Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

MANUAL ON MUTATION BREEDING

THIRD EDITION

Manual on Mutation Breeding

Third Edition

Edited by

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Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture
Vienna, Austria

Food and Agriculture Organization of the United Nations
International Atomic Energy Agency
Vienna, 2018

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Recommended citation

FAO/IAEA. 2018. Manual on Mutation Breeding - Third edition. Spencer-Lopes, M.M., Forster, B.P. and Jankuloski, L. (eds.), Food and Agriculture Organization of the United Nations. Rome, Italy. 301 pp.

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ISBN 978-92-5-130526-3

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FOREWORD

FAO estimates that global food production must increase by 70% to feed the projected growth in the world's population from about seven to nine billion people by 2050. This cannot be done without increasing land productivity and conserving natural resources in the face of the multitude of severe challenges posed, including climate change, drought and flooding, soil erosion and salinization – all of which contribute to hampering crop productivity and making it an increasingly high-risk venture. Global cutting-edge R&D efforts are accelerating to both develop and put into practice sustainable, climate-smart agricultural practices that are of distinct benefits not only to agricultural productivity, farming communities and food security but also to the environment and the natural resource base that must necessarily sustain these agricultural practices.

As demonstrated during the Green Revolution of the 1960s and 1970s, which resulted in numerous new high-yielding varieties, particularly of cereals, plant mutation breeding can indeed play a crucial and most valuable role in meeting challenges relating to food security. Whereas fifty years ago, mutation breeding focussed predominantly on increasing yields, especially dwarf wheats and rice, the challenges today are primarily related to increasing the tolerance of crops to environmental and weather associated hazards and to driving opportunities for climate-smart agriculture.

The 2nd edition of the *Manual on Mutation Breeding* was published in 1977. After nearly 40 years, and considering the numerous technological advances in this field, a completely revised and updated version has now been long overdue. The third edition, the *Manual on Mutation Breeding* that you now peruse, describes advances in plant mutation breeding, in irradiation techniques as well as in the use of chemical mutagenesis, in seed-propagated and vegetatively propagated crops, and in the types of traits that we believe warrant urgent attention to achieve the set target of global and nutritious food security for all. It also provides a comprehensive overview and guidelines for new high-throughput screening methods – both phenotypic and genotypic – that are currently available to enable the detection of rare and valuable mutant traits and reviews techniques for increasing the efficiency of crop mutation breeding.

Since its establishment in 1964, when the Food and Agriculture Organization of the United Nations (FAO) and the International Atomic Energy Agency (IAEA) took the visionary step of merging their mandates to create the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, the Joint FAO/IAEA Division has remained a global leader in the application of irradiation for plant mutation breeding

and crop improvement. Over 3275 mutant varieties in more than 220 plant species have to-date been officially released worldwide (see http://mvd.iaea.org/). Their value is measured in billions of dollars of additional revenue, in millions of cultivated hectares and – most importantly – in innumerable people leading happy and healthy lives. It is our sincere hope that this third edition will help the global community in its endeavour to provide sustenance also for the 10% of the global population that currently suffer from hunger and malnutrition.

Qu Liang
Director
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture

ACKNOWLEDGEMENTS

This manual is largely based on the previous edition coordinated by Alexander Micke and published as a Technical Series N°120 in 1977 by the Division of Publications, IAEA Vienna. This edition resulted of the work of numerous pioneers in mutation induction and mutation breeding including technical officers of the Plant Breeding and Genetics section and laboratory and their collaborators from various parts of the world. So, we wish here to gratefully thank these forerunners for their work but also for having paved the way to the work of all the technical officers who followed in their foot-steps. All the scientific topics covered in the 2nd edition were revisited, updated and enhanced with the addition of new technologies and new scientific results. More importantly the new technologies, such as *in vitro* and molecular techniques, which appear to clearly and notably speed-up and widen the outcomes of mutation breeding have been added.

We also wish to thank our counterparts and collaborators under the coordinated research and the technical projects for their invaluable contributions to mutation breeding. In fact, if the IAEA mutant variety database can now include more that 3200 new priceless mutant varieties, which cover thousands, if not millions of Ha and bring additional income to farmers and agricultural systems all over the world, it is thank to each, and every one of these passionate scientists and breeders.

Among the major additions in this edition are the practical examples and photographs provided by some of the counterparts and collaborators and by active staff members. Their contributions are sincerely appreciated and gratefully acknowledged. Thus, the section would like to express their appreciations and indebtedness to the following persons: Ms Luz Gomez-Pando; Ms Joanna Jankowicz-Cieslak; Mr Burak Kunter; Ms Süreyya Sekerci; Ms Kadriye Yaprak Kantoğlu, and Mr Penna Suprasanna and their collaborators for providing some inspiring practical examples of successful mutation breeding programmes.

The completion of this undertaking could not have been possible without the assistance of Mr Indra Giri who assembled and so artfully crafted the schemes and figures to better showcase the examples and/or methodologies provided, and Ms Katayoun Allaf for her kind assistance in formatting the document according to the FAO and IAEA Publication’s standards.

INTRODUCTION

Spencer-Lopes, M.M., Forster, B.P., Mba, C., and Jankuloski, L.

Mutation, i.e. the heritable change to an individual's genetic makeup, results in new traits which are passed on from parent to offspring and thereby, drives evolution. In nature, mutations are caused by errors in the replication of deoxyribonucleic acid (DNA). This hereditary material could also be changed due to exposure to surroundings' natural radiations. A resulting modified individual is then known as a spontaneous mutant.

Mutation is the underlying cause of evolution as an individual with a novel trait may be preferentially selected for in nature – because its superior fitness arising from novel (mutant) adaptive features – or artificially by man – because of the desirability of the novelty.

Following the discoveries of X-rays by Roentgen in 1895; radioactivity by Becquerel in 1896; and radioactive elements by Marie and Pierre Curie in 1898, it was shortly afterwards demonstrated that radiation caused mutations in fruit flies (Muller, 1927) and in the crop plants – maize and barley (Staedler, 1928). The subsequent rapid and widespread adoption of induced mutations as a crop improvement tool derives directly from these pioneering discoveries. It became evident that man did not have to wait for chance discoveries of desirable off-type plants as was the case for our forebear hunter-gatherers. Man could, in fact, induce mutations at will!

Mutation breeding has witnessed spectacular successes since the release of the first induced mutant variety – a light green mutant of tobacco released in Indonesia in the mid-1930s. Easy targets for plant mutation breeding are annual, inbred, seed propagated crops: seeds are ideal for mutation induction and short life-cycles mean mutant generations can be produced quickly and desirable mutant lines can be developed into varieties rapidly. Thus, early successes were made in crops such as rice, barley and tobacco and these have been sustained ever since. Next up are the annual, outbred seed crops, these have a slightly more complex breeding system, but nevertheless early successes in developing mutant varieties were reported and there has been a continual production of new mutant varieties in crops such as maize by the late 60s in various countries. More problematic are the vegetatively propagated crops which have lagged, behind seed propagated crops in mutation breeding. This group was targeted by Frantisek Novak *circa* 1980s. Working at the FAO/IAEA's Plant Breeding and Genetics Laboratory, Novak and his team pioneered tissue culture methods needed for banana micro-propagation. Micro-propagation is essential as it allows large numbers of cuttings to be produced for both mutation induction and subsequent mutant lines development. The vegetatively propagated crops are

currently undergoing a renaissance with respect to plant mutation breeding as numerous biotechnologies can be applied for efficient mutagenesis and mutant screening, particularly tissue culture techniques. And last, but not least, are the perennial crops, these naturally have long juvenile stages and have been neglected as it takes many years for them to bear fruit. A further hindrance in plant mutation breeding has been the reliance on phenotypic screening which is normally applied in the second generation after mutation induction at the earliest. This *modus operandi* is changing with the emergence of DNA analytical tools. Genotypic screening has the potential to accelerate both mutation detection and mutant line development. This can be applied to all crops, but has special relevance to (orphaned) perennial and plantation crops such as oil palm, cocoa, rubber, tea and coffee.

According to the International Grains Council (www.igc.int) world estimates for Maize production, for 2017/2018 stands above 1050 million tonnes. This grain cereal together with wheat, barley and rice covers most of the food supply for the current world population of 7.6 billion people. However, not everyone knows that this incredible cereal found in so many various forms from pop-corn to porridge has been manufactured by civilisations all over the world for about 10 000 years. In fact, it was through the accumulations of several spontaneous mutations that *Zea mexicana* (Schrad.) Kuntze or the Mexican teosinte evolved to become *Zea mays* (maize or corn), the most widely produced cereal crop (FAOSTAT, 2014).

However, the greatest impetus for induced mutation breeding would come from the establishment, under the aegis of the United Nations, in 1957 of the International Atomic Energy Agency (IAEA) with its headquarters in Vienna, Austria in keeping with the notion of "Atoms for Peace and Development". In 1964, the Food and Agriculture Organisation (FAO), another UN specialized agency – with a mandate to eradicate hunger and malnutrition, and the IAEA established the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture. The aim was to pool the resources of both organizations to more effectively leverage peaceful uses of atomic energy in assisting their member countries produce more, better and safer food. One major activity of the Joint Division was the provision of support to Member States of both organizations in establishing gamma-ray irradiators for plant mutation breeding.

The Plant Breeding and Genetics Section (PBGS), one of the 5 sections under the Joint FAO/IAEA Subprogramme has, since then, devoted its effort towards promoting the use of mutation induction for improving both productivity and crop nutritional values using physical mutagens such as: X- and gamma-rays, neutrons, α, β and other accelerators particles, ion or laser beam, etc.

The PBGS provides technical assistance to Members States in activities dealing with mutation induction for crop improvement, organising trainings and expert missions

and coordinating meetings all relating to mutation breeding for crop improvement. This support is provided through Coordinated Research Programmes (CRPs) and Technical Cooperation Programmes (TCPs), in the form of research projects and capacity building (e.g. laboratory equipment and training). The outputs of these activities are generally published under different information formats from research articles, to newsletters, protocols and manuals. Considering that there is an upsurge in the use of induced mutations – for crop improvement and genomics – and at the same time, there is the urgent need to generate novel useful heritable variations for developing the nutritious, resilient, input use-efficient and productive crop varieties, the Joint FAO/IAEA Division made the decision to update the existing manual. The Manual on Mutation Breeding is founded on the experience and knowledge of the PBGS staff, but has been written in collaboration with external experts. The eight chapters of this 3rd edition provide up-to-date insights on the use of physical and chemical mutagenesis on seed and vegetatively propagated crops. It provides guidance on practical ways for conducting mutation induction to take a full advantage of the power of this technique for unmasking novel alleles that could be incorporated into plant breeding programmes or for studying the functions and patterns of inheritance of genes and the traits that they control.

Among the most notable examples of mutation breeding is the development of barley (*Hordeum vulgare* L.) semi-dwarf mutant varieties: Golden Promise and Diamant in the early 1970s. These founding mutant varieties revolutionised the crop as they were better adapted to mechanical (combine) harvesting, had higher yields (especially under adverse weather conditions) and set a new standard for quality. The first chapters describe physical and chemical mutagenesis and cover the action modes of the different mutagenic agents with details of application procedures, the expected outcomes as well as precautionary measures necessary for practical use. Chapters 3 and 4 explore the various types of mutations, which serve practical crop improvement (mutant descriptors) and the more fundamental aspects of the exploration, identification and understanding of mutations in DNA sequences. Chapters 5 and 6 deal with the practicalities of mutation breeding in seed and vegetatively propagated crops, including the new processes in developing mutant populations, screening and selection. The main factors affecting the success of mutation breeding such as population size, propagation and isolation of mutant plants and examples of screening for desired mutants are described in detail and illustrated with schematic diagrams and photographs to assist practical mutation breeding. Chapter 7 attempts to provide the most accurate and extensive examples of development of new mutant varieties displaying superior traits and wider adaptability to ever-changing environmental conditions. Finally, Chapter 8 considers new and emerging concepts and technologies that are expected to impact on future plant mutation breeding. These focus on more efficient methods in accelerating the mutation breeding process by embracing new developments in pertinent biotechnologies, particularly in the areas of DNA analysis

and doubled haploidy. This 3rd edition demonstrates the practicalities of applied mutation breeding, now more than ever before, to all crop species.

1. PHYSICAL MUTAGENESIS

1.1.RADIATION TYPES AND SERVICES

This chapter is an update, largely based on the chapter on Mutagenic Radiation presented in the second edition of the Manual on Mutation Breeding published in 1977. Physical mutagens comprise all nuclear radiations and sources of radio-activity including ultraviolet light (a non-ionising radiation), several types of ionizing radiations, namely X- and gamma-rays, alpha and beta particles, protons and neutrons. An overview of the main physical mutagens used in plant mutation breeding is presented here, covering their physical characteristics, their mode of action and all general principles and consideration on how they may be applied for mutation induction in plants.

Several types of ionizing radiation are available for plant mutation induction. Each of these has the common feature of releasing ionizing energy. However, there are several differences among ionizing radiations regarding the energy deployed the penetrating capability and the level of hazard involved for operators (Table 1.1 and Figure 1.1).

1.1.1. **X-rays**

X-rays are known to originate from electrons and not from nuclear energy. Like gamma-rays and ultraviolet (UV) light, X-rays are electro-magnetic radiations emitted as quanta, their difference is based on the wavelengths; 0.001 – 10 nm for gamma- and X-rays compared to 2000 – 3000 nm for UV light. In an X-ray machine, electrons are electrically accelerated in a high vacuum and then stopped abruptly by striking a target, e.g. a tungsten, gold or molybdenum barrier resulting in the emission of radiation (Figures 1.2a,b). For mutation induction hard X-rays (short wavelength) are usually preferred since their penetration is greater than soft X-rays (which have a longer wavelength). The shortest wavelength emitted (except for constant potential machines) is related to the peak operating voltage (kVp) of the X-ray tube, the higher the kVp, the shorter the wavelength. Specific filters, e.g. aluminium filter: 0.5nm, are often used in hard X-ray production to absorb unwanted soft radiation. The kVp, milliamperes (mA), thickness and type of filter, distance of tube to target, dose and dose rate affect the results and should always be recorded (Mehta and Parker, 2011).

1.1.2. **Gamma-rays**

In general, gamma-rays emitted by decay of an unstable nucleus of an atom, have a shorter wavelength and therefore possess more energy per photon than X-rays. Mono energetic gamma radiation is usually obtained from radio-isotopes, in contrast to X-

rays. A gamma irradiation facility can be used in a similar manner as an X-ray machine for acute or semi–acute exposures. Gamma cells are the most commonly used emitters for plant mutation induction, as of 2004 there were about 200 gamma-cells in use world–wide (IAEA, 2004). However, the gamma radiation source has a distinct advantage for prolonged treatments in that it may be placed in a controlled environment chamber (Figures 1.3a,b), in a greenhouse (Figures 1.4a,b), or in a field so that plants may be exposed at various times and at various developmental stages.

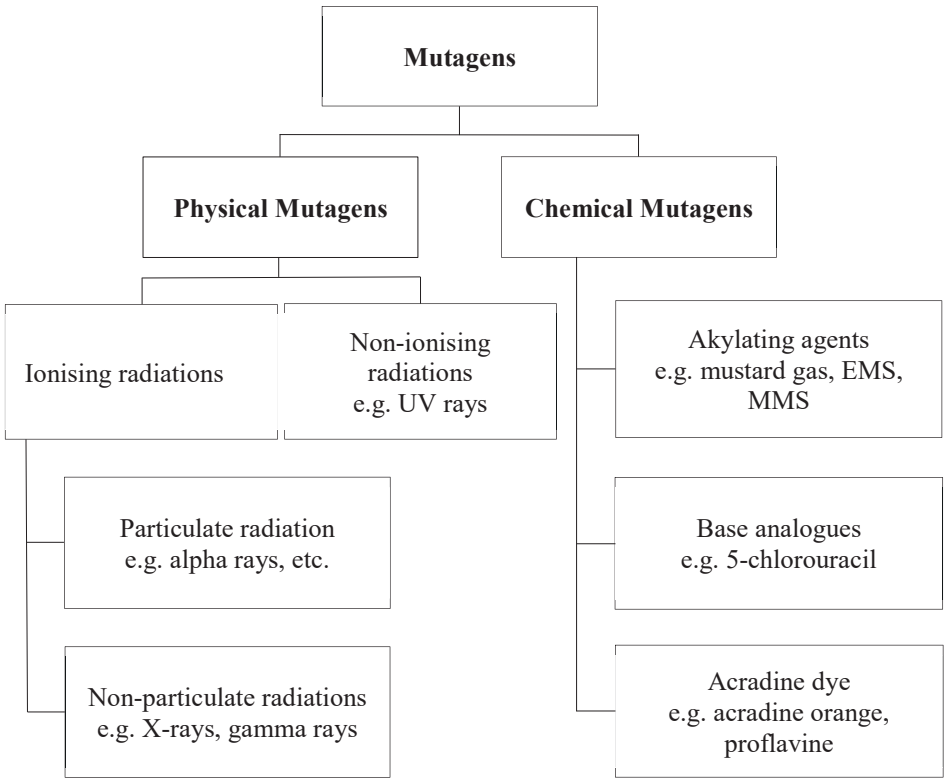
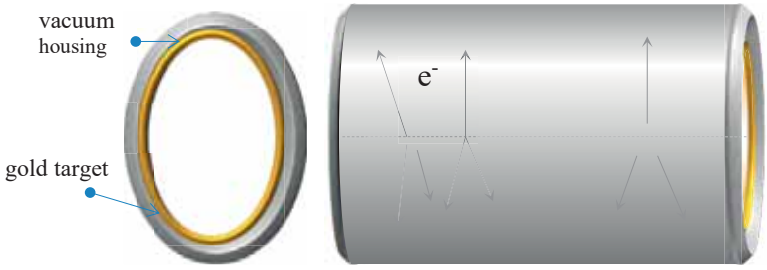
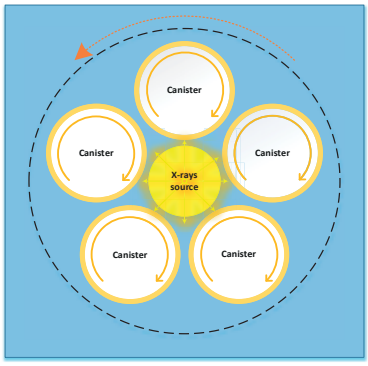


Figure 1.1. Common mutagens used in plant mutation induction.

Figure 1.2a. RS-2400 self-shielded X-ray irradiator (Produced by RAD Source Technologies Inc., USA) in the Entomology Unit - Seibersdorf Laboratories - Joint FAO/IAEA - Programme of Nuclear Techniques in Food and Agriculture, Vienna, Austria (Mehta and Parker, 2011).



X-ray tube principle



X-ray tube (centre) surrounded by orbiting and rotating sample canisters.

Figure 1.2b. X-ray tube principle and structure

TABLE 1.1. PROPERTIES OF RADIATIONS AND THEIR APPLICATIONS IN MUTATION BREEDING

Types of radiation	Source	Description	Energy	Hazard
X-rays	X-ray tube with cylindrical anod (Kirk and Gorzen, 2008)	Electro-magnetic radiation potent Mutagen agent	1 to 500 KeV. for mutation breeding: 50-300 keV	Dangerous, penetrating
Gamma rays	Radio-isotopes and nuclear reactions (^{60}Co and ^{137}Cs)	Electro-magnetic radiation like X-rays	Up to several MeV	Dangerous, highly penetrating
Neutrons (fast, slow, and thermal)	Nuclear reactors /atomic piles or accelerator [Californium (^{252}Cf) and Curium (^{248}Cm)]	Uncharged particles slightly heavier than a proton, not observable except through interaction with nuclei in the material it traverses.	From less than 1eV to several million	Very hazardous
Beta particles (ß$^{-}$ - negatron; ß$^{+}$ - positron and EC -electror capture)	Radioactive isotopes or accelerators (^{7}Be)	Emitted by the nucleus of radio-nuclide with neutron/proton imbalance efficient only when incorporated directly into cells	Up to several MeV	May be dangerous
Alpha particles (α) emitted by nucleus with atomic number >81	Radio-isotopes from radio-nuclide (^{32}P)	Two protons and two neutrons emitted as nucleus decay products, not efficient in mutation due to very low tissue penetration	2 – 9 MeV	Very dangerous internally
Particles from accelerators	Nuclear reactors or particle accelerators	Beam of fast-moving, electrically charged atomic or sub-atomic particles (i.e., Quarks) Application ion implantation and/or mutation	600 MeV to 2.75 GeV	Very hazardous

Figure 1.3a. Gamma-rays machine in the Joint FAO/IAEA Plant Breeding and Genetics Laboratory, Seibersdorf – Austria. Courtesy of M. Matijevic.

Figure 1.3b. Loading of seed samples. Courtesy of M. Matijevic.

Figure 1.4a. External view of the greenhouse field in Kuala Lumpur, Malaysia. Courtesy of L. Jankuloski.

Figure 1.4b. View of the plants arrangement in the greenhouse with the ^{137}Cs source. Courtesy of S. Nielen.

The isotopes Cobalt-60 (^{60}Co) and Caesium-137 (^{137}Cs) are the main sources of gamma rays. In addition to naturally occurring radioactive isotopes, artificial gamma-rays may be produced using cyclotrons (IAEA, 2004). Caesium-137 has a half–life of 30.17 years and is used in many installations as this is much longer than the half–life of Cobalt-60, which is of 5.26 years. It should be noted that, both these radio-isotopes

must, at all time, be shielded in lead containers, for safety and security purposes. The International Safety Standards for Protection against Ionizing Sources or Basic Safety Standard Handbook, published by the IAEA in 2016 provides details for the safe handling of these gamma sources.

1.1.3. **Ultraviolet light**

Ultraviolet light or UV light is a non-ionizing radiation at the wavelength commonly employed (e.g. the 2537 nm line of mercury germicidal lamps), but it will be included in this discussion because it has frequently been used in plant mutation induction especially in pollen grains, cell and/or plant tissue cultures. UV radiations are generally divided into three classes: UV-A, UV-B and UV-C. The UV-C region of the UV spectrum includes wavelengths below 280 nm; UV radiation in the UV-B region those from 280 to 320 nm, and UV wavelengths from 320 to 390 nm make up the UV-A region of the spectrum.

Ultraviolet light has limited tissue penetration and its use is restricted to treating sensitive materials, often single cells or single layer tissues, such as spores, suspension cell cultures and pollen grains. However, the increasing use of cell and tissue culture for mutation breeding of plants has led to increased use of UV light as a mutagenic agent, especially when single mutant genes are sought (see Chapter 8). In order to make quantitative assessments of experimental results, it proved necessary to use monochromatic (or near monochromatic) UV-C light because it has confirmed biological effects on photosynthesis, dark respiration and transpiration (Castronuovo *et al.*, 2014).

Initial research on UV light focussed on DNA damage, DNA repair, and pollen irradiation. The latter showed the reactivation of transposable elements and thereby indirect gene mutation, for example in maize (Jardim *et al.*, 2015). Descriptions of equipment and procedures for treating plant materials with UV light could be found in Mba, (2013) and Mba *et al.*, (2012). UV-B light has a strong effect on surface or near-to-surface zones of plant cells including plastid structure (mostly thylakoid membranes) and thus on photosynthesis (Kovacs and Keresztes, 2002).

1.1.4. **Alpha particles**

The alpha particles, are structurally equivalent to the nucleus of a helium atom, and are emitted from radionuclides with atomic numbers larger than 82 such as Radium and Plutonium (L'Annunziata, 2016). They are considered potential health hazards when ingested or inhaled, but their low tissue penetration power, e.g. through epidermis, renders them quite inefficient for mutation induction in plants (van Harten, 1998).

1.1.5. **Beta particles**

Beta particles are emitted from the nucleus of an atom during radioactive decay (L'Annunziata, 2016), and are effective in inducing mutations. Beta particles, such as those from ^{3}H, ^{32}P and ^{35}S produce in target tissue similar effects to those of X- or gamma-rays, although the penetration of beta particles is lower than that of X- or gamma-rays. However, this difficulty may be resolved by placing the radioisotopes in a solution administered directly to the target plant material. Thus, ^{32}P or ^{35}S may be incorporated directly into cell nuclei and induce mutations as observed in rice and cotton (Mba *et al.*, 2012). Due to variability from tissue to tissue and cell to cell, it is difficult to determine the exact internal beta particle dose, and thus its use in mutation breeding has been limited. A successful example is the mutation induction in rice seeds using a solution of ^{32}P as reported by Kharkwal, Pandey and Pawar, (2004).

1.1.6. **Particles from accelerators**

Amaldi, (2000) listed around 15 000 different types of particles accelerators world-wide, e.g. the Cockroft-Walton and Van de Gruff accelerators, the betatrons, the cyclotrons, the synchrocyclotrons, the synchrotrons and the linear accelerators. In general practice, they are used to accelerate protons, deuterons and electrons. Particle accelerators produce beams of energetic ions and electrons which may be deployed for various purposes, including mutation induction in plants. Particle induced X-ray emissions (micro–PIXE) with focused ion beams have recently been applied in plant mutation investigations (see also section below on ion beam irradiation).

1.1.7. **Neutrons**

According to Byrne (2013), Pauli (1930) was the first to propose the idea that, to have a better conception of the relations in the nucleus, in addition to protons and electrons, there should be neutral particles, which he then called "neutrons". The neutron is stable only in the confines of atom nucleus, once separated from the nucleus the neutron decays with a mean lifetime of about 15 minutes releasing various kinetic energies. Table 1.2, below presents the different neutrons as per the energy released; thermal (0.4 – 100 eV) and fast neutrons (200keV – 10MeV) are the most currently used for plant mutation induction.

TABLE 1.2. CATEGORIZATION OF NEUTRONS ACCORDING TO THE ENERGY RELEASED (L'Annunziata, 2016)

Cold neutrons	< 0.003 eV
Slow (thermal) thermal neutrons	0.003 – 0.4 eV
Slow (epithermal) neutrons	0.4 – 00 eV
Intermediate neutrons	100 – 200 keV
Fast neutrons	200 KeV – 10 MeV
High energy (relativistic) neutrons	>10 MeV

The radio-nuclide Californium-252 (^{252}Cf) is currently the most commonly used as a spontaneous neutron source (Karelin *et al.*, 1997). Neutrons have been shown to be highly effective for the induction of mutations in plants. Even though, their practical use has been hampered by a lack of adequate dosimetry techniques, by a lack of uniform dosimetry among various reactor facilities, and by the range of procedures to report the dose and spectrum of neutrons used. Hence, a certain degree of confusion exists concerning the results of early neutron experiments. In the past decade, however, situation has improved considerably and recommended procedures are now available for mutation induction, e.g. in the report on *Neutron irradiation of seeds* (IAEA, 1973).

1.1.8. **Ion beam irradiation and ion beam implantation**

The demonstration of the effectiveness of ion beam irradiation for mutation induction in tobacco embryos during fertilization without any damage to other plant tissue led to the widespread use of this technology, for instance in Japan (Abe *et al.*, 2007).

Ion beam implantation is a method where atoms are injected into the surface layers; a process mostly used in industry, but it can also be applied on plant tissues and this turned out to be an effective tool in mutation induction. Feng and Yu reported in Shu *et al.*, (2012) that the effects of ion beam implantation on plants were first revealed in the late-80s by Ziegler and Manoyan (1988). The latter author extensively described the process of ion beam implantation and the resulting effects. It has been shown that this method has many outstanding advantages including: lower damage rate; higher mutation rate and wider and novel mutational spectrum. Many new mutant lines of rice with higher yield, broader disease resistance, shorter growing period but higher grain quality have been bred; several new lines of cotton, wheat and other crops have also been developed (Zengquan *et al.*, 2003; Shu *et al.*, 2012).

1.1.9. **Cosmic irradiation**

Cosmic rays were discovered by Victor Hess in 1911–1913, as reported by L'Annunziata, (2016). These so called "air showers", which constantly strike the top of the atmosphere, consist of cascades of sub-atomic particles and electro-magnetic radiations resulting from the various collisions occurring in space (L'Annunziata, 2016).

Cosmic radiations have been largely investigated in plant biology and mutation induction. For example, in maize, somatic mutations, including white-yellow stripes on leaves, dwarfing, change of leaf sheath or seedling colour were observed in plants developed from seeds flown into space. In China, several new mutants (≈ 66) in rice, wheat, cotton, rapeseed, sesame, pepper, tomato and alfalfa were obtained and released as new varieties from seeds subjected to space travel (Liu *et al.*, 2009) . This has led to the establishment of new techniques and methods of mutation induction by simulating the space environment in laboratories.

1.1.10. **Laser beam irradiation**

In recent years several research groups have investigated the efficiency of laser beams in radiation induced mutation. Interesting findings have been presented mainly on the changes in cells, organelles and genomes caused by laser beam. Rybianski (2000) demonstrated the effectiveness of a helium-neon laser (He-Ne) with a wavelength of 632.8 nm and a power density of $1 mWcm^{-2}$ in inducing phenotypical mutations in barley; some of the mutants showed good agronomic traits such as high yield.

1.2.RADIO-BIOLOGY

1.2.1. **Absorption of ionizing radiation**

As described earlier, there are many types of radiations, but the two most common are electro-magnetic radiations and ionizing radiations. Electromagnetic radiations are described as waves of photons. Ionizing radiations refer to radioactive particles, such as alpha and beta particles, but include also some electro-magnetic waves, such as X- or gamma-rays, which have sufficient energy to detach electrons from atoms and create ions, hence the name "ionizing radiation." Absorbed energy from ionizing radiation induces changes in plants at the molecular level, i.e. the macro-molecules such as DNA or enzymes or even some smaller molecules such as ATP and co–enzymes (Harrison, 2013). The effect of radiation involves two mechanisms: i) the direct (physical) action, which reflects on the molecule damage and ii) the indirect (chemical) action from the highly reactive free radicals derived from ionized water molecules (Lagoda *et al.*, 2012). The process of irradiation involves physical, physico-chemical, chemical and biochemical, and biological effects and the ionizing

radiations can thus, affect plant growth and development, the severity or magnitude of the observable modifications is strongly dependent upon several factors including species, genotype, plant age, physiology and morphology as well as the plant genome size and organization (Lagoda, 2009).

