150, 11g07220, miR1514, miR1509, 06g14180, 08g02290, 09g00490, 12g37050, PDS, DD, ALS, FE, SHR, Rj4*	(Jacobs *et al.*, 2015, Sun *et al.*, 2015, Du *et al.*, 2016, Li *et al.*, 2015, Cai *et al.*, 2015, Tang *et al.*, 2016)
Oryza sativa	*eIF$_4$G, AAP$_3$, ALS, NRT1.1B, CDC48, SLR1, ACC-T, PDS, BADH2, MPK, 02g23823, ROC5, SPP, YSA, MYB, DERF1, EPSPS, MSH1, PDS, PMS3, SWEET, CAO1, LAZY1, BEL, CPS4, CYP99A2, CYP76M5, CYP76M6, KO1, KOL5, CDKA2, CDKB1, CDKB2, GSTU, MRP15, ANP, WAXY, 7 FTL genes, AOX1, BEL, YSA, DL, P450, DWD1, RAV2, DMC, NAL1, LPA1, LG1, GL1-1, DEP1, ROC5, Gn1a, GS3, IPA1, ERF922, OST2, CSA, RUPO, EPSPS, TMS5, PMR, MEGs, PEGs, Hd, SBEI, SBEIIB, ACT, GST, RBOHH, EPFL9*	(Wang *et al.*, 2015a, Shan *et al.*, 2013c, Xie and Yang, 2013, Zhang *et al.*, 2014, Miao *et al.*, 2013, Xu *et al.*, 2014, Zhou *et al.*, 2014, Endo *et al.*, 2015, Xie *et al.*, 2015, Sun *et al.*, 2016, Ma *et al.*, 2015b, Yin *et al.*, 2017, Yamauchi *et al.*, 2017, Wang *et al.*, 2017)

Lycopersicum esculentum	*CycB, JAZ2, ALC, SHR, AGO, 08g041770, 07g021170, 12g044760, RIN, PDS, PIF4, SIAGL6, SP5G, SIBOP, SIIAA9, MLO*	(Ron *et al.*, 2014, Brooks *et al.*, 2014, Ito *et al.*, 2015, Pan *et al.*, 2016, Klap *et al.*, 2017, Soyk *et al.*, 2016, Xu *et al.*, 2016, Ueta *et al.*, 2017, Nekrasov *et al.*, 2017)
Solanum tuberosum	*16DOX, IAA2, ALS, GBSS, MYB44*	(Wang *et al.*, 2015c)
Arabidopsis thaliana	*PDS3, FLS2, RACK1b, RACK1c, BRI1, GAI, JAZ1, CHLI, TT4, AP1, GL2, ADH1, RTEL, FT, SPL4, ABP1, Cru3, TRY, CPC, ETC2, CHIL, 1g03180, 1g16210, 5g55580, 1g56650, PHYB, BRI1, PDS3, PYR1, PYL, SH3P3, eIF(iso)4E, CBF, DM2, UGT79B, CWIN1, MIR169a, MIR827a, TFL1, TTG1*	(Li *et al.*, 2013, Jiang *et al.*, 2013, Mao *et al.*, 2013, Feng *et al.*, 2014)
Nicotiana tabacum	*PDS, PDR6*	(Gao *et al.*, 2015)
Zea mays	*PSY1, ZB7, 2g332562, 2g080129, 2g099580, 2g170586, 2g438243, ARGOS8, AGO18a, Ago18b, a1, a4*	(Liang *et al.*, 2014, Svitashev *et al.*, 2015)
Triticum aestivum	*MLO, INOX, GASR7, GW2, DEP1, NAC2, PIN1, LOX2, Ubi, GASR7*	(Wang *et al.*, 2014, Upadhyay *et al.*, 2013, Shan *et al.*, 2013b, Zhang *et al.*, 2016b, Gil-Humanes *et al.*, 2017)
Hordeum vulgare	*HvPM19*	(Lawrenson *et al.*, 2015)
Brassica oleracea	*BoIC.GA4.a*	(Lawrenson *et al.*, 2015)
Gossypium sp.	*MYB25-like A, MYB25-like D, CLA1, VP*	(Li *et al.*, 2017, Chen *et al.*, 2017)

15.6 Applications of targeted mutagenesis

15.6.1 Study functional genomics

Targeted mutagenesis has been used to achieve modification in the plant genomes via ZFNs, TALEN and CRISPR. Several modifications such as gene insertion, point mutation, substitution and deletion of a large fragment

of a gene are made to introduce mutation in the plant genome. The main purpose of targeted mutagenesis is to study the function of a specific gene in plants. Scientists use targeted mutagenesis to study the role of an individual gene, not only in a single cell but also in the whole organism. By studying functional genomics of crop plants with different targeted mutagenesis techniques, we can increase the molecular breeding of crop plants. Targeted mutagenesis can be used for single-gene knockout and for multiplex gene knockout to check the effect of multiple genes simultaneously. Large fragment deletion by introducing two double-strand breaks in the target DNA has become possible. Gene knockout in polyploid plants that was very difficult to achieve with traditional methods of mutagenesis, has become quite easy by using targeted mutagenesis. In *Triticum aestivum,* all six alleles of *MLO* gene have been mutated by using TALEN and CRISPR systems.

15.6.2 Enhanced yield

Crop plant quantity and quality improvement are the ultimate objectives of targeted mutagenesis. The yield of a crop is dependent on the number of grains and their weight. Genes are found that are related to crop yield and these genes are mutated by targeted mutagenesis. For example, in rice plants there are a few genes (*GS3*, *DEP1*, *GS5*, *GW2*, *Gn1a*, and *TGW6*) that negatively affect the yield of rice (Li *et al.*, 2016a). By using the CRISPR technique, these genes were mutated which ultimately increased rice yield. Similarly, in wheat those genes that negatively regulate the kernel width and weight were knocked out by CRISPR to improve the yield (Zhang *et al.*, 2016b).

15.6.3 Disease resistance

Scientists were trying to produce disease-resistant plants and have successfully produced disease-resistant rice by targeted mutagenesis in the TAL binding sequences of DNA. When pathogens attack plants, they translocate their virulence protein into the nucleus of the plants and bind with the promoter gene (S) and activate the gene expression that develops a vulnerable reaction between plants and pathogens. These promoter elements are mutated by TALEN or other target mutagenesis introducing tools and then these promotors are no more available for protein binding. Powdery mildew resistant wheat varieties have been produced via targeted mutagenesis. Powdery mildew when it attacks wheat targets the *MLO* locus that encodes the G-protein and disrupts the wheat defense mechanism. In

the powdery mildew resistant wheat, the mildew-resistance locus (*MLO*) gene is no longer available for the pathogen that is mutated by using the TALEN (Wang *et al.*, 2014).

15.6.4 Herbicide resistance

Herbicides are used to kill herbs that not only kill the herbs but also affect the growth of a crop. 5'-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) and Acetolactate synthase (*ALS)* are the genes whose products are targeted during the herbicide synthesis. Both genes products are involved in the biosynthesis of amino acids, so when these enzymes are inhibited by the herbicide, the plants die due to starvation. The aAcetolactate synthase (*ALS)* gene has been mutated by ZFNs that is targeted by the imidazolinon and sulfonylureas, triazolopyrimidines, pyrimidinyl oxybenzoates, and sulfonylamino carbonyl triazolinones herbicides. In rice, maize and soybeans this gene has also been mutated by using CRISPR and TALEN (Li *et al.*, 2016b). This technology can be used in future to create herbicide-resistant plants. The *EPSPS* gene product is inhibited by the herbicide glyphosate. In flax and rice plants *EPSPS* genes have been mutated by CRISPR and oligonucleotide-directed mutagenesis to make them tolerant against the herbicide.

15.6.5 Seed oil content improvement

Oil content in seeds can be improved by targeted mutagenesis. Zinc finger nucleases have been designed to improve C_{18} and decrease palmitic acid. The enhanced multiplication of two genes via b-ketoacyl ACP synthase II (*KASII*) in which the VP16 transcriptional activator domain had been connected. TALEN has been used to improve the oleic acid content of the soybean by controlling the two-desaturase genes activity i.e. *FAD2* and *FAD3*, these convert oleic acid to linoleic acid.

Instead of the above mentioned application of targeted mutagenesis, there are some other applications of these approaches such as aromatic rice which has been developed and in which the betaine aldehyde dehydrogenase 2*(BADH2*) gene is disrupted by TALEN and the 2-acetyl-1-pyrroline (*2AP)* level is increased that gives fragrance to the rice. In maize, phosphorus is present in the form of phytic acid that is an anti-nutrient compound and inhibits the digestion of food. So, to reduce the phytic acid content of maize, ZFNs have been designed to modify the gene involved in the production of phytic acid.

15.6.6 Safety issues related to targeted mutagenesis

Targeted mutagenesis is a good way to keep the native structure of the genome intact as compared to random mutagenesis and is considered harmless for improving the crops. Despite this consideration, there are some biosafety issues related to crops using targeted mutagenesis. The most dominant issue is the off-target effects of nuclease during the mutagenesis. The off-target effects of a technique can be reduced by increasing the efficiency and specificity of that technique. The specificity of the nuclease to introduce the doubled-strand break at the targeted site can be checked before the experiment, in silico. Different software is available that we can use to check the specificity of the technique to minimize the off-target effect of that technique.

15.6.7 Conclusion

Targeted mutagenesis of the plant genome is very important for investigating the function of genes and to genetically modify crop plants for their trait improvement. Targeted mutagenesis could be used to improve the crop plants that lack the transgenic DNA. It has been found that targeted mutagenesis is preferable to random mutagenesis due to its precise and effective results on plant genomes to study plant biology. Site-directed mutagenesis has become more efficient and easier with the engineered nucleases and guided RNA which are designed in such a way that they can induce mutation at the target site and the chance of off-target effects is reduced. Finally, targeted mutagenesis will help us to identify new alleles and improve the quality and quantity of crops to deal with the upcoming challenges

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CHAPTER 16

MUTAGENESIS AND PLANT BREEDING IN THE TWENTY-FIRST CENTURY

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Abstract: In the recent era, global challenges of rapidly increasing population, abrupt climate changes, global warming, reduction in soil fertility and agricultural lands have been the main contributors toward reduced crop productivity and subsequent food security. Plant breeding is one of the tools used for altering plant characters to improve the quality of human life. Mutagenesis in plant breeding is the source for incorporating desirable traits in to crops to produce new varieties. These modified varieties are usually superior to previous ones in terms of their yield, quality, and resistance against biotic and abiotic stresses. An increase of about 60% in food demand is anticipated to provide for the 9.8 billion projected human population by the year 2050 (UN Department of Economic and Social Affairs 2017). Mutational breeding to develop new crop varieties with improved characteristics might be an important source to meet the new challenges soon. The traditional plant breeding techniques are responsible for developing new varieties in crop plants. In certain cases where conventional plant breeding techniques fail, induced mutations are the sources of bringing desirable traits in crop plants (Khan and Wani, 2004).

