Mutagenesis, Cytotoxicity and Crop Improvement

Mutagenesis, Cytotoxicity and Crop Improvement:

Revolutionizing Food Science

Edited by

Tariq Ahmad Bhat

Cambridge Scholars Publishing

Mutagenesis, Cytotoxicity and Crop Improvement:
Revolutionizing Food Science

Edited by Tariq Ahmad Bhat

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.

CONTENTS

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
Dr Tariq Ahmad Bhat

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.

Dr Khalid Ul Rehman Hakeem
Professor
Department of Biological Sciences,
Faculty of Science,
King Abdulaziz University,
Jeddah, Makkah Province
Kingdom of Saudi Arabia
Kur.hakeem@gmail.com

CHAPTER 1

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

AAMIR RAINA[1,2*], SHAZIA BI ANSARI[1], SHAHNAWAZ KHURSHEED[1], MOHAMMAD RAFIQ WANI[3], SAMIULLAH KHAN[1], TARIQ AHMAD BHAT[4]

[1]Mutation Breeding Laboratory, Department of Botany, Aligarh Muslim University, Aligarh
[2]Botany Section, Women's College, Aligarh Muslim University, Aligarh
[3]Department of Botany, Abdul Ahad Azad Memorial Degree College Bemina-190 018, Cluster University Srinagar, Jammu and Kashmir, India
[4]Department of Education, Govt. of Jammu & Kashmir, India
*Email: aamir854@gmail.com

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.