The ionization events caused by X-rays, gamma-rays and beta particles occur sparsely along the track of the ionizing units. When the ionizing particle consists of an atomic nucleus, alpha-rays or recoil nuclei knock-out by fast neutrons, the ionization events are relatively dense. Each radiation is characterized by its ion density, which is expressed in terms of Linear Energy Transfer (LET); the energy dissipated per unit length along the tracks of the ionizing particles. Gamma- and X-rays are examples of low LET radiations, alpha–rays and fast neutrons are high LET radiations. De Micco *et al.*, (2011) provided a comprehensive review on the different effects of irradiation in plants focusing on genetic alterations, modifications of growth and reproduction and changes in biochemical pathways especially photosynthetic behaviour.

1.2.2. **Chemical effects of ionizing radiation: DNA damage and DNA repair**

Mutation induced by physical mutagens is brought about by the chemical effects in the living cell. In the ionization process positive radical ions and free electrons are produced. When penetrating the biological systems, the electron is trapped in the polar surroundings and then the radical ion, which is both unstable and reactive, may either react with other molecules or pass through internal rearrangements. In water solutions the free electron can polarize several water molecules and become a "hydrated electron" (e^-_{aq}). The free radicals generated in solution will sooner or later recombine with each other to form stable products. There are several definitions for the terms: free radical. Halliwell and Gutteridge, (2015) in a review on free radicals proposed a simple definition – a free radical is any reactive species capable of independent existence that contains one or more impaired electrons – and a superscript dot is used to denote a free radical, e.g. $H^\bullet$ and $O_2^\bullet$. A free radical can be formed by losing a single electron from a molecule, leaving behind an impaired electron and a positive charge:

- $X - e^- \longrightarrow X^{\bullet +}$ (radical cation)

or by gaining a single electron

- $Y + e^- \longrightarrow Y^{\bullet -}$ (radical anion)

Radicals can also be formed if a covalent bond is broken to leave one electron from the bonding pair on each atom; a process called "homolytic fission" (Halliwell and Gutteridge, 2015). If molecular oxygen (a bi-radical) is present, it reacts readily with radiation induced free radicals to form peroxy-radicals. In the solid state, where

molecular movements are restricted, the radiation-induced radicals are stable. This is the case in plant seeds of low water content. The higher the water content of the irradiated target (e.g. seed) the greater the related damage.

1.2.3. **Lethal effects of ionizing radiation: DNA damage and repair**

The lethal action of ionizing radiations on cells is often measured as the loss of cell division, i.e. mitotic activity. Indirect evidence that this is the result of chromosomal aberrations has been obtained in several systems. Muller and then Staedler in the 1920s were the first to demonstrate that applying mutagenic agent, such as X-rays, on living cells resulted in phenotypic mutations. These observations were rapidly connected with the DNA described in the 50s, as resulting from DNA damages. Chromosomal aberrations were soon detected in the dividing cells at anaphase or as micro-nuclei, but non-dividing cells also died after irradiation, this is termed "interphase death", although this generally requires larger doses of radiation than for reproductive cells (especially those undergoing meiosis). DNA damages can be broadly classified into three types of lesions: mismatched bases, double-strand breaks, and chemically modified bases.

When the effects of gamma-rays and, for example, fast neutrons are compared, it is found that gamma-ray treatments have a greater biological effect, also called Relative Biological Effectiveness (RBE). Several investigations have shown that the RBE is a function of the linear energy transfer (LET). Later, it became largely recognized that all organisms, which very often recover after irradiation and/or show DNA damages, also possess a range of conserved biochemical activities responsible in restoring the DNA to its undamaged state, this is called: "DNA repair" (Croteau and Bohr, 2013). DNA repair studies often focus on chemical lesions, but the repair pathways for the other two classes of damage are particularly interesting to the plant geneticist and have been investigated thoroughly. In both bacteria and animal cells, this repair is known as efficient and fast, similar findings have been reported in higher plants (Gill *et al.*, 2015). It is, however, interesting to note that a fraction of the breaks remains unrepaired. This fraction of unrepaired breaks is significantly higher after fast neutron irradiation than after gamma-rays (Lagoda *et al.*, 2012); Croteau and Bohr, 2013). One reason for this could be the high ion density after fast neutron irradiation, which leads to a locally more extensive or "clustered" damage, and is therefore more difficult to repair.

1.3.DOSIMETRY

Radiation dosimetry deals with methods for the quantitative determination of absorbed dose (of the target material) and its physical interpretation. Absorbed dose is the energy which is deposited in a unit mass of matter, because of interaction of

radiation with this matter. From the symposium co-organized by the IAEA and the World Health Organization (WHO) in the early 70s, some classical measurements systems were thoroughly evaluated and presented and regularly revisited and the results published by the IAEA in a series of Technical Reports (IAEA,1973). Since then, a substantial development has been observed due, both to the various radiations sources developed, the multitude of uses and applications of these radiations, and expected outcomes.

1.3.1. Exposure and dose determination

In many practical cases absorbed dose is not measured directly but calculated from the measured number of ions produced in air by the ionizing radiation. Measurements of this kind are done with ionization chambers and the quantity measured in this way is called 'exposure'. Exposure is symbolized by "*X"* and has been defined by the ICRU (1998), as "the quotient of the electric charge ΔQ (of either sign) produced in dry air in standard conditions by X- or gamma-radiation to the mass Δm of the air" (when all the secondary electrons liberated by the photons are completely stopped in the corresponding air volume).

$$X = \frac{\Delta Q}{\Delta m_{air}}$$

The SI unit is: C kg^{-1}

Another important measure is the exposure rate, which expresses the variation of exposure over time:

The exposure rate $\dot{X}$ is defined as:

$$\dot{X} = \frac{\Delta X}{D\Delta t}$$

Usual unit of exposure rate is: C $Kg^{-1}s^{-1}$

The exposure rate in air, $\dot{X}$, is inversely proportional to the squared distance (r^2) from the point source of activity, Δ(t):

$$\dot{X} = \frac{\Gamma\Delta(t)}{r^2}$$

Γ is the specific exposure gamma-ray constant at 1 m distance from the source and is defined for each radioactive nuclide (ICRU, 1998; Adlienė and Adlytė, 2017).

1.3.2. **Absorbed dose in irradiated targets**

In quantitative radiation biology it is desirable to relate the observed biological effect to a well-defined and easily measurable physical quantity characterising the amount of radiation responsible for the effect. It was generally accepted that the "radiation absorbed dose or rad" best meets this demand. Therefore, it is common and convenient to describe radiation effects in biological targets in terms of dose-effect relationships. The absorbed dose or, briefly, dose: D for any ionizing radiation is defined as the amount of energy absorbed per mass of irradiated matter at the point of interest. In 1925, the First International Congress for Radiology in London saw the Foundation of the "International Commission on Radiation Units and Measurement" this organisation regularly publishes reports and provides updates on dosimetry for various types of radiations (ICRU, 1998). As reported by Podgorsak (2005) the absorbed dose corresponds to the quotient of ΔE_{ab} by Δm, where ΔE_{ab} is the mean absorbed energy imparted by the ionising radiation to the mass of the medium used.

$$D = \frac{\Delta E_{ab}}{\Delta m}$$

However, the rad is not any more in use, the current SI unit of absorbed dose is the gray (Gy), which corresponds to 1 Joule per kg ($1Gy = 1Jkg^{-1}$), and 1gray = 100 rads.

It should be noted that, although the concept of absorbed dose is independent of any specified material, irradiation of two different materials under exactly equal conditions will generally lead to different absorbed doses in the two materials; this is because of the different absorption coefficients of the two materials.

Podgorsak (2005) defined a dosimeter as: "any device that is capable of providing a reading that is a measure of the average absorbed dose deposited in a sensitive volume by an ionizing radiation". A dosimeter can generally be considered as consisting of a sensitive volume filled with a given medium lying in a container of another medium to ensure accuracy. The dosimeter and the associated reader are generally referred to as: dosimeter system.

Following thorough reviews of the various existing dosimetry systems applied in radio-biology and mutation breeding; some requirements of dosimetry systems as well as practical sequence of steps required for efficient plant mutation induction and have been established and have been largely followed by breeders over the years as refer to by Legrand, Hartmann and Karger (2012). These studies also recognized the work by (Draganić and Gupta, 1973) who specified that to serve as a measure of absorbed dose, any dosimetry systems must satisfy a number of criteria.

a. The amount of chemical changes should be linearly proportional to the dose absorbed, or at least, the calculation of the corresponding dose must be simple.

b. Any side events during the irradiation, i.e., accumulation of radiolysis products, should not interfere with the calculation of the absorbed dose which must remain simple.

c. The amount of chemical changes must be independent over a wide range of LET of radiation, of dose rates and of temperatures, when it is not the case there must be a sure way to integrate those factors.

d. Chemical dosimeters require chemically pure substances, not needing any additional purification prior to be used in the dosimeter and the sample should allow a normal handling under laboratory conditions.

e. A method that is accurate, but also simple should be used for determining the chemical change which serves as measure of the absorbed dose of energy.

1.3.3. **Dosimeters**

A major objective in plant mutation breeding programme is to obtain the highest mutagenic efficiency, i.e. a maximum number of desired mutations, while preserving the viability of the plants and a low background mutation rate. Thus, the plant breeder must assess the most appropriate mutagen dose, because the damages involved may create an additional cost in terms of manpower, time and field area, and the rounds of crossing/backcrossing needed to clean up unwanted background mutations. These considerations lead to careful consideration of dosimetry, meaning practical measurements of the mutagen quantities applied as well as that received by the target plant material. Brunner, 1995 proposed a set of steps that should be considered both at the level of the mutagen and at the level of the plant itself (Table 1.3).

Different dosimetry systems are used for different purposes in industrial and research irradiation facilities. They may have different requirements for dose determinations. Radiation safety standards and issues involving the radiation protection of humans against radiation exposure have their own dosimetry metrology (Adlienė and Adlytė, 2017). Some dosimeters commonly used in mutation induction are briefly described below.

TABLE 1.3. STEPS INVOLVED IN DOSE ASSESSMENT FOR CROPS SPECIES IN A MUTATION BREEDING EXPERIMENT (Brunner, 1995)

A. Radiation source characteristics	
High or low LET radiation	
Energy distribution	
Degree of contamination with other radiations	
Dose gradients, requirements of dose homogeneity	
Methods to monitor radiation dose and/or dose rate:	
(i) Physical ⟶	Ionization chambers, threshold detectors, etc.
(ii) Chemical ⟶	Determination of ionic (chemical) yield, e.g. Fricke
(iii) Biological ⟶	Determination of an index of primary damage, e.g. seedling height, epicotyl length in comparison with non-irradiated control materials
B. Characteristics of the biological target	
Seeds	Whole plants
Pollen grains	Vegetative organs
Gametophytes and zygotes	Cells and/or tissue in culture
Criteria of radio-sensitivity	
Biological factors, environmental factors, etc.	
C. Prediction of dose effects	
Early assessable criteria of primary damage in e.g. seedling height of the first leaf, epicotyl length, etc. and their correlation to mutation frequency in M_2, e.g. Usually to chlorophyll indicator mutations.	

1.3.3.1.The Fricke dosimeter

The Fricke dosimeter is an aqueous solution containing ferrous ions (Fe^{2+}), which are oxidized to ferric ions (Fe^{3+}) under ionizing radiation proportionally to the absorbed dose (Fricke and Hart, 1966). The Fricke or ferrous sulphate dosimeter is a very useful chemical system for gamma irradiation dose measurements. With some modifications it can also be used for neutrons and mixed gamma/neutron fields, but with a reduced accuracy. Combined use of ionization chambers and the Fricke dosimeter is recommended whenever it is desirable to have two independent dose measurements (Boudou *et al.*, 2004).

The standard Fricke solution for measurement of gamma doses consists of

- Ferrous ammonium sulphate: Fe $(NH4)_2$, 6 (H_20) at 10^{-3} mol/l
- Sodium chloride: NaCl at 10^{-3} mol/l
- Sulphuric acid: H_2S0_4 at 0.4 mol/l concentration in aqueous solution.

The standard Fricke dosimeter can be used in the dose range 40 – 400 Gy for gamma radiation and for doses rates up to 10^6 Gy/s (deAlmeida *et al.*, 2014). Since it provides an accurate and direct dose determination, it can be used for calibration of other systems, e.g. ionization chambers. However, it such cases, one has to take into account the factor G, which measures the radiation chemical yield and may vary with the types of radiation (Klassen *et al.*, 1999).

1.3.3.2.The FeCu dosimeter – (ferrous sulphate–copper sulphate dosimeter)

As stated above, the Fricke dosimetry is applicable in the dose range between 40 Gy and 0.4 kGy. For higher doses, the system goes into saturation. With a modification of the solution it is possible to shift this measuring range to higher doses; raising the Fe^{2+} concentration delays the saturation effect up to 1.5kGy. The addition of Cu^{2+} enables dose measurements up to 25kGy. This is used mainly when the gamma and fast neutron doses from a mixed field are to be determined separately (Haninger and Henniger, 2016).

Composition of the solution:

- Ferrous sulphate: $FeSO_4$ 10^{-3} mol/l
- Copper sulphate: $CuSO_4$ 10^{-2} mol/l
- Sulphuric acid: H_2S0_4 $5x10^{-3}$ mol/l

The following G values have been reported for cobalt 60 and neutrons (deAlmeida *et al.*, 2014) :

G (FeCu) = 0.66 for ^{60}Co radiation

G (FeCu) = 2 for fission neutrons.

1.3.3.3.The Fricke-gel dosimeters

Even though the above chemical dosimeters have been used routinely, modern users have found several limitations, for example in terms of stability of the air after irradiation, moreover because these were mostly one- and two- dimensional

measuring devices. Thus, there has been much interest in the development of a three-dimensional (3D) radiation measuring device. The most promising development in 3D dosimetry has been the addition of polymers and gels, which is then measured by magnetic resonance imaging. The first developments in this field were carried out with gels infused with Fricke–gel solution (Schreiner, 2004).

1.3.3.4.Neutron dosimetry

Neutrons, as well as photons ionize indirectly, so the methods of dosimetry used are, in general, similar. However, there will be a wide range of variation depending on the target; live tissue or physical material. The challenge in using ionization chamber is to have a phantom material as close as possible to the target tissue. The usual phantom material is water and the calculation is made using the ICRU (1993) recommendations.

1.3.3.5.Personal dosimetry

The applications of X-rays, gamma-rays, neutrons and electron beams have proven to be of great benefit in medicine, biology, industries, etc. However, these applications also mean substantial acute or recurrent exposure for the operator/personnel involved. The latest IAEA recommendations for radiation protection are provided in the Basic Safety Standards series (IAEA, 2010, 2014 and 2016). They are based on the knowledge of radiation effects and on established principles of radiation protection recommended by the International Commission on Radiological Protection (Adlienė and Adlytė, 2017).

Different methods are used to assess the occupational dose of an individual staff depending on the different exposure situations in the radiation fields (external exposure) and/or on the different radionuclides (intake), which might contribute to the dose. The ICRU (1993) defined several units for assessing the exposure of workers to radiation, among which; the dose equivalent (H) measuring the dose absorbed at a point by an organ or tissue multiplied by the relevant weighing or quality factor (Q) depending on the type of radiation, which is pre-established for each one of them: alpha particles, electrons, photons or neutrons. The dose equivalent unit is the Sievert (Sv), which is equal to 1 joule of energy deposited in a kilogram of human tissue (Adlienė and Adlytė, 2017).

Moiseenko *et al.*, (2016) in their study on the effects of radiation on workers and population 30 years after the Chernobyl disaster (1986), highlighted the main requirements for biological dosimetry methods for a reliable and useful assessment:

- Low detection threshold.
- Low person– to– person variation in dose–response for healthy individuals.
- Ability to obtain calibration curves in laboratory conditions, e.g. *in vitro*.
- Stability of the biological effect so that dose can be reconstructed at long time periods, years or decades, after being exposed.

The authors mostly referred to the evaluation of the different types of biological dosimetry methods established by the IAEA (2011).

1.4.OBJECTIVES AND TREATMENTS

All plant parts can be irradiated by one method or another, but some are easier to treat than others. Besides the commonly treated seeds and pollen, whole plants, cuttings, tubers, corms, bulbs, stolons and organs tissues, or cells cultured *in vitro* may also be irradiated.

There are wide differences in the radio-sensitivity of the various plant parts; the reaction of a given type of cell to radiation depends on its physiological conditions at the time of irradiation as well as on the pre- and post-irradiation conditions. The decision to be made by the investigator concerning the most appropriate plant part or stage to expose requires a thorough knowledge of the organism and clear objectives for the experiment.

1.4.1. **Target plant materials**

1.4.1.1.Whole plants

Large plants have often been irradiated in a gamma field, a gamma greenhouse or a gamma room. Seedlings or small plants, on the other hand, can easily be irradiated by most X-ray machines or by gamma sources in a greenhouse or shielded rooms. Today, the use of (open) gamma fields is extremely reduced considering the restrictions associated with environmental and human health concerns; other more contained methods of treatments are available and have proven to be more economic for plant breeding, at least for seed propagated plant species.

1.4.1.2.Seeds

Seeds are the favoured material for irradiation in many mutation induction experiments and in practical mutation breeding. Seeds can be irradiated in many physical environments and they can be desiccated, soaked, heated or frozen prior to the treatments. They can be stored for extended periods of time in air-tight, vacuum

and refrigerated conditions. When dry, the seeds are almost inert biologically (quiescent), they are also easiest to handle and can be shipped over large distances. However, larger radiation doses are required to produce sufficient genetic mutations than when other plant materials are irradiated. On the other hand, soaking of seeds before irradiation can reduce the dose level required, but might also introduce some complicating factors as it will promote germination.

1.4.1.3.Pollen

A great advantage of pollen irradiation as opposed to the irradiation of seeds or growing plants is the fact that the former rarely produces chimeras, i.e. the M_1 plants resulting from fertilization by irradiated pollen of a non-irradiated egg cells will be fully homozygous for any induced mutation (see Chapter 8.2). The disadvantages of pollen irradiation include the difficulty of obtaining sufficient material from some species and the short viability of pollen grain in many plant species. By using proper techniques, however, pollen of some species can be kept alive for several months (or years for species that have bi-nucleate pollen, e.g. oil palm) and can be used for germplasm conservation. Large quantities of pollen are obtainable from most naturally cross-pollinated plants, for example a maize plant can produce 14 to 50 million of pollen grains. Pollen grains are generally considered to be the most suitable plant part for UV irradiation treatment.

1.4.1.4.Meristems

Seed irradiation is essentially a treatment of the embryo meristems. The anatomy and pattern of embryo meristems is important for mutagenic treatments of seeds (as well as other plant material) since it determines whether a mutated cell will be lost during differentiation or produce sufficient cell progenies to be found throughout much of the plant including germ cells.

For most vegetatively propagated crops (VPCs), seeds are not available and therefore other plant parts are needed as targets for mutation induction. The structure of meristematic regions and the development of new meristems from differentiated tissue are particularly important when investigating radiation induced mutation of VPCs. In most cases, the new shoots originate from a single epidermal cell from a tissue and this could directly lead to homohistant mutant plants whose genetics may be investigated further. For more information (see Chapter 4. 6 and 8).

1.4.1.5.Plant cells and in vitro tissue culture

The use of plant cells and tissues in culture offers exciting applications in crop mutation breeding. The *in vitro* methods for plants micro-propagation launched in the

early 60s, rapidly became a powerful tool for scientists working on plant mutation induction, especially in VPCs. Plant organ, tissue and cell culture provided ways for:

- rapid and mass propagation of target M_0 plants;
- rapid and mass propagation of any mutant populations;
- thorough and easy methods for analysing mutations morphogenetic and physiologic changes associated with mutation induction;
- easy and rapid ways of dissociating chimeras;
- *in vitro* mutagenesis; and
- *in vitro* screening.

1.4.2. **Irradiation treatments and conditions**

There are several experimental variables to be considered for irradiation treatments.

1.4.2.1.Irradiation dose and dose rate

The dose percentage at which a given dose of radiation is administered – dose rate – frequently has a significant effect, both qualitatively and quantitatively, on the results obtained. For this reason, the dose rate should be carefully chosen and recorded in all experiments. For most species/targets recommended dose treatments are available from the literature (Table 1.4) and may be applied in every specific condition on the same crop with some precautionary measures, such as using the given value X and two additional ones: X ± 20%.

1.4.2.2.Acute versus chronic irradiation

The frequency of mutations induced by ionizing radiation in a genome appears to be directly proportional to the dose. Different results might be expected depending on whether the induced mutations are caused as a-one-hit event, such as simple chromosome deficiency or deletion, or if they appear as point mutations resulting from several one-hit events distributed throughout the genome. There is, therefore, a need to weighing the need for high dose during a short period versus low dose during a longer time. Kovalchuk *et al.*, (2000) reported observing a strong and significant correlation between the frequency of homologous recombination (HR) in plants, the radio-activity of the soil samples and the doses of radiation absorbed by plants.

Exposures that are continued over long periods of time (usually weeks, months or years) are referred to as “chronic”, exposures delivered in minutes or a few hours are referred to as “acute”. Almost any source of radiation can be used for acute exposures.

A comparison of acute versus chronic irradiation usually implies a comparison of high versus low dose rates and/or high versus low radioactivity for gamma emitters.

Comparative studies on the effects of acute and chronic doses of X- and gamma-rays have been made in terms of growth, survival, fertility, and yield and mutation induction after exposure of both seeds and plants. Acute irradiation of seeds might be more effective in inhibiting growth and decreasing survival and fertility because of the recovery phenomena at low irradiation intensities with chronic irradiation.

1.4.2.3.Recurrent irradiation treatments

The procedure of recurrent irradiation – irradiating plant material that had already been irradiated in one or more subsequent generations – has been proposed as a method of accumulating and expanding genetic variability to be utilized in plant breeding. Appropriate strategies in mutation induction as application of fractionated doses and recurrent irradiation combined with *in vitro* culture techniques have been chosen in assessing their cost-effective applicability in ornamental plants.

Since various physical and chemical agents are known to cause different types of mutations; recurrent treatments using various chemical mutagens such as ethyl methanesulphonate (EMS) or hydroxylamine (HA) separately in a combination or alternatively associated with physical mutagen such as X-rays and also the combination of UV light and X-rays have also been investigated in several plants (Chopra, 2005).

TABLE 1.4. RADIO-SENSITIVITY OF SOME CROP SPECIES TO GAMMA AND FAST NEUTRON RADIATION (Brunner, 1985)

Genus or Family (common name)	Species	Range of GR 50* (dose in Gy) ɣ	Typical doses (dose in Gy) ɣ
POACEAE			
Oat	*Avena sativa*	300-450	100-250
Barley	*Hordeum vulgare*	300-450	100-250
Rice	*Oryza sativa*		
	japonica	250-400	100-280
	indica	350-500	150-350
Rice	*Oryza glaberrima*	300-400	150-300
Bread wheat	*Triticum aestivum*	450-600	150-350
Durum wheat	*Triticum durum*	350-500	150-300
FABACEAE			
Peanut	*Arachis hypogaea*	300-450	100-350
Pigeon pea	*Cajanus cajan*	150-240	80-150
Chickpea	*Cicer arietinum*	180-300	100-200
POLYGONACEAE			
Buckwheat	*Fagopyrum esculentum*	300-500	150-300
BRASSICACEAE			
White mustard	*Sinapis alba*	900-1500	500-1000
Wild turnip	*Brassica campestris ollifera*	800-1600	500-1000
Indian mustard	*Brassica juncea*	1600-2000	1000-1500
SOLANACEAE			
Red pepper (chilli)	*Capsicum annuum*	250-500	100-350
Tomato	*Lycopersicon esculentum*	450-600	200-400
LILIACEAE			
Onion	*Allium cepa*	160-280	80-180
Leek	*Allium scorodoprasum*	200-250	80-140
Asparagus	*Asparagus officinalis*	300-400	150-250
UMBELLIFERAE			
Carrot	*Daucus carota*	550-700	250-400
CHENOPODIACEAE			
Spinach	*Spinacea oleracea*	300-500	150-300
Quinoa	*Chenopodium quinoa*	300-500	150-300
MALVACEAE			
Cotton	*Gossypium arboretrum*	140-250	80-150
Cotton	*Gossypium hirsutum*	300-500	150-300
Okra	*Hibiscus esculentus*	600-850	300-500
ASTERACEAE			
Sunflower	*Helianthus annuus*	250-500	100-300
Safflower	*Carthamus tinctorius*	600-700	200-450
Niger	*Guizota abyssinica*	200-260	80-160
TILIACEAE			
Jute	*Corchorus Olitorius 2n*	700-850	300-550
	Corchorus Olitorius 4n	550-700	250-450

Genus or Family (common name)	Species	Range of GR 50* (dose in Gy) ɣ	Typical doses (dose in Gy) ɣ
CUCURBITACEAE			
Squash	*Cucurbita maxima*	500-700	250-450
Cucumber	*Cucumis sativus*	450-600	200-400
Melon	*Cucumis melo*	350-500	200-350
PEDALIACEAE			
Sesame	*Sesamum indicum*	700-900	400-700
LINACEAE			
Flax	*Linum usitatissimum*	600-1000	300-600

*GR 50 = 50% seedling height (or epicotyl height) reduction after irradiation of quiescent seeds equilibrated to 12-14% moisture with ^{60}Co ɣ radiation (dose rate varied between 60 and 7 Gy min^{-1}) or with fast neutrons derived from the SNIF (Standard Neutron Irradiation Facility). Precision of the applied doses: ± 5% and irradiation of dry, quiescent seeds with 12 14% moisture.

1.5.RADIATION SENSITIVITY AND MODIFYING FACTORS

The response of cells of higher plants to physical and chemical mutagens is influenced to a varying degree by numerous biological, environmental and chemical factors. These factors modify the effectiveness and efficiency of mutagens in the cells of higher plants. The mechanisms involved are poorly understood, but it is very important to monitor these factors closely, as they may interfere with the process of radiation.

The two most important modifying factors for seed irradiation are oxygen and water content, whereas for active tissues, factors such as stage of development including the relation to DNA synthesis and dose rate are more important. Factors such as nuclear and interphase chromosome volumes are also important for, both, resting and active tissues. Specific problems related to the mutation breeding of VPCs are discussed in Chapter 6.

A protocol for radio-sensitivity testing is given in Section 1.7. below. Here we discuss the factors that modify the response of seeds to ionizing radiations, they may be grouped into two major categories: (1) environmental factors such as atmosphere (oxic versus anoxic), seed water content, post-irradiation storage, and temperature; and (2) biological factors such as genetic differences, nuclear and interphase chromosome volumes, etc.

1.5.1. **Environmental factors**

1.5.1.1.Oxygen

Oxygen is one of the best-known modifiers of radiation sensitivity and the biological effects of irradiation are usually greater in the presence of oxygen. Other factors, such as water content, temperature and post-irradiation storage conditions appear to be

secondary. Environmental factors are less important with densely ionizing radiations such as fast neutrons. Using yeast (*Saccharomyces cerivisae*), Nairy *et al.*, (2014) conducted a comprehensive study of Oxygen Enhancement Ratio (OER) and their variation as a function of the radiation doses.

Oxygen enhancement effect of gamma radiation induced damage is greatest in very dry seeds (≤ 3 percent water content). The degree of enhancement, however, is not the same for all species. In general, higher mutagenic efficiency (less damage in terms of seedling injury and chromosome aberrations in relation to mutation frequency) can be obtained if oxygen effects are minimized. Data from various experiments show that this can be accomplished by irradiating seeds in an anoxic atmosphere – in nitrogen filled medium or under partial vacuum – or by adjusting the seed water content to 12 – 14 percent.

However, the control of oxygen is often impractical for general mutation induction in plant breeding and is often ignored. Additionally, the oxygen applied under pressure can, by itself, act as a mutagen.

1.5.1.2.Water content

In all cases of mutation induction, including seed irradiation, water content is an important and easily regulated factor (van Harten, 1998). Various methods have been used for determining the water content of seeds: weight difference between fresh and dehydrated seeds using oven or desiccator systems; use of saturated solutions of various salt; and the use of the glycerol-water solutions (Forney and Brandl, 1992).

Even minor differences in water content can have a very pronounced influence on the end biological effect. Seeds stored under normal laboratory conditions are often in the range of 10.0 and 11.5 percent water and a difference of only 0.2 to 0.3 percent might greatly alter radio-sensitivity of some species. This should be weighed in, when choosing an effective mutagenic dose; a dose too low might not induce any mutations, and a dose too high might result in excess sterility or no surviving plants. Seeds of different species equilibrate water content at different rates and may differ not only in water content but also in radio-sensitivity when equilibrated at the same relative humidity (IAEA, 1977). Thus, pre-irradiation desiccation is generally considered a routine treatment for seeds.

1.5.1.3.Temperature

The temperature of plant cells before, during and after irradiation may affect the total amount of genetic damage induced by X- or gamma-rays. However, the effect of temperature as a modifying factor of radiation damage is not clearly understood and appears unimportant to the plant breeder. A combination of heat shock and oxygen-

free hydration was found to be most protective against the post-irradiation oxygen-dependent damage (scored as seedling injury and chromosomal aberrations).

1.5.2. **Biological factors**

1.5.2.1.Nuclear and interphase chromosome volume

Since the cell nucleus is generally considered to be the primary site of radiation damage, it seems logical to look for the factors influencing radio-sensitivity in the nuclei of different species. The previous edition of the IAEA Manual on Mutation Breeding (1977), thoroughly explored the correlation between the nuclear volume of a species and its radio-sensitivity. Several studies have shown that neither DNA content, chromosome number, nor chromosome arm number could be responsible for the differences in radio-sensitivity (Leonard, Jacquet and Lauwerys, 1983; Bakri *et al.*, 2005) , but there exists a relationship between average interphase nuclear volume (INV) and cell sensitivity to radiation. It was concluded, that the higher the chromosome number, the higher the resistance to radiation, resulting from the fact that other chromosomes or parts of chromosomes might compensate for the mutations, this is particularly true for polyploid species (Datta, 2014). Chromosomal organisation, number and position of centromeres and chromosome size are also associated with radio-sensitivity (large chromosomes are generally more radio-sensitive than small chromosomes).

1.5.2.2.Cell cycle

The sensitivity of the cell or more precisely of the nucleus is dependant of the length of the cell cycle, and the stage of the cell division: mitosis or meiosis. A thorough and very informative review of the effects of radiation on cell division and plant growth could be found in Lagoda *et al.*, (2012).