Keywords: Genetic engineering, mutagen, RNA-dependent DNA methylation, CRISPR-cas, Oligonucleotide-directed mutagenesis, mutation breeding

16.1 Historical Background

In plant breeding techniques, mutations are the primary source for bringing variability and diversity in the genetic make-up of any organism (Kharkwal, 2012). Mutation breeding is the technique of producing smart crop varieties by bringing genetic variability using certain chemical or physical mutagens. The concept of mutation breeding came from the start of the twentieth century with the discovery of the techniques of using different chemical and physical agents to bring genetic variability such as using X-rays (Shu *et al*., 2012). Mutation breeding brought a great revolution in the world of conventional plant breeding techniques. During the last five decades, these discoveries of induced mutations led to the development of smart crop varieties all around the world. In this century, mutation breeding is one of the most powerful tools in the hands of plant breeders among other modern breeding techniques such as transgenic and recombinant breeding techniques. However, Novak and Brunner (1992) described that in seed propagated plants, breeding through induced mutations can be a better way to achieve desirable traits in plants. Whereas in seedless crops (seedless grapes and banana) mutation breeding is the only way to achieve new varieties with novel characters (Ahloowalia and Maluszynski, 2001; Broertjes and Van Harten, 1988; Chai *et al*., 2004). Moreover, crops which propagate through roots or tubers and in ornamental plants, cross-breeding has many constraints of clonal identity and time consumption (Predieri, 2004; Hensz, 1978) and mutational breeding is used to produce variation in colors and other traits (Kondo, 2009). A brief description of contributors toward mutation breeding is described in Table 16.1.

Name	Era	Contribution toward Mutation breeding
Carl von Linné	1707-1778	Father of Taxonomy, described many mutants, and heritable variations in plants.
Gregor Johann Mendel	1822-1884	Father of Genetics, developed Mendalian genetics.
Wilhelm Conrad von Röntgen	1845-1923	German Physicist, Father of Diagnostic radiology, discovered X-rays, Nobel Prize in Physics (1901).

Antoine Henri Becquerel	1852–1908	French Physicist, discovered radioactivity. Nobel Prize in Physics (1903).
Charles Robert Darwin	1809 - 1882	Wrote “On the origin of species”, developed “the evolutionary theory of natural selection” to describe heritable genetic variations (mutations) among organisms.
Hugo de Vries	1848 – 1935	Developed the concept of “pangenes” and mutation. Also discovered that mutations can be induced artificially, published “The mutation theory” and “Species and varieties: their origin by mutation”.
Nikolai Ivanovich Vavilov	1887 – 1943	Russian Botanist who proposed the concept of “Centers of diversity” in which he described the occurrence of wild type alleles at the center while those of recessive alleles (produced as result of mutation) at periphery at certain geographic boundaries. His most famous statement is “Plant breeding was evolution in human hands”.
Herman Joseph Muller	1890 – 1967	American geneticist, provided foundations toward mutation breeding by proving that X-rays can induce mutations in genes using Drosophila as model, Nobel Prize in Physiology and Medicine (1946).
Lewis John Stadler	1896 – 1954	Muller’s contemporary, worked on induced mutations monocot seed crops (wheat, rice and barley). Described that mutations can be induced in plants for the first time but was critical about their benefits as he was of the view that most of them were “junk”, and they also require laborious work for screening.

Herman Nilsson-Ehle	1879-1949	A Swedish plant physiologist, geneticist and breeder. Was first to produce improved barley varieties through mutation induction.
Reinhold von Sengbusch	1898-1985	A German scientist who set the foundations for the efficient methods of mass screening in mutation detection.
Hans Stubbe	1902-1989	German plant breeder described the importance of mutation to enhance diversity. Founder of the Institute of Plant Genetics and Crop Research and also Genebank at Gatersleben, Germany.
Ake Gustafson	1908-1998	Swedish geneticist and plant breeder known as Father of mutation breeding. In contrast to the views of Stadler, he described mutation as a useful and powerful tool in the hands of plant breeders. Also developed a basic research program to improve the methods of mutation breeding in crop plants.
Iosif Abramovich Rapoport	1912-1990	A Russian scientist, pioneer of induced mutations in plant breeding through chemicals. Also was responsible for mutation induction in almost 3,000 various crop varieties out of which 366 were selected with improved characteristics.
Auerbach and Robson	1944-46	Discovered the mutagenic effects of certain chemical mutants.

Table 16.1 Contributors to Mutation breeding.

16.2 Types of mutations

Mutations in plants can mainly be divided into three broad categories i.e. point mutations, structural mutations and alterations in the number of chromosome within a species. Point mutations or intragenic mutations deal with the changes in the specific DNA sequence. Structural mutations or

intergenic mutations involve translocation, inversion, duplications or deletions (Kharkwal and Shu, 2009; Forster and Shu, 2012) while alteration in chromosome numbers is associated with the induction of haploidy, polyploidy or aneuploidy (Pathirana, 2012). In addition to these, certain extra-nuclear mutations, specifically mutation in the chloroplast or mitochondrial genome are also capable of playing an important role in altering the crop plant's characteristics. Whatever the kind of mutation, the basic thing in mutation breeding is the identification of targeted mutants from the whole population which mainly involves two stages of screening and subsequent confirmation of mutants (Forster and Shu, 2012). In mutation screening, mutants with the desired characteristics such as resistance against certain diseases or biotic and abiotic stresses are selected from among the individuals of a large population subjected to mutation. In spite of all these processes of screening, the mutants may be false or putative. The process of re-screening and re-evaluating the large populations of putative mutants to select the organism with desired characteristics is referred to as mutant confirmation.

16.3 Kinds of Mutagens

The agents used for mutation induction are known as mutagens. In a broader sense, mutagens can be categorized into two main classes of physical mutagens and chemical mutagens (Acquaah, 2006; Mba *et al*., 2010; Oldach, 2011). Mutations can be induced through physical mutagens such as exposure to irradiation including X-rays, gamma rays or ion beams etc. or by using chemical mutagens. Mostly chemical mutagens are used for the induction of point mutations. Physical mutagenic agents are responsible for the induction of lesions or chromosomal aberrations in plants. In addition to the kind of mutagen, time of exposure and its dosage directly affects the rate and frequency of mutation in plants.

In crop plants, the source plant is usually treated with some suitable mutagen to develop a new variety for human benefit. The source plant can either be the seed or any other vegetative tissue. Although the seeds are more preferably used, other parts such as seedlings can also be used to achieve the goal. In plant breeding *in vitro* cultured somatic cells or calluses can also be used for mutation induction as they are capable of totipotency. Moreover, other vegetative propagules such as tubers, rhizomes, corms, bulbs, and stolon can also be used as the source plant for mutation induction (Wani *et al*., 2014). In recent years, another type of source i.e. plants showing vegetative propagation is directly being used for mutation induction. Other

vegetative tissues i.e. cuttings, scions, anthers, inflorescence and spikes etc., having the ability to develop into the whole plant can also be the source for mutation induction. Whatever the plant material is, it is then given exposure to the mutagenic agent.

16.4 Physical mutagens

Since the development of mutation breeding, physical mutagens, especially ionizing radiation are being most widely used. Mba *et al*. (2012) stated that more than 70% of mutant varieties have been developed only through these mutagenic agents. All the types of ionizing radiation may be used, including gamma rays, cosmic rays and X-rays (Mba, 2013). Radioactive particles of gamma rays are mostly obtained from radioactive isotopes of cobalt-60 and caesium-137. In addition to these, subatomic particles i.e. alpha particles, beta particles, protons and neutrons have also been widely used to induce mutations. Despite all their benefits, they can also cause hereditary aberrations. Physical mutagens require complicated tools and facilities to induce mutations. Some of the physical mutagens being used in mutation breeding with their characteristic features are given in Table16. 2.

Mutagen	Penetrance	Source	Hazardous Effect
Alpha particles	Very shallow	Radioisotopes	Very strong
Beta particles	Shallow	P^{32} and C^{14}	Mild
X-rays	Penetrating	X-ray machine	Strong
Gamma rays	Very penetrating	Co^{60} and Cs^{137}	Strong
Ultraviolet rays	Penetrating	UV source lamp	Strong
Protons	Up to several centimeters	Hydrogen nucleus	Very strong
Neutrons	Up to several centimeters	U^{235}	Very strong
Ion beams	Very penetrating	Particle accelerator	Strong

Table 16. 2 A brief description of physical mutagens.

16.5 Chemical mutagens

The most widely used chemical mutagens are a group of alkylating compounds. On the mutant varieties database among the registered varieties more than 80% have been developed using alkylating agents. These agents are mostly found in classes of compounds including alkyl halides, alkyl phosphates, alkyl sulfates, alkylnitrosoamides, diazoalkanes, mustards of nitrogen and sulfur, ethyleneimines, ethyleneimides, epoxides, alkyl methanesulfonates and many others. The most effective alkylating agents are 1-methyl-1-nitrosourea and 1-ethyl-1nitrosourea and ethyl methanesulfonate (EMS) which are the most widely used. Wani *et al*. (2014) described that among the alkylating agents some are capable of reacting with DNA to alkylate the phosphate group, purines and pyrimidines of nucleic acids. In addition to these, another group of compounds are the base analogs, e.g., 5-bromouracil incorporated during DNA replication are also widely used for mutation induction. In many plants, chemical mutagens have generated many phenotypic modifications by affecting the activities of the proteins. In crop plants, resistance against the herbicide glyphosate has been developed through mutation induction which altered the protein binding sites (Bradshaw *et al.,* 1997; Wakelin and Preston, 2006). Similarly, in a mutant variety of leguminous plant *Medicago truncatula*, resistance against the herbicide sulphonylurea has been generated through mutation breeding (Oldach *et al*., 2008). Some antibiotics such as mitomycin C, actinomycin D, azaserine and streptonigrin are also used as chemical mutagens. Thus, chemical mutagens are a class of compounds that have brought great advances in mutation breeding programs of crops.

Chemical mutagens have an advantage over physical mutagens not only due to their milder effects, but they are also relatively easier in their application. Using chemical mutagens, the mutation induction rate is relatively higher, and they are thus more effective than physical mutagens (Acquaah, 2006). Although most of the chemical mutagens are synthetic in origin a few are biological in origin e.g. the strong mutagen streptozotocin has been isolated from the bacterium species *Streptomyces achromogenes*. They are usually applied to the source material for mutation induction only by soaking it in the solution of the respective mutagen. However, they are usually hazardous to health and extra care is needed for their application. Before the application of the chemical mutagenic agent, its choice, method of application, hazardous effects and methods of disposal are the important factors which the breeder must give consideration to. Several chemical mutagens are known but only a few have been used in plant breeding programs for mutation induction.

Some of the widely used mutagens with their characteristic features are given in Table 16.3.

Name of Mutagen	Mode of action
Alkylating agents	React with bases by adding methyl or ethyl groups which induces mutation during DNA replication.
Base analogs	Replaces nitrogenous bases during DNA replication.
Antibiotics	Chromosomal aberrations.

Table 16. 3 Mode of action of some chemical mutagens.

Chemical mutagens cause less damage to chromosomes compared to physical mutagens. These mutagens are also the source of the high rate of mutation along with allelic mutations at target sites and they mostly cause point mutations which are usually uniformly spread over the whole genome. Another advantage of using chemical mutagens is the availability of standardized protocols for mutation induction in almost all important agronomic crops which can either be applied to seeds, *in vitro* grown calli or any other tissue. Although chemical mutagens are most widely used, due to the high mutation frequency, the selection of desirable mutations is a tedious job as it requires several backcrosses. In woody tissues, mutation induction is relatively difficult due to the low penetration of these mutagens in the plant tissue. Moreover, these tissues have low regeneration capacity. Mutation induction in recalcitrant seeds as well as in dormant tissues is also difficult. In dormant seeds, some pretreatments are often required to break the dormancy. Chemical mutagens are not capable of inducing large chromosomal mutations which are heritable, thus have limitations in their use. Additionally, they have safety concerns, not only in their usage, but also in their storage and disposal due to their carcinogenic or toxic properties.