It appears that tissues which are growing rapidly, and have a high mitotic index are much more sensitive to irradiation than sessile or dormant targets, such as seeds. In addition the more complex mechanisms of meiosis make this process more sensitive to irradiation (van Harten, 1998; Datta, 2014).

1.5.2.3.Genetics

Differences in mutagen sensitivity among genotypes are common. Amongst the more recent studies, the investigation of radio-sensitivity of the wild grass *Roegneria* spp. seeds, confirmed genotypic effects on: seed germination, seedlings growth, plant height, and plant survival (Luo *et al.*, 2013). However, differences in radio-sensitivity among genotypes within a species are usually much less than between species. Therefore, for plant breeders wishing to induce mutations, the genotype factors can

be ignored, and a routine radio-sensitivity test will determine an appropriate dose rate for mutation induction.

1.6.PRE- AND POST-IRRADIATION TREATMENTS

1.6.1. **Pre-treatment**

As stated in the previous section, it is important that the plant breeder ascertains practical conditions for materials in preparation for X- and gamma- irradiation of seeds to obtain optimal mutagenic efficiency and repeatability.

1.6.2. **Adjustment of seed water content**

Water content is a crucial factor to take into consideration when initiating radiation induced mutagenesis. Thus, before the irradiation treatment, seeds should be brought to standard water content for that specific species as described in relating literature, using various physical and/or chemical technologies. An easy, less damaging, stable and reliable method is the use of glycerol/water solutions (Figure 1.5).

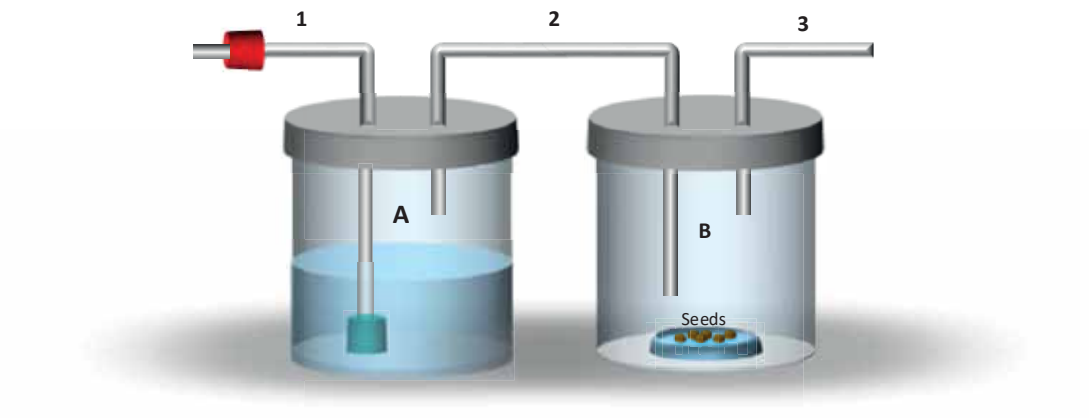


Figure 1.5. Humidity (water content) adjustment in seeds. The system for adjusting seed moisture (water content) uses flow-through chambers. The gas (or air) to be humidified) flows through tube 1 into the glycerol-water solution in jar A. The humidified gas then flows through tube 2 into the controlled-environment chamber (jar B) and exits into the atmosphere through tube 3. (Modified from Forney and Brandl, 1992).

1.6.3. **Post-irradiation storage**

Undesirable post-irradiation storage effects may be avoided by proper adjustment of seed water content, for example to 12 – 14 percent for small grain cereals such as wheat, rice, barley, etc., before irradiation and by storing the seeds for no more than 2 to 4 weeks at temperatures not exceeding 18 – 23°C (Table 1.5). However, it is

usually possible to store seeds that have been desiccated at less than 14 percent water content in a freezer (-20 or -80°C) for several months. A post-irradiation storage period in a vacuum for about one to two weeks at room temperature will extend storage life. Seeds of more than 14 percent water content may not be shipped successfully, but may be shipped frozen.

TABLE 1.5. WATER CONTENT (%) OF BARLEY SEEDS STORED OVER DIFFERENT CONCENTRATIONS OF GLYCEROL AT 22°C

Glycerol in solution (vol. %)	Calculated vapour pressure of solution (mm Hg)	Relative humidity (%)	Seed water content (%)	Storage period (days)
100	0.0	0.0	8	7
95	3.5	17.6	9	7
85	8.3	41.7	10	4
75	11.4	57.5	11	4
70	12.6	63.5	12	4
65	13.6	68.6	13	4
60	14.5	73.0	14	4

1.7.PROTOCOL FOR RADIO-SENSITIVITY TESTING

This protocol is intended for scientists who want to determine effective doses of gamma irradiation to generate induced mutants in seed propagated crops. The information given is based on protocols established at the Plant Breeding and Genetics Laboratory of the Joint FAO/IAEA Agriculture and Biotechnologies Laboratories, Seibersdorf, Austria.

1.7.1. **List of equipment, supplies and facilities needed**

- Seeds
- Water permeable bags
- Paper clips or stapler
- Desiccator
- 60 percent glycerol: distilled water mixture (v/v)
- Gamma cell machine available on-site or easily contacted

- Ruler (starting with zero) or measuring board (millimeter graph paper on a piece of plywood, best sealed inside transparent plastic film)
- Greenhouse and/or laboratory facility
- Trays and heat /steam sterilized potting mix or germination containers with filter paper, bleach, sterile water and Tween 20
- Pots and potting mix, if cultivation to maturity of the plants is planned

1.7.1.1.Seeds

The number of seeds will depend mainly on the level of information available on the radio-sensitivity of the crop genotype of interest.

Seeds should be:

- genetically uniform;
- representative of the genotype;
- dry;
- quiescent[1];
- with a high germination rate;
- and have a water permeable seed coat[2].

[1]If seeds are dormant, any procedures required for breaking dormancy should precede the radiation treatment.

[2]If the seed coat is not water permeable it must be removed or chemically/mechanically modified (scarified). For this purpose, seed coats can be rubbed with sandpaper, nicked with a knife or filed with a metal file.

1.7.1.2.Water permeable bag

Bags can be made of mesh, and can be made from simple materials available at local markets. The number and size of the bags will depend on the amount of seeds. The bags must have a size that fits into the irradiation compartment of the gamma cell (see Figure 1.3).

1.7.1.3.Desiccator (vacuum tight) with 60 percent glycerol: water mixture (v/v)

The desiccator should be large enough to hold 1000 ml of liquid in the base. To prepare a 1000 ml of the mixture use 600 ml glycerol and 400 ml distilled water. Inside the desiccator the relative humidity should be about 73 percent which can be

monitored with a hygrometer. In a desiccator with 1000 ml glycerol/water mixture, up to 500 g of small seeds can be treated.

1.7.1.4.Trays

Trays with a size of 400 × 600 × 120mm can accommodate 4 – 7 rows of seeds of cereal and bean species (Figure 1.6). Alternatively, compartmentalized seed trays (commercially available) can be used. Trays need to have holes in their base for drainage.

1.7.1.5.Germination containers

Choose a germination container (Petri dish, clear box, etc.) which is large enough to contain all the seeds without over-crowding.

1.7.2. **Radio-sensitivity testing procedure**

Keep detailed records of material, treatment dates, radiation source, dose, dose rate, the treatment conditions, growing season, seed storage and growing conditions, in order to be able to eventually repeat successfully the experiment.

1.7.2.1.Project planning

1. Check for recommended doses in the literature or refer to Shu *et al.*, (2012)

2. If reliable data are available then move on to point 4 of this protocol. If no information is known for your target plant, run a preliminary test.

3. For a preliminary test use 20 seeds per dose, one replication only. Use several doses at a wide range at even intervals e.g. 0, 150, 300, 450, 600 Gy; or 0, 200, 400, 600, 800 Gy. Most plants will fall into the range between 100 Gy to 700 Gy.

4. For the radio-sensitivity test use 20 – 25 seeds, with 3 replications (a total of 60 – 75 seeds per dose). Increase the number of seeds per treatment if the germination rate in the control is low to compensate for the probable loss. Use appropriate doses cited in literature on the crop of interest as a point of reference and extend treatments 25 to 50 percent higher and lower at a narrow dose range (intervals of e.g. 50 or 100 Gy). For example: if the literature consulted states for 50 percent seedling height reduction at a range of 250 – 400 Gy, you may use 100, 200, 300, 400, 500, 600 Gy plus a control (0 Gy).

5. Always include a same-sized control (non-radiated) population to gauge treatment effects and to assess phenotypic variability of the parent stock. The control is treated like the material to be mutagenized except for the radiation exposure.

6. Identify a gamma facility that offers radiation service (see Section 1.9) and inquire about their specific requirements and procedures. Discuss with specialists your required doses and the amount of the material to be treated to ensure the facility is suited for your needs. Check if a phytosanitary certificate and quarantine procedures are needed for shipment.

1.7.2.2. Pre-conditioning of seeds and gamma radiation treatment

1. Discard any injured, atypical or diseased seed.

2. Count out the number of seeds required per dose and pack them loosely in water-permeable bags.

3. Label the bags with information on species, variety/genotype, date and the treatment dose.

4. Fold the tops of the bags over and close with a paper clip or staples to avoid spillage.

5. Place the bags on the plate of the desiccator above a 60% glycerol-water mixture at room temperature for a minimum of seven days for small grain cereals such as rice and wheat. This is extended for up to 14 days for greater quantities, larger size seeds (e.g. beans) and/or seed with thick seed coats. The bags should not come in contact with the liquid.

6. Remove the seeds from the desiccator just before radiation treatment. If the moisture equilibration cannot be followed immediately by radiation treatment or seeds need to be shipped to the irradiation facility, pack the seeds in air-tight containers or sealed plastic bags to maintain the desired moisture content of 12 – 14%.

7. Hand moisture equilibrated seeds in bags or containers over to the operator of the gamma cell for irradiation. The gamma source must be operated by experienced and trained personnel who will calculate the required exposure time based on the present dose rate.

8. Receive treated seeds which can be handled without precautions as gamma irradiation does not leave any radioactivity behind.

9. Record information on the present dose rate, the type of gamma source and the exposure time.

1.7.2.3. Post irradiation storage

Plant seeds as soon as possible after irradiation to avoid an increase of damage arising from prolonged post-irradiation storage. If seeds are shipped or not planted immediately, store them at room temperature only for up to four weeks. If longer storage is required, store them dry in sealed airtight bags or vials, in the dark at a temperature range of –5°C to 2°C to minimize metabolic activity. Most of all choose storage conditions that suit the seed material.

1.7.2.4. Planting

Choose one of the two planting techniques bellow according to the requirements of the crop or your preference. Field experiments are not recommended at this stage, mainly because they are more difficult to control and interpret, due to additional biotic and abiotic stresses and other varying environmental conditions.

- Flat method: Ideal for beans, cereals and larger sized seeds. This planting technique warrants organized measurement conditions. The effect of the dose is easily ascertained by visual assessment of the sowing trays. (Figure 1.6) Continue under point 1 in *Flat method* below of the protocol.

    1. Sow seeds in trays containing potting mix in the greenhouse. Keep the sowing depth uniform.

    2. Plant seeds in rows keeping in mind the requirements of the species.

    3. The seeds are planted in order of increasing dose with replications sown in different trays.

    4. Keep the moisture adequate to ensure germination and all environmental conditions uniform for all treatments. Move on to data collection (1.7.2.5).

- Petri dish method: Ideal for cereals or smaller sized seeds and for seeds that require light for germination. Fungal contamination can become a problem. Continue under point 1 below in *Petri dish method* of the protocol.

    1. Disinfect seeds e.g. by placing them in 10 percent bleach for 10 minutes. Prepare 200 ml of bleach by using 20 ml of sodium hypochlorite (NaOCl - approx. 5 percent w/v active ingredient), a few

drops of Tween 20 and 180 ml of distilled water. Rinse the seeds three times with sterilized water to remove disinfectant solution.

2. Wet filter paper in a germination container with sterilized water and place seeds on filter paper in a grid using forceps. Use a separate container for each replication.
3. Wrap the container in foil if darkness is required for germination.
4. Tightly seal containers inside a plastic bag to prevent moisture loss.
5. Remove any foil and lift off the lid of the container when seedlings reach the top.
6. Ensure the paper remains always moist as germination is strongly influenced by water uptake.

1.7.2.5. Data collection

Collect data on the germination percentage, seedling height, survival, fertility, sterility in M_1 and M_2 populations. For optimal results all these measurements should be performed on the same day for all treatments and replications to reduce bias.

Germination percentage

Count the number of seedlings when germination is completed. Be aware that delayed germination may be observed in irradiated seeds as compared to the control. Count all germinated seeds, also the ones that have died. When using the flat method, seedling emergence rather than germination is taken. Calculate Germination (seedling emergence) percentage for each replication (Figure 1.7).

$$\text{Germination percentage} = \frac{\text{Number of germinated seeds} \times 100}{\text{Number of seeds planted}}$$

Seedling height variation

Determine the time at which the first true leaves in the control plants cease to expand. When the first true leaves in control plants have stopped growing, determine seedling height for the control and for the radiated material. It may be useful to take two full sets of measurements a few days apart to determine afterwards what the optimal day was. Use a ruler or measuring board and collect data to the nearest millimeter.

In monocotyledons, measures are taken from soil level to the tip of the first or secondary leaf. In the Petri dish method start measuring from the root-shoot-junction instead of the soil level. In cereals, take measurements when shoots of control seedlings are between 11 and 20cm, usually after 10 to 14 days.

Make sure to identify the first leaf correctly and not the cotyledons, which may resemble leaves in some plant species. In cereals, the first true leaf emerges through the coleoptile (leaf sheath). Do not measure seedlings where only the coleoptile has developed.

In dicotyledons, distinguish between epigeous and hypogeous germination. In epigeous germination the cotyledons are pulled upward through the soil during germination, e.g. common bean; *Phaseolus vulgaris*. In hypogeous germination the cotyledons remain below the soil surface, e.g. pea; *Pisum sativum*.

Measure the seedling height from soil level to the tip of the primary leaf or to the stem apex manually by straightening out the plant, or in the petri dish from the root-shoot junction. Include in the measurements and calculations only the plants with a fully developed primary leaf. With dicotyledons and epigeous germination, alternatively you can measure the epicotyl between the points of attachment of the cotyledons to the tip of the primary leaves or to the stem apex. The parameter would then be referred to as epicotyl height rather than seedling height. However, make sure you measure all plants with the same method and keep a record of it, see example in Table 1.6. Calculate average seedling height (epicotyl height) for each replication.

$$\text{Average seedling height} = \frac{\text{Sum of seedling height in mm}}{\text{Number of plants measured}}$$

Figure 1.6. Radiosensitivity test in rice (Oryza sativa) using ^{60}Co gamma-rays at doses: 0,150, 200, 300, 400, and 500 Gy. Note the seedling height decrease, the poor germination rate and/or lethality at the higher doses: 400 and 500 Gy. Courtesy of A. Kodym.

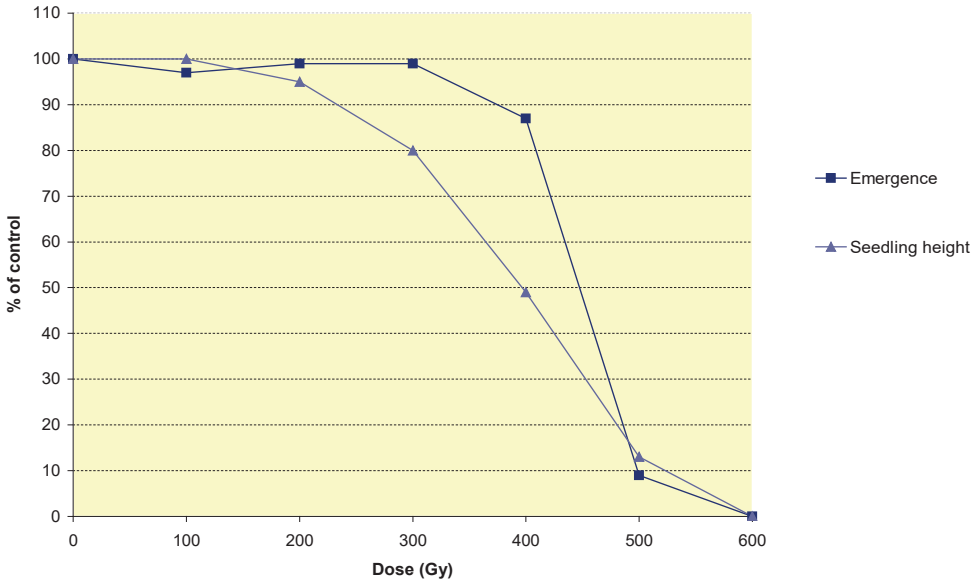


Figure 1.7. Schematic presentation of emergence and seedling height data after gamma radiation (control=100%).

Survival

Count all healthy seedlings. Record the time and stage of plant development at which data are taken, generally at the time that seedling height is measured. However, it should be noted that actual plant death might occur at any time between the onset of germination and maturity. If plants are grown to full maturity it is best to score plant survival again at the time of harvest, as a plant that successfully produced seeds. Calculate average plant survival for each replication using the equation below.

$$\text{Survival percentage} = \frac{\text{Number of surviving seedlings}}{\text{Number of seeds planted}} \times 100$$

Check leaves for visible leaf spotting or leaf streaks, caused by arrested pigment development in some cells. It is frequently generated in plants of the Fabaceae family. Record the degree (number and/or size of the leaf spots), where feasible, as that can be difficult to quantify.

Decide if you have the time, facilities and the need to carry on with the experiment. Most data collections could stop here. If you are running a preliminary test, always stop here and go to data analysis (Section 1.7.2.6). If you decide to carry on, replant plants into pots while meeting all their specific requirements after the determination of germination/emergence, seedling height and leaf spotting.

Fertility

Record the number of flowers/spikes/inflorescences, fruits and seeds produced to assess fertility at the time of maturity of seeds/fruits. These parameters are determined according to the development of the plants. Calculate the average for each characteristic per replication: (example here it is given for seeds harvested).

$$\text{Average number of seeds per plant} = \frac{\text{Number of seeds harvested}}{\text{Total number of plants (including plants that did not produce seed)}}$$

Sterility in the M_2 population

Germinate about 50 seeds, according to the availability, of the harvested seeds using the method described under ‘Planting’ to evaluate sterility in the M_2 population. Calculate the germination percentage in the M_2 population:

$$\text{Germination percentage in the } M_2 = \frac{\text{Number of germinated seeds}}{\text{Number of seeds planted}} \times 100$$

1.7.2.6. Data analysis

Compare the germination percentages of the replications. If values are within a reasonable range, calculate the average per treatment from the three replications. If not, look for off-types and try to see where the variation might come from (e.g. problems with watering) and critically decide what values are representative and should be included for further analyzing. Express the reduction/increase of the radiated materials as a percentage of the non-irradiated control in percentages (Table 1.7).

$$\text{Percent of control} = \frac{\text{Average of irradiated treatment}}{\text{Average of control treatment}} \times 100$$

Draw the calculated values as a dose-response curve using the dose as x-axis and the percentages on the y-axis (Figure 1.7). The control treatment is set as 100 percent.

Repeat these steps with the other parameters (seedling height, survival, fertility, sterility in the M_2). Note that at low dose seedling height of irradiated material may exceed control values.

Compare the graphs from the different parameters (reduction in germination percentage, seedling height, survival and possibly fertility and sterility in the M_2). They may show different percent reductions at the same dose, but an overall trend can be derived by combining the data.

In breeding programs, fertility and sterility are very important criteria as they will determine the size of the M_2 population, which will be available for evaluation and screening of the mutations. Seeds may form after radiation treatment but fail to germinate properly or die after germination.

Keep in mind when planning later field trials that emergence and survival obtained under glasshouse testing conditions may differ considerably from that under field conditions due to environmental stresses.

TABLE 1.6. SEEDLING HEIGHT MEASUREMENTS IN MM AND SURVIVAL DATA OF RICE VARIETY XY, RAW DATA: 25 SEEDS PER REPLICATION AND SEVEN DIFFERENT DOSES

No.	Ctrl	Ctrl	Ctrl	100Gy	100Gy	100Gy	200Gy	200Gy	200Gy	300Gy	300Gy	300Gy	400Gy	400Gy	400Gy	500Gy	500Gy	500Gy	600Gy	600Gy	600Gy
	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
1	195	230	210	225	210	190	225	180	175	170	150	335	160	90	120		30	60			
2	175	210	195	195	200	210	225	145	200	150	155	170	95	125	110			40			
3	130	245	200	185	230	200	215	170	200	155	160	150	40	115	70			40			
4	165	240	235	210	240	220	210	165	205	185	180	45	130	140	70						
5	200	230	230	125	235	220	190	165	210	100	140	190	100	145	2						
6	115	210	200	190	220	190	175	165	115	125	140	205	145	50	70						
7	225	205	225	215	165	220	220	195	190	175	140	165	120	95	85						
8	200	215	200	235	190	225	210	175	180	165	155	185	105	35	115						
9	190	210	200	215	230	220	240	190	195	190	150	180	100	95	110						
10	240	215	215	200	90	225	240	190	165	155	145	190	70	90	80						
11	195	205	200	190	210	215	220	180	150	155	175	165	95	130	95						
12	170	225	225	220	220	100	240	195	175	95	180	145	80	30	115						
13	200	220	160	240	175	180	240	165	200	195	140	140	100	90	120						
14	200	190	185	220	225	230	220	210	155	165	170	165	150	75	60						
15	215	230	210	220	230	200	260	175	185	195	185	185	90	45	130						
16	120	220	215	215	230	210	165	185	200	160	180	195	110	90	115						
17	170	200	225	225	195	215	190	235	190	185	170	140	85	115	2						
18	140	165	220	225	200	215	175	215	185	170	165	125	15	130	120						
19	120	200	225	215	210	165	195	205	120	140	170	140	130	100	120						
20	180	210	185	190	185	200	175	210	170	160	60	150	90	140	5						
21	130	225	210	175	160	180	155	205	210	175	160	160	130	140	40						
22	195	240	210	165	180	230	165	215	175	165	180	140	115		125						
23	220	230	215	195	220	185	185	200	220	160	185	120									
24	210	225	205	160	215		160	205	210	180	140	140									
25	200	225	210	215					190		155	155									

Data in gray cells were included for the evaluation of survival but not considered for seedling height. The primary leaf did not develop, only the coleoptile was measured.

TABLE 1.7. EMERGENCE PERCENTAGE (RAW DATA NOT PRESENTED), SURVIVAL PERCENTAGE AND SEEDLING HEIGHT OF RICE VARIETY XY BASED ON TABLE 1.6 AVERAGE FROM THREE REPLICATIONS (I; II; III) AT SEVEN DIFFERENT DOSES AND EXPRESSED AS A PERCENTAGE OF RADIATED MATERIAL OVER THE NON-IRRADIATED CONTROL (=100%)

	Replication/Dose	Control	100 Gy	200 Gy	300 Gy	400 Gy	500 Gy	600 Gy
Emergence	I	100	100	100	96	88	12	0
Percentage	II	100	96	96	100	84	4	0
	III	100	96	100	100	88	12	0
	Average	100	97.3	98.7	98.7	86.7	9.3	0
	% of control	100	97	99	99	87	9	0
Survival	I	100	100	96	96	88	0	0
Percentage	II	100	96	96	100	84	4	0
	III	100	92	100	100	88	12	0
	Average	100	96	97.3	98.7	86.7	5.3	0
	% of control	100	96	97	99	87	5	0
Seedling height	I	180	203	204	161	103	0	0
mm	II	217	203	189	157	98	30	0
	III	208	202	183	163	98	47	0
	Average	201.7	202.7	192	160.3	99.7	25.7	0
	% of control	100	100	95	79	49	12	0

1.8. STANDARD PROTOCOLS FOR SEED MUTAGENESIS USING X-RAY IRRADIATORS

The protocol given below is for small grain cereals, or plants with seeds of similar size and may be adapted for other types of seeds.

1.8.1. **Pre-treatment preparations of seed samples**

- Screen and decide on the specific variety/genotype to be treated (preferably fresh breeder or foundation seeds).
- Clean the fresh seeds from shaft, remove broken, shrivelled or under-size ones, and if needed, disinfect to eliminate any contaminant.
- Test the viability of the seeds, i.e. germination rate on a small sample (10 – 20 seeds) according to the species specificities (dormancy, temperature/light and time) and enter, if possible, only seeds with more than 85 percent germination rate should be preferred.
- For inbreeding species, it is preferable to select homozygous material; doubled-haploids are ideal, when available.
- Seeds should come from a seed increase field experiment with vigilant growing conditions to prevent outcrossing (minimal heterozygosity) and mixing (heterogeneity) with other seed sources of the same species.

1.8.2. **Radiosensitivity tests**

- Assess and equilibrate the seed moisture, if necessary using 60 percent glycerol solution (Figure 1.5) to 12 – 14 percent, which is considered optimal for irradiation treatment.
- Pack 15 – 20 seeds in an envelope for each specific dose, the dose range should be between 6 – 10 depending on the literature information for the target species, the growing media (trays, Petri dishes, pots or fields) and the available space for testing.
- Carefully label each sample with the variety/genotype name, the replication number, the dose amount, the source of irradiation, and the date. Note that the amount of seeds per treatment and the number of treatments vary with the crop, i.e. type of seeds, growing conditions, etc. At the PBGL, planning for 6 – 7 treatments including the control (untreated) are standard procedure.

1.8.3. **Irradiation treatment of the seeds**

Below is the description of treatment procedures for two types of irradiators available for mutation induction (vertical and horizontal rotating machines); the RS2400 Bio-Rad X-ray machine and the Faxitron and Hitachi Irradiators (Figure 1.8). For any of these machines, the treatments may be replicated 2 – 3 times depending on the seed availability and the planting space. As for any manipulation of hazardous tools, e.g. irradiators, the steps given in this section should be carried out by a qualified irradiator operator.

1.8.3.1.Vertical rotating RS2400 X-rays irradiator

- Place the seed samples into the appropriate containers, and depending on the size and the amount of seeds, fill the remaining space firmly with instant rice, then use appropriate fixing device to ensure stability of the samples during the rotating motion.

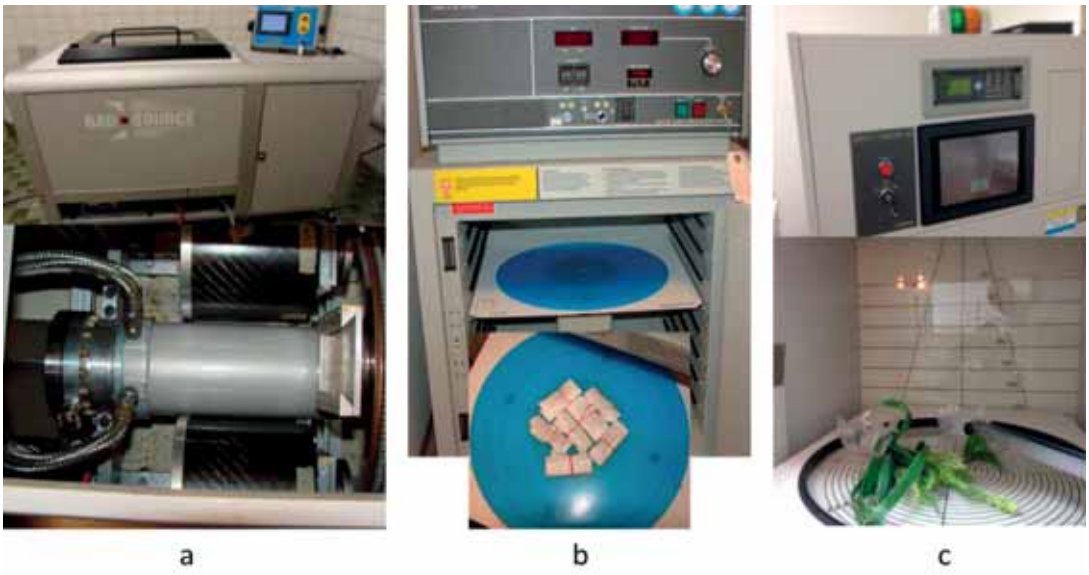


Figure 1.8. Representative X-ray machines; a) vertical rotating Rad Source RS2400 showing the machine (upper) and the sample chamber with the upper most Canister removed for ease view, b) Faxitron 650 (Faxitron bioptics LLC, Tuscon, AZ, USA) showing horizontally rotating tray with seed bag samples placed in the centre, and c) Hitachi Standard MBR-1520R-3 machine showing the sample chamber at the lower most position with green wheat spikes ready for irradiation. Courtesy of A. Mukhtar Ali Ghanim.

- Place samples into the canister and fix the containers with the specific brackets, fill the containers with instant rice again to avoid vacuum condition during the rotation and firmly close the canisters with the lid.
- Switch on the machine, open the shielding window of the irradiation chamber, place canister in each one of the 5 canister holders, and close the shielding window of the irradiator chamber (Figure 1.8. a).
- Set the exposure time, by imputing the amount of kW needed to produce the required dose and run until the countdown monitor displays: 0 kW. The machine may then, be turned off.
- Open the shielding window take out and open each canister.
- Gently remove the rice filling and extract the samples and adaptors.
- Mark the envelopes/bags contained the treatment M_1 seeds together with the untreated control samples to be entered in the next steps of the experiment (laboratory, greenhouse or field) with appropriate information.

1.8.3.2.Horizontal rotating Hitachi and Faxitron X-ray irradiators

In both machines Hitachi (MBR-1520R-3) and Faxitron bioptics (LLC, Tucson, AZ – USA) the loading and rotation are horizontal and the irradiation source emits vertically, usually from top to bottom. (Figure 1.8 b, c)

- Turn on the X-ray cabinet (power button) and follow the warm up procedure as described by the manufacturer.
- Set the energy (display "Tube Voltage"; kVp) by turning the "kVp Control" button if not using pre-stored settings. Tube current (mA) is usually pre-adjusted. If using filtration by Aluminium filter (typically 0.5mm), make sure it is placed into the filter holder below the X-ray tube to cut off the soft X-ray and leave only hard X-rays.
- Choose the tray position according to the planned dose rate and space needed to accommodate the samples. These positions vary according to the source (X-ray tube), the sample distance, and may be adjusted to the dose rate.
- Place the samples onto the sample tray within the marked area for the chosen position to ensure desired irradiation. Make sure to properly turn on the rotation of the sample tray located below the tray.
- Adjust the timer setting (Time set; minutes and seconds) to the desired irradiation duration, which is calculated with the following equation:

- $\text{Time (min)} = \dfrac{\text{Dose (Gy)}}{\text{Dose rate (Gy/min)}}$

- Appropriately close the X-ray cabinet and start the irradiation process. The lamp “X-ray on” turns automatically on and the elapsing count down time starts. The machine will automatically turn off when the irradiation process is completed.
- Mark the envelops/bags with the proper information: date, irradiation dose and duration, operator name, etc.

1.8.4. **Post irradiation treatments and handling**

These procedures are identical to those described earlier for gamma-ray irradiation (section 1.7).

1.8.PRACTICAL EXAMPLE OF APPLICATIONS USING PHYSICAL MUTAGENS

Example on mutation breeding for barley (*Hordeum vulgare* L.); cultivar UNALM96, (Gómez-Pando *et al.*, 2009) from the Cereal and Native Grain Research Program, *Universidad Agraria La Molina*, Lima, Peru (Figure 1.9).

Figure 1.9. Steps in barley (Hordeum vulgare L.) mutation breeding: ***1.*** *1997 - Preliminary radiosensitivity test were conducted to determine the LD50 and select the most efficient dose;* ***2.*** *Same year: two batches of 250g of M_0 barley seeds (cv. UNALM96) were irradiated using a gamma ray machine at 200 and 300 Gy;* ***3****. 1998 - M_1 seeds were sown in the field along with non-irradiated control seeds and grown to maturity;* ***4.*** *1999 - M_2 Seeds from individual spikes were planted in 342958 rows and inspected for signs of mutation: off-types i.e. albino, viridis, altered spikes, etc., which were carefully recorded;* ***5.*** *2000 - All M_3 seeds were separately harvested and sown as M_3 family/row;* ***6.*** *2001 to 2005 - From M_4 to M_8 generations of putative mutant lines selected on the basis of their agronomical performances were grown at two locations under standard agronomic conditions [in irrigated fields (Coast) and rain-fed (Highland)];* ***7.*** *2006 - Release of the new barley mutant cultivar 'Centenario' from the 12 mutant lines outperforming the parental checks. Courtesy of L. Gomez-Pando.*

1.9.SEED IRRADIATION SERVICES AT THE JOINT FAO/IAEA PLANT BREEDING AND GENETICS LABORATORY

The Joint FAO/IAEA Plant Breeding and Genetics Laboratory, Seibersdorf, Austria provides an irradiation service for plant mutation induction for Member States. The requestor must follow the following procedures:

1. Select high quality seeds. The seeds should be disease-free, uniform and representative of the variety/line/genotype. The seeds should have a high germination rate (90 percent or more).

2. The samples should be bagged and clearly labelled.

3. The size of the seed sample should be determined prior to sending.

4. If the irradiation dose is not known, about 100 seeds are needed for a radio-sensitivity test.

5. A Standard Material Transfer Agreement (SMTA) should be signed.

6. Seed samples should be inspected by local Quarantine Officers prior to sending and sent with a Phytosanitary Certificate.

7. Import Quarantine regulations may also apply.

For a Laboratory Mutagenesis Service Request form and further details contact:

Head, Plant Breeding and Genetics Laboratory
FAO/IAEA Agriculture and Biotechnologies Laboratories
Friedenstrasse 1
A-2444 SEIBERSDORF
AUSTRIA

1.10. OTHER MUTAGENS

In addition to physical mutagens, there are also chemical mutagens (Chapter 2) and number of biological mutagens that can induce mutations in plants either experimentally or naturally. A close analysis of the so-called “spontaneous mutations” frequently observed in nature, show that they result in fact, from intrinsic and/or extrinsic mutagenic factors affecting the organism. The genetic constitution of the organism appears as the main factor, comprising variation in chromosomes number (polyploidy, aneuploidy, etc.) and structural changes in the chromosomes such as inversions and translocations resulting from chromosomes cross-overs during cell

division. It is also known that some genes present in the genome, might induce these mutations (see also Chapter 3 on transposons).

The physiological conditions, such as age and sex, have been shown to have strong effects on chromosomes in living cells, which often increase with age. As to the origin of mutations in ageing seeds various evidence indicate that they are the result of chemical action of metabolites and /or waste products that accumulate in seeds over time.

2. CHEMICAL MUTAGENESIS

This chapter reviews commonly used plant chemical mutagens with particular attention to alkylating agents and sodium azide, the main chemical mutagens in current use for practical crop improvement or experimental plant mutagenesis. This chapter also provides guidelines on methods of application and the various parameters that can influence the outcome of a plant chemical mutagenesis experiment. Basic information on health and safety considerations to ensure the safe use of chemical mutagenesis is provided. Chemical mutagenesis in reverse genetics has enjoyed a renaissance since the early 2000s due to technological innovations including Targeting Induced Local Lesions in Genomes (TILLING) and, more recently, Next Generation DNA Sequencing (NGS) technologies. These advances have yielded important new insights into the mechanism and spectrum of chemically-induced mutations. Example spectra for key mutagens and crops are included to help guide plant breeders and/or researchers in designing plant mutagenesis experiments. Likewise, advances in *in vitro* plant tissue culture created new opportunities in expanding chemical mutagenesis to *in vitro* tissues. This is particularly important for the vegetatively propagated crops (VPCs) which have lagged behind annual seed propagated crops for mutation breeding. The chapter includes detailed protocols for the use of ethyl methanesulphonate (EMS) mutagenesis of banana (*Musa acuminata*) *in vitro* shoot tips and barley (*Hordeum vulgare*) seeds. Both protocols may be adapted for various vegetatively and seed propagated crops. In addition, an efficient protocol for combined mutagenesis treatment of barley seeds using sodium azide (SA) and *N*-methyl *N*-nitrosourea (MNU) is provided as an example of increasing the mutation spectrum.

2.1.MAIN CHEMICAL MUTAGENS

The use of chemicals as mutagens dates back to the 1940's with the treatment of *Drosophila melanogaster* with mustard gas (Auerbach, 1946; Auerbach and Robson, 1946). To date, the number of chemicals known to have mutagenic effects on living organisms - animals, plants or microorganisms - is enormous. By contrast, only a few number of different chemicals are routinely used for experimental plant mutagenesis or crop mutation breeding. Figure 2.1. summarizes the number of varieties that have been released following chemical mutagenesis along with the type of mutagen that was used (data extracted from the IAEA/FAO Mutant Varieties Database; http://mvd.iaea.org, November 2017). As shown in Figure 2.1a, the top seven most frequently used chemical mutagens all belong to the class of alkylating agents, except

for colchicine. Figure 2.1.b shows that rice, barley, wheat and maize constitute nearly half of the released mutant varieties.

2.1.1. **Alkylating agents**

A1kylating agents are known to be mutagenic in plants for many decades (Ehrenberg, Lundqvist and Ström, 1958). They are by far the most successful from the perspective of producing new mutant cultivars because of their effectiveness and ease of handling, and most importantly the convenient detoxification process through simple hydrolysis for disposal. Alkylating agents are electron deficient (i.e. electrophilic) compounds with one or more alkyl groups, which can be transferred to biological molecules such as DNA that contain nucleophilic groups. Most alkylating agents are pro-mutagens, i.e. they undergo transformation to produce reactive intermediates. These intermediates can react with DNA by alkylating the phosphate groups in the phosphodiester backbone as well as the various imino- or carbonyl- groups present on the purine (adenine, guanine) or pyrimidine (cytosine, thymine) bases.

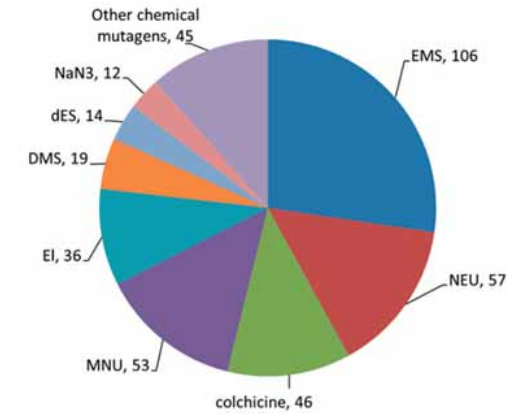


Figure a

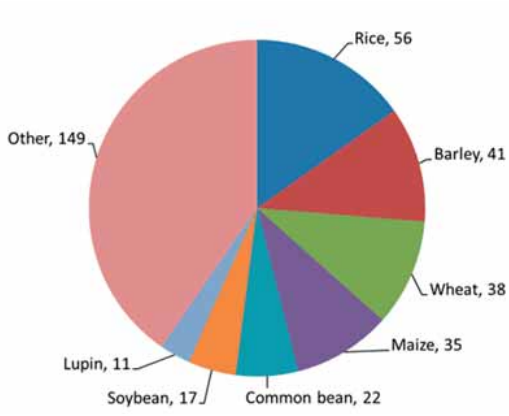


Figure b

Figure 2.1a. Chemical mutagens most frequently applied in generating mutant varieties. Among top agents are EMS (ethyl methanesulphonate), with 106 officially registered mutant varieties, NEU (nitrosoethyl urea) (57), MNU (N-methyl N-nitrosourea) (53), colchicine (46), and EI (ethylenimine) (36). Figure 2.1b. Officially released mutant crop varieties registered in the MVD produced via chemical mutagenesis.

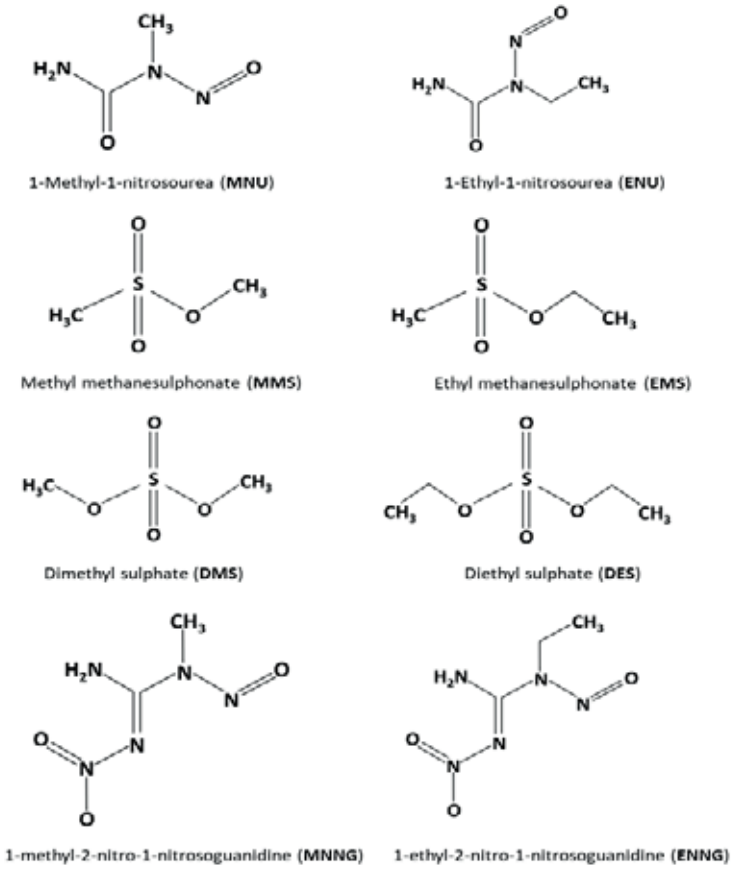


Figure 2.2. Molecular structure of alkylating agents commonly used in chemical mutagenesis of plants.

Alkylating agents can be classified as mono-, bi- or poly-functional types, depending on the number of alkyl groups present in the compound. The alkylating agents most commonly used in plant mutation breeding are mono-functional. Among these, EMS, MNU and diepoxybutane are frequently used (Figure 2.2) Bifunctional alkylating agents can induce inter- and intra-strand DNA-DNA cross-links causing the inhibition of DNA replication.

2.1.2. **Sodium azide**

Sodium azide (NaN_3; SA) is an inorganic, highly toxic compound and the only other chemical mutagen besides the alkylating agents that has been used frequently for practical crop improvement (Figure 2.1). SA is a well-known inhibitor of cellular respiratory processes in living cells (Tsubaki *et al.*, 1993), and has proven to be an effective mutagen in many crop species such as barley, rice, soybean and maize but not in other plants such as *Arabidopsis thaliana* (Kleinhofs *et al.*, 1975).

Sodium azide is also considered a pro-mutagen as it is metabolized *in vivo* to a powerful chemical mutagen through an organic intermediate, identified in barley as L-azido-alanine. It appears that L-azido-alanine itself does not directly interact with DNA, but that mutagenesis is mediated by the host-plant cellular processes involved in DNA excision-repair (Owais and Kleinhofs, 1988; Sadiq and Owais, 2000). These findings may also explain the lack of SA mutagenic effects in some plant species such as Arabidopsis. Hence, initial experiments are required to assess the effectiveness of SA in a new plant species prior to conducting large-scale mutagenesis. In plants, SA affects multiple metabolic pathways explaining its cytotoxic and physiological effects in addition to its mutagenic effects (Gruszka, Szarejko and Maluszynski, 2012).

The mutagenic effects of SA have been extensively studied in barley (Olsen, Wang and von Wettstein, 1993; Maluszynski and Szarejko, 2003; Lababidi *et al.*, 2009) as well as in other crops, such as tomato (Abdulrazaq and Ammar, 2015), the oat species *Avena longiglumis* (Khan, Al-Qurainy and Anwar, 2009), and rice where an advanced mutant line with enhanced amylose content was developed (Suzuki *et al.*, 2008).

The mutagenic effect of SA depends greatly on the acidity of the treatment solution (Nilan *et al.*, 1973). The mutagen should be applied at low pH (< 4), e.g. for barley mutagenesis it is being dissolved in a phosphate buffer at pH 3 (see also Section 2.5. for examples of treatment procedures).

2.1.3. **Other chemical mutagens**

In addition to the alkylating agents and azides, the previous (second) edition of the IAEA Manual on Mutation Breeding (IAEA, 1977) described the following groups of chemical mutagens: (i) base analogues; (ii) antibiotics; (iii) acridines; (iv) nitrous acid; and, (v) hydroxylamine. Leitão, (2012) described the acridines under a more general category of intercalating agents together with topoisomerase inhibitors and poisons. The exploitation of these chemical mutagens for plant genetic improvement is much more restricted compared to the alkylating agents and azide, because they are either less effective, less well studied or more challenging to handle from a health or safety perspective. Hence, selected groups are reviewed below. Colchicine is considered a chemical mutagen *senso latu*, because its main effect is on ploidy and not on genes (see Chapter 3) and is briefly described below. For additional information on the types, properties, and mutagenic effects of these additional chemical mutagens, the reader is referred to (Leitão, 2012).

2.1.3.1.Base analogues and related compounds

True base analogues are closely related to the DNA bases: adenine, guanine, cytosine or thymine and can be incorporated into the DNA molecule without hindering its replication. However, since these analogues differ subtly, occasional base pairing

errors can occur during DNA synthesis or DNA replication. The most frequently used analogues are 5-bromo-uracil (BU) and 5-bromo-deoxyuridine (BUdR), which are analogues of thymine and adenine, respectively. BU is able to induce mutations in higher plants but the mutation frequency remains low (Handro, 2014; Gautam, Saxena and Kumar, 2016). Overall, base analogues have not been extensively tested as agents for mutation induction in plants.

2.1.3.2.Antibiotics

Antibiotics are functionally defined as having anti-microbial effects, but structurally they comprise a very diverse range of compounds. Antibiotics also differ with regards to their cytotoxic or mutagenic properties, which have been primarily studied in microbial and animal systems. For example, streptozoocin (STZ) is a potent mutagen and carcinogenic and is used as an anti-neoplastic agent. STZ causes primarily point mutations while other antibiotics have chromosome-breaking properties. Mitomycin C (MMC) is a naturally occurring antibiotic isolated from *Streptomyces caespitosus*. MMC is a bi-functional alkylating agent that reacts with guanosine residues to form cross-links between DNA strands (see also 2.1.1. and Palom *et al.*, 2002).

While antibiotics have had limited use in plant mutation breeding, streptomycin has been successfully used to induce male sterility in several plant species including rice, sorghum, pearl millet, sugar beet, and sunflower, the latter resulting in the official release of several mutants (Hu and Rutger, 1991; Jan and Vick, 2006; Elkonin and Tsvetova, 2008).

2.1.3.3.Intercalating agents

Intercalating agents can reversibly intercalate with double-stranded DNA, but do not covalently bind to it. Classical intercalating agents include ethidium bromide, 4', 6-diamidino-2-phenylindole (DAPI) and acridines which are widely used as dyes in biological or biochemical studies. Acridines and their derivatives have light absorbing properties, and display photo-enhanced cytotoxic and mutagenic effects. The mutagenic effects induced by acridines can range from base-pair substitutions to frameshift mutations to chromosome-breaks depending on the type of acridine used and are well demonstrated in prokaryotic and mammalian systems.

However, these compounds have rarely been studied in plants. Recent experiments include wild ginger species (family *Zingiberaceae*) with ornamental potential, (Prabhukumar *et al.*, 2015) and flax (*Linum usitatissimum* L.), (Bhat *et al.*, 2017). These authors have compared the mutagenic effects of acridine to other chemical or physical mutagens such as EMS, colchicine, gamma- or X-rays. The studies clearly demonstrated the mutagenic effects of acridines on plant growth and development. For example, treatment of the wild ginger species *Larsenianthus careyanus* with

1 percent acridine produced white variegation on the leaves. The strong mutagenic capabilities of these compounds as demonstrated in prokaryotic and mammalian cells, warrant further investigation in plants in view of their potential to induce new and unique mutations for plant scientists and breeders.

2.1.3.4.Colchicine

Colchicine is a toxic alkaloid derived from the meadow plant: C*olchicum autumnale* (Autumn crocus). Colchicine is widely used in plant breeding work to produce changes in ploidy. The increased number of chromosomes usually brings about changes in plant morphology and functions. Colchicine treatments of meristem-containing propagules or tissues can be performed in many ways using concentrations ranging from 0.005 percent to 1.5 percent (van Harten, 1998). Common methods for chromosome doubling involve soaking the seeds in a colchicine solution, applying colchicine using a brush on growing shoot apices, or culturing (*in vitro*) plantlets in colchicine-containing medium (Hamill, Smith and Dodd, 1992). A major use for colchicine is the treatment of haploids to produce doubled haploids which are completely homozygous, i.e. genetically pure. Practical methods in doubled haploid production in a wide range of plant species are available in Maluszynski *et al.,* (2003); see also Chapter 8-B in this manual.

2.2.MODE OF ACTION AND MUTATION SPECTRA

The mutagenic effect of a chemical depends on the lesion initially induced in the DNA as well as on any DNA repair mechanisms present in the host plant cells. Therefore, both the characteristics of the chemical and the host DNA repair processes play an important role for determining the ultimate mutagenicity of a chemical mutagen. Details on plant host repair mechanisms can be found in Chapters 1, 3 and 4.

2.2.1. **Alkylating agents**

The DNA breakage and clastogenic effects, i.e. disruption or breakage of chromosomes, induced by alkylating agents have been documented for over 80 years (Auerbach and Robson, 1946). To date, alkylating agents are widely applied to induce single base pair changes to alter protein function or structure.

Alkylation is defined as the transfer of an alkyl group from one molecule to another. The alkyl group may be transferred as an alkyl carbocation, a free radical, a carbanion or a carbene (or their equivalents). The dialkyl nitroso amines (e.g. diethyl nitroso amine) are stable compounds, which apparently act on DNA only after enzymatic activation (removal of one alkyl group). The major DNA alkylation mechanisms present in host plants have been reviewed by Leitão, (2012).

Lee *et al.*, (2014) reported that EMS induces alkylation on guanine resulting in GC>AT transitions, which can lead to single nucleotide mutations. Resulting data sets from Targeting Induced Local Lesions in Genomes experiments (TILLING) in over 15 plant species have shown that EMS primarily causes GC to AT transitions as expected for alkylation of guanine at the ^{6}O position (Jankowicz-Cieslak and Till, 2015).

Several studies have shown that EMS mutations are distributed randomly across the genome (Greene *et al.*, 2003; Till *et al.*, 2003). According to these authors, a bulk mutagenesis experiment producing a population of 3 000 to 6 000 lines is typically sufficient to recover multiple alleles in any gene in case of diploids. An extensive characterization of EMS-induced rice mutants demonstrated that EMS-induced mutagenesis has a strong local sequence context bias specifically targeting guanine residues in the context R**G**CG (R is A or G; the mutated guanine indicated in bold), (Henry *et al.*, 2014b).

While the high mutation densities achieved in these studies enables the recovery of allelic variants in any gene, this high mutational load may present a challenge for functional genomics studies, and for practical crop improvement. This is especially the case when attempting to improve elite materials for one or a few traits as the presence of the numerous undesirable mutations in the background may disrupt the finely tuned genetic architecture of the elite variety (see Section 2.3.2).

2.2.2. **Sodium azide**

Sodium azide induces chromosome aberrations at a very low rate. The type and number of mutations induced by SA has been studied in barley (Talamè *et al.*, 2008; Kurowska *et al.*, 2011) and more recently also in rice (Tai *et al.*, 2016). These studies showed that SA is a powerful mutagen for inducing point mutations. Both in barley and rice, GC to AT transitions were the predominant mutation type. It appears that sodium azide-induced mutagenesis has a different local sequence context bias (G**G**R) compared to EMS (Tai *et al.*, 2016). Therefore, combining different mutagenic compounds may expand the spectrum of induced mutations and the resulting mutant phenotypes.

An overview of the types and density of mutations induced by different chemical mutagens based on DNA sequence analysis is provided in Table 2.1.

TABLE 2.1. SPECTRUM OF CHEMICALLY INDUCED POINT MUTATIONS IN DIFFERENT SEED-PROPAGATED SPECIES AND BANANA, A VPC

Species (common name), ploidy level	Mutagen	Mutation density (kb)	Transitions G/C > A/T (%)	Transitions A/T > G/C (%)	Trans-versions (%)	Reference
Arabidopsis thaliana, 2x	EMS	1/200	100	0	0	Greene *et al.*, 2003
Avena sativa (oat), 6x	EMS	1/24	94.4	0	5.6	Chawade *et al.*, 2010
Brassica rapa (field mustard), 2x	EMS	1/56 and 1/67	-	-	-	Stephenson *et al.*, 2010
Cucumis melo (melon), 2x	EMS	1/573	97.8	0	2.2	Dahmani-Mardas *et al.*, 2010
Glycine max (soybean), 4x	EMS (repeated)	1/74	84.3	-	23 to 47	Tsuda *et al.*, 2015
Hordeum vulgare (barley), 2x	EMS	1/500	n. a	n. a	n. a	Gottwald *et al.*, 2009
Hordeum vulgare (barley), 2x	EMS	1/1,000	70	10	20	(Caldwell *et al.*, 2004)
Musa acuminata (banana), 3x	EMS	1/57	100	0	0	Jankowicz-Cieslak *et al.*, 2012
Oryza sativa japonica (rice), 2x	EMS	1/147	88	-	-	Henry *et al.*, 2014
Solanum lycopersicum (tomato), 2x	EMS	-	-	-	55	Minoia *et al.*, 2010
Triticum aestivum (bread wheat), 6x	EMS	1/23.3 to 1/37.5	99.2	0	0.8	Dong *et al.*, 2009
Triticum durum (durum wheat), 4x	EMS	1/51	-	-	-	Uauy *et al.*, 2009
Triticum durum (durum wheat), 4x	EMS	1/50	-	-	-	Henry *et al*., 2014
Glycine max (soybean), 4x	EMS or MNU	1/140 to 1/550	90	-	-	Cooper *et al.*, 2008
Oryza sativa japonica (rice), 2x	EMS and SA-MNU	1/265 to 1/294	70.4 to 66.7	0	29.6 to 33.3	Till *et al.*, 2007
Oryza sativa (rice), 2x	MNU	1/135	92	-	-	Suzuki *et al.*, 2008a
Hordeum vulgare (barley), 2x	MNU	1/504	23	33	37	Kurowska *et al.*, 2011
Hordeum vulgare (barley), 2x	SA	-	86	14	-	Olsen, Wang and von Wettstein, 1993
Hordeum vulgare (barley), 2x	SA	1/374	95.5	-	4.5	Talamè *et al.*, 2008
Hordeum vulgare (barley), 2x	SA-MNU	1/477	88	4.5	7.5	Szarejko *et al.*, 2017

2.3.GUIDELINES FOR CHEMICAL MUTAGENESIS

This section provides general guidelines for chemical mutagenesis of both seeds and vegetative propagules, including *in vitro* cell cultures. Many factors can influence the outcome of chemical mutagenesis including the characteristics of the target plant material, the dose of the chemical applied, the physico-chemical properties of the chemical mutagen, the nature of the mutagenic solution (e.g. pH), the environmental conditions of the laboratory (e.g. temperature) as well as the growing conditions (greenhouse, nursery, field, *in vitro*, etc.) of the plant seeds and/or propagules before and after the mutagenic treatment.

2.3.1. **Target plant materials**

The choice of the most suitable material depends on the objectives of the mutagenic treatment and the plant species. Different types of plant propagules are described further below, including seeds, so called '*in vivo*' vegetative propagules and *in vitro* explants or tissues. The genetic constitution of the target material such as heterozygosity or ploidy level is also a critical consideration for applied mutagenesis studies. This aspect is covered in Chapters 1, 2, 3, and 4 of this manual.

Seeds are the most commonly used target tissue for chemical mutagenesis. For example, seeds of cereals or legumes can be easily stored and shipped and treated in large quantities. Over the past few decades, standardized protocols for EMS mutagenesis of many seed crops have been established and routinely used in various laboratories.

Soaking the seeds in the mutagen solution is the most convenient and most widely used method, thus small grain cereals and other seeds that imbibe rapidly are easy targets. Plant species and varieties may respond differently to a certain chemical mutagenic treatment. Likewise, the actual experimental conditions may vary from laboratory to laboratory. Therefore, it is highly recommended to always perform dose-response experiments for seed mutagenesis of new species or varieties prior to conducting a large-scale mutagenesis experiment. Two examples of protocols for EMS mutagenesis and one protocol for combined MNU and sodium azide (SA, NaN_3) mutagenesis of barley seeds are described below (see examples of treatment procedures section 2.5.3).

It should be noted that one can also treat *in vivo* vegetative propagules and explants such as tubers, bulbs or corms, ramets, cuttings or scion woods, rooted cuttings or growing plants, bud woods, or stolons (see Chapter 6). The treatment procedures for such vegetative propagules, however, are less well established compared to seed mutagenesis protocols mainly because of technical challenges regarding the uptake

and penetration of the chemical in the plant tissue resulting in an uneven distribution of the chemical mutagen within the target meristem. Consequently such *in vivo* treatments may lack reproducibility. When the target material is small such as small cuttings or shoot tips, the challenges faced may be fewer.

In vitro explants are currently becoming a useful target for chemical mutagenesis. *In vitro* systems may offer several advantages such as the availability of more standardized conditions and the possibility to prevent or restrict the formation of chimeras (see Chapter 8). For example, successful EMS mutagenesis of banana shoot tip explants (Jankowicz-Cieslak *et al.*, 2012), and callus tissue of rice (Serrat *et al.*, 2014), wheat (Simonson, Baenziger *et al.,* 1991) and sugarcane (Purnamaningsih and Hutami, 2016) have been reported. Callus tissue was induced from bahiagrass seeds treated with sodium azide (Kannan *et al.*, 2015). A large mutant population of 19 630 plants was regenerated from these calli via somatic embryogenesis and a superior mutant line with improved traits was later identified in multi-locational field trials. Collectively, these studies demonstrate that EMS mutagenesis can be like in the case of seeds applied to *in vitro* explants for the recovery of superior mutant crops with improved traits. A protocol for EMS mutagenesis of *in vitro* banana shoot tips is described in section 2.5.

To our knowledge, the frequency and types of mutations induced using *in vitro* cell cultures has only been determined in a few cases, e.g. banana (Jankowicz-Cieslak *et al.*, 2012) and rice. The molecular mutation spectra in populations derived from *in vitro* explants in these studies were consistent with results obtained from EMS mutagenized seeds.

A wide range of plants can be regenerated from single cells via *in vitro* tissue culture (see Chapter 8-A). This provides an excellent opportunity for combining tissue culture protocols with mutagenesis techniques. As is the case with radiation treatment of VPCs, appropriate tissue culture techniques will greatly facilitate the regeneration of a whole homohistont plant from a single cell to avoid the development of chimeras.

The ideal plant material for chemical mutagenesis would be haploid cells, especially those that can be manipulated to produce doubled haploids (this is discussed further in Chapter 8-B). Chemical mutagenesis of pollen has been extensively described for maize (Neuffer, 1994). In fact, this has become the method of choice for chemically induced mutations in maize for several decades, primarily because it avoids the creation of chimeric plants that may or may not transmit induced mutations to progeny.

2.3.2. Dose, dose determination and mutational load

The two most commonly used experimental variables to describe dose in the context of chemical mutagenesis are the concentration of the chemical in the mutagenic solution and the duration of the treatment (dose = concentration × duration).

In practice, when the optimal dose for a specific crop (or cultivar), target tissue, or chemical mutagen cannot be found in the literature, a dose-response curve needs to be established. This is equivalent to conducting a radio-sensitivity experiment for physical mutagenesis. The dose-response curve, also called 'kill curve' or 'chemical toxicity test', establishes the relationship between the survival rate or growth reduction of the propagules after treatment with increasing concentrations of the mutagenic chemical(s) during specific periods of time.

In the case of seeds, a seedling test is conducted whereby seed germination and seedling survival or growth can be measured after the mutagenic treatment. For vegetative propagules such as *in vivo* cuttings or *in vitro* cell cultures, similar methods to measure growth or survival of the propagules can be followed. Example of dose-response curves for *in vitro* banana shoot tips and barley seeds are described in Section 2.5.

It should be noted that the dose-response curve for chemical mutagenesis may considerably differ from the one from radio-sensitivity tests, this is due to the specificity of chemical toxicity on cells. According to van Harten, (1998), a 20 – 30 percent growth reduction (which may correspond to a survival rate of 70 – 80 percent) may produce an optimal mutation yield in cereal crops. The duration of the treatment is also relevant, it should enable proper uptake of the chemical mutagen by the plant tissues. In case of seeds, the duration may be shortened when using pre-soaked seeds (see also section 2.3.5.). Typically, in a dose-response experiment, the seeds or explants will be exposed to increasing concentrations of the mutagen over different durations.