16.6 Different Techniques being used in Gene Mutagenesis

16.6.1 Insertion mutagenesis

Insertion mutations are the method of introducing foreign nucleotides in the DNA either to express a gene by inserting its sequence or to disrupt the gene function by altering the reading frame. This technique is extensively used in molecular biology to study the gene function. In nature, insertions occur

during evolution. The insertion can also be induced artificially using viruses or transposons.

16.6.2 RNA-dependent DNA methylation

RNA-dependent DNA methylation (RdDM) is a gene-silencing technique also known as shutting down the gene expression (Zhang *et al*., 2013). In this technique, instead of incorporating mutation in DNA, double-stranded RNA is processed as small interfering RNA (siRNA) which in turn leads to DNA methylation. This technique is widely used in plant molecular studies with no extra DNA being incorporated in the plant genome. For the production of a protein, the DNA of an organism that carries all genetic information is first transcribed into mRNA and then is translated into that specific protein. Gene silencing for the biosynthesis of a specific protein can be achieved either by disrupting the RNA intermediate molecules, known as post-transcriptional gene silencing, or by interrupting the transcription, also known as transcriptional gene silencing or RNA-dependent DNA methylation (Matzke and Mosher, 2014). In RNA-dependent DNA methylation, the methyl group binds to DNA. For example, the methylation of promoter DNA of a specific gene prevents messenger RNA synthesis. Consequently, the gene will be silenced, and this gene silencing can also be inherited into the progeny of the plant (Jones *et al.*, 2001). This technique is advantageous over conventional breeding, being site-specific. This can be made more precise if non-coding RNA instead of mRNA is being subjected to methylation due to gene expression interruption.

The RNA-dependent DNA methylation technique can be used to improve the varieties of economically important crop plants through gene silencing without altering the genome sequence. For example, to combat the ever-changing environmental conditions, heat and drought tolerant crop plants can be produced. Similarly, insect or pest resistance can also be introduced in certain plants using this technique. Moreover, RdDM can also be used, not only for improving the nutritional value or the taste in crop plants, but also for the production of various colored flowers with increased shelf life in horticultural plants which can be of great economic importance. For example, the silencing of granule bound starch synthase (GBSS) in potato altered the composition of starch within the potato (Andersson *et al*., 2017).

16.6.3 Oligonucleotide-directed mutagenesis

Oligonucleotide-directed mutagenesis (ODM) is one of the successful techniques in the recent era of mutation breeding. Several crop plant varieties have been developed using this technique. This technique results in site-specific mutation in which a single-stranded oligonucleotide (20 – 100 bps) with the desired sequence is synthesized (Breyer *et al*., 2009). This oligonucleotide then binds to the specific site (Gocal *et al.,* 2015). The schematic representation of the ODM system is given in Figure 16.2. The oligonucleotide-directed mutagenesis technique can induce either additions or deletions which are heritable. Through this technique, several crop varieties with modified characters such as herbicide resistance have been produced through the Rapid Trait Development System (RTDS). Some of the plants developed through the ODM system with modified traits are tobacco (Ruiter *et al*., 2003), rice (Okuzaki and Toriyama, 2004) and *Brassica napus* (Gocal *et al*., 2015).

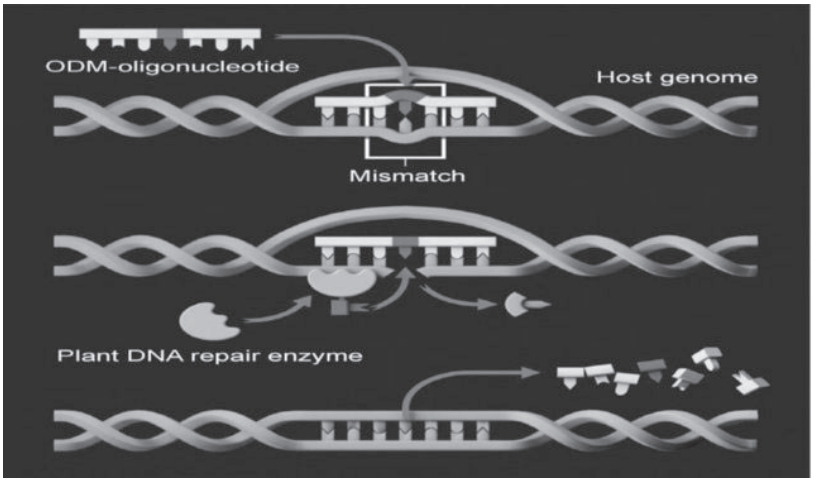

Figure 16.1: Schematic representation of oligonucleotide-directed mutagenesis (Source: http://cibus.com/).

16.6.4 CRISPR-cas

Like the ODM system Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-cas) and CRISPR associated cas genes are also widely used to induce site-directed mutagenesis which is primarily based on the activity of site-directed nuclease. These nucleases snip the DNA after

specific site recognitions. In archaea and bacteria, the CRISPR-cas genes are associated with adaptive immunity. Although these repeats were discovered in *E. coli* in the 1980s (Ishino, *et al*., 1987) but in 2007 their function was described by Barrangou and his co-workers (Barrangou *et al.,* 2007). In this technique, the foreign DNA is spliced down by nuclease activity into shorter fragments of about 20 base pairs. These spliced DNA fragments are incorporated into genomic DNA at CRISPR loci of a prokaryote. These loci are transcribed into CRISPR RNA which is then used to trigger endonuclease activity to target foreign DNA.

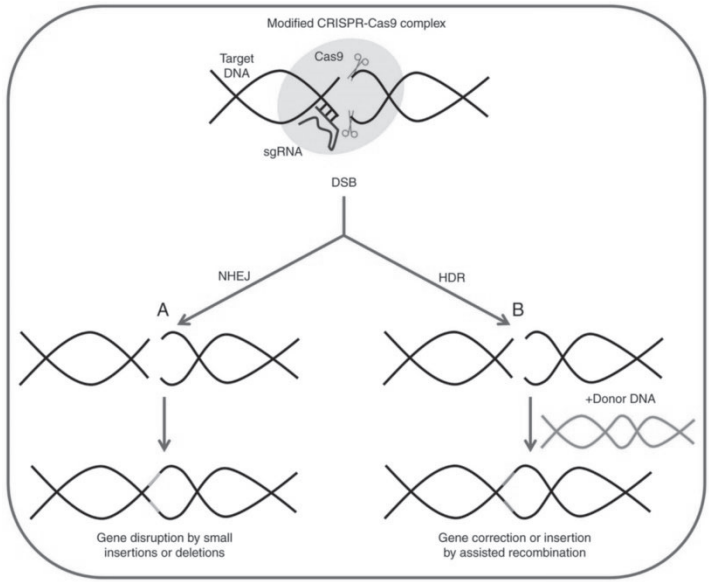

Figure 16.2: A schematic representation of CRISPR-cas system (Rodríguez-Rodríguez *et al.*, 2019).

The precision in the CRISPR-cas system can be increased by the online tools for computational analyses that can predict the non-specific targets as well. Some examples of these tools are Zinc Finger Targeter - ZiFiT (Sander *et al.,* 2010) and CRISPR Design Tool (Hsu *et al.,* 2013) which are quite helpful in non-specific cleavage detection.

Through the CRISPR-cas system, a large number of desirable traits have been introduced, not only in plants, but also in animals, including human beings too. This system has great potential for developing desirable

characteristics within organisms which are beyond our imaginations. This technique is also of great potential as it can target the proteins directly, thus can act as transcriptional regulation.

16.7 Mutation breeding in crops

Mutation breeding has become a successful tool in plant breeding programs as more than 3300 mutant varieties of 314 plant species have been produced officially by more than 70 countries all over the world (IAEA mutant variety database, 2019). Among these, more than 1,000 varieties of staple crops are being widely cultivated all over the world. Asia Pacific is the leading region in the production of new mutant varieties. According to the FOA/IAEA mutant variety database, 2019, among 3300 globally introduced mutant varieties, approximately 2000 have been reported from the region. In the year 2017-18 alone, the Asia Pacific area introduced fifteen new varieties. In the same time, through mutation breeding, the release of four new crop varieties and the pre-release of eleven varieties have been reported from Africa. Most of these newly introduced varieties have resistance against heat and drought stress. Among these mutant varieties wheat, rice, cotton, some fruit trees and various ornamentals are more prominent. Figure 16.4 shows the breakdown of all the mutant crops that have been developed so far.

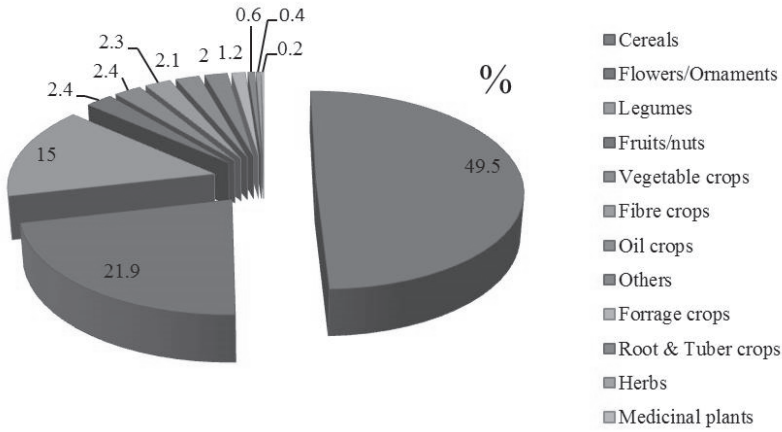

Figure 16.3 Percentage of mutant crops (IAEA mutant database, http:// mvgs.iaea.org, Accessed on August 2019).

16.8 Development of Mutant wheat varieties

Almost 42.5% of the total food supply is provided by cereals (wheat and rice) all around the world. Wheat is the main source of food for a population of about 1.2 billion. It provides around 20% of energy when consumed as a food source. It is grown mostly in temperate regions of the world. Its yield is mainly reduced due to disease attack and changing weather conditions. Wheat blast is a new threat to wheat plants in addition to already occurring common diseases. There is a strong need for mutation breeding programs or projects to introduce new resistant varieties against these diseases.

16.9 Development of Mutant rice varieties

Rice is cultivated globally and is the main food source of about half of the world's population (IRRI, 2015; AfricaRice, 2015). Changing environmental conditions and several diseases are responsible for reducing the annual yield of the crop. Some diseases such as sheath blight, rice blast, false smut of rice and bacterial leaf blight are the major threats of rice yield. All over the world, much work has been done on the development of mutant varieties to enhance the quality and quantity of the rice crop. During the last five decades, approximately 800 mutant rice varieties have been produced either through mutation breeding or by crossing these mutant lines with other plants (Kharkwal and Shu, 2009). In 1957 China introduced two rice varieties developed through mutation breeding techniques. Another rice variety was also introduced the same year which was developed by the cross-breeding of a mutant variety with other rice-breeding lines (Rutger, 1992). Afterwards, a semi-dwarf mutant variety of rice resistant to lodging with relatively high yield was developed in Japan (Futsuhara, 1968). All over the world, production of semi-dwarf cultivars of rice was responsible for a revolution in rice yield. In Pakistan, another variety of Basmati rice i.e. "Kashmir Basmati" is early maturing and tolerant to low temperatures and has been developed through mutation breeding programs. This rice variety also has additional characteristics of better aroma and cooking (Awan, 1991). Similarly, in India several semi-dwarf rice mutant cultivars with high yield have also been reported (Chakrabarti, 1995). On the IAEA database, more than 820 mutant rice varieties have been registered and most of them have been produced through the use of physical mutagens.