The volume of the treatment solution may also play a role: the volume should be sufficiently large to provide each seed (or propagule) the opportunity to absorb the same amount of mutagen. For example, 0.5 – 1ml is recommended per seed in the case of small grain cereals. To ensure a uniform concentration of the mutagen throughout the treatment, the solution should be gently shaken.

The temperature of the mutagenic solution greatly influences the treatment, mainly because of the effect of temperature on the reactivity of the chemical (see section 2.3.4.). Some authors recommend short time treatments of 0.5 – 2h at temperatures of about 20 – 25°C to seeds that have been pre-soaked for different times at room temperature (= pulse treatment). These conditions facilitate the absorption of

the mutagen, increase the metabolic activity of the seed and enhance the reaction between the chemical and the genetic target. The optimal dose ultimately depends upon the desired goal of the mutagenesis experiment or breeding programme. As illustrated in Table 2.1, mutant populations with a high density of mutations have now been produced for reverse genetic studies of the major cereals and legumes.

In fact, in reverse genetics one identifies an altered sequence and then proceeds to determine its effect, if any, on the phenotype. To increase the efficiency of this process, it becomes imperative to induce a high mutational load per line to reduce the size of the mutant population needed. For example, in the case of wheat, single mutant lines can carry, on average, several hundred thousand mutations.

This high mutational load can have significant implications for practical plant breeding because these background mutations may disrupt the finely tuned genetic constitution of the elite parental line. From a practical perspective, plant breeders may therefore, consider applying lower doses and increase the size of the mutant population similarly to the mutant population development methods described when using physical mutagenesis (see Chapters 1, 4 and 6). When attempting to modify just one or two characteristics, doses inducing less than 30 percent growth reduction have been recommended for plant breeding projects (Maluszynski *et al.*, 2009).

Different chemical mutagens can be combined to broaden the mutation spectrum. An example of a combined treatment of SA with MNU for barley seeds is given in section 2.5.3. Similarly, chemical mutagenesis can be combined with physical mutagenesis treatments to broaden the spectrum of mutations.

2.3.3. **Status of plant materials**

It is usually preferable for seeds as well as for vegetative propagules to apply the treatments while they are in an actively growing stage. Various methods exist to stimulate or enhance the efficiency of chemical mutagenesis in case soaking the seeds or plant propagules is not feasible or effective, as illustrated below.

a. Seeds of bahiagrass were scarified, surface sterilized and treated with SA. Thereafter, callus tissue was induced *in vitro* and plants were regenerated via somatic embryogenesis to produce M_2 mutant progeny (Kannan *et al.*, 2015).

b. Treatment of an inflorescence (or bud) can be performed by covering these plant parts with a wad of cotton wool soaked in the chemical (van Harten, 1998).

c. A mutagen may be applied in low concentrations to the growth medium and allowed to enter the plant through the roots. This simple method offers

advantages when studying chronic mutagen exposures, or for determining the sensitivity of different stages of growth and development to the chemical mutagen.

d. A frequently used protocol for EMS mutagenesis of pollen in maize involves the use of paraffin oil to prepare an emulsion of the mutagen and the pollen thereby avoiding lysis of the pollen in the aqueous solutions (Weil and Monde, 2009).

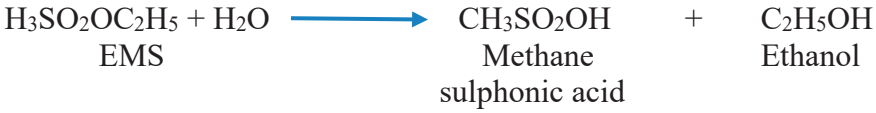
Plant materials less suited to chemical mutagenesis include those that do not readily imbibe the chemical solution; this includes woody tissues and seeds with thick shells (e.g. nuts) and plant parts that are dormant. Still, various pre-treatments can be utilized to break dormancy or to increase cell permeability and adsorption of the chemical mutagen such as scarification or similar methods.

2.3.4. **Physico-chemical properties of chemical mutagens and mutagen solutions**

Properties of mutagens that limit their effectiveness are their (a) solubility, (b) toxicity, and (c) chemical reactivity. The useful range of concentrations is restricted by the solubility of the mutagen in the treatment solution as well as by its toxic effects on the plant propagule.

Mutagens vary widely in their toxicity. In general, the methylating agents, e.g. MMS, are more toxic than their corresponding ethylating agents, e.g. EMS. Even though methylating agents are more mutagenic than ethylating agents, the efficiency of e.g. MMS is lower than EMS because of the higher toxicity of the MMS resulting in a higher level of damage to the plant propagule, which in turn results in a lower survival rate after the mutagenic treatment.

The alkylating agents are very reactive agents and will hydrolyse in a water solution. This implies that solutions must be prepared fresh and never stored. The reaction with water usually gives rise to compounds that are no longer mutagenic but can still be harmful or toxic for the operator. For EMS, the hydrolysis reaction is as follows:



The rate of hydrolysis of a chemical mutagen is usually measured by its half-life. For a given compound, the half-life is a function of the temperature and sometimes of the pH. For example, in case of the alkylating agents, the hydrolysis rate decreases with decreasing temperature thus, a mutagen will be stable for a longer time at lower

temperature, ensuring its reactivity with the nucleophilic centres in the target. The pH is especially important for ethyleneimine derivatives, sulphur and nitrogen mustards and some nitroso-compounds, which should always be dissolved in buffers of well-defined pH, usually below 7.

In terms of chemical reactivity, the alkyl alkane sulphonates and alkyl sulphates produce strong acidic products upon hydrolysis in the mutagenic solution, as well as, inside the cell. Therefore, significant physiological damage may occur in unbuffered solutions. This can lower the mutagenic efficiency through reduced M_1 plant survival. The negative effects of hydrolysis can be greatly reduced by properly balancing the solutions with buffers. The pH of the solution should hence be monitored before and after the treatment.

DMSO (dimethyl sulphoxide) is known to increase cell permeability and enhance absorption through biological membranes and thus, it has been tested as a carrier in chemical mutagenesis. At the PBGL in the Joint FAO/IAEA Agriculture and Biotechnologies Laboratories in Seibersdorf, Austria, the mutagenesis is conducted in a 2 percent DMSO solution to ensure solubilization of the EMS. Amin, Laskar and Khan, (2015) showed that the action of the chemical mutagen MMS alone and in combination with DMSO induces physiological, biochemical, metabolic, and genetic disturbances which results in significant bio-morphology and quantitative variations in lentil (*Lens culinaris*) but they also demonstrated enhancing effects of DMSO on MMS mutagenicity.

2.3.5. **Pre- and post-treatment procedures**

In general pre-soaking the seeds prior to mutagen treatment increases the efficiency of mutation induction by activating metabolic processes and DNA synthesis in the cells (IAEA, 1977). Thus, pre-soaking seeds or buds triggers the transition from a dormant status to an actively metabolizing and synthesizing stage. Pre-soaking may also speed up the uptake of the mutagen by increasing the cell membrane permeability. Several important changes take place in seeds when they are soaked. These depend to some extent on the conditions of soaking (duration, temperature, soaking solution) and on the type of seed or plant propagule. The duration of pre-soaking of seeds in water can be estimated experimentally. Seeds should stay in the solution, as long as, they actively absorb it. To optimize the duration of pre-soaking a pilot experiment can be carried out whereby the incubated seeds are weighed every hour to determine when their increasing weight reaches a plateau. The pre-soaking should not be shorter than the time estimated experimentally.

Post-treatment handling procedures up to initiation of growth can also affect the mutagenic efficiency of a treatment. Important factors are the duration and temperature of storage of the treated seeds. Storing of mutagen-treated (M_1) seeds

mostly enhances injury. However, post-washed and rapidly re-dried seeds can be stored at 0°C to 4°C for long periods without seriously altering the mutagenic effects, since the post-washing rapidly removes both non-reacted chemicals and their hydrolytic by-products from the seeds.

Various methods of post-treatment washing and/or drying have been used. Treated and post-washed seeds may be simply air-dried by laying them out on a blotting paper. Drying time may be reduced using an electric fan blowing over the seeds or under a ventilated fume hood. Elevated temperature drying may also be convenient, but in this case the temperature used should not exceed 35°C and uncontrolled heating should not be used. Post-treatment drying is especially desirable for convenient handling and shipping of mutagen-treated M_1 seeds. With most alkylating agents increased damage may occur on re-drying and storage of seeds. Several factors seem responsible for the phenomena observed including: (1) the hydrolysis rate of the mutagenic agent; (2) the enzymatic actions in the biological system; (3) and the uptake of hydrolysis by-products by the treated seeds or explants. Scientists and breeders must therefore consider the specific requirements of the specific crop and carefully plan any further laboratory, greenhouse or field activities before initiating a mutation breeding programme using chemical mutagens.

2.3.6. **Advantages and limitations of chemical mutagenesis**

The advantages and limitations of chemical mutagenesis for experimental plant mutagenesis or plant breeding have been previously summarised (van Harten, 1998). Taking into consideration the more recent findings pertaining to plant chemical mutagenesis, these can be updated as stated below.

Advantages

- Well characterized mutation spectrum producing mainly point mutations.
- Less chromosomal damage when compared to physical mutagens.
- High mutation frequency allows to create allelic variation at any target gene.
- Mutations appear to be evenly spread across the entire genome.
- Standardized protocols for seed treatment of the major seed propagated food crops.
- Can be equally applied to *in vitro* tissues or explants.
- EMS mutagenesis can be applied in a standard laboratory setting.

Limitations

- Dense mutation rate, this may require several rounds of backcrossing to remove undesirable mutations.
- Penetration in multi-cellular or woody plant tissues is often difficult or has low reproducibility.
- Materials or seeds that are dormant or have long germination times, e.g. nuts, may require special pre-treatments or manipulations.
- Limited repertoire of well-characterized chemical mutagens for plant mutagenesis.
- May not be effective to induce large chromosomal variations that are heritable.
- Health and safety concerns due to toxic or carcinogenic properties.

2.4.STORAGE, HANDLING AND DECONTAMINATION PROCEDURES OF CHEMICAL MUTAGENS

Most chemical mutagens are potential carcinogens and therefore, appropriate health and safety issues need to be fully understood and complied with. The purpose of this section is to provide basic information relative to the storage, handling and clean-up of the commonly used chemical mutagens described in this chapter, i.e. EMS, MNU and SA.

It is highly recommended that chemical mutagenesis is carried out by trained personnel in specialised facilities.

One should always practise the following laboratory safety procedures.

- Wear the correct personal protective equipment such as gloves, safety goggles, and long-sleeved lab coats.
- Perform chemical mutagenesis under a functional fume hood to ensure disposal of chemical vapours.
- Store the chemicals in a designated area with the appropriate hazards sign and ventilation if required.
- Consult the Material Safety Data sheet (MSDS) which is an important component of occupational safety and health and product stewardship.

Additional information on the safety, biological activity and properties of other chemical mutagens can be retrieved from the Pubchem database (https://pubchem.ncbi.nlm.nih.gov/).

2.4.1. Alkyl alkanesulphonates and alkyl sulphates

Common example

- Ethyl methanesulphonate (EMS)

Physico-chemical properties

- Generally, liquids, highly soluble in organic solvents, slightly soluble in water; undergo hydrolysis forming strong acid with the rate of hydrolysis largely dependent on the alkyl group. The reactivity of alkylating agents varies greatly and this is influenced by the nature of the mutagen solution or reaction medium.

Storage

- Store in a small, airtight bottle in a refrigerator, inside a sealed chamber containing a desiccant and protected from the light.

Clean-up

- At the Joint FAO/IAEA Plant Breeding and Genetics Laboratory, decontamination of the working surface, laboratory equipment or glassware that has come into contact with EMS is done using a freshly prepared 1 M sodium thiosulphate stock solution ($Na_2S_2O_3.10H_20$) diluted to 100mM. Special care should be taken when gram amounts or more of EMS or MMS need to be destroyed as violent reactions can occur with sodium thiosulphate solutions. In this case, large volumes of aqueous bicarbonate solutions are recommended.

Health hazards

- If the mutagen is accidently swallowed, induce vomiting. Drink saline or other alkaline solution. Get a physician to carefully examine the functions of the liver and kidney. Person with diseases of the central nervous system, kidney and liver should not work with these compounds.

2.4.2. **Nitroso compounds**

Common example

- *N*-methyl *N*-nitrosourea (MNU)

Physico-chemical properties

- Generally, exists in solid state, highly soluble in organic solvents, and reactivity dependent on pH of the solution.

Storage

- Store in small units (50-100 g) in a refrigerator. Avoid exposure to heat, friction, or impact. Hazardous above room temperature and therefore must always be kept cool.

Clean-up

- If the powder is spilled, dampen the powder and carefully brush onto a pan, empty it in a plastic bag. If the liquid is spilled, absorb with paper or vermiculite and scoop into a plastic bag. Sponge up the spillage with water followed by decontamination with a 10 percent ceric ammonium nitrate solution.

Dangerous reactions

- Nitroso compounds can undergo violent thermal decomposition. Above 200°C the vapour is known to explode.
- In the presence of alkali nitroso guanidine evolves diazomethane, which can explode even at low temperatures if traces of organic matter are present.

Health hazards

- Exposure to nitroso compounds can cause corneal ulcers, asthma, contact dermatitis, etc. Some of the diagnostic tests include prominent hilar shadows in chest X-rays and non-specific changes in electrocardiograms.

2.4.3. **Azides**

Common examples

- Mainly sodium and potassium azide

Physico-chemical properties

- Mainly exists in the form of crystalline salts. The alkali metals salts are relatively stable, but when in contact with water or acids they are readily converted to hydrazoic acid (HN_3). The acid form is volatile, boiling at 36°C.

Storage

- Store as alkali metal salts in small quantities in glass containers inside a refrigerator at 4°C. Do not store where accidental spillage or breakage of containers could cause inadvertent mixing with acids.

Clean-up

- Mop any spillage with excess water and soap or detergent. If spill is under acidic conditions, also ventilate the area. Wear self-contained protective breathing apparatus.

Health hazards

- Sodium azide is highly and acutely toxic by all routes of exposure, e.g. oral LD50 for rat was determined at 27 mg/kg. Hydrazoic acid is a toxic gas with a pungent odour.

Precautions

- For seed mutagenesis, sodium azide is most effective at acid pH and during the treatment the solutions are bubbled with oxygen or air. Under these conditions, HN_3 is readily volatilized. Therefore, all the treatments should be exclusively performed in a well-ventilated fume hood.

2.5.EXAMPLES OF TREATMENT PROCEDURES

As stated previously, chemical mutagenesis can be performed in both sexually propagated plants as well as in VPCs. Treatment procedures need to be optimised based on the plant material chosen, the type of mutagen as well as on the objectives of the mutagenesis experiment or breeding programme. For better understanding,

three detailed treatment procedures for seed propagated crops and VPCs chemical mutagenesis, which may be adapted to other experimental situations are described here.

1. A protocol for EMS mutagenesis of shoot apical meristems of banana (*Musa acuminata*).

2. A treatment procedure for mutagenesis of barley (*Hordeum vulgare*) seeds using EMS.

3. An efficient combined mutagenesis treatment procedure for barley seeds using sodium azide and *N*-nitroso-*N*-methylurea (NMU).

2.5.1. **Ethyl methanesulphonate (EMS) mutagenesis of *in vitro* banana meristem explants**

Below is presented the description of the treatment procedures, as modified and adapted from Jankowicz-Cieslak and Till, (2016).

2.5.1.1.Preparation

Prepare a sufficiently large number of *in vitro* shoot tip explants of banana (e.g. 1000 for bulk mutagenesis or 50 for establishing the dose-response curve) for each EMS dosage (concentration + duration). Select uniformly sized, healthy looking explants and distribute into autoclaved bottles (Figure 2.3). It is important to note that all items coming into contact with the *in vitro* tissues, including the mutagen, must have been adequately sterilised prior to the initiation of the experiment. Consider from the onset that three to four cycles of *in vitro* micro-propagation subcultures will be required to dissolve any chimeras. As a result, the original population size will increase significantly during this process. This may, however, be balanced by the loss of propagules due to injuries resulting from the mutagenic treatment. Thus, space, time and labour resources should be considered accordingly before initiating any chemical mutagenesis experiment.

2.5.1.2.Establishing the dose-response curve

In the absence of reliable information on optimal dose for conducting a mutagenesis experiment, a dose-response curve is typically established prior to conducting bulk mutagenesis. It is important to note that the frequency of induced mutations may be different in different genotypes and due to variations in experimental procedures. In the case of banana shoot tips, the experimental variable measured for determining the optimal dose is the reduction in fresh weight in relation to the different EMS concentrations and different incubation periods.

Figure 2.4 illustrates the growth reduction and lethality of *in vitro* banana shoot tips with increasing EMS concentrations. Based on these results, the optimal doses for bulk mutagenic treatment were selected. This protocol has been successfully applied to other vegetative propagules from Jatropha, potato and cassava and could be further adapted to other *in vitro* explants such as embryogenic callus, nodal cuttings, *in vivo* cuttings, etc.

2.5.1.3.Mutagenesis treatment

Prepare fresh 1M sodium thiosulphate stock solution and dilute to 100mM. This solution will be used for deactivation of EMS as well as for the decontamination of working surface and laboratory equipment that has come into contact with EMS.

Calculate the volumes of EMS and DMSO needed, dispense required volumes of distilled water and autoclave. Let the liquid cool to room temperature; add DMSO using a sterile pipette tip and required volume of EMS using a sterile syringe and filter membrane. Shake the EMS/DMSO solution vigorously for 15 seconds for optimal solubilization.

Pour EMS mixture into each bottle containing the *in vitro* plant material ensuring that the tissue is fully immersed in the liquid.

Incubate at room temperature on a rotary shaker at 150 rpm for the pre-determined length of time. If needed, adjust the rotation speed so that tissues are gently and regularly moving.

2.5.1.4.Post-treatment

After incubation, fill the bottles with sterile water, mix gently and immediately decant carefully into an empty beaker using a sterile sieve to capture any material that may accidentally fall out of the bottle (Figure 2.5.).

It is important to dilute and remove as much EMS solution as possible while maintaining a sterile environment. It is however advisable to leave a small amount of liquid in the bottle rather than risk having material drop into the sieve and become contaminated. Repeat this washing step four times. After the final wash, pour tissue into a sterile sieve over a beaker. Note that although the tissue is washed, a strong smell of DMSO may remain. Using a sterile forceps transfer tissue to a Petri plate containing sterile water and then, carefully transfer rinsed tissue culture material into the growth medium. Incubate mutagenized material following standard procedures established for the investigated crop. The next day transfer all treated plantlets into fresh growth medium to remove any residual DMSO. Tissue growth should be monitored regularly.

Wipe the laminar flow hood with a damp paper towel soaked in sodium thiosulphate followed by a water rinse to ensure there is no remaining trace of EMS contamination in the work area. Decontaminate also all laboratory equipment that has come into contact with EMS.

2.5.1.5.Conclusions

The treatment procedures described here are based on several studies at the Joint FAO/IAEA Plant Breeding and Genetics Laboratory, Seibersdorf, Austria. Figure 2.6 shows a stable mutant phenotype induced with EMS mutagenesis using this protocol. With modifications these treatments can be successfully adapted to other VPCs or other *in vitro* plant materials.

Because the target meristematic tissues are multi-cellular in origin, tissues with a genetic mosaic structure may arise immediately post-mutagenesis. Repeated sub-culturing is performed to reduce genotypic heterogeneity. Eventually, sub-culturing will result in clonal propagation of fixed mutant alleles (see Chapter 6). This should be considered when developing a mutant population.

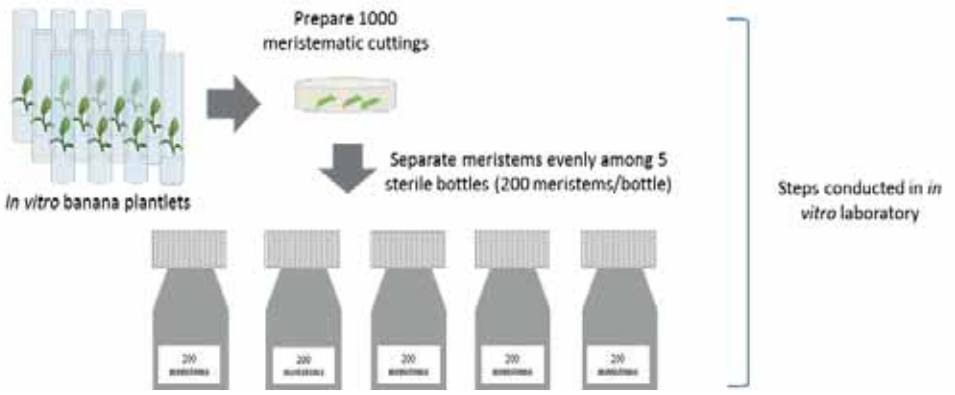


Figure 2.3. Preparation of banana in vitro materials for chemical mutagenesis. Steps include tissue multiplication under aseptic conditions and transfer of explants into autoclaved bottles, here 200 meristems/bottle, for transfer to the chemical mutagenesis laboratory.

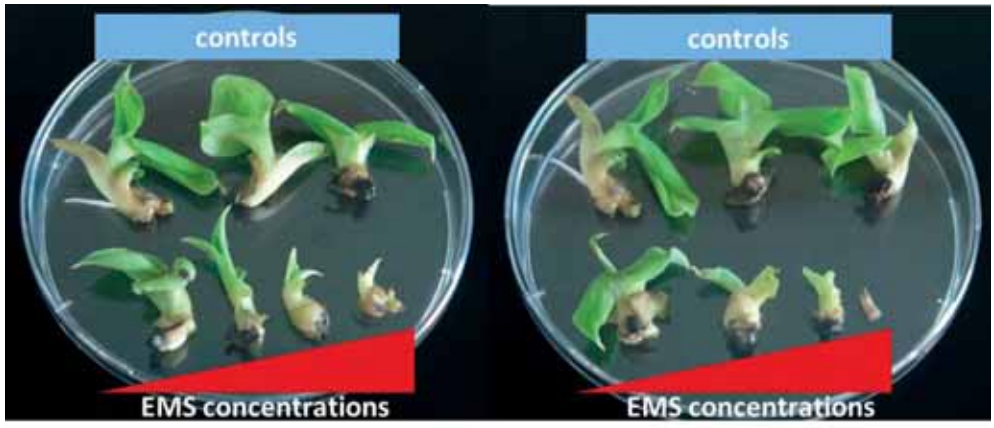


Figure 2.4. Establishing EMS dose-response curve for in vitro banana shoot tip explants. Explants below: different EMS concentrations, from left to right: 0.25%; 0.5%; 1% and 1.5% EMS. Explants on top: different types of controls, from left to right: water, DMSO and untreated explants. Plate left: 2 hr incubation; Plate right: 4 hr incubation. Figure adapted from (Jankowicz-Cieslak and Till, 2016).

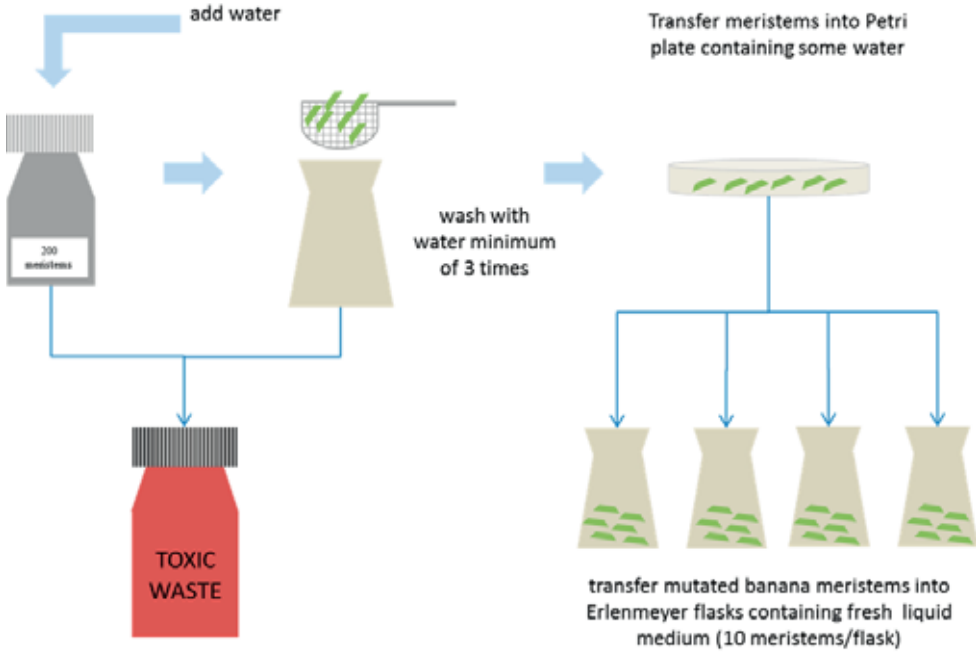


Figure 2.5. Post-treatment washing of banana in vitro explants. Treated meristems need to be carefully washed after EMS treatment to remove the remaining EMS solution. After a minimum of 3 washes, the explants are placed on Petri plates, sealed with parafilm and moved back into the in vitro laboratory. Mutated banana explants should be immediately transferred to fresh liquid growth medium. Figure adapted from Jankowicz-Cieslak et al., 2012.

Figure 2.6. A mutant banana plant exhibiting a stable rolled leaf phenotype induced via EMS mutagenesis. Courtesy of J. Jankowicz-Cieslak.

2.5.2. **Ethyl methanesulphonate (EMS) mutagenesis treatment of barley seeds**

This is a three-day protocol consisting of three major steps as modified and adapted from Konzak and Mikaelsen, (1977) and Jankowicz-Cieslak and Till, (2016). The first step involves pre-soaking the seeds overnight in water. On day 2, EMS is diluted to the desired concentration and added to the seeds for an overnight incubation. The EMS solution is removed on day 3. Seeds are then washed with a deactivating solution (100mM sodium thiosulphate) and rinsed with water before planting.

All steps involving EMS treatment and the post-treatment washes on day 2 and 3 are performed under a ventilated fume hood.

2.5.2.1.Preparation

Select uniformly sized seeds with 95 – 100 percent germination rate. If the information on seed viability is not available, it is advised to determine the germination percentage of the seed stock prior to the treatment with EMS.

The total number of seeds treated depends on the scale of the experiment, and whether a chemical toxicity test is being performed or a bulk mutagenesis. For chemical toxicity testing, approximately 200 seeds per treatment are sufficient. As mentioned previously, for a bulk mutagenesis for a reverse-genetic screen of diploid plants, 3000 to 6000 lines are typically sufficient for the recovery of mutant alleles in any gene. When attempting to improve one or two traits in an elite germplasm in a mutation breeding programme, this number may need to be adjusted as well as the optimal dose to reduce the high mutational load observed in reverse-genetic screens, as discussed in section 2.3.2. Either way, an excess of seeds should be treated considering that a percentage of the M_1 seeds will not germinate, and, in addition, a percentage of the M_1 plants produced will be sterile following the EMS treatment.

2.5.2.2.Pre-soaking

Estimate the best ratio of seeds to liquid for the EMS incubation. Add seeds to approximately 1/5th of the total beaker's volume. Add distilled (or deionized) water to approximately 1/3rd of the total volume and place on the orbital shaker. Adjust the rotation speed of the shaker so that all seeds can freely move in the water. Split seeds into multiple beakers with reduced volume to avoid spillage. Soak the seeds for 12 – 20 hours at 20 – 22°C (~room temperature).

After this period of pre-soaking the uptake or diffusion of the mutagen has reached its optimal rate or speed, which means that the maximum amount of mutagen can penetrate the embryo in the shortest possible time. At this stage the coleoptile and radicle have begun to emerge in case of barley or other small grain cereals.

Drain the water carefully measuring the volume poured-off to estimate the volume of the EMS solution as well as the wash solution to be added to the seeds during and post-treatment, respectively.

2.5.2.3.Mutagenesis treatment

All steps are to be carried out under a well-ventilated Biohazard fume hood.

Prepare fresh 1M sodium thiosulphate stock solution and dilute to 100mM which will be used for deactivation of EMS.

The concentrations of EMS to be used can be estimated based on previously published studies of the species you work on. However, it is important to note that the frequency of accumulated mutations may be different in different genotypes or due to variations in experimental procedures. It is therefore advisable to perform a kill curve experiment using different doses of mutagen. An example calculation for EMS mixtures of different concentrations containing 2 percent DMSO (volume/volume) in a final volume of 1 L is shown in Table 2.2. Some publications use molarity of EMS rather than percentage. Conversion between percentage and molarity is accomplished using the formula weight for EMS (124.16 g/mol). EMS is not easily soluble in water so DMSO is added to 2 percent to improve solubility.

The concentrations of EMS to use can be estimated based on previously published studies of the species you work on. However, it is important to note that the frequency of accumulated mutations may be different in different genotypes or due to variations in experimental procedures. It is therefore advisable to always perform a kill-curve experiment using different doses of the mutagen. EMS is not easily soluble in water so a 2 percent (volume/volume) DMSO is added to improve EMS solubility. An example calculation for EMS mixtures of different concentrations (in mM) containing 2 percent DMSO in a final volume of 1 L is shown in Table 2.2. Some publications use percentage of EMS rather than molarity. Conversion between percentage and molarity is accomplished using the formula weight for EMS (124.16 g/mol). The EMS/DMSO solution should be thoroughly mixed. The mixture is prepared in a bottle sealed with a screw cap, and then shaken vigorously before adding to the seeds. Test the bottle first by mimicking the shaking procedure using water in the ventilated fume hood to ensure that the bottle does not leak. Carefully add the required volume of EMS solution to the beaker containing the seeds. Avoid adding excess EMS/DMSO solution to the beaker with seeds as this may result in spills during orbital rotation. Set the orbital shaker to the appropriate speed and incubate for the set length of time.

TABLE 2.2. DIFFERENT CONCENTRATIONS OF EMS MIXTURE CONTAINING DMSO

Final EMS concentration (mM)	0	20	30	40	50	60
Volume EMS (ml)	0	2.1	3.1	4.1	5.2	6.2
Volume DMSO (ml)	20	20	20	20	20	20
Volume Water (ml)	980.0	977.9	976.9	975.9	974.8	973.8

2.5.2.4.Post-wash

Decant the EMS solution and pour into the toxic waste bottle. Be very careful when pouring off liquid to avoid splashes. A mesh screen can be placed into a funnel to capture seeds that may be unintentionally poured out of the beaker. Add 100mM sodium thiosulphate to the mutagenized seeds and incubate for 15 minutes on the orbital shaker. Repeat this step for a total of 2 washes with sodium thiosulphate. Add deionized water to the beaker and incubate for 10 minutes under orbital rotation, repeat this step for a total of two rinses.