16.10 Development of mutant cotton

Cotton is the leading crop and plays a vital role in the economy of Australia, Bangladesh, China, India, Iran and Pakistan. That is why it is also known as white gold. It is grown over 20 million hectares of land in only three of the leading cotton-producing countries i.e. China, India and Pakistan, which contribute about 60-65% of the net global cotton yield. It is cultivated primarily for its fiber. It is the second best natural source of plant proteins while it is ranked fifth in terms of oil production. The other by-products of the plant including hulls, seed cake and other vegetative parts of the plant are used for several products. Many factors influence the yield. These factors include increasing temperatures, soil fertility loss, salinity and biotic stresses. Mutation breeding is the technique for improving the quality and yield of the crop by introducing new traits including resistance against many biotic and abiotic stresses, relatively high adaptability to a changing environment, increased number of bolls, early boll ripening and high oil content.

In Pakistan through mutation breeding programs, many mutant lines have been developed with fine fiber quality that are also tolerant of drought. In 2017 Pakistan successfully introduced a mutant variety, NIAB787, while in 2018 two mutant lines i.e. NIAB 1048 and NIAB545 have been released. Thailand and Indonesia are at the very initial stages of mutation breeding programs for cotton development.

16.11 Advantages of mutation breeding

Some of the main advantages of mutation induction in breeding programs are:

1. A large number of crop varieties to be used for cultivation have been produced utilizing mutation breeding techniques. These varieties have modified characteristics for better yield, improved quality, increased resistance against certain biotic and abiotic stresses.
2. The characters introduced into crop plants are mostly heritable and well adapted to the existing environmental conditions.
3. Through mutation breeding techniques site-specific mutations can be induced which are otherwise difficult to achieve through conventional breeding techniques.

16.12 Disadvantages of mutation breeding

Some of the disadvantages of mutation induction are:

1. Most of the mutations are lethal and are thus undesirable to the breeder.
2. In plant breeding, the mutation induction rate is usually very low. The breeder has to grow a large number of plants for screening for the desired mutation among the whole population. Thus, screening is a quite laborious and time-consuming process.
3. As the mutations are mostly reversible, so the stability of the mutant can also be a factor strongly affecting the mutation breeding program and require testing before releasing new cultivars.
4. Most of the mutations incorporated into the genome are recessive, so for their phenotypic expression, they are required in a double dose.
5. Pure breeding lines can only be prepared if mutation is induced in gametes.

16.13 Future concerns in mutation breeding

Presently there is a need for developing new crop varieties that can combat the challenges of increased food supply due to the explosive population growth rate. Moreover, such varieties might also have modifications against many biotic and abiotic stresses including harsh environment, drought and salinity resistance etc. Mutation breeding techniques are environmentally friendly ways to develop new crop varieties to combat all these challenges. Therefore, there is a need for focusing on the developed germplasm to be exchanged by plant breeders to accelerate the production of new varieties even with relatively better traits. This will also help diversify the germplasm. The plant breeders also need to focus on discovering the mutant traits that can be further used as molecular markers to identify them in other crops as well. The Food and Agriculture Organization (FAO) of the United Nations in collaboration with IAEA help improve sustainable agriculture by using nuclear tools. Many countries, including Pakistan's Nuclear Institute for Agriculture and Biotechnology are using their nuclear tools to develop new mutant varieties to achieve the goal of ensuring stable food quality and "Zero Hunger" by 2030. It is estimated that all these techniques will not only be environmentally friendly but also be helpful in the conservation of natural resources.

The future concerns of plant mutant breeders mainly include sustainable agriculture to feed the huge population. The goal may only be achieved by

utilizing all the nuclear and other related techniques of mutation breeding that might be environmentally friendly by leaving the least prints on the environment. The other objectives of twenty-first century mutant breeders include developing disease-resistant crop varieties which can also bear harsh environmental conditions. Fortified crops might also be a product of mutation breeding to combat the problems of malnutrition. These foods might be enriched with some vitamins or mineral elements. In addition to combating malnutrition, initiatives are being taken to produce foods which can combat the problems of obesity as well. Natural resources can also be preserved by improving the germplasm of livestock through breeding techniques. Another important future concern of mutation breeders is the use of nuclear and irradiation techniques by IAEA and FAO for the production of sustainable agricultural products. The objective of the Farm to Fork approach is the production of food with better quality, having a relatively long shelf life. All these practices will be helpful in the economy of a country.

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CHAPTER 17

CYTOGENETICS OF TWO GIANT PILL-MILLIPEDES OF THE GENUS *ARTHROSPHAERA* (SPHAEROTHERIIDA) IN THE WESTERN GHATS OF INDIA

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Abstract: Cytological observations on two endemic giant pill-millipedes of Gondwanan origin of the Western Ghats of India (*Arthrosphaera carinata* Attems and *Arthrosphaera hendersoni* Pocock) were carried out based on the male meiotic analysis. Among the Diplopoda, so far about ten species of the genus *Arthrosphaera* showed the highest number of chromosomes ($2n = 30$), but their chromosomal evolutionary pattern is still poorly known. The spermatogonial metaphase of the present study revealed $2n = 30$ chromosomes in *Arthrosphaera* spp. with all acrocentrics. The sex chromosome pair was more conspicuous than the autosomes with a large size in all stages of meiosis and their behavior was similar in both species. Karyotypes revealed the chromosomal relative length ranged from 19.03–117.7 in *A. carinata* and 21.9–183.7 in *A. hendersoni*. Besides karyological studies, sub-stages of meiotic prophase, the behavior of chromosomes, heteropycnosis, pre-metaphase stretch and evolution of sex chromosomes in the genus *Arthrosphaera* have been emphasized.

Keywords: Chromosomes, Cytology, Diplopoda, Karyotype, Male meiosis, Sex chromosomes.

17.1 Introduction

Millipedes belong to the class Diplopoda and are the major soil saprophagous fauna which enhance soil fertility by mechanical fragmentation and decomposition of organic matter through soil and gut microflora (Hopkin and Read, 1992). The order Sphaerotheriida in Diplopoda, composed of the giant pill-millipedes, currently exhibit patchy distribution in Australian, Ethiopian and Oriental zoogeographic regions (Wesener and van den Spiegel, 2009). Recent morphological and molecular phylogenetic studies on the pill-millipede genus *Arthrosphaera* relates to the paraphyletic group of another pill-millipede genus *Sphaeromimus*, which simultaneously evolved in Madagascar and the Western Ghats of India during the Gondwanan period (Wesener and van den Spiegel, 2009; Kadamannaya and Sridhar, 2009; Wesener *et al*., 2010; Kadamannaya *et al*., 2010, 2012). The current estimates reveal about 46 species of *Arthrosphaera* occur in forests of the Eastern Ghats (states of Andhra Pradesh and Tamil Nadu), Western Ghats (states of Maharashtra, Karnataka, Kerala and Tamil Nadu) and the west coast of India (state of Karnataka) (Sridhar and Kadamannaya, 2009; Sridhar and Ashwini, 2011; Ambarish and Sridhar, 2013, 2016, 2018). Owing to the small number as well as the microendemic distribution of *Arthrosphaera* spp. they are under high risk of extinction and warrant habitat conservation (Wesener, 2009; David and Handa, 2010).

Cytological investigations on *Arthrosphaera* spp. were initiated in India by Chowdaiah (1966a) followed by Achar (1984), Kadamannaya (2010) and Ambarish *et al.* (2013). Nearly one-third of the *Arthrosphaera* spp. occurring in the Indian subcontinent have been subjected to cytological studies (Kadamannaya *et al.,* 2010). In the class Diplopoda, the *Arthrosphaera* spp. has immense evolutionary significance as they possess a high number of chromosomes $2n = 30$ in ten species, $2n = 28$ in two species and $2n = 26$ in three species (Kadamannaya *et al*., 2010; Ambarish and Sridhar, 2014). Achar (1987) opined that the Robertsonian translocations and polyploidy have an important role in chromosomal evolution of Diplopoda. However, cytogenetics of pill-millipedes attracted less attention owing to difficulties in acquiring mitotic chromosomes (Fontanetti *et al*., 2002). A few species of *Arthrosphaera* have been assessed cytogenetically for chromosome dimorphism and the nature of male meiosis

(Kadamannaya *et al*., 2010; Ambarish *et al*., 2013; Ambarish and Sridhar, 2014).

Arthrosphaera carinata and *Arthrosphaera hendersoni* were recovered from the foothills of the Western Ghats and from the coastal region of Southwest India, respectively. There are no cytological studies so far on *A. carinata*, while *A. hendersoni* has been studied by Chowdaiah and Kanaka (1974), based on camera-lucida drawings. The present study evaluates the spermatogonial metaphase of two *Arthrosphaera* spp. emphasizing different stages of prophase of male meiosis. Chromosomal heteropycnosis, a stretch of pre-metaphase chromosomes and sex chromosomal evolution in the genus *Arthrosphaera* have been discussed.

17.2 Pill-millipedes and Process

Adult individuals of *Arthrosphaera carinata* Attems (from a mixed plantation in Adyanadka, located in the foothills of the Western Ghats, Karnataka: 12°41′N, 75°6′E) and *Arthrosphaera hendersoni* Pocock (from a mixed plantation in Uppala, located on the west coast, Kerala: 12°41' N, 74°56'E) were gathered during the monsoon period (July-September) in 2012. Taxonomic identification was based on matching with descriptions and keys provided by Pocock (1899) and Attems (1936). Representative specimens were deposited in the Museum, Department of Zoology, Mangalore University (*A. carinata*: MUAZPMAc-03; *A. hendersoni*: MUAZPMAh-04). Pill-millipedes were acclimatized by maintaining in laboratory mesocosms for up to two weeks and by offering wet mixed leaf litter periodically on a soil bed in glass containers. Adult males were selected for the cytological study.

The protocol proposed by Achar and Chowdaiah (1979) was employed with a slight modification to prepare the chromosomes of the male pill-millipedes. Colchicine (0.5 ml; 0.5%) was injected into adult males. After 5 hr, in normal saline, the testes were dissected out. Different molarities of KCl (0.16–0.21M) were used to treat the whole testes for up to 1 hr at 37°C. Pre-treated tissues of testes were minced in normal saline, followed by centrifugation (1100 rpm; 5 min). The pellet of cells obtained was resuspended in hypotonic solution with two changes. The suspension was centrifuged to fix in a fresh fixative (1:3 acetic acid-methanol, v/v) for up to 30 min. After two changes of fixative, it was resuspended in a small amount of fresh fixative and a few drops of cell suspension were dropped on to ice-cold slides, followed by air-drying and preservation (27°C) until staining. Air-dried slides were stained with Giemsa stain (2.5%) followed

by observation under 100X objective microscope (Olympus CX41RF, USA) to screen different meiotic stages to follow the topography as well as the behavior of chromosomes.

Methods proposed by Levan *et al*. (1964) and Achar (1986) were adapted for the construction of karyotypes from the spermatogonial metaphase chromosomes and stages and sub-stages of male meiosis were analyzed to follow chromosomal behavior. The length of each chromosome in the plates was measured in a pixel scale using the Olympus microscope and later converted into centimeters. Each measurement of the individual chromosomes was used to evaluate the relative length (RL): RL = (Length of each chromosome / Total length of a haploid set) × 1000. The arrangement of autosomes was in descending order of length followed by placing X and Y chromosomes for karyotype construction.

17.3 Chromosomes

Spermatogonial metaphase showed diploid chromosome $2n = 30$ (Figure 17.1a) with sex determination by the XY system. Chromosomes of both millipedes were acrocentric with gradual seriation. Sex chromosomes are the largest acrocentric pair and organized at the periphery of the plate of spermatogonia (Figure 17.1a). The dissimilarity between X and Y chromosomes was not distinguishable. Chromosomes were thread-like with a few thickenings in the pachytene stage (Figure 17.1b) and showed prominent dark-stained regions in the zygotene stage (Figure 17.1c). The bivalents exhibited characteristic fuzziness in the diplotene stage (Figure 17.1d). Due to the large size with terminal chiasma, the sex bivalents were more conspicuous. The diakinesis stage was represented by 15 highly condensed bivalents (Figure 17.1e, f). The 15 conspicuous bivalents exhibited almost spherical shapes in the metaphase I stage (Figure 17.1g). The first meiotic division stage was represented by laggards of sex chromosomes (Figure 17.1h), whereas in the second equational division stage they were normal (Figure 17.1i). In the meiosis II stage, autosomes, as well as sex chromosomes, were equational with clear cut 15 half bivalents with separation of sister chromatids (Figure 17.1j, k, l).

The metaphase stage of spermatogonia of the male possesses diploid chromosomes ($2n = 30$) with heterogametic X and Y chromosomes (Figure 17.2a). The autosomes represented by acrocentrics with gradual seriation, while the largest X and Y acrocentric chromosomes showed up as a separate group with slightly larger X compared to the Y chromosome (Figure 17.2a). Chromosomes become thick due to pairing in the pachytene stage (Figure

17.2b). In the sub-stages of prophase, heteropycnotic regions were prominent with several dark-stained parts in threads of chromosomes (Figure 17.2c). Typical bivalents possessing characteristic fuzziness with conspicuous unstained gaps show up in the diplotene stage (Figure 17.2d). In the diakinesis stage, the sex bivalents become conspicuous owing to their large size as well as dark appearance (Figure 17.2e). The late diakinesis stage was represented by terminalization of chromosomes and the sex chromosomes held together by chiasmata (Figure 17.2e). The dia-metaphase stage was conspicuous (Figure 17.2i). Autosomes and allosomes attained an almost dumbbell shape in the metaphase I stage (Figure 17.2f). All chromosomes, including the sex chromosomes, were reductional for the meiosis I stage. The equational meiosis II stage exhibited 15 conspicuous dyads (or half bivalents) (Figure 17.2j, k). Separated sister chromatids and lagging of sex chromosomes were seen during the anaphase I stage (Figure 17.2g, h). Prominent chromosome separation in clumps was seen in the telophase stage (Figure 12.2l).

Karyotypes of the spermatogonial metaphase of *A. carinata* and *A. hendersoni* are presented in Figure 17.3 (a, b). The nature of all chromosomes in both species of *Arthrosphaera* was acrocentric. In *A. carinata*, the X and Y were less distinguishable, and both were smaller compared to *A. hendersoni*. The relative length (RL) of autosomes in *A. carinata* ranged from 19.03–89.7, whereas for X and Y the RL was 117.7 and 106.3, respectively (Table 17.1). In *A. hendersoni*, the RL ranged from 21.9–81.45 for autosomes, while it was 183.7 and 173.2 for the X and Y chromosomes, respectively (Table 17.1).

17.4 Discussion

The current cytological observation on two endemic pill-millipedes *A. carinata* and *A. hendersoni* gives insight for comparison with earlier studies on other species of *Arthrosphaera*. The following sub-sections emphasize the chromosome features, heteropycnosis, pre-metaphase stretch, sex chromosome ring, karyotypes and evolution of sex chromosomes in *Arthrosphaera*.

So far, 15 species of *Arthrosphaera* have been evaluated for chromosome dimorphism (Kadamannaya *et al*., 2010). In *Arthrosphaera* spp., the diploid chromosome number varied from $2n = 26$ (3 species); $2n = 28$ (2 species) and $2n = 30$ (10 species) (Kadamannaya *et al*., 2010). Spermatogonial metaphase revealed that the entire autosomes including allosomes in the majority of *Arthrosphaera* were acrocentrics (e.g. *Arthosphaera bicolor*, *A.*

craspedota, *A. dalyi, A. fumosa, A. gracilis, A. hendersoni, A. lutescens, A. magna* and *A. zebraica*) (Ambarish and Sridhar, 2014). However, there are some deviations and ambiguity in the nature of chromosomes studied by various authors. For instance, the spermatogonial metaphase of *A. dalyi* also showed 21 telocentric, 7 sub-telocentric and 2 sub-metacentric chromosomes (Kadamannaya *et al*., 2010), while the mitotic metaphase revealed 16 acrocentric, 12 sub-metacentric and 2 dot-chromosomes (Achar, 1983). Likewise, the spermatogonial metaphase of *A. magna* showed 22 acrocentric, 4 sub-metacentric and 2 metacentric chromosomes (Ambarish and Sridhar, 2014). The spermatogonial metaphase of *A. zebraica* also consists of 26 telocentric chromosomes (Kadamannaya *et al*., 2010). The mitotic metaphase of *A. davisoni* and *A. nitida* also showed 4 and 1 sub-metacentric chromosomes, respectively (Achar, 1983). In the present study, all chromosomes ($2n = 30$) of *A. carinata* and *A. hendersoni* were acrocentrics as seen in other species, which was supported by the earlier observations by Achar (1987) that the higher chromosome number is represented by more acrocentrics.

Heteropycnosis of chromosomes in Diplopoda is common during prophase (Achar, 1987). The thin, granular and thread-like appearance of chromosomes in the prophase stage is striking evidence in several diplopods including the pill-millipedes belonging to *Arthrosphaera* (Chowdaiah and Kanaka, 1974; Achar, 1986; Ambarish and Sridhar, 2014). Dark-stained pycnotic condensations are common at the end of each chromatin in many *Arthrosphaera* spp., which corroborate with the number of chromosomes in each species (see Figure 17.2b). Condensation and coiling initiate from the terminal part and result in pycnotic knobs at one end of the chromatid. The mechanism of pycnotic condensation, as well as its significance in the meiotic stage and its sub-stages of *Arthrosphaera,* deserves further investigation.

In the genus *Arthrosphaera*, a chromosomal stretch during pro-metaphase as well as pre-metaphase was prominent. Such events are less clear in *A. carinata* (see Figure 17.1e, f, g), while it was clearer in *A. hendersoni* (see Figure 17.2e, f). Other *Arthrosphaera* (e.g. *A. dalyi* and *A. nitida*) during meiosis show clear chromosomal stretch (Achar, 1986). Congregation of bivalents on the equator subsequent to the diakinesis stage without the formation of the metaphase plate is common. Such behavior of chromosomes has been envisaged as being to satisfy the tensions during the pre-metaphase stage before final positioning of the spindle (Achar, 1987). In some instances, initiation of chromosome stretch before spindle formation results in the repulsion of centromeres of bivalents in pre-

metaphase stretch (Schrader, 1944). Such chromosomal pre-metaphase stretch has significance in normal disjunctions in pill-millipedes with non-chiasmatic meiosis (Achar, 1986).

Ring formation by X and Y chromosomes was seen in *A. carinata* and *A. hendersoni* during the first meiotic prophase stages (diplotene and diakinesis) (see Figure 17.1d, e, f; Figure 17.2d). In *A. magna* and *A. lutescens* also such behavior of sex chromosomes was reported by Achar (1986). In *A. disticta*, the ring formation during the diakinesis stage and the open-cross of sex chromosomes were reported (Chowdaiah and Kanaka, 1974). Such sex chromosomal configurations were due to the formation of chiasmata on both sides of the centromere, which are less distinguishable in several *Arthrosphaera* spp. However, these events have been demonstrated by Achar and Chowdaiah (1980) in the millipede *Carlogonus ascifer* (Sphaerostreptida, Harpagophoridae). In *Arthrosphaera*, the ring formation may be due to the mutual attraction of heterochromatin components of sex chromosomes.

Analysis of male meiosis of *A. carinata* and *A. hendersoni* resulted in 30 acrocentric chromosomes. The sex chromosome pair is the largest, and is relatively smaller in *A. carinata* than *A. hendersoni.* Such difference in the sex chromosome pair has also been reported in *A. dalyi* and *A. magna* (Achar, 1986). Compared to the mitotic metaphase spread, the spermatogonial metaphase spread has less resolution of chromosomes. Thus, karyotype analysis will be better using the mitotic metaphase than the meiotic metaphase. However, in male or female *Arthrosphaera*, acquiring the mitotic chromosome is difficult as well as tedious. Besides, too little difference between the X and Y chromosomes further hampers the karyotype organization. However, Achar (1983) used epithelial cells of male testes for mitotic metaphase preparations and karyotype study of three species of *Arthrosphaera*. Recently, Ambarish and Sridhar (2014) also observed a few plates of mitotic metaphase along with spermatogonial metaphase in *A. fumosa*.

In the genus *Arthrosphaera*, chromosomal relative length (RL) is not comparable between species (Ambarish *et al*., 2013). For instance, the autosomal RL in *A. craspedota, A. dalyi* and *A. fumosa* ranges between 20.6–103, 39.4–99.9 and 21.9–109.3, respectively; while the X and Y chromosomal RL ranges between 170.1 and 159.7, 194.7 and 161, 130.1 and 113.6, respectively (Kadamannaya *et al*., 2010, Ambarish *et al*., 2013; Ambarish and Sridhar, 2014). In our study, the RL of autosomes (19.03–89.7), X (117.7) and Y (106.3) chromosomes of *A. carinata* were least

compared to *A. craspedota, A. dalyi* and *A. fumosa*. However, *A. hendersoni* has intermediate results in RL of autosomes (21.9–81.45), X (183.7) and Y (173.2) chromosomes.

The sex chromosomes are laggards in the first meiotic division, while they show normal segregation in the second meiotic division. Laggards of sex chromosomes were seen in *A. carinata* and *A. hendersoni,* similar to *A. disticta*, *A. fumosa, A. lutescens* and *A. magna*. Kaiser and Bachtrog (2010) suggested that the lagging is due to mechanical difficulties during segregation of X and Y chromosomes owing to their high mass. However, it is still not clear about the synchronous movement of sex chromosomes in the second meiotic division. In the diplotene stage, sex chromosomes frequently attain a cruciform configuration during chiasma formation in many *Arthrosphaera* spp. (Achar, 1987). It is assumed that the sex determination with XY type in *Arthrosphaera* is most primitive, thus recognition of sex chromosomes from autosomes is difficult. Moreover, the sex chromosomes exhibit a minor difference in size, but they are most conspicuous in male meiosis.