Pour all liquid into the toxic waste bottle. Decontaminate the entire working area as well as all the tools and the glassware that came into contact with EMS using a100mM sodium thiosulphate solution.

After post-washing the seeds should either be given a short surface drying or be planted in the field as soon as possible. This is called the wet treatment. If the seeds cannot be planted soon after post-washing, they should be readily dried to a moisture content of approximately 13 percent to prevent any further physiological damage. A simple practical procedure is to let the seeds dry on filter paper on a laboratory bench at room temperature (20 – 25°C). This procedure is called the dry-back treatment. Under these conditions the seeds will remain dormant and can maintain a good germination capability for several weeks. If longer storage time is required, storage at very low temperature is advisable.

The treatment procedures described here are based on several studies in the Joint FAO/IAEA Plant Breeding and Genetics Laboratory using barley and can be readily adapted to other small grains.

2.5.3. **Combined treatment of barley seeds with sodium azide and N-nitroso N-methylurea**

This example describes the application of a combined mutagenic treatment of barley seeds using sodium azide (SA) and *N*-nitroso *N*-methylurea (MNU) with a period of germination in between the two mutagenic treatments. This protocol results in a high frequency of point mutations in barley and was utilized to create a TILLING population of barley cv. 'Sebastian' (Szarejko *et al.*, 2017) and rice (Till *et al.*, 2007). Both mutagens induce mostly GC to AT transitions but in a different local sequence context (Kurowska *et al.*, 2011; Tai *et al.*, 2016). The objective of using two different mutagenic compounds in a combined treatment is to broaden the spectrum of induced mutations.

2.5.3.1.Preparation

Calculate the amount of seeds needed for the treatment. For a large-scale treatment use the number of seeds estimated on the basis of the evaluation of the mutagen effects on somatic cells. It is crucial to select well-filled and uniform barley seeds from a batch with a high germination rate (~100 percent).

Remember that in addition to DNA lesions in the nucleus and cytoplasmic organelles, mutagens can generate damage in all components of the cytosol and disturbances of the cell cycle. Therefore, mutagenic treatment can impair metabolism of cells in various tissues and organs and influence the growth and development of the M_1 plants. These effects, called 'somatic effects' are manifested by a delay in seed germination, reduction of plant emergence, growth reduction, appearance of chlorophyll defects, reduction of fertility and plant survival. The size of the M_1 population should thus, be calculated bearing in mind the lethality and sterility of the M_1 plants to guarantee sufficient seeds for the subsequent M_2 generation. It is worthwhile to organize a pilot experiment to compare the somatic and genetic effects induced by a range of doses. Such a pilot experiment will expand the procedure but will surely help in a proper selection of the optimal doses for the large-scale treatment.

If a complete pilot experiment is not feasible before performing a large-scale mutagenesis, it is essential to conduct a preliminary mutagenic treatment with different doses of mutagens to create a kill-curve and evaluate an optimal mutagen dose. For barley use the standard seedling assay of measuring seedling emergence and growth reduction. To perform such assay plant the seeds treated with a range of mutagen doses into pots filled with soil and covered with a 3-cm layer of sand. Seven to ten days after the mutagenic treatment cut-off all the seedlings near the surface of the sand, count their number and measure their length. Calculate the growth reduction separately for each variety, dose and replication (see Chapter 1). If you cannot plant M_1 seeds immediately after the treatment, completely dry the seeds on a filter paper and store them in plastic bags at 4°C until sowing time. The response to mutagens may differ between barley genotypes, therefore it is recommended to evaluate the optimal dose of mutagens separately for each genotype (Figure 2.7.). As mentioned earlier, the optimal doses may be different depending on the objectives of the mutagenesis experiment or breeding programme.

2.5.3.2.Pre-soaking

The seeds should be pre-soaked in distilled water before treatment with the mutagen for physiological activation. The amount of distilled water used in pre-soaking should be at least two to three times the volume of the dry seeds. Eight hours of pre-soaking at room temperature (20 – 24°C) is optimal for barley, but for convenience the seeds

can be pre-soaked overnight. The pre-soaking reduces the somatic effects of the chemical mutagen.

2.5.3.3.Mutagenic treatment

It should be stressed that most chemical mutagens are also strong carcinogens. For this reason, all steps of mutagenic treatment should be carried out under a well-ventilated Biohazard fume hood. Disposable gloves and a laboratory coat should be worn at all times when performing the treatments and dealing with treated seeds. Taking these precautions is especially important during treatment with MNU – a strong mutagen and carcinogen.

The mutagenic effect of SA depends on the acidic pH of the treatment solution (Nilan *et al.*, 1973). The doses of SA that are routinely used for the mutagenic treatment of barley seeds are within the range of 0.5 – 4mM for 3 – 5 hours (Nilan *et al.*, 1973; Maluszynski *et al.*, 2003), note that a dose as high as 10mM for 2 hours has been applied for the creation of a TILLmore population of cv. 'Morex' (Talamè *et al.*, 2008).

When a combined treatment with two mutagens is performed, the first regular protocol of mutagenic treatment is followed with the addition of a 5 – 6 hours inter-incubation germination (iig) period between treatments during which the seeds are incubated on a wet filter paper at room temperature.

Calculate the amount of solutions needed for the treatments (for all testing doses). For small grain seeds such as barley, prepare a volume ensuring a 0.5ml solution per one seed.

Prepare appropriate amount of fresh solutions of sodium azide and of MNU. MNU should be dissolved in dH_2O, whereas SA should be dissolved in a phosphate buffer with pH=3.0. To prepare the phosphate buffer at pH=3.0, use 54.436 g KH_2PO_4 to which 3.67 ml H_3PO_4 is added per 1L buffer.

When evaluating an optimal dose prepare the mutagenic solutions starting from the basic solution (the highest concentration used for treatment). Leave part of this solution for the treatment and dilute the rest to the other required concentrations. You can use a formula: $C1 \times V1 = C2 \times V2$, where: C1 is the concentration of the basic solution, V1 – volume of the basic solution, C2 – concentration of the solution required, V2 – volume of the solution required. Use a ventilated fume hood for the preparation of the mutagenic solutions. Before the treatment, pour out the dH_2O from the beakers containing thee seeds and rinse twice with tap water. Be cautious to completely remove the water after rinsing.

Perform the treatment, i.e. pour the mutagen solutions into the beakers with the pre-soaked and rinsed seeds. Keep the same order of combinations during the whole procedure, i.e. pre-soaking, rinsing, treatment, and rinsing after treatment. Perform the mutagenic treatment at room temperature.

After 3 hours of treatment with SA (the first applied mutagen), pour off the mutagen solution and thoroughly rinse the seeds (3 – 4 times) in tap water. Then put the seeds into trays containing a few layers of a filter paper, cover them with a wet sheet of the filter paper and keep for 6 hours at room temperature. Next, transfer the seeds into the labelled beakers and add the second mutagenic solution to be applied, i.e. MNU. Treat the seeds for 3 hours and then pour off the mutagen and rinse the seeds again 3 – 4 times in running tap water. The mutagen solutions should always be poured off into the toxic waste bottles and appropriately handled.

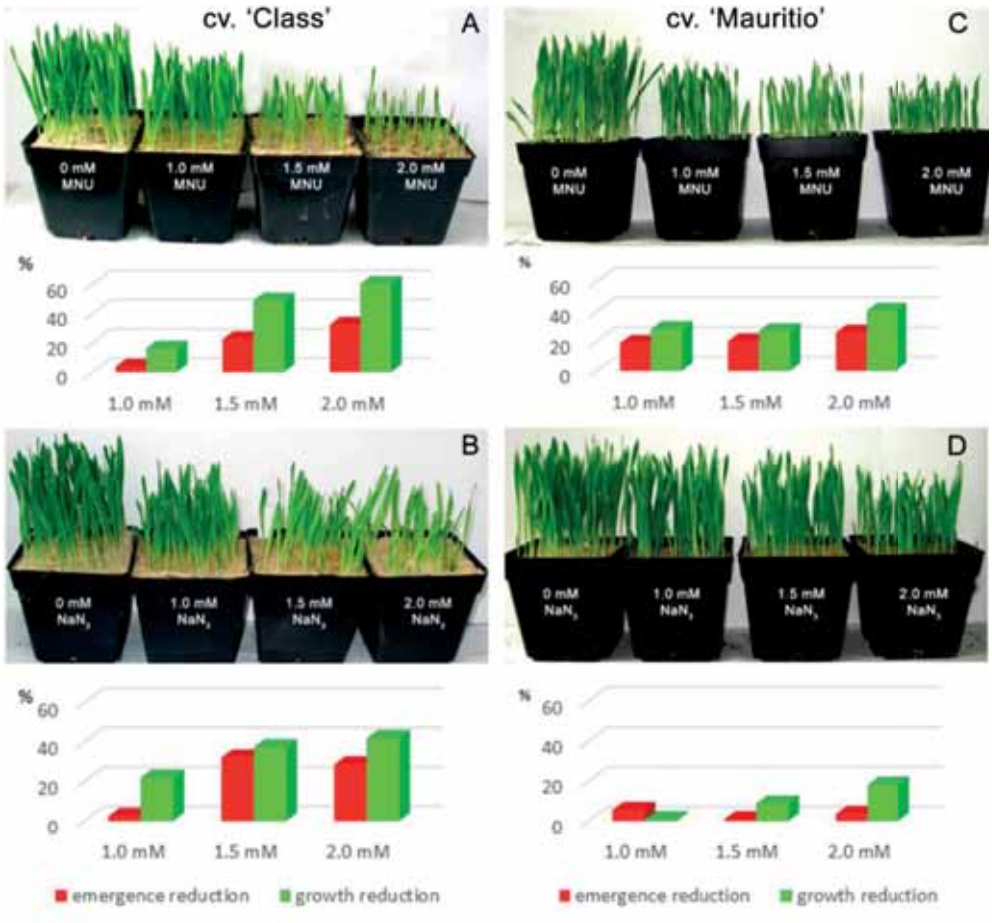


Figure 2.7. Differential sensitivity of barley cultivars 'Class' (A, B) and 'Mauritio' (C, D) to mutagenic treatment with MNU (A, C) and NaN_3 (B, D) based on seedling emergence and growth reduction.

For the barley cultivar ‘Sebastian’ that was used to create a TILLING population, we applied two different treatment combinations:

1. 1.5mM NaN_3/3 h – 6 h iig – 0.75mM MNU/3 h

2. 1.5mM NaN_3/3 h – 6 h iig – 0.5mM MNU/3 h

In both combinations the same dose of SA was used (1.5mM/3 h) while the dose of MNU was different. The treatment with the higher dose of MNU (0.75mM/3 h) caused a higher mutation frequency than the 0.5mM, but also resulted in a much higher sterility of M_1 plants (Szurman-Zubrzycka, pers. comm).

2.5.3.4.Post-treatment

An extensive post-treatment of rinsing in tap water is necessary to terminate the action of the mutagen and to remove any mutagenic residues from the surface of the seeds. To facilitate sowing, the treated seeds can be allowed to dry on a filter paper under a ventilated fume hood. Not, however, that a too intensive drying, especially at increased air temperature, can enhance the somatic damage effects of the mutagen.

2.5.4. **Conclusion**

Chemical mutagenesis has proven extremely useful to create new allelic variants that then can be used in functional genomics studies and/or plant breeding. Advantages include low-cost, a high density of variation and the technique can be applied to many species. Point mutations induced by EMS treatment can have varying effects on gene expression ranging from knockouts to (subtle) changes in protein function. Therefore, EMS can produce a range of phenotypes and provide an in-depth characterization of gene function. Mutagenesis using two different mutagens such as SA with MNU may lead to a broader spectrum and different types of mutations. Furthermore, relatively small populations are required to recover desired traits. However, the accumulation of a high density of induced mutations does imply that each plant line will harbour numerous mutations. Therefore, additional steps need to be taken, such as backcrossing, for the unambiguous assignment of the mutant gene causing the altered trait and to reduce or eliminate undesired mutations in the genetic background.

3. TYPES OF MUTATIONS

Mutations are heritable changes that occur in the genetic material of living organisms. These changes occur for various reasons and may be natural or induced. They can be recognized as phenotypic variants at different stages in the life cycle, but the primary effects are genetic (Lundqvist, Franckowiak and Forster, 2012).

3.1.PHENOTYPIC MUTANTS

All mutations occur in the DNA and various classes are listed in Section 3.2. However, for practical purposes including plant breeding the expressed mutant phenotype is usually the first description of a mutant. Phenotypic selection is a fundamental part of plant breeding and therefore, there has been a natural link between mutant phenotypes and their selection by plant breeders, which traditionally has been based on phenotypic selection. The exploitation of mutants via phenotypic selection often occurs long before the underlying genetic change is understood. Thus, for example, induced semi-dwarf mutants in rice in the USA were exploited in breeding new short stature varieties before the direct cause, a mutation in the gibberellin biosynthesis, gene was discovered (see Section 3.2.2).

Forster *et al.*, (2012) using barley as an example described a wide range of induced phenotypic mutants and how these may be classed and catalogued in various developmental stages.

- Seed
- Seedling development
- Vegetative growth
- Reproductive growth
- Inflorescence formation
- Spike development
- Meiosis and flowering
- Adult plant ripening

In the hands of plant breeders novel and desirable mutant traits are quickly detected and selected from field observations, they include agronomic traits, e.g. semi-dwarf stature and flowering time, pest and disease resistance and yield variation. Selected lines are then incorporated into breeding programmes using phenotypic mutant

descriptors. The classification of mutants at the phenotypic level also includes quality and nutritional traits (composition and content of proteins, oil, minerals, vitamins etc.).

Phenotypic mutants should not be confused with physiological disorders, which are non-heritable; these can often mimic mutations (Lundqvist, Franckowiak and Forster, 2012). This is particularly important in mutation induction and detection at the first mutant generation (M_1) is often weak and suffers from physiological disorders as a result of mutagen treatments. Thus, the M_1 cannot be used for mutant phenotypic selection. The M_2 generation is the first opportunity to apply phenotypic mutant screens (though, this is limited to single plant selection, see Chapters 4 and 5).

3.2.GENOTYPIC MUTATIONS

Genotypic mutations describe the primary mutant event in the genome, i.e. changes to the DNA sequences and are described in the sections below, but may also include epigenetic modifications, which often do not result in heritable mutations (Text Box 3.1).

Text Box 3.1.

Mutations can also be mimicked through epigenetic modifications. The most common modification is DNA methylation/demethylation, which, often triggered through environmental effects, can interfere with gene expression. Numerous examples are reported on DNA methylation changes in response to abiotic stresses, such as cold, salinity, drought, osmolality, or imbalance of mineral nutrition. One example is a methylated gene (Asr2) that in plants, such as tomato, is linked to alleviation of water stress response. Induction of artificial drought has led to demethylation in the regulatory region of this gene and thus, to its expression and adaption of the plant to the water-deficit stress condition (González, Ricardi and Iusem, 2013). Epigenetic changes in response to biotic stress are documented as well, for instance, the demethylation of resistance genes in rice after infection with the bacterial blight disease pathogen Xanthomonas oryzae (Li et al., 2012). Epigenetic modifications can be stably transmitted through mitosis or meiosis in the absence of the original inducing signal. In contrast to animals, the methylome in plants is maintained during sexual reproduction (Eichten, Schmitz and Springer, 2014; Quadrana and Colot, 2016). In a mutation breeding programme, it is important not to confound epigenetic changes with the nucleotide or chromosome changes that have been induced through the use of physical or chemical mutagens. In some cases, however, the borders between both may be unclear. Mutation of the ddm1 gene that is required to maintain normal cytosine methylation pattern in Arabidopsis has led to demethylation of mainly repetitive sequences and, subsequently, to activation of transposable elements (Jeddeloh, Stokes and Richards, 1999). In the next chapter the effects of transposable elements, which can induce new mutations in the genome, are described (see section 4.5).

The genome of the cell comprises DNA embedded in chromosomes, which are in the nucleus, but also in the organelles. In plants, both mitochondria and plastids also carry DNA and these are in the cytoplasm of the cell.

Mutations may thus happen in any DNA, those occurring in the nucleus will be transmitted to male and female germlines (sperm and eggs), whereas mutations in cytoplasmic (organelles) DNA may only be transmitted via the cytoplasm of the egg (though, in some rare cases, e.g. banana the cytoplasm of off-spring is donated via the sperm).

3.2.1. **Genome mutations**

The cell karyotype encompasses the entirety of the chromosomes in the nucleus, thus defining the organism with a number (n) of chromosome sets. Ploidy mutations comprise changes in the genome number, either in the form of a subtraction or an addition of a complete set of chromosomes, and/or a reduction of diploid (2n) to haploid (n) genomes which, can, for example, be spontaneous or experimentally induced (see Chapter 8.2.). In polyploid species, the haploids have more than one set of chromosomes and are called “polyhaploids”. Spontaneous or colchicine induced chromosome doubling in developing doubled haploid (DH) plants results in the generation of stable homozygous lines. These are genetically pure and fully fertile (having a balanced set of paired chromosomes at meiosis) and are thus invaluable in plant breeding and genetics. They are of outmost relevance in mutation breeding for fixing any recessive mutant allele (see Chapter 8-B). Detailed protocols for DH production in more than 20 crop species including cereals, vegetables and fruits have been described in Maluszynski *et al.*, (2003). One method of producing haploid embryos is via aberrant pollination using pollen killed or made impotent by irradiation. Here the pollen may stimulate the egg to develop as a haploid embryo without fertilisation (parthenogenesis). The method is fairly universal and can be applied to a wide range of species (see Germana, (2012) for practical examples).

Polyploidy induced either by genome duplication (autoploidy) or genome addition (alloploidy) has occurred naturally during the evolution of many plant species including crops, and has been used to produce new species, e.g. triticale, which combines genomes from wheat and rye. One effect of polyploidy is to increase the volume of the nucleus; this in turn increases the size of the cells, tissues, and organs and consequently of the entire plant, which at that stage may out-perform their diploid relatives. Polyploids can originate through doubling of somatic chromosome numbers from diploids, but it is suggested that in the wild, the most likely origin of polyploidy are 2n gametes exhibiting a modified gametogenesis due to errors in meiosis (Heslop-Harrison and Schwarzacher, 2007; Ortiz and Peloquin, 1992). Alloploids have additional advantages as the different genomes contain dissimilar sets of genes and

thereby enrich the gene diversity – by adding new genes – promote heterosis (hybrid vigour) and buffer deleterious mutations. Polyploids, spontaneous or induced have therefore been attractive for domestication and crop improvement. Examples of polyploid crops include:

- triploids: banana, watermelon and apple;
- tetraploids: cotton, groundnut, brassicas, durum wheat, leek, potato and tobacco;
- hexaploids: bread wheat, oat, triticale and chrysanthemum;
- octaploids: dahlia, strawberry, triticale and pansies.

In some species the polyploidy event is so ancient that it cannot be recognised by cytological techniques and can only be detected at the molecular level, e.g. by discovering gene duplications. Such paleopolyploids include: oil palm, maize, rice, rubber and soybean.

Deliberate induction of polyploidy began in the mid-20th century and was successful in vegetable crops where organ size is of particular importance (Akerberg and Hagberg, 1963). A more recent example is the development of the wheat/rye hybrid; triticale, which combines productivity traits of wheat with abiotic stress tolerance of rye. Triticale has been produced at the hexaploid and octoploid levels. The hexaploids are now established as a crop for marginal lands, usually not suitable for wheat cultivation (Hao *et al.*, 2013).

3.2.1.1.Chromosome mutations

The euploid (normal) number of chromosomes, their arrangement and their structure are generally established for each species. However, apart from the variation in the number of chromosome sets, i.e. n = × 1, × 2, × 3, × 4, etc., described above, several other types of alterations may appear as a result of mutations.

Aneuploidy

Aneuploidy is a category of chromosome mutation in which the chromosome number is abnormal (non-euploid). Generally, the aneuploid chromosome set differs from wild type by only one or a small number of chromosomes. Aneuploids can have a chromosome number either greater or smaller than the euploid chromosome complement. Aneuploid nomenclature is based on the number of copies of specific chromosomes. For example, the aneuploid condition 2n − 1 is called a monosomic (meaning “one chromosome”) because only one copy of some specific chromosome is present instead of the usual two (a pair) found in the euploid. The aneuploid 2n + 1

is called trisomic, 2n − 2 is nullisomic, and n + 1 is disomic, which is an aberration of a haploid organism. (Griffiths *et al.*, 2000). Such aneuploidy sets are common in polyploid species such as bread wheat.

Aneuploids can occur naturally, but are also produced in abundance in progenies of crosses where parents contribute unequal numbers of genomes and chromosomes. For a detailed discussion on the use of aneuploids in wheat see Law, Snape and Worland, (1987). Aneuploids such as monosomics can be generated by the application of low doses of X-rays, gamma-rays and fast neutrons to seeds, flower organs or pollen grains and have proven to be of great value in a number of plant species, particularly crop species, in developing genetic stocks and locating genes (Sanamyan *et al.*, 2011)

In triploid *Musa* cultivars, treatment of cell cultures with gamma-rays has been shown to result in a significant reduction of the chromosome number (Shepherd and Bakry, 2000). This demonstrates the need for determining the karyotype of mutagenized plant cells, particularly when they are derived from *in vitro* mutagenesis experiments. Roux *et al.*, (2003) demonstrated that flow cytometry can be applied to facilitate screening of mutagenized plant material. They irradiated shoot tips of the triploid (2n = 3x = 33) *Musa* clone 'Grande Naine' with gamma rays at 35 Gy from a ^{60}Co source and proceeded to ensuing sub-cultures up to M_1V_4 and identified aneuploid plants with chromosome numbers of 2n = 31 or 32.

Chromosomal rearrangements

Hermann Josef Muller in his Nobel Lecture entitled "The Production of Mutations" (1946) pointed out the effect of ionizing radiation on rearrangements of parts of chromosomes caused by "breakages of the chromosomes, followed afterwards by attachments occurring between the adhesive broken ends that joined in a different order than before." The Nobel Peace prize winner also reported on a clear dose-effect relationship that he and his co-workers detected.

DNA double strand breaks (DSB) are subjected to the cell's own repair mechanisms, which preserve the genetic stability/integrity. The main DSB repair pathways are: 1) homologous recombination (HR), and 2) non-homologous end joining (NHEJ) (Puchta, Dujon and Hohn, 1996; Waterworth *et al.*, 2011). For a comprehensive review on repair processes in plants see Manova and Gruszka (2015). The most prominent pathway for DSB repair is NHEJ. HR functions mainly during S and G2 phases of the cell cycle. However, both pathways are error prone and it is ultimately these "mistakes" that lead to chromosomal rearrangements; these can be deletions, duplications, inversions, insertions, or translocations (Figure 3.1). An example of chromosomal rearrangements analysed by genomic *in situ* hybridization (GISH) is shown in Figure 3.2.

Translocations

Translocations are of two main types: intra- or inter-chromosomal rearrangements. In intra-chromosomal translocations a transposition or shift of an interstitial segment within an arm or from one arm to the opposite occurs. Inter-chromosomal translocations can be terminal, whole arm, reciprocal or intercalary. Wang *et al.*, (2012) explained these four types of translocations as described below.

- Terminal translocations (Figure 3.1e) are the most common type, these involve the distal segment of a chromosome replacement by a segment of another (e.g. an alien) chromosome.
- When breaks occur within the centromeric region, it may result in a whole arm translocation, in which a whole new arm of a chromosome replaces that of another chromosome.
- A reciprocal translocation (Figure 3.1e-*i*) is a type of chromosome rearrangement involving the exchange of chromosome segments between two chromosomes that do not belong to the same pair of homologous chromosomes.
- A compensating translocation, in which a desired alien segment replaces an equivalent segment of a homoeologous (related) chromosome, is more likely to be beneficial. In an intercalary translocation, a chromosome segment is inserted into another; however, this seldom occurs as this requires several simultaneous breakage and reunion events. This type of translocation will be desirable when an alien segment containing desirable genes is inserted into a host chromosome without loss of host genes. This requires one break in the host chromosome and two breaks in the alien chromosome, with the desirable genes between the breaks. The excised alien segment has unstable ends, which can unite with the unstable ends of the host chromosome at the break point. If the inserted alien segment is quite short, it should not interfere with the pairing of the homologous host chromosomes, particularly if the chromosome with the insertion is made homozygous, e.g. by selfing.

Irradiation is used efficiently for inducing translocations in amphiploids or chromosome addition lines in order to introgress alien chromosome segments with useful genes, such as genes for disease resistance. In particular for wheat, numerous examples for the transfer of genes from alien chromosome using ^{60}Co-gamma irradiation of seeds or gametes are available (Friebe *et al.*, 1991; Mukai *et al.*, 1993; Liu, Chen and Liu, 1998; Liu, Chen and Liu, 2000; Chen *et al.*, 2013).

Inversions

In the case of inversions, a fragment of chromosome is re-ligated in the original place but after a 180° rotation (Figure 3.1c). Thus, the linear order of the genes is opposite of that of wild type. If the inversion involves the centromere it is known as a pericentric inversion (Figure 3.1c-*ii*), if not it is called a paracentric inversion (Figure 3.1c-*i*). Meiotic recombination events involving inversions can result in recombinants either carrying duplications or displaying deficiencies for parts of that chromosome. Pericentric inversions often give rise to new karyotypes. Paracentric heterozygotes usually form reverse loop pairing at prophase of meiosis. As a result of crossing-over within the loop, dicentric bridges and acentric fragments may appear at anaphase 1, leading to the formation of aberrant gametes that carry deletions, insertions, and either zero or two centromeres. Paracentrics are further characterized by a reduced recombination frequency due to abortion of cross-over chromatids and imperfect pairing of homologues. The paracentric heterozygotes give rise to pollen abortion, as shown in maize (Morgan Jr, 1950), bean (Sjödin, 1971), or barley (Ekberg, 1974) .

Chromosomal inversions have greatly contributed to species differentiation in nature. They are found as fixed differences between species and as polymorphisms within species in many groups of animals and plants. In some groups, speciation is associated with inversions and other changes in the karyotype (White, 1978). The evolutionary role of chromosomal inversions in plants has been extensively studied by Hoffmann and Rieseberg, (2008) and Lowry and Willis, (2010).

Segmental duplications

As described earlier, duplication of DNA sequences is one common form of mutation that occurred during the evolution of all organisms. Generally, if the size of the duplication is bigger than 1 kb in length it is called a segmental duplication (Figure 3.1b). Such duplications are found in maps of diploid and polyploid species, which for example could provide evidence for the hypothetical development of a diploid *Brassica* spp. from an ancestor with a lower than expected original basic chromosome number. Likewise, through comparative genomics, duplications of chromosomal regions in sorghum (Paterson *et al.*, 2009) and oil palm (Singh *et al.*, 2013b) were proven.

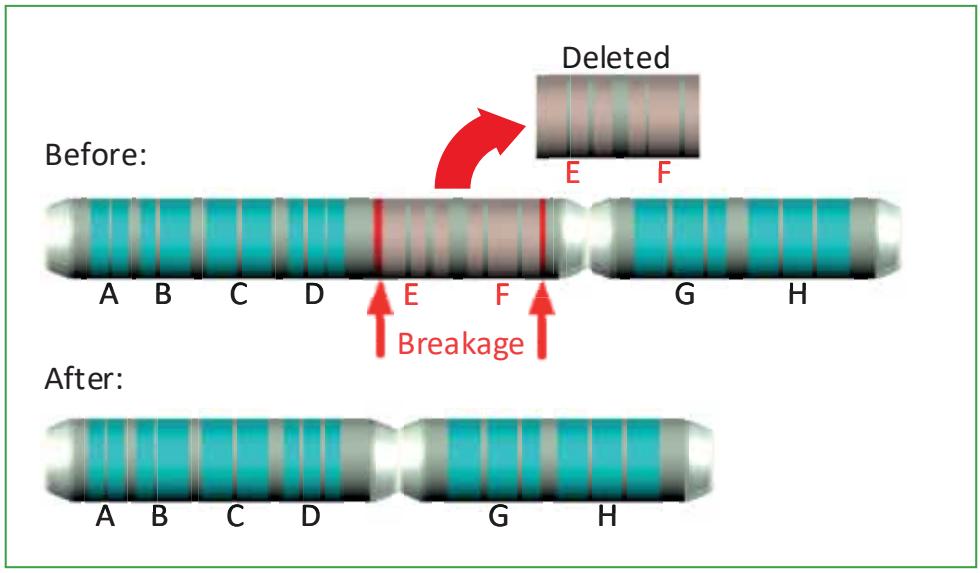


Figure 3.1a. Deletion: An interstitial part of a chromosome is lost after double breaks. After the end re-joined, the chromosome is shortened. The deleted fragment is acentric and will be lost if not inserted into another chromosome (see Insertion 3.1d. below).