Sex chromosomes are known for their major evolutionary role, such as determination of sex, adaptability to the native environment and speciation (Kaiser and Bachtrog, 2010). They are conspicuous in all stages of meiosis of *Arthrosphaera* spp. *Arthrosphaera* are heterogametic and their sex determination is governed by XY type in males and XX type in females (however, studies in females are meager) (Achar, 1986). Interestingly, XO type or XXO type of sex determination were reported in other genera of Diplopoda (e.g. *Gonoplectus malayus* and *Phyllogonostreptus nigrolabiatus*), but not in the genus *Arthrosphaera* (Bessire, 1948; Natarajan, 1959; Sharma and Handa, 1974). However, in *Arthrosphaera* such observations are still too fragmentary to understand the mechanism of sex determination as well as chromosome behavior. The XY type of sex determination in males of *Arthrosphaera* was confirmed in the present study as well as in earlier investigations (Chowdaiah, 1966a, b; Achar, 1986; Kadamannaya *et al*., 2010; Ambarish *et al*., 2013). Charlesworth (1996) pointed out that X and Y chromosomes independently evolved to determine the sex. It is believed that initially the sex chromosomes evolved from the identical chromosome pair and subsequently differentiation (morphological and genetic) occurred due to possible degeneration of the chromosome resulting in genetic recombination. The sex chromosomes in the genus *Arthrosphaera* possessing ancestral genes are possibly to facilitate tolerance or coping with unusual or inhospitable challenges posed by the environment.

17.5 Conclusions and Outlook

The giant Indian pill-millipedes belonging to the genus *Arthrosphaera* have Gondwanan origin and the sister-taxon *Sphaeromimus* is known only from Madagascar, which separated around 88 million years ago (Wesener *et al*., 2010). The diploid chromosome number is $2n = 26$ to 30 in *Arthrosphaera* with the XY pattern of sex determination. The number, as well as the nature of chromosomes in *Sphaeromimus* (the closest relative of *Arthrosphaera*) from Madagascar, is yet to be investigated. Major cytological investigations on the genus *Arthrosphaera* were carried out only in the Indian subcontinent, but the molecular studies are still lacking. A large number of pill-millipedes belong to the order Sphaerotheriida distributed in the India, Sri Lanka and Afro-Madagascar eco-regions. Precise caryological and molecular studies are necessary to understand the chromosomal evolution in *Arthrosphaera*. Due to lack of detailed morphological, cytological and molecular evidence, it is difficult to trace the pattern of evolution of Indian giant pill-millipedes dispersed in the Australian, Ethiopian and Oriental continents.

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Table 17.1 Relative length (RL) of chromosomes of *Arthrosphaera* spp. (*n* = 3 ± SD).

Chromosome pair	Relative length (RL)		Nature of chromosome
	Arthrosphaera carinata	*Arthrosphaera hendersoni*	
1	89.70±0.34	81.45±0.56	Acrocentric
2	75.60±0.75	78.90±0.36	Acrocentric
3	70.04±0.04	62.60±0.12	Acrocentric
4	69.76±0.63	59.50±0.69	Acrocentric
5	66.98±0.38	56.30±0.12	Acrocentric
6	58.42±0.14	53.20±0.11	Acrocentric
7	53.21±0.84	50.10±0.05	Acrocentric
8	44.08±0.33	47.60±0.21	Acrocentric
9	39.06±0.20	45.70±0.09	Acrocentric
10	33.13±0.69	44.40±0.08	Acrocentric
11	29.32±0.27	40.70±0.10	Acrocentric
12	24.12±0.31	31.20±0.02	Acrocentric
13	21.06±0.48	27.50±0.09	Acrocentric
14	19.03±0.29	21.90±0.23	Acrocentric
15	X = 117.70±0.56	X = 183.70±0.87	Acrocentric
	Y = 106.30±0.78	Y = 173.20±0.93	Acrocentric

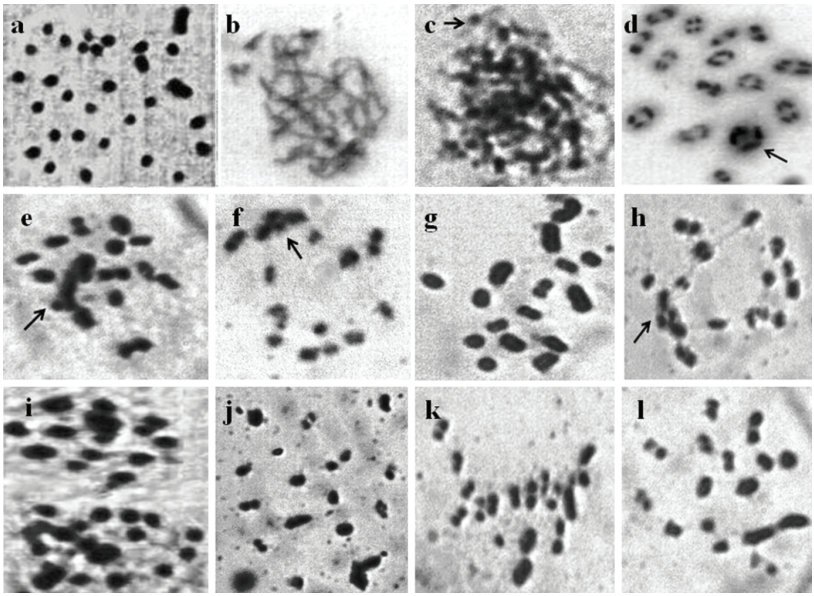

Fig. 17.1 Photomicrographs of chromosomes in male meiosis of *Arthrosphaera carinata* ($2n = 30$): (a) spermatogonial metaphase; (b) pachytene; (c) zygotene (arrow, darkly stained region); (d) diplotene (arrow, ring formation by X and Y); (e) and (f) diakinesis (arrow, incomplete chiasma); (g) metaphase I (polar view); (h) early anaphase (arrow, lagging of XY); (i) anaphase I; (j) dia-metaphase; (k) and (l) metaphase II.

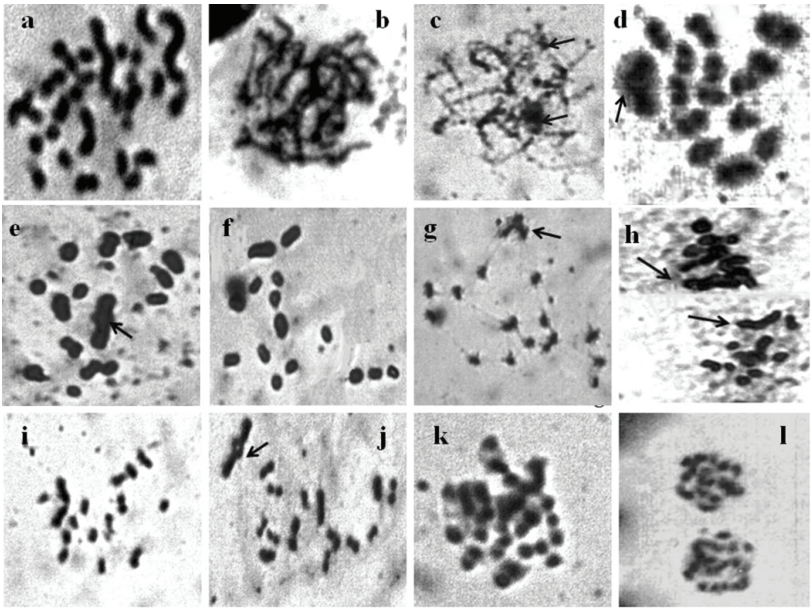

Fig. 17.2 Photomicrographs of chromosomes in male meiosis of *Arthrosphaera hendersoni* ($2n = 30$): (a) spermatogonial metaphase; (b) pachytene; (c) zygotene (arrow, darkly stained region); (d) diplotene (arrow, ring formation by X and Y); (e) diakinesis (dumbbell shape; arrow, chiasma); (f) metaphase I; (g) early anaphase (arrow, lagging of XY); (h) anaphase I (arrows, lagging of XY); (i) dia-metaphase I; (j) metaphase II (polar view; arrow, XY); (k) metaphase II (side view); and (l) telophase.

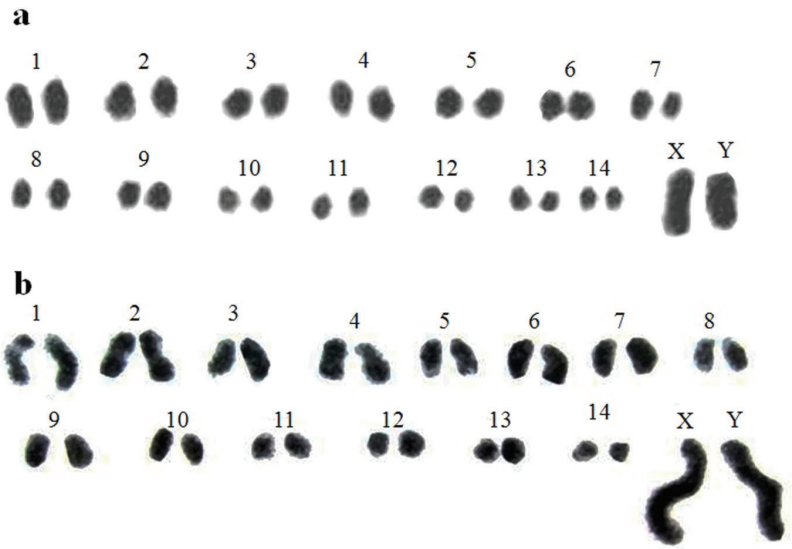

Fig. 17.3 Karyotypes of spermatogonial metaphase of (a) *Arthrosphaera carinata*; (b) *Arthrosphaera hendersoni*.

CHAPTER 18

CYTOLOGY OF PILL-MILLIPEDES (DIPLOPODA) WITH AN EMPHASIS ON *ARTHROSPHAERA DISTICTA*

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Abstract: Pill-millipedes belonging to the mega-diverse group of Arthropoda (class, Diplopoda) have attracted less attention for cytological investigations. Most of the studies carried out on pill-millipedes belonging to the genus *Arthrosphaera* are confined to the Indian subcontinent. The 2*n* chromosome number of pill-millipedes of the genera *Arthrosphaera* and *Glomeris* ranges from 16-30 with XY of type sex determination. The chromosomal reshuffling, especially the Robertsonian modifications and pericentric inversions, appear to have significantly contributed to polyploidy as well as karyotypic evolution in pill-millipedes. Because of meager information on the cytogenetics, the present chapter consolidates studies on cytogenetics of certain pill-millipedes (*Arthrosphaera* and *Glomeris*) with a brief note on diplopod cytology. Cytology and karyotype of one of the endemic pill-millipedes, *Arthrosphaera disticta* of the Western Ghats of India have been emphasized.

Keywords: Chromosomes, Cytology, Glomerida, Sphaerotheriida, Western Ghats.

18.1 Introduction

The millipede fauna belong to the class Diplopoda and have worldwide distribution engaged in recycling the organic matter in several habitats through mechanical disintegration and conversion of organic matter into fecal pellets to facilitate nutrient cycling (Hopkin and Read, 1992). Approximate estimation of the global diversity of millipedes is 80,000 (Hoffman, 1979). Although so far only about 12,000 species belonging to 16 orders have been described, their ecology, life history and phylogeny are less studied (Sierwald and Bond, 2007; Shear, 2011). The order Sphaerotheriida (pill-millipedes) of the class Diplopoda is composed of 325 species with discontinuous geographic distribution in Australia, Madagascar, New Zealand, the Oriental regions and South Africa (Jeekel, 1974; Hoffman, 1982; Shelly, 1999; Wesener and van den Spiegel, 2009; Wesener *et al*., 2010). Among the five genera of pill-millipedes, the *Glomeris* is known from temperate regions, the *Arthrosphaera* showed tropical disjunct distribution, while the *Sphaeromimus,* as well as *Zoosphaerium,* have distribution in Madagascar and the *Cynotelopus* is confined to Western Australia (Main *et al.,* 2002; Wesener and Sierwald, 2005a, 2005b; Sierwald and Bond, 2007; Wesener *et al*., 2010).

Studies on the Indian giant pill-millipedes were initiated by Pocock (1882), which resulted in a morphology-based monograph (Pocock, 1899). Subsequently, a key has been developed for pill-millipedes based on the morphology by using museum-preserved specimen (Attems, 1936). Cytological and ecological studies were initiated in Southern India on the genus *Arthrosphaera* (Kadamannaya *et al.,* 2010; Ambarish and Sridhar, 2013). Even though morphology serves as the basic component in the identification of Diplopoda, cytological and molecular studies help in precise characterization to develop phylogenetic relationships.

The cytology of Diplopoda is attractive owing to low vagility as well as the endemic distribution of pill-millipedes, which reveals speciation and the evolutionary blueprint (Fontanetti, 1996a). The cytogenetics of millipedes failed to attract the interest of researchers due to difficulties in the preparation of mitotic chromosomal stages (Fontanetti *et al*., 2002). Most of the cytological studies were confined to a few species of pill-millipedes of the Indian subcontinent and few reports are available from regions other than Asia-Pacific (Kadamannaya *et al*., 2010). Therefore, this paper deals with a brief outline on the cytogenetics of Diplopoda along with the orders Sphaerotheriidae and Glomeridae with an emphasis

on the cytology of *Arthrosphaera disticta,* endemic to the Western Ghats of India.

18.2 Cytology of Diplopoda

Studies on the class Diplopoda before 1940 have long historical significance. Subsequently, the door of cytology of Indian diplopods was opened by Natarajan (Natarajan, 1959) and later notable contributions were made by Chowdaiah and co-workers (Chowdaiah, 1966a, 1966b, 1966c, 1967; Chowdaiah and Kanaka, 1969; Achar and Chowdaiah, 1979). Chowdaiah and Kanaka (1974, 1979) have applied the classical cytological techniques to study millipedes belonging to the Indian Diplopoda. Achar and Chowdaiah (1979) launched a modified air-drying technique to evaluate the chromosomes of Diplopoda. An additional cytological technique like Giemsa differential banding has an added advantage to study pill-millipedes (Achar and Chowdaiah, 1980; Achar, 1983a). Subsequently, karyotype analysis of several species of pill-millipedes of the Western Ghats was undertaken by Achar (1983b) as well as Kadamannaya *et al*., (2010). These studies suggested that the largest chromosome was composed of sex bivalents in many pill-millipedes. For the first time, Achar (1983a) employed the acetic saline Giemsa (ASG) banding technique to evaluate the banding pattern of chromosomes of *Spirostreptus asthenes*. This study showed that each chromosome of pill-millipedes possesses alternating dark and light bands (~36 bands). However, no such attempts were made to study the chromosome banding pattern of the pill-millipedes of Western Ghats.

The 2*n* chromosome number in Diplopoda ranges between 8 and 30 with a high number in pill-millipedes (*Arthrosphaera*, 26-30; *Glomeris*, 16). With a few exceptions, it is interesting to note that the chromosomal complements of Diplopoda are multiples of 4 or 6 (Achar, 1987). The mechanism of sex determination in Diplopoda is still in the primitive state because the sex chromosomes are poorly differentiated from those of autosomes in most of the species (Achar, 1983a). This observation is valid because a variety of mammalian studies revealed that the X and Y chromosomes have evolved from the autosomes without morphological differentiation. The X and Y chromosomes have been depicted as the largest pair chromosomes in spite of a slight difference in size in several diplopods as reported by Achar (1987) from India.

The Robertsonian changes that occur in the form of centric fusion or fission generally occur during the evolution of animal karyotype is also

applicable to the diplopods. The Robertsonian changes and pericentric inversions seem to have a significant role in polyploidy as well as karyotype evolution of diplopods (Achar, 1987). Achar (1983b) has reported that pericentric inversions usually appear to operate in two opposite directions during karyotype evolution in millipedes such as *Carlogonus acifer* and *C. palmatus*. It may be assumed that the pericentric inversions might have played a critical role in the transformation of a metacentric condition in *C. acifer* from sub-metacentric type in *C. palmatus*. However, such clues or reports are not found so far in pill-millipedes.

Studies on the millipede *Polydesmus gracilis* by Achar (1984) revealed many critical points of cytological interest. This study provided evidence for the occurrence of pycnotic condensation of chromatin at the end of each thread in prophase as well as that the pycnotic knob numbers correspond to the 2*n* number of chromosomes. Such heteropycnosis was also reported by Chowdaiah and Kanaka (1979) in their studies on several species of millipedes. Since the sex determination mechanism of *P. gracilis* is in a primitive state, it is hard to distinguish X and Y chromosomes based on the shape and/or size. The polyploid nature of nuclei is also known in *P. gracilis.* Another significant observation made on the millipede *Aulacobolus excellens* by Achar (1985) revealed the formation of the translocation chain of six chromosomes (comprising four autosomes and two sex chromosomes). That was the first report on diplopod cytological investigations on translocation to provide a strong basis for speciation.

Cytological studies on 16 Brazilian diplopods by Fontanetti (1991, 1996a, 1996b, 1996c, 1998, 2000) demonstrated the chromosome number and sex determination mechanism. Another study by Fontanetti (1990) showed the formation of bouquet arrangements during early meiotic prophase and diffused states were seen in the pachytene stage. The latter stages showed more constitutive heterochromatin and the rest of the regions were euchromatic. The bouquet arrangement of chromosomes was frequently found in many diplopods. The genome of two diplopod species studied by Vetturi *et al*., (1997) from Spain was found to consist of 60% heterochromatin.

According to Achar (1987), the hypothesis proposed by Imai *et al.*, (1977) that explains the karyotypic evolution in Australian ants is also applicable to the Diplopoda. This hypothesis states that ancestral haploid number (n) is a model for the karyotype evolution in animals. Hence, the basic

chromosome numbers (4 and 6) may be assumed to be the ancestral haploid numbers for diplopods. Ahe similar prediction has also been made for *Drosophila*, Orthoptera and Mammalia (Patterson and Stone, 1952; Matthey, 1973; White, 1973). The proposed hypothesis states that the karyotype evolution leads to both augmentation or decrease in the chromosome number and hence runs in both directions. However, Robertsonian translocation, pericentric inversions and polyploidy played a significant role in the karyotype evolution.

18.3 Cytology of *Arthrosphaera*

The pill-millipedes acquired the highest pairs of chromosome number among the Diplopoda. Chowdaiah (1966c) reported the diploid chromosome number as 26 in *Arthrosphaera zebraica*. All the chromosomes are acrocentric, and the sex chromosome pairs are recognizably based on the size, heteromorphic feature and segregation behavior. Chowdaiah and Kanaka (1974) revealed that the 2*n* chromosome number in *A. disticta* and *A. gracilis w*as 28. But, in other species such as *A. craspedota, A. bicolor, A. dalyi* and *A. handersoni* the 2*n* chromosome number was 30. Chowdaiah (1967) demonstrated an interesting fact that the sex chromosomes exhibit unorthodox behavior in males, which provided a clue for possible coexistence of pre-reduction and post-reduction mechanisms in chromosomes in single species. Chowdaiah (1969) also reported on the chromatid bridges in pill-millipedes. Kadamannaya *et al*., (2010) established that the 2*n* chromosome number is 30 in *A. dalyi,* possessing the relative length (RL) range between 39 and 195, however, in *A. zebraica*, the 2*n* chromosome number is 26 telocentrics with RL ranging from 36-178. *A. dalyi* and *A. zebraica* distributed in the Western Ghats of India also showed heteropycnosis (Matthey, 1973). The number of heteropycnotic knobs are equal to the number of chromosomes of a particular species because only one end of each chromosome consists of heteropycnotic knobs. The meiotic behavior of chromosomes in both species is almost similar and in metaphase I the chromosomes are highly condensed to form clusters. This type of cluster formation was observed in earlier studies on *Arthrosphaera* species (Chowdaiah and Kanaka, 1974; Achar, 1987). Among the 25 spp. of *Arthrosphaera* known from India, 15 spp. have been investigated for chromosome number, sex determination mechanism and description of karyotypes for a few of them (Table 18.1).

18.4 Cytology of *Glomeris*

Up to 100 spp. belonging to the genus *Glomeris* (Family, Glomeridae) have been reported from the Canaries, Europe, North Africa and Northwestern Antolia (Mauries, 2006). Among them, cytogenetics of only three species was investigated and revealed cytological uncertainties (Table 18.1). Warchałowska-Śliwa *et al*., 2004 studied the chromosomes in two species of *Glomeris* and demonstrated that the 2*n* chromosome number is 16, consisting of metacentric/submetacentric autosomes showing XY sex determination type. The chromosome sizes between X and Y in *Glomeris* spp. are similar or different. For example, in *G. connexa* both the sex chromosomes possess the same size, whereas the X chromosome is larger than the Y chromosome in *G. hexasticha*. For the first time, the existence of supernumerary chromosomes in the millipede *Plusioporus setiger* from Brazil was demonstrated by Fontanetti (1998). In *G. hexasticha* also similar unstable B chromosomes have been reported by Warchałowska-Śliwa *et al.* (2004). Interestingly, the size of the B chromosome is smaller than the smallest autosome and possesses similar condensation patterns like autosomes or sex chromosomes. To demonstrate the heterochromatin content in *G. connexa* and *G. hexasticha*, the C-banding technique was followed. The chromosome number in karyotype matched with the number of C-bands seen. The nucleolar organizing regions (NOR) of early spermatogonial metaphase as well as meiotic prophase until following the pachytene stage was demonstrated by the silver staining technique. However, the C-banding and NOR staining methods have not been attempted to study the chromosomes of the Indian pill-millipedes.

18.5 Cytology of *Arthrosphaera disticta* Pocock

So far karyotype studies on the *Arthrosphaera disticta* have not been made. Individuals of *A. disticta* were collected from semi-evergreen forests (altitude, 651 m) of Shankaraghatta, Shimoga (Karnataka, India) (13°35′N 75°48′E). They were maintained on decomposed leaf litter in the laboratory. The present study includes karyotypic analysis of the fifth species of *Arthrosphaera* (Table 18.1).