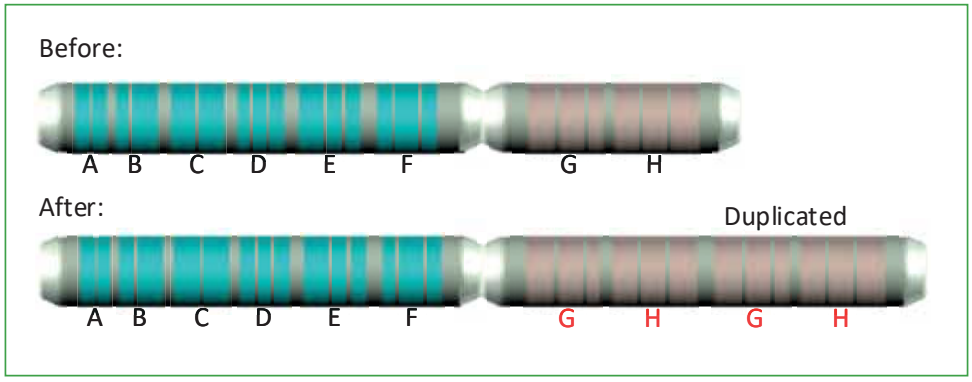
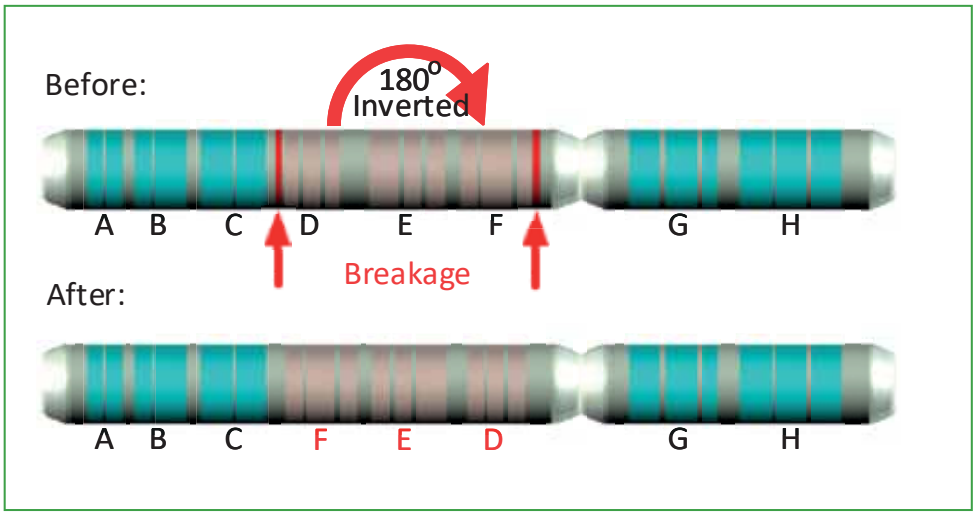
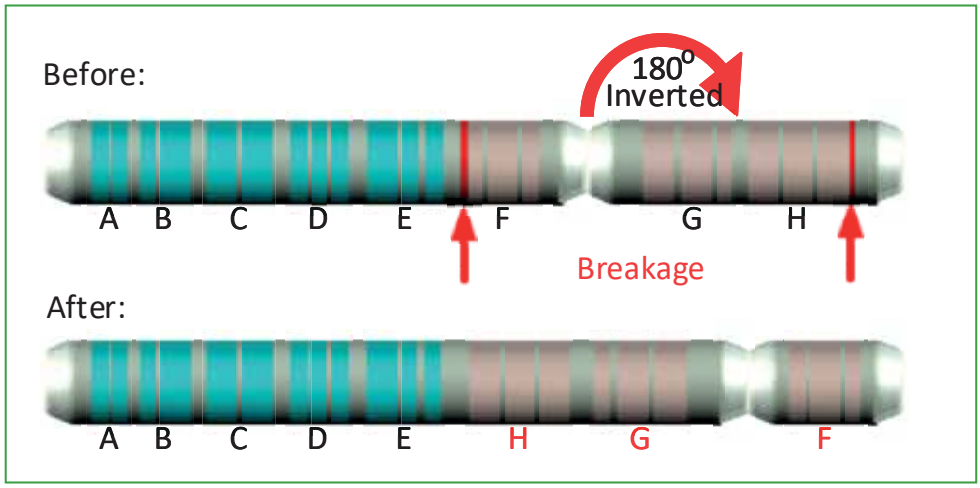


Figure 3.1b. Duplication: An extra copy of a chromosome region is produced. The duplicated region can be located adjacent to each other or can be unlinked.



(i) Paracentric inversion



(ii) Pericentric inversion

Figure 3.1c. Inversion: After two breaks in a chromosome the region between the breaks rotates 180 degrees before re-joining with the two end fragments. Since no genetic material has been deleted or duplicated, inversions generally do not cause phenotypic changes. Two types of inversions are differentiated: paracentric and pericentric. In paracentric inversions (i) the centromere is outside the inversion, whereas pericentric inversions (ii) include the centromere.

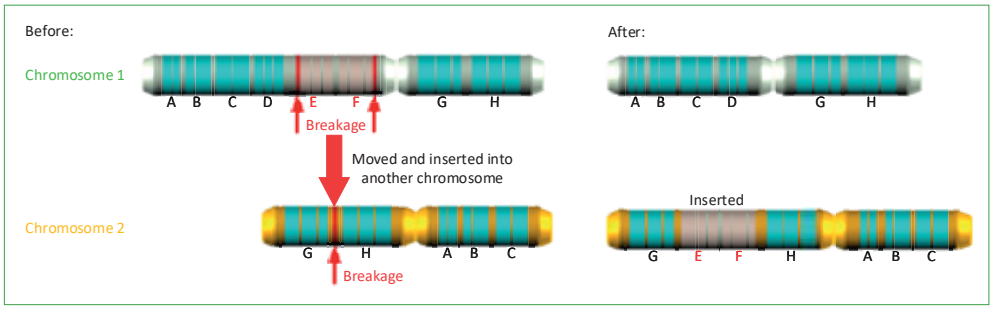
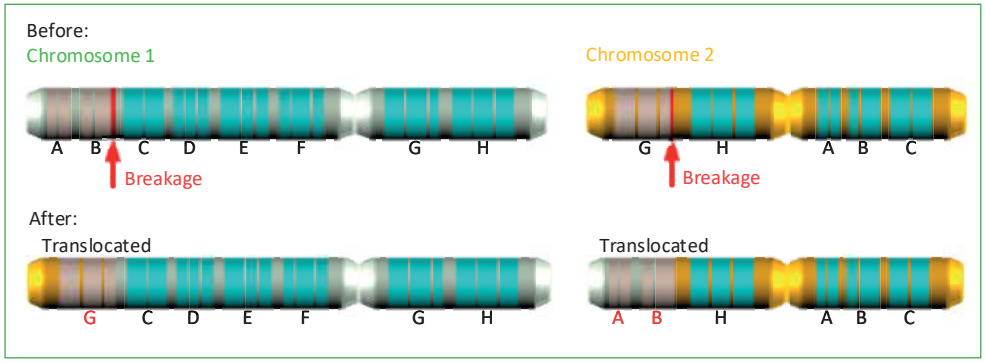
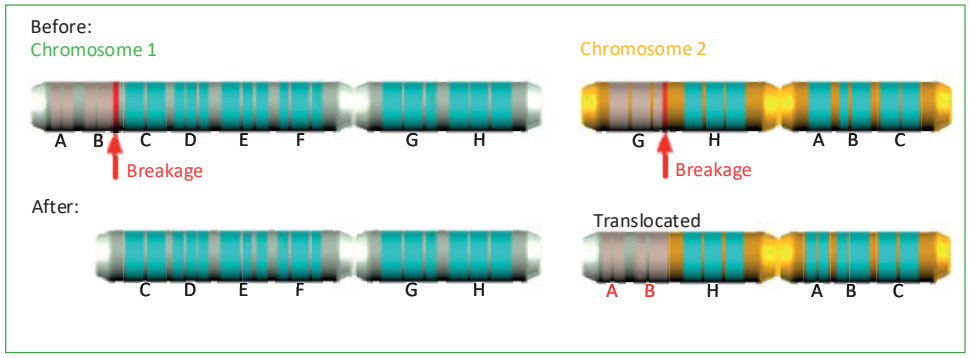


Figure 3.1d. Insertion: A deleted chromosome fragment resulting from a double break is inserted into another chromosome, which requires a single break in that chromosome and subsequent re-joining of the ends.



(i) Reciprocal translocation



(ii) Non-reciprocal translocation

Figure 3.1e. Translocations: Two non-homologous chromosomes exchange segments after breaks have been induced in both chromosomes; here they result in a terminal translocation. This exchange can be either reciprocal (i) or non-reciprocal (ii), if the segment of one chromosome is lost.

Figure 3.1. Major types of chromosomal mutations: (a) deletion, (b) duplication, (c) inversions, (d) insertion, and (e) translocation.

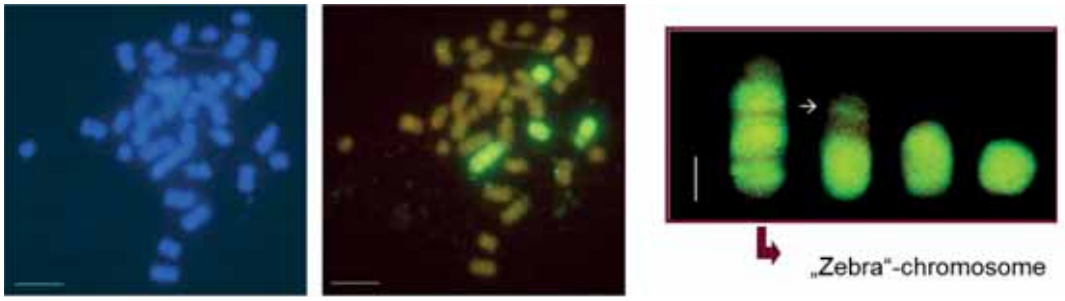


Figure 3.2. Example of chromosomal rearrangements, as a consequence of X-ray irradiation in a somatic hybridization experiment between Brassica napus and irradiated B. nigra protoplasts. Metaphase chromosome spreads are analysed by genomic in situ hybridization (GISH) (left: DAPI counterstaining; middle: GISH, B. napus orange, B. nigra yellow-green; right: detail with "zebra" chromosome resulting from multiple insertions of B. nigra fragments, arrow showing small B. nigra insertion in B. napus background), (Nielen et al., 1998).

3.2.1.2.Extra-nuclear mutations

As stated earlier in the chapter, apart from the nuclear genome of eukaryotes, genes are also located in cytoplasmic organelles: mitochondria and plastids. These organelles are called semi-autonomous because only a part of the proteins they need for their function is encoded by their own genome, the rest is encoded by the nuclear genome. In plants we differentiate between the chloroplast genome (plastome) and the mitochondria genome (chondriome). In most higher plants they encode for 100 – 150 genes, among them the large sub-unit of ribulose: 1.5-bisphosphate carboxylase/oxygenase (RuBisCO). The chondriome is more complex and has circular and linear DNA molecules and its size ranges from 200 – 2000 kbp, depending on the species.

Organelles characteristically display a non-Mendelian inheritance, which is predominantly uni-parental, usually maternal. Another characteristic is that organelle inheritance includes somatic segregation (sorting-out) of genetically distinct organelles. For more details on organelle inheritance, see Greiner *et al*., (2015). In general, mutation induction of organelle genes is not well documented and it is assumed that it is far more difficult than for nuclear genes. Prina, Pacheco and Landau, (2012) reported on such mutated genomes and their function. A main character of interest, due to its importance in hybrid production, is cytoplasmic male sterility (CMS), encoded by mitochondrial genes (Wang *et al.*, 2006). This can be exploited to produce female only (seed bearing) plants that must be cross pollinated to yield F_1 hybrid seeds. Cytoplasmic male sterility for F_1 hybrid production was first exploited in maize, which resulted in spectacular yield increases. F_1 hybrid breeding is currently of great interest and several major crops are being converted to F_1 hybrids, notably rice, but also rye, and with active development programmes in many other crops.

3.2.2. **Gene mutations**

Gene mutations can be divided in structural variations that encompass copy number mutations, and nucleotide point mutations.

As in chromosomal mutations, where the number of chromosomes of a genome or of single chromosomes is abnormal, the copy number of DNA sequences can also change due to mutation. This refers to regions typically larger than 1 kb, but also to short insertions and deletions (indels) with a size of 20 – 50 bp. A typical example of an increase in copy number is active retro-elements, which multiply through a "copy and paste" mechanism (see Chapter 4). Whereas retro-elements are well-defined entities that encode the complete machinery for reverse transcription and re-integration of the new copy into the genome, other copy number variations (CNVs) are thought to result from non-allelic homologous recombination between DNA segments of high similarity that are not alleles. Żmieńko *et al.*, (2014) reviewed the current knowledge on copy number polymorphism in plant genomes, including associations of CNVs with plant phenotypes. One example given for such association is the diversity of flowering time and plant height in wheat (*Triticum aestivum*), where CNVs association resulted in the alteration of two major genes; the gene for photoperiod response (*Ppd-B1*) and the gene for vernalisation requirement (*Vrn-A1*) creating new alleles for each (Díaz *et al.*, 2012).

In point mutations, which comprise all DNA mutations where only one base pair is affected, the differentiation is between base substitution and base additions or deletions. A base substitution can be either a transition or a transversion. Whereas in a transition, a purine (A or G) is changed to another purine, or a pyrimidine (C or T) to another pyrimidine; in a transversion a purine is exchanged by a pyrimidine and vice versa. If these substitutions are within the coding region of a gene, they can result in:

- a missense mutation, where a changed triplet codes for a different amino acid, or
- a nonsense mutation, the new triplet creates a stop codon inducing a premature stop in translation, or
- a silent mutation in which the change has no consequence to the amino acid sequence of the synthetized peptides/proteins (Table 3.1).

The effect of point mutations on a phenotypic character can be wide ranging from null (no effect) to abolishing all function or inducing partial malfunction of an enzyme, or even producing an entirely new trait that was not part of the prior plant portfolio. As explained earlier (Chapter 2) point mutations, i.e. changes in one nucleotide, are

primarily induced by chemical mutagens. However, the example of the semi-dwarf rice mutant Calrose 76 that has been developed through gamma irradiation (Rutger *et al.*, 1976; Rutger, Peterson and Hu, 1977) has shown, that the phenotype was the result of a point mutation, namely a C to T transition in a gene coding for the gibberellin synthesis pathway, forming the *Sd1* locus (Spielmeyer, Ellis and Chandler, 2002).

Base additions and deletions engender frameshift mutations that can add up to a completely new protein being synthesised, and this may lead to the recovery of one or several new traits. Frameshift mutations can be suppressed by another insertion or deletion that reconstitutes the original frame (see Figure 3.3).

3.2.3. **Gene(s) mutations expression at the trait level**

Traits may be classed into two types: qualitative traits (single gene traits) and quantitative (multi-gene traits). Selection for each of the two groups of traits should be considered carefully prior to the initiation of a mutation breeding programme. Any proposal to use induced mutations in plant improvement must first consider the likelihood of success when compared with conventional techniques and the effort required to obtain the desired genotype. This likelihood of success can be measured in relation to the breeding system of the species and the genetic control of the character to be improved. The selection for a specific trait is usually the first option, e.g. yield, height, flowering time, disease resistance, etc., and this is often done without any consideration of the genes involved. Single genes, especially developmental genes such as those controlling flowering time (photoperiod and vernalisation genes) and height (semi-dwarfing genes) can have major pleiotropic effects on other traits, especially yield, as they are major genetic factors that adapt a cultivar to its growing environment. However, these major genes are often fixed in elite germplasm and if a breeder wants to alter such traits subtly he may be forced to develop mutations in minor quantitative genes that influence the trait and this is more challenging for the breeder.

3.2.3.1. Qualitative traits

Qualitative traits are controlled by one or two genes and are characterized by their simple Mendelian inheritance. Typical examples for qualitative traits include plant stature, flowering time, kernel size, flower and leaf colours, shell thickness, seed amino acid content, etc. The segregants from crosses typically separate into distinct classes – non-continuous variation – such as green or yellow seeds in peas, thin or thick shell in oil palm.

Disease resistance is often considered as a qualitative trait when it is controlled by race specific major R-genes. The plant becomes vulnerable if a new race of the

pathogen arises. Also, if a particular cultivar with a qualitative resistance is cultivated continuously, the pressure on the pathogen becomes high and new races evolve that break down plant resistance. It is therefore desirable, for a sustainable breeding programme to combine several major resistance genes into new cultivars in order to achieve broad resistance. This strategy has been successfully applied to control wheat stem rust (Singh *et al.*, 2011). The resistance, however, may break down when a new aggressive race of the pathogen appears, e.g. the race of *Puccinia graminis* that appeared in Uganda in 1999, Ug99 (Pretorius *et al.*, 2000). Ug99 is spread via wind borne spores and quickly moved out of East Africa, to the south into Yemen, and as far east as Iran. It is a threat to worldwide wheat production and wheat production in affected areas can only be achieved through the high use of expensive fungicides. New resistant mutant varieties were developed under an IAEA Interregional TC Project INT/5/150 (see examples in Section 3.3.1).

TABLE 3.1. TYPES OF MUTATIONS WITHIN THE CODING REGION OF DNA

Type of Change	Mutation in the DNA	Example
None	None	5’ - A - T - G - A - C - C - G - A - C - C - C - G - A - A - A - G - G - G - A - C - C - 3’ *
		Met Thr Asp Pro Lys Gly Thr
Silent	Base substitution	5’ - A - T - G - A - C - C - G - A - C - C - C - **C** - A - A - A - G - G - G - A - C - C - 3’
		Met Thr Asp Pro Lys Gly Thr
Missense	Base substitution	5’ - A - T - G - **C** - C - C - G - A - C - C - C - G - A - A - A - G - G - G - A - C - C - 3’
		Met Pro Asp Pro Lys Gly Thr
Nonsense	Base substitution	5’ - A - T - G - A - C - C - G - A - C - C - C - G - **T** - A - A - G - G - G - A - C - C - 3’
		Met Thr Asp Pro STOP!
Frameshift	Addition/ deletion	5’ - A - T - G - A - C - C - G - A - C - **G** - C - C - G - A - A - A - G - G - G - A - C - C - 3’
		Met Thr Asp Ala Glu Arg Asp

Frameshift mutations: Insertions or deletions of nucleotides that cause a shift in the translational reading frame.

Suppressor mutations: Mutations that counteract or suppress the effects of another mutations.

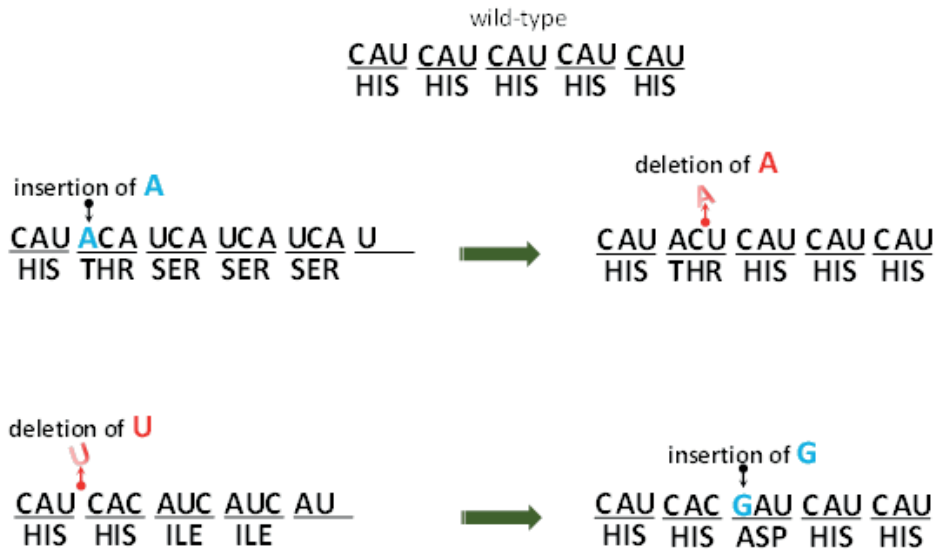


Figure 3.3. Frameshift mutations and their possible suppression.

3.2.3.2.Quantitative traits

Quantitative traits are characterised by continuous variation such as normal frequency distributions in progenies. Theoretically it takes only a small number of genes (4) with equal effects to produce a normal distribution; therefore, quantitative traits can be controlled by a small number (4 or 5) or more genes (polygenes). Segregation of genes for a quantitative trait, each contributing a portion of the total variation would modify their expression by interacting with other genes and with the environment (Paran and Zamir, 2003). The net effects of each gene affecting the trait can be partitioned into components attributable to additive, dominance and epistatic genetic variance, variance of G×E interactions and other environmental variances and are highly specific to the population under study.

In the context of increased complexities of the underlying gene actions involving several loci with unknown effects and interactions, many quantitative trait loci (QTLs) remain unknown. Many traits of interest such as grain/ fodder yield, seed weight, panicle weight, stem diameter, plant height and seed quality parameters are quantitative in nature and are important to the plant breeders. In order to enhance the genetic variation for quantitative traits, mutagenic agents can be used, as it is practiced for qualitative traits.

The frequency and effects of segregating genes for a QTL decides the quantum of variances and heritability of a population and environment (Kharkwal, 2012). Such genetic variances induced by mutations generally show narrow ranges over many

traits and crop species (Keightley and Halligan, 2009). Under the stabilizing selection, segregating genes with large effects would show low expected heterozygosity (Turelli, 1984), such that the predicted variance maintained is proportional to the total mutation rate of genes controlling a quantitative trait and inverse of the selection strength.

Text Box 3.2.

Among studies on efficient methods for the selection of mutants for QTLs, Ukai and Nakagawa, (2012) demonstrated a one-spike-one-grain selection in cereals. The reasoning is that assuming a quantitative trait has a phenotypic value that follows a normal distribution (A) with a mean N and a standard deviation s, where: N is the genotypic value of the normal plant and s^2 is the environmental variance.

If one supposes that a targeted mutation (A→a') is induced at a locus with a high genetic effect with a mutation rate per cell p_1, then, assuming also that the phenotypic value of the homozygote a'a' follows another normal distribution (B) of mean M (>N) with the same magnitude of standard deviation as a normal plant (σ). In other words, the mutant has a genotypic value of M and an environmental variance of σ^2. Finally, the authors also assume that the mutated allele is completely recessive to the original allele, and that the phenotypic value of the heterozygote (Aa') follows the distribution A. Then, the phenotypic values of plants in M_2 population is thought to follow a distribution which is a combination of two normal distributions A and B with a ratio of 1–0.25 p_1 and 0.25 p_1. In such cases, all plants with phenotypic values above a threshold point (Th) may be selected. In this type of selection, the value of Th in relation to the mutant genotype and environmental variation is therefore, important.

Many genetical theories have been proposed to dissect the complex traits governed by polygenes and statistical methodologies were developed for improving our ability to understand the effect of these genes (Text Box 3.2). Statistical genetics has focused on methods leading to partitioning of the components of variation, parameter estimation and prediction of breeding value for phenotypic data.

Phenotypic manifestation of quantitative characters altered by the mutagenic treatment can be detected by measures of central tendency (mean) and dispersion (variance) instead of ratios or inheritance patterns. The mean values for quantitative traits in populations obtained from irradiated gametes (pollen grains) or embryos (dormant seeds) are in most instances lower in treated than in untreated populations. Khan, Wani and Parveen, (2004) in a study on induced genetic variability for

quantitative traits in mung bean (*Vigna radiata*) demonstrated that the assessment of variance has been the most dependable statistical measure to find the mutagenic effect on the QTLs. Estimation of various genetic parameters viz., genotypic coefficient of variation (GCV), heritability (h^2) and genetic advance (GA) for three quantitative characters of the two varieties of mung bean provided ample evidence that mutagenic treatments could alter mean values and create additional genetic variability for quantitative traits.

With the advent of genomic tools, QTLs are being identified using a diverse array of molecular markers and sequencing facilities. Molecular markers are used to know the inheritance of the phenotype for a quantitative trait, which are useful in locating the specific genes in the genome and the magnitude of their effects on these traits. High density genetic maps with DNA marker based techniques will help in understanding the nature of these QTLs, such as knowing the effects and chromosomal location of gene/s affecting a trait, nature and effect of multiple copies of a single gene, interaction between and among genes governing a trait, the pleiotropic nature and the stability of gene action under different environmental conditions (Paterson *et al.*, 1988). QTL mapping has been very efficient for identifying the genetic regions linked to quantitative agronomic traits, in conjunction with high-throughput genotyping and phenotyping techniques. In addition, two or more QTLs identified in different varieties or compatible species and responsible for different traits are introgressed into the same elite line (Ashikari and Matsuoka, 2006) through marker assisted selection (MAS). In this way, the molecular basis of a quantitative trait can be inferred.

3.3.PRACTICAL EXAMPLES

3.3.1. **Example 1 - Selection of a single gene mutation: Ug99 rust resistance in wheat**

- In 1999 a new virulent race, Ug99 (known as race TTKSK), of the black stem rust disease caused by the fungus *Puccinia graminis* appeared on wheat crops in Uganda. The disease can cause complete failure of wheat crops.
- No wheat varieties carried resistance to Ug99 and the disease increased in severity and since the spores are wind-borne began to spread rapidly.
- Ug99 accounts for 8.3M tonnes of lost wheat grain per year (US$ 1.23 billion), thus threatening the global multi-billion-dollars wheat production.
- In 2009 the growing concern of Ug99 led to the establishment of the IAEA Technical Cooperation project INT/5/150 entitled: ‘Responding to the Transboundary Threat of Wheat Black Stem Rust (Ug99), which involved over 20 countries.

- This project has involved over 18 countries and 5 national and international institutions and examined possible mutation induction treatments to deal with the challenges posed by Ug99. Meetings and workshops to facilitate the project efforts were held in Kenya and Turkey.
- Mutation induction treatments were carried out at the Joint FAO/IAEA Plant Breeding and Genetics Laboratory (PBGL) in Seibersdorf (Austria) in 2009 by determining radio-sensitivity of each wheat variety and then irradiating seeds at optimum dose levels for mutation induction. The treatment provided enhanced biodiversity and treated seeds were sent for testing at Eldoret, Kenya, a hot spot for the disease. IAEA support to Kenya also included the establishment of irrigation systems, which allowed two generations of wheat to be grown and tested per year.
- In 2013, 13 resistant advanced mutant lines were developed in wheat varieties from 6 Member States (Algeria, Iraq, Kenya, Syria, Uganda and Yemen).
- In February 2014 the first mutant wheat variety resistant to Ug99 was officially released to farmers. It was named, “Eldo Ngano1” (Swahili for “Eldoret Story1”) and combines disease resistance with high yield. The short time taken from mutation induction to variety release (less than 5 years) is unprecedented. Six tonnes of seed were raised in 2013 and multiplied further at Eldoret, Kenya. In 2014 local farmers received sufficient seeds of “Eldo Ngano1” for 400 – 500 hectares in the first year of release. “Eldo Ngano1” has been followed swiftly by the mutant variety “Eldo Mavuno” (“Eldoret Harvest”) which received Kenyan Government approval in May 2014.

TABLE 3.2. SUMMARY OF THE DEVELOPMENT OF "ELDO NGANO1" RESISTANT MUTANT VARIETY: FROM MUTATION INDUCTION TO OFFICIAL VARIETY RELEASE

Date	Development of variety
March 2009	Selection of parental variety: Chozi and Njoro II (Kenya)
April 2009	Mutation induction – gamma ray treatment at the PBGL, Austria
June 2009 – September 2009	First mutant generation (M_1 bulk grown in Eldoret, Kenya)
October 2009 – February 2010	M_2 population (about 10 000 head rows) for each variety grown at Eldoret, Kenya (hotspot for the disease) 16 resistant plants selected from 5 head rows
May 2010 – September 2010	M_3 selections grown as head rows at Eldoret, Kenya 5 resistant lines selected
October 2010 – February 2011	M_4 selections re-tested by artificial infection of seedlings in glasshouse tests, Eldoret, Kenya Selected lines grown as field plots and confirmed as resistant
June – September 2011	M_5 selected lines (head rows) grown in infected fields, Eldoret, Kenya as plots 5 lines confirmed as resistant
October 2011 – February 2012	M_6 selected lines grown in infected fields, Eldoret, Kenya
March 2012 – October 2012	M_7 selected lines entered into official National Performance Trials 4 of 5 selected mutant lines (3 from Chozi and 1 from Njoro II)
2012 – 2013	Multiplication of seeds of 4 selected mutant lines, at Eldoret, Kenya
March 2013	Start 2nd year National Performance Trials 1 line dropped due to low yield, 3 continued, 1 new line included
August 2013	Official release of first approved variety: Eldo Ngano1

3.3.2. Example 2 - Induced mutations for genetic improvement of quantitative traits in sorghum

Sorghum is the fifth most important cereal crop worldwide and grown on marginal soils of arid and semi-arid regions. African and Asian countries are major sorghum producing countries (83 percent of the area and 57 percent of the world production) with a predominance of local landraces and varieties used mainly for food purposes (Rakshit *et al.*, 2014). In India, Southern and Central States account for 70 percent of the total sorghum production. Most of the area is under rainfed conditions and local landraces of the durra type are predominantly grown. They are tall, late maturing, photosensitive and possess low harvest indices compared to irrigated cultivars. In order to improve the locally adapted landraces for food and fodder qualities, biotic and abiotic stress tolerance, attempts have been made to improve these traits using mutation breeding methods. Various physical (X-rays, gamma rays, electron beams and fast neutrons) and chemical mutagens (EMS, MMS, SA and NEU) and various

combinations of both types have been used to induce mutations in sorghum (Reddy and Rao, 1981). Recently, carbon ion beam mutagenesis has been used to improve stem juiciness in sweet sorghum (Dong *et al.*, 2017). Gamma rays have been used to induce mutations in quantitative traits in sorghum such as increased panicle size, grain yield and fodder quality (Soeranto, *et al.,* 2001); grain numbers/panicle, seed weight and grain yield in Co-S-28 (Jayaramachandran *et al.*, 2010), and Yezin -7 genotypes (Htun, Min and Win, 2015).

Popular sorghum landraces such as Maldandi, TC-2, TH-11-10 and TJP-1-5 have been grown widely for food and fodder purpose in southern Indian states. They produce marginal yields with lustrous pearl yellow seeds and are susceptible to shoot fly and charcoal rot. In 2012, a research programme was initiated to improve the local landraces for quantitative traits using gamma rays and EMS. Briefly, 3000 self-fertilized seeds of each landraces having 10-12 percent moisture content were selected for induced mutagenesis. They were subjected to gamma irradiation of 300 Gy (dose rate of 38 Gy/min.). Half of the irradiated seeds were used for additional EMS treatment. 1 500 seeds were soaked with agitation in 250 ml water for 16 hrs and then treated with 0.1 percent (v/v) of EMS for 8 hrs at room temperature. The treated seeds were washed with tap water for four hours at room temperature and air dried. These air-dried seeds were used for direct planting in the field, one seed per Hill plot with a spacing of 45cm × 10cm. Gamma ray and EMS treated seeds were separately sown in 15m × 3m blocks along with the untreated parents as control. Local cultural and management practices were followed to raise a healthy crop in M_1 generation. Individual panicles were bagged before anthesis to ensure selfing, and harvested separately. Each fertile panicle was planted as a head to row progeny in the M_2 generation and harvested separately.