Chromosomes of *A. disticta* were prepared by following the air-dry technique described by Rothfels and Siminovitch (Rothfels and Siminovitch, 1958) with a slight modification. On injection of colchicine (0.5%, 0.5-1 ml) to two adult males, after 3-4 hr, testes were isolated in

normal saline (NaCl, 0.65%) and pre-treated with hypotonic solution (KCl, 0.13M) at laboratory temperature for 30 min. Pre-treated material was thoroughly minced in the hypotonic solution to achieve a uniform turbid suspension, incubated (37°C, 30 min) and centrifuged (800-1000 rpm, 5 min). After discarding the supernatant, the pellet of cells formed was re-suspended (in hypotonic solution). After two changes (in the hypotonic solution), it was centrifuged to fix in fresh fixative (acetic acid and methanol, 1:3; 30 min). Two changes were given in the fixative, the cell pellet formed was re-suspended (in a small quantity of fixative). A few drops of the cell suspension in a Pasteur pipette was dropped (~30cm height) onto the alcohol cleaned microscope slides chilled in ice-cold water. The slides were air-dried and preserved. The prepared slides were stained with Giemsa working solution (2.5%). They were then rinsed in distilled water and air-dried. Spermatogonial metaphase and different meiotic stages were examined to construct a karyotype as per the method outlined by Levan *et al.*, 1964). The relative length (RL) was calculated from the karyotype. The relative length = (Length of the chromosome /Total length of a haploid set) ×1000.

The karyotype of male *A. disticta* is composed of 28 chromosomes along with a heteromorphic sex pair (XY) (Fig. 18.1A-L). Both autosomes and XY chromosomes are acrocentric, while excluding the sex pair the acrocentric pairs of autosomes are homomorphic. The X chromosome is slightly larger and darker than the Y chromosome (Fig. 18.1A). At the zygotene stage, a bouquet formation of chromosomes is evident (Fig. 18.1B) and a similar bouquet formation was also reported in *Arthrosphaera craspedota* by Chowdaiah and Kanaka (1974). The bouquet formation in pill-millipedes is depicted owing to the polarization of chromosomes (in the leptotene stage) and attachment of the chromosomes at the centrosome region to the nuclear membrane. According to Fontanetti (Fontanetti, 1990), such bouquet formation helps in the union of homologous chromosomes with synapsis at zygotene stage. Yunis and Yasmineh (1971) interpreted that bouquet formation occurs owing to non-homologous heterochromatin clumping. The pachytene stages could be recognized by the pairing of the homologous chromosome along their length (Fig. 18.1C). Highly condensed bivalents with terminalization of chiasmata with the incomplete stage in sex chromosome were seen at late diakinesis (Fig. 18.1D). In the meiotic metaphase-1 stage, 14 bivalents were highly conspicuous by attaining the dumb-bell shape (Fig. 18.1E, F), while clumping of chromosomes was also seen occasionally.

The lagging behavior of sex chromosomes was seen during meiotic division (Fig. 18.1G). The laggards also showed segregation during the meiotic II division in the equatorial region along with other chromosomes in metaphase-II (Fig. 18.1H). Most of the meiotic chromosomal pattern and behavior correspond to the earlier reports on pill-millipedes. As shown by Chowdaiah (1966c), the male pill-millipede is heterogametic in sex and the mechanism of sex determination is of the XY type. The sex chromosomes are larger in size and more conspicuous than the autosomes, these features are typical and characteristic of pill-millipede karyotype and it is assumed to be an adaptation to the spindle mechanism. The male karyotype of *A. disticta* is composed of 28 chromosomes along with a heteromorphic sex pair (XY) (Fig. 18.2). The acrocentric autosomes and XY chromosomes are relatively homomorphic except for the sex pair. The X chromosome is darkly stained and slightly larger compared to the Y chromosome (Fig. 18.2). The relative lengths of the chromosomes range between 30.1 and 138.7 (Table 18.2).

18.6 Outlook

Logical conclusions on the cytology of pill-millipedes can be drawn only after studying more species for a clear understanding of the evolutionary pattern. The techniques such as G-, C- and Q-banding enable resolving the chromosome architecture of pill-millipedes. Determination of the position of heterochromatin, as well as NOR in chromosomes, will open up the possibilities of recognizing the gene localization, chromosome rearrangements and cytogenetics of related species of millipedes. Recently, the morphology-based phylogeny of four species of *Arthrosphaera* from Madagascar and Sri Lanka has been studied by considering 89 morphological characteristics (Wesener and van den Spiegel, 2009). Only one study is available on the molecular basis of evolution among *Arthrosphaera* (*Arthrosphaera brandtii*), the giant pill-millipede from Madagascar, which is known as the sister-taxon of *Arthrosphaera* located in the Western Ghats of India (Wesener *et al*., 2010). Further, molecular studies have to be undertaken to solve the cytotaxonomical uncertainties of *Arthrosphaera*. The molecular basis of phylogeny has been applied mainly to re-evaluate the existing classification schemes for updating different groups of organisms. A blend of morphology (including scanning electron microscopy), cytology and molecular systematics will help by providing further scope to unravel the history, phylogeny and evolution of pill-millipedes.

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Table 18.1 Details of cytological studies carried out on pill-millipedes.

	2*n*	Sex mechanism	Karyo-type	Sampling location	Reference
Sphaerotheriidae					
Arthrosphaera davisoni Pocock, 1895	26	XY	Yes	Khandala hill, Maharashtra, India	Achar (1986)
A. lutescens Butler, 1872	26	XY	-	Thirthahalli, Karnataka, India	Achar (1986)
A. zebraica Butler, 1872	26	XY	Yes	Karnataka, India	Chowdaiah (1966c), Kadamannaya *et al*. (2010)
A. disticta Pocock, 1895	28	XY	Yes*	Alagarkovil hilly tracts, Tamil Nadu, India; Shankaraghatta, Shimoga, Karnataia, India	Chowdaiah and Kanaka (1974), Achar (1984), Present study
A. gracilis Attems, 1936	28	XY	-	Southern India	Chowdaiah and Kanaka (1974)
A. craspedota Attems, 1936	30	XY	-	Southern India	Chowdaiah and Kanaka (1974)
A. bicolor Pocock, 1895	30	XY	-	Southern India	Chowdaiah and Kanaka (1974)
A. dalyi Pocock, 1895	30	XY	Yes	Alagarkovil hilly tracts, Tamil Nadu, India	Chowdaiah and Kanaka (1974), Achar (1986), Kadamannaya *et al*. [34]
A. handersoni Pocock, 1895	30	XY	-	Southern India	Chowdaiah and Kanaka (1974)
A. magna Attems, 1936	30	XY	-	Karnataka, India	Achar (1986)
A. nitida Pocock, 1895	30	XY	Yes	Wyanad, Kerala, India	Achar (1986)
Arthrosphaera sp. 1	30	XY	-	Southern India	Chowdaiah and Kanaka (1974)
Arthrosphaera sp. 2	30	XY	-	Southern India	Chowdaiah and Kanaka (1974)

Arthrosphaera sp. (M)	30	XY	-	Madikeri, Karnataka, India	Achar (1986)
Arthrosphaera sp. (C)	30	XY	-	Chenganacherri, Kerala, India	Achar (1980)
Glomeridae					
Glomeris annulata Brandt, 1833	20	XY	?	?	Bessiere (1948)
Glomeris connexa C.L. Koch, 1847	16	XY	Yes	Rogozno, Poland	Warchalowska-Sliwa *et al*. (2004)
Glomeris hexasticha Brandt, 1833	16	XY	Yes	Zemborzyce, Poland	Warchalowska-Sliwa *et al*. (2004)

-, Not available; *, Present study; ?, Not defined

Table 18.2 Relative length (RL) (n=5±SD) and nature of chromosomes of *Arthrosphaera disticta* based on karyotype analysis (2*n*=28).

Chromosome Pair	Relative length (RL)	Nature of chromosome
1	113.7±0.43	Acrocentric
2	101.6±0.57	Acrocentric
3	91.04±0.40	Acrocentric
4	79.76±0.63	Acrocentric
5	66.98±0.38	Acrocentric
6	55.42±0.41	Acrocentric
7	50.84±0.48	Acrocentric
8	48.08±0.34	Acrocentric
9	44.06±0.20	Acrocentric
10	40.13±0.69	Acrocentric
11	37.318±0.175	Acrocentric
12	34.12±0.31	Acrocentric
13	30.06±0.48	Acrocentric
14	X - 138.72±0.68	Acrocentric
	Y - 117.12±0.58	Acrocentric

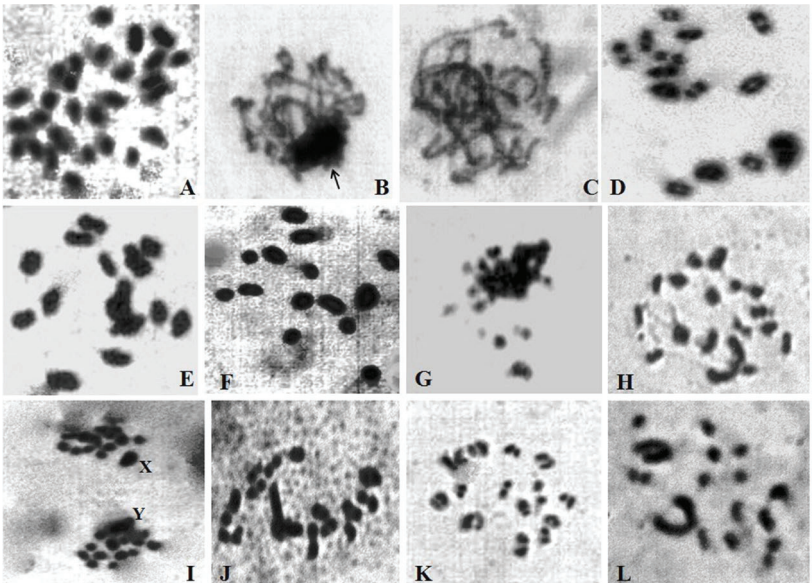

Fig. 18.1 Photomicrographs of chromosomes in male meiosis of *Arthrosphaera disticta* (2*n*=28): A, spermatogonial metaphase; B, zygotene (arrow, polarized material); C, pachytene; D, diplotene; E, diakinesis; F, metaphase I (polar view); G, metaphase I (clumped chromosomes); H, early anaphase I; I, anaphase I; J, metaphase-II; K, metaphase-II (side view); L, early anaphase II.

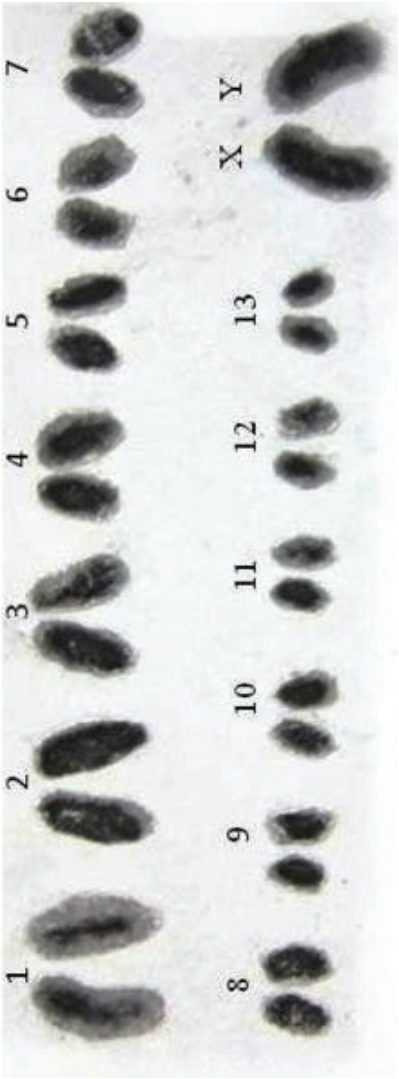

Fig. 18.2 Karyotype of *Arthrosphaera disticta* (2*n*=28) with heteromorphic chromosomes X and Y.