All morphological and other variants (albino, xantha, viridis and chlorina) were recorded as the plants developed. In subsequent generations, 15 – 20 percent of the elite variants with the most outstanding features were selected and forwarded as plant-to-row progenies until M_5 in comparison with control plants. In the M_6 generation, promising mutants showing ten days earlier flowering time were identified in TC-2 and TJP-1-5 populations with high grain yield (2600kg/ha against check 1600kg/ha), large seeds (4.1 g/100 seeds against check, 3.5 g/100 seeds, see Table 3.3). Wide variability was also observed for other quantitative traits such as plant height (115 – 338cm), stem diameter (0.8 – 2.2cm), panicle length (8 – 34cm) and width (7 – 25cm) in these mutants (Badigannavar *et al.*, 2017). Bold lustrous pearl yellow seeds are preferred by consumers and fetch premium prices in the market. Further, induced mutagenesis of two parents TC-2 and TJP1-5 resulted in the identification of five mutants for enhanced iron content (17 – 21 mg/100g) and four for zinc content (2.5 – 4.1 mg/100g) compared to their parents. Currently, these mutant lines, TC-2 and TJP1-5 are being tested in official National Performance Trials of ICAR (Indian

Council for Agriculture Research) for post rainy season conditions. For more details on chemical mutagenesis see Chapter 2.

TABLE 3.3. MEAN PERFORMANCES OF FOUR ELITE SORGHUM MUTANTS AT TWO LOCATIONS OVER THREE SEASONS

Quantitative traits	TC-2	TJP-1-5	TH-11-10	M-35-1 (check)
Days to flower	53	52	57	62
Panicle length (cm)	23.5	20.5	19.2	14.5
Panicle width (cm)	6.5	5.8	7.2	4.5
Seed weight (g/100 seeds)	4.0	3.95	4.0	3.6
Grain yield (kg/ha)	2700	2500	2450	1550

4. MUTAGEN EFFECTS IN THE FIRST GENERATION AFTER SEED TREATMENT: BIOLOGICAL EFFECTS OF MUTATION TREATMENTS

4.1.PLANT INJURY AND LETHALITY

As discussed in Chapter 3, the first mutation population (M_1) suffers from physiological disorders as a result of the mutagen treatment. This is a major reason why phenotypic selection for mutation cannot be done in the M_1 generation. In addition, most induced mutations are recessive and therefore the mutant phenotype cannot be observed until the mutation is homozygous. Moreover, the mutation induced is originally a one-cell event and is not present in every cell of the plant. Thus, M_1 plants must be regarded as chimeric plants (see Section 4.4.). For practical purposes the most important effects are growth retardation, sterility and death of the M_1 plants. Physiological disorders may be linked to chromosomal and/or extra-chromosomal damage, but a separation of the two causes is usually not possible. Regardless of these effects, the general weakened state of M_1 plants usually means that the M_1 population should be grown in benign (stress-free) environments to maximise growth, fertility and the production of the next (M_2) generation. All effects of the mutation treatment depend on the mutagen doses applied. For a review on the effects of gamma irradiation on morphological, physiological, and biochemical aspects in plants see Jan, Parween and Siddiqi, (2012).

In mutagenesis experiments, the resulting physiological damage sets practical limits to dose increase; in fact, an end point is reached with 100 percent lethality of the irradiated material and/or no germination of the M_1 seeds. In the case of seeds, the disruption and disorganisation of the tunica, or seed layers, or operculum (germination pore) or impaired mitosis or even complete elimination of cell division have been described as causes of failed germination after gamma irradiation (Lokesha *et al.*, 1992). Gamma irradiation of seeds also affects the survival rates of plants at maturity in a dose dependent relationship, this is also true to a lesser extent for EMS mutagenesis (Mahamune and Kothekar, 2012). Plant death might occur at any time between the onset of germination and maturity; however, there are critical phases during plant development at which lethal effects are more prominent, and M_1 plants are frequently sterile because of aberrations at meiosis, pollen, embryo sac or seed development (Micke and Wöhrmann, 1960). Therefore, when reporting lethality rates or survival percentages it is essential to note the time and stage of plant development at which data are collected. Survival rates obtained under laboratory conditions may differ considerably from those observed under field conditions, due to the possible occurrence of environmental stress at critical phases of the plant development.

At the chemical level, the generation of reactive oxygen species (ROSs) is a major factor responsible for physiological disorders following mutation induction treatments. ROSs have toxic properties and cause oxidations of poly-unsaturated fatty acids in lipids (lipid peroxidation) or oxidative deactivation of specific enzymes and may also cause damage on DNA or RNA (Figure 4.1). In addition, ROSs may trigger transcription of specific genes as a response to the stress caused by the treatment. This is currently discussed as one of the reasons for the often-observed stimulated germination rate at lower irradiation doses (in the range of about 10 to 50 Gy, depending on the species). An increase of assimilatory pigments, chlorophyll a, chlorophyll b, and carotenoids, in response to increasing irradiation dose has also been described (Marcu, Cristea and Daraban, 2013). The stimulating effect of gamma rays on cell division, growth and development is called "hormesis". It should be noted, however, that hormetic effects (those that surpass normal performance of non-irradiated controls) are usually minor and short-lived, and do not cause significant increases in yield (Miller and Miller, 1987). With increasing doses, the inhibitory and toxic effects of ROSs as well as the direct impact of ionizing radiation on DNA prevail over hormesis stimulation (Marcu, Cristea and Daraban, 2013).

Generation of ROSs is dependent on the water content of the irradiated tissue; this is one reason *in vitro* explants or tissue cultured materials are irradiated at much lower dosages as compared to (dry) seeds. The water content of seeds at the time of irradiation plays an important role as regards to the effect of the mutagen, and it is normal practice to standardise this by placing seeds in a desiccator. The higher the water content in the seeds, the greater the susceptibility to ionizing radiation. This can clearly be perceived in radio-sensitivity tests using seeds adjusted to various water content levels. Therefore, it is imperative to adjust the water content of seeds before mutation induction, normally to about 12 – 15 percent (for further details Chapter 1).

For any mutation induction in plant breeding a mutagen at a given dose should be applied that results in low plant injury, but induces a workable frequency of mutation induction for target traits with a low mutational (background) load. A balance is therefore needed, which is often determined by the size of the M_1 and subsequent mutant populations that can be handled by the breeder. For any given mutagenic treatment there is a correlation, for example, in cereals between M_1 seedling height and survival on one hand and M_1 mutation frequency on the other Gaul, (1959) as cited by (Suresh *et al.*, 2017).

The quantitative determination of M_1 injury and radio-sensitivity testing should be routine procedures in mutation breeding programmes. The easiest and most routinely applied methods to assess M_1 injury are: 1) the determination of germination frequency, and 2) shoot and/or root growth rates at a particular plant developmental stage (usually in seedlings). These tests are typically performed in the laboratory or a

greenhouse. Parameters that can be measured and compared to the control (untreated) under field and/or laboratory conditions include, germination, seedling establishment, height, root growth, leaf colour, flowering time, flower production, seed set and yield per plant (these are discussed in detail in Chapter 5).

4.2.CYTOLOGICAL EFFECTS

Some mutagenic treatment effects can be observed at the cytological level and the frequency of chromosomal aberrations in the M_1 can be determined. After treatment of seeds, the analysis of the first mitotic cycle in shoots or root cells offers a quick test to determine the effect of the mutagen (Figure 4.2). This test is more laborious than measuring seedling traits, e.g. height reduction; however, it adds additional information and should be applied whenever a new mutagenic treatment is introduced in a breeding or research programme. In many crops seedling roots are routinely used for chromosome studies and protocols are well established to examine metaphase chromosome spreads (see e.g. Maluszynska, 2003).

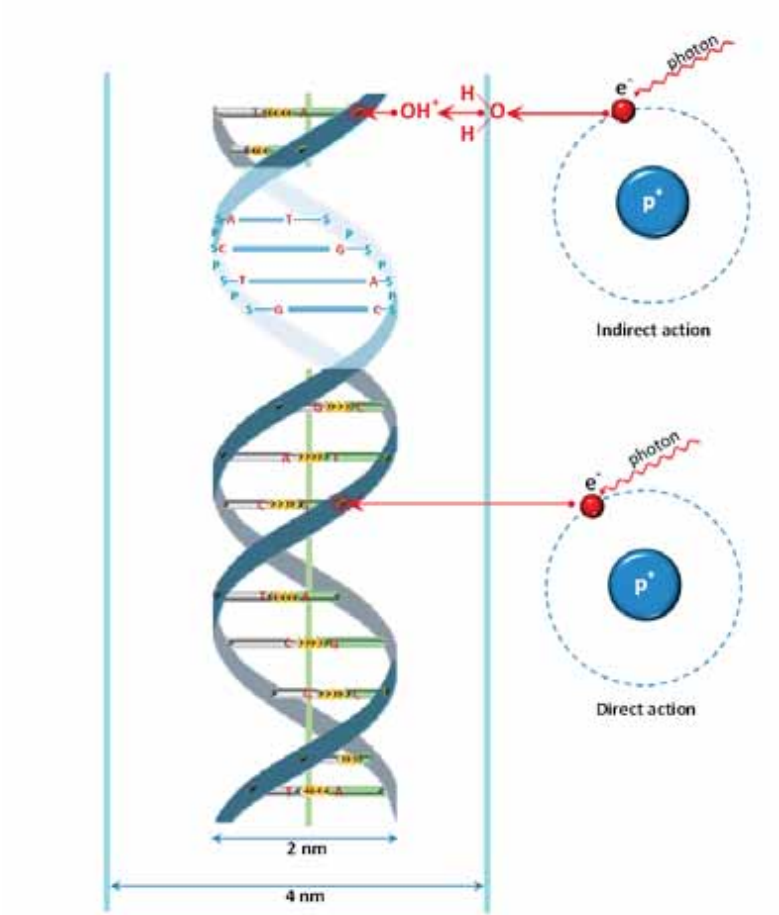


Figure 4.1. Direct and indirect action of gamma and X-rays on DNA. Top: reactive oxygen species (ROSs, here: hydoxyl radical) generated through ionizing radiation causing DNA strand breaks. Bottom: strand break is a direct effect of ionizing radiation on DNA.

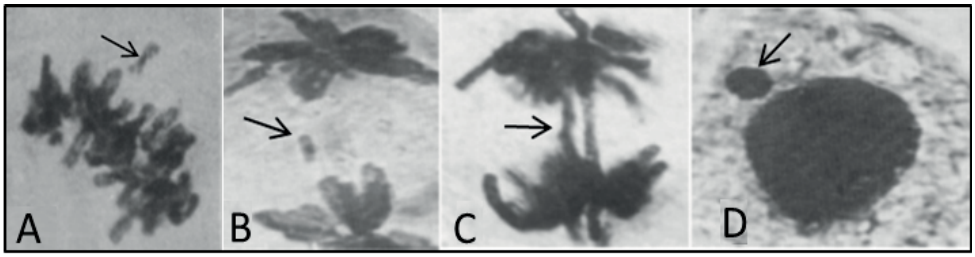


Figure 4.2. Chromosomal aberrations in onion Allium cepa L. induced by gamma irradiation. (A) Non-congression onto metaphase plate. (B) Laggards (C) Chromosome bridges. (D) Interphase with micro-nucleus. (Images are taken from Kumar et al., (2011) ; copyright © University of Florence, reprinted by permission of Taylor & Francis Ltd, www.tandfonline.com on behalf of University of Florence, Italy).

4.2.1. **Chromosome observations**

Chromosomal aberrations can be visualized in mitotically active tissues (root tips, leaf) of young seedlings derived from mutagenized seeds (Figure 4.2). In anaphase cells chromosome bridges and fragments are observed and interphases often show micronuclei as an effect of the treatment. For examples see Maluszynski *et al.*, (2009) and Kumar *et al.*, (2011).

Mutagens often delay the germination of treated seeds; they may also delay the onset of cell divisions and slow down the mitotic cycle. This fact needs to be taken into consideration when root or shoot tips are to be treated and fixed for cytological examination. In barley the nuclear divisions start in the seminal roots; they are followed somewhat later in the coleoptile, leaves and apical bud of the shoot (Wertz, 1940).

4.2.2. **The comet assay**

The comet assay is an elegant method of measuring the degree of DNA strand breaks and thereby chromosomal aberrations after mutagenic treatment (Figure 4.3). In practice, nuclei are released from young leaves by chopping with a razor blade in cold Tris-HCl buffer. The nuclei are then embedded on agarose coated slides. By treatment with high-salt lysis solution the membranes and nucleoplasm are removed, the nucleosomes disrupted and the histones solubilized. The remaining nucleoids with negatively super-coiled DNA are subjected to electrophoresis. After electrophoresis and staining with a fluorescent dye, such as ethidium bromide (EB) or 4.6-diamidino-2-phenylindole (DAPI) the slides are observed under a florescence microscope using UV excitation. DNA breakage leads to a loss of super-coiling and enables the debris to migrate towards the anode thereby forming a structure resembling the tail of a comet. Unaffected super-coiled DNA in contrast does not move and forms the head of the comet. There is a clear correlation between the length of the comet tail, the DNA damage and the dose applied for mutation induction. For a critical review on the comet assay, its principle, applications and limitations, see Collins, 2004. Interestingly, the comet assay allows monitoring of the kinetics of DNA repair by analysing samples taken at certain times after treatment. Gichner *et al.*, (2000) irradiated tobacco seedlings with low dosages of gamma rays (20 or 40 Gy) and determined complete DNA repair after 24h. An interesting modification of the assay is the application of fluorescent *in situ* hybridization (comet-FISH). This allows, in addition to quantitative measurements, a qualitative evaluation on the chromosomal

regions that have been affected by mutagenic treatment. For example, using comet-FISH, Kwasniewska and Kwasniewski (2013) showed that telomeric DNA sequences are more frequently present in the comet tail than centromeric ones, which is explained by the different number of breaks needed for the chromosomal aberrations: one break for a deletion at the end of the chromosome, two breaks for a deletion in a centromeric region.

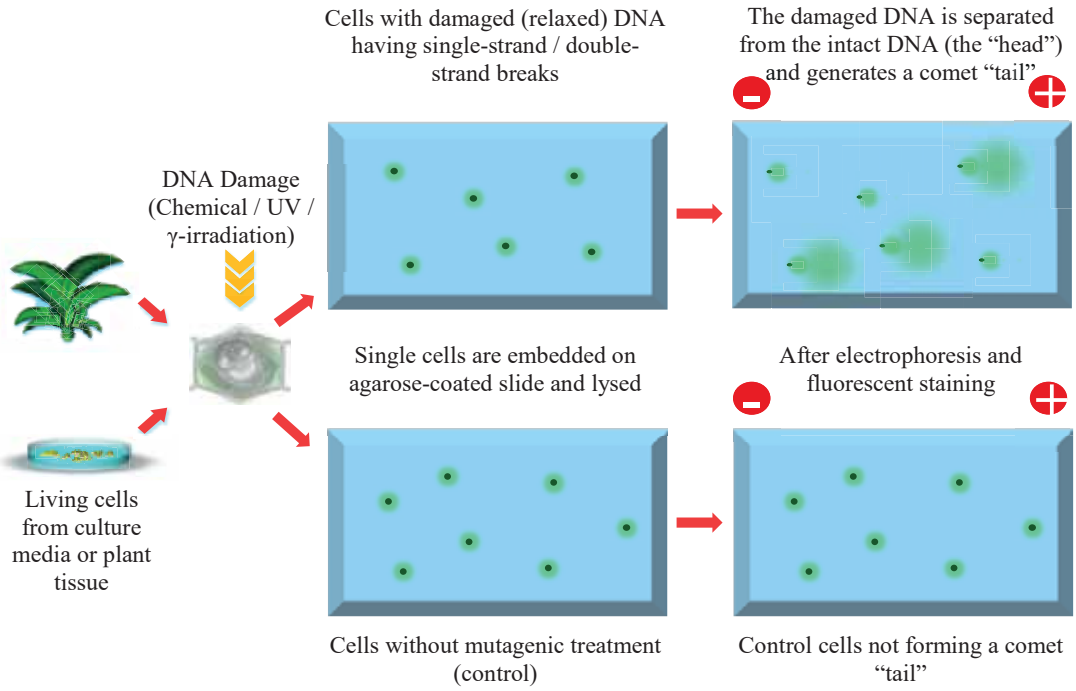


Figure 4.3. Comet assay – principle (modified for plant tissue, http://www.sigmaaldrich.com).

4.2.3. **Low dose stimulation**

The low dose stimulatory effect of gamma irradiation causes an increase of mitotic activity, but increasing the dose also reduces cell division. For example, in cowpea chromosomal abnormalities analysis (Badr, El-Shazly and Halawa, 2014; Kozgar *et al.*, 2014) at metaphase, four types of induced abnormalities were observed:

- c-metaphase configurations, with complete inhibition of spindle fibre formation;
- chromosomal stickiness where the chromosomes appear clumped together;

- disturbed metaphase configurations and;
- polyploid cells, however less frequently.

At anaphase-telophase chromosome bridges and lagging chromosomes were observed. In chromosome preparations of irradiated ginger rhizomes, Kamaruddin *et al*. (2016) observed clumping of chromosomes at prophase, metaphase and telophase; and sticky chromosomes were detected at anaphase and metaphase.

4.2.4. **Effects on meiosis**

Meiosis is a sensitive stage in plant development and is influenced by both genetic and environmental factors (Wijnker and de Jong, 2008). The control of meiosis is a "holy grail" for plant breeding but has rarely been achieved in practical conditions with the exceptions of the use of apomixis for seed production (e.g. *Brachiaria* forage grasses), through bridge crosses, and polyploidization. Mutagenic treatments (physical, chemical and biological) affect meiotic chromosome pairing and recombination, and can induce chromosome translocations in plants, sometimes with desirable results (Puchta, Dujon and Hohn, 1996; Lagoda *et al.*, 2012). Mutagen treatments targeting meiosis have been used to induce alien chromosome segment introgression into crop species (Wang *et al.* in Shu *et al.*, 2012). For example, irradiation at meiosis of aneuploid stocks of wheat carrying additional chromosomes of: 1) *T. umbellulatum* carrying rust resistance – X-ray irradiation (Sears *et al.,* 1956) and 2) *Leymus racemosus* carrying scab head blight resistance – gamma irradiation (Chen *et al.*, 2005), were used to induce chromosome breaks and provide opportunities for unions with other chromosomes in creating wheat/alien translocation lines. The irradiation induced double-strand breaks and the repair mechanism produced non-homologous chromosome recombination. Sadly, there are few examples of irradiation treatments to manipulated meiosis and recombination; this may be due to the ultra-sensitivity of the meiotic processes. More research is needed in this area.

4.3.STERILITY

Mutagen-induced reduction of reproductive capacity encompasses various phenomena and various sources. The phenomena include: (1) severe stunting or growth inhibition which prevents flowering; (2) flowers are formed but lack reproductive structures; (3) reproductive structures are present but pollen and/or ovules abort; (4) fertilization occurs but embryos abort before maturity; and/or (5) seeds form but fail to germinate properly or die after germination. Most common is the occurrence of non-functional gametes as a result of failed meiosis. Mutagen-induced sterility may be caused by (1) chromosome mutations, (2) gene mutations (3) cytoplasmic mutations, and (4) physiological effects. Chromosome mutations are

probably the major origin of all mutagen-induced sterility. As with induced physiological disorders, sterility issues set practical limits on increasing dose treatments.

Following chemical (EMS) treatment in *Arabidopsis,* it was observed that the mutation frequency was limited by sterility in M_1 plants, and not by the increased M_1 lethality. Several mutants were studied in detail with defects that included the establishment of anther morphology, microspore production, pollen differentiation, and anther dehiscence, leading to male sterility (Sanders *et al.*, 1999). Several reports have presented data on genes involved in mutation induced sterility for both pollen (male) and ovule (female), (Sanders *et al.*, 1999; Robinson-Beers, Pruitt and Gasser, 1992). This is an issue for breeding as, although, plant breeders want to produce a reasonable number of lines carrying a desired mutation, they do not want a high background mutational load as the target material is usually an elite line with a finely tuned and valuable genetic background, and the mutational load requires lengthy backcrossing procedures to clean up.

While, sterility may be an issue in mutant line development, it can also be exploited, especially in the production of F_1 hybrids. In the context of hybrid breeding the development of male sterile plants (female lines) is highly desirable in commercial production of hybrid seeds. Chaudhury, (1993) in a review on male fertility in plants has described various male-sterile mutants and concluded that several genes are involved in controlling male fertility.

4.4.CHIMERAS

In a simple definition a chimeric plant is a plant that is composed of cells harbouring more than one genotype. In Chapters 2, 3, 5, 6 and 8 the generation and dissolution of chimeras are discussed in detail. The occurrence of chimeras after mutagen treatment is of great importance for the implementation of a mutation breeding programme, in particular with regards to the handling of the mutated populations.

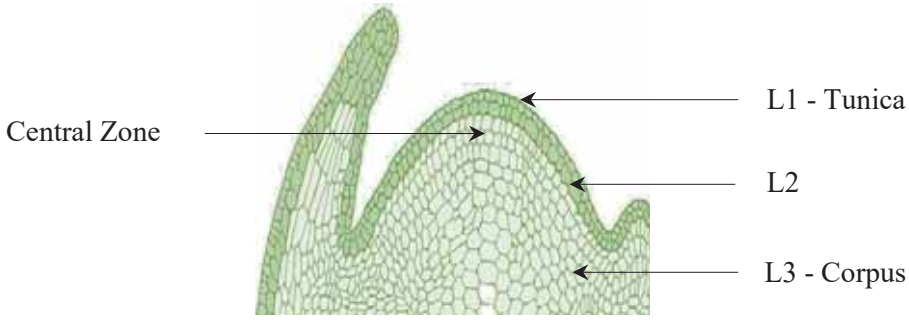


Figure 4.4. Organization of cell layers in the apical dome of a shoot meristem.

Chimera types are broadly classed as either somatic or reproductive (see Chapter 5). The most visible indication of somatic chimerism is chlorophyll variegation in leaves with chlorophyll deficient sectors forming longitudinal streaks in monocotyledons and irregular patches in dicotyledons. Chimeras can persist in vegetatively propagated crops (VPCs) but may be dissolved quickly in seed crops. However, even in seed crops chimeras can be transmitted to the next generation at a low frequency. The segregation of chimeras in M_2 plants depends on the number of initial cells giving rise to the germline (genetically effective cells) and thus to the formation of the gametes, as well as on the mutations induced or not in each individual cell. For more information see text box 4.1.

Text box 4.1.

For barley it has been established that each of the main 4-5 spikes originate from 2-4 initial cells (Gaul, 1964). Ukai and Nakagawa in (Shu et al., 2012) presented an example of mutant segregation using the M_1 spike method that was previously developed by Stadler (1928) and is based on separate harvests of M_2 seeds from each spike of M_1 plants. The segregation frequency of mutants in the M_2 then appeared to depend on the number of initial cells that gave rise to a spike. In case of only one initial diploid mutated cell the segregation frequency in the M_2 is equal to the segregation ratio of a recessive mutant (a'a'), namely 0.25. If in addition to the mutated cell, more cells are involved in the development of the spike, it will have a chimeric sector and the segregation frequency in the M_2 will be lower than 0.25. The relationship between initial cells and segregation frequency can be described by the formula 0.25/k, with k being the number of initial cells. This means, in practical terms that with a higher number of initial mutant cells the chances of finding a homozygous recessive mutant in the M_2 becomes lower. It should be noted that mutagenic treatments can kill initial cells and that more than one initial cell can be mutated differently. In the latter case the breeder will obtain a larger number of M_2 mutant plants with different characters. An additional influence on the segregation frequency is given by so-called diplontic- or haplontic selection, which refers to the competition among cells with different genotypes, specifically between mutated and non-mutated cells, either in the diplophase between somatic cells or the haplophase during gamete production and forming the zygotes (see Chapters 3, 5, 6 and 8 for more information).

In vegetatively propagated crops (VPCs), the issue of chimeras can be more problematic as it takes considerable effort to eliminate chimeras and obtain homohistont plant material (see Chapter 6).

Chimeras in VPCs result from mutations generated in the shoot apical meristem. The specific nature of chimeric results from mutations in the shoot apical meristem known to be organized in layered structures (Figure 4.4). Shoot apical meristems of

angiosperms typically have three distinct layers of cells, L1, L2 and L3. Layer L1 is the single layer of cells making up the epidermis, layer L2 the single cell sub-epidermal layer that gives rise to the sub-epidermal mesophyll cells in the leaves and to the gametes and layer L3 constitutes the rest of the internal cells including the vascular system. The type of chimera depends from the layer in which the mutation was induced: sectorial-, mericlinal- and periclinal chimeras (see Chapter 8-A). Plants that have one layer of cells that is genetically distinct from another layer are called periclinal chimeras. Periclinal chimeras are the most stable type of chimeras and are derived from a mutation in a single cell of the layer that through anticlinal division forms a uniform layer bearing this mutation. In a mericlinal chimera a mutation in one layer does not spread over the entire layer, these are unstable and often loose the mutation or develop into periclinal chimeras. Sectorial chimeras are characterized by a mutated sector being spread over more than one layer. This type of chimeras is unstable and often develops into shoots which are not chimeras and may give rise to genetically homogenous types. An art in mutation breeding of VPCs, such as the triploid Cavendish banana, is the dissociation of chimeras after mutation induction, with the aim to develop homohistont clones that can be screened for the trait of interest as soon as possible. (Roux *et al.*, 2001a) tested different tissue culture methods for chimera dissociation in diploid and triploid Musa acuminata and used polyploid cytochimeras as a model system for mutations, which can be easily followed using flow cytometry. Three different techniques were applied for three subcultures each (shoot tip culture- ST, multi-apexing culture - MA, and corm slice culture- CS). The average percentage of cytochimeras was reduced from 100 percent to 36 percent using ST, from 100 percent to 24 percent when propagating by CS technique and from 100 percent to 8 percent using the multi-apexing technique.

In the case of chimeric sectors in ornamental horticultural plants with attractive mutations such as new flower colour or shape, the mutant tissue can be isolated and subjected to tissue culture techniques in order to regenerate plants that carry the favourable mutation. This has been shown for example by (Mandal, Chakrabarty and Datta, 2000) who regenerated mutated sectorial floret tissue from chrysanthemum through organogenesis thereby retaining the mutation.

4.5.SECONDARY EFFECTS: TRANSPOSON ACTIVATION

The vast majority of variation induced by all forms of mutagens is a loss of gene function, and hence most mutations are recessive. In contrast, transposable element (TE) insertions can produce a much broader range of phenotypes (Lisch, 2013). TEs also called “jumping genes” are endogenous mobile elements able to move and insert themselves at different positions in the genome. In doing so, they often produce mutations when they re-insert into genes. In contrast to other sources of genetic variation TE insertions have the potential to induce homeotic transformations,

dominant ectopic expression mutations and environmentally induced changes in gene expression (Naito *et al.*, 2009; Lisch, 2013). There are several well documented examples of a direct involvement of these endogenous mutagens in plant domestication and improvement. Importantly, several of these mutations would not have arisen via simple point mutations or insertions and deletions (INDELs). This is due to the regulatory sequences carried by TE insertions, as well as epigenetic regulation of TEs, that can alter the expression of neighbouring genes. Thus, although they can certainly create null mutations, TEs have the additional capacity to create a new spectrum of regulatory mutations.

Stress, including radiation exposure can activate otherwise silent TEs (Bui and Grandbastien, 2012; Bradshaw and McEntee, 1989; Sacerdot *et al.*, 2005; Farkash *et al.*, 2006; Qüesta, Fina and Casati, 2013), see also: (https://www.cospar-assembly.org/abstractcd/COSPAR-12/abstracts/F4.6-0009-12.pdf).

The involvement of TEs activated by ion beam treatments in the induction of flower mutants has been documented by Okamura *et al.* (2006). At the Joint FAO/IAEA Plant Breeding and Genetics Laboratory, Seibersdorf, Austria, molecular analysis of rice plants derived from radio-sensitivity tests indicated that the retroelement Tos17 was activated by gamma irradiation of rice seeds (Nielen, Guzman and Zapata-Arias, 2000). These results indicate that TEs have a major role in mutation induction, the full potential of exploiting TEs in mutation breeding has not yet been realised.

4.5.1. Examples of TE-induced mutations in plant breeding

There are a number of success stories in TE-induced mutation in crop improvement. For example, the reduced branching, associated with maize domestication from *Teosintes,* is due to the introduction of an enhancer element carried by a retrotransposon that inserted upstream of the *tb1* gene (Studer *et al.*, 2011). The temperature-sensitive expression of red pigment in the flesh of blood oranges is due to regulatory elements encoded by a retrotransposon inserted upstream of a colour gene (Butelli *et al.*, 2012); this provides a useful dominant gain-of-function mutation in a species propagated by cuttings or by apomictic seeds. Interestingly, a secondary rearrangement leaving behind a small portion of the transposable element, leads to an intensification of that red flesh colour.

The propensity for TEs to mobilize coding sequences via retro-transpositions lead to a change in tissue specificity in the *IQD12* gene in tomatoes (*Solanum lycopersicum*), which is responsible for the oval shape of most modern tomatoes (Xiao *et al.*, 2008). The white colour of grape berry (*Vitis vinifera*) is also due to an insertion of a retrotransposon upstream of a *Myb*-related gene that regulates anthocyanin biosynthesis (Kobayashi, Goto-Yamamoto and Hirochika, 2004) and the phenotype

is only partially reversed to a pink berry colour after internal recombination leaving a solo-LTR at the insertion site (Pelsy, 2010).

Thus, TE insertions can exert various subtle regulatory changes that can produce beneficial traits for plant breeding purposes. Adaptive changes mediated by TE insertions have also been reported. A retrotransposon insertion in the soybean phytochrome A photoreceptor gene *GmphyA2* conferred photoperiod insensitivity, which has contributed to the expansion of soybean cultivation to higher latitudes of East Asia (Kanazawa *et al.*, 2009). Also, the Pit disease resistance gene has been transcriptionally reactivated in a resistant rice cultivar because of an insertion of a new promoter provided by a retrotransposon (Hayashi and Yoshida, 2009).

Several examples of similar adaptive mutations caused by recurrent insertions have also been reported including the peculiar 'hose-in-hose' primrose mutant (Text Box 4.2). These are important because they suggest that without the presence of TEs these mutations may not have been recovered. In a line of hexaploid bread wheat, the *Vrn3* vernalization gene is upregulated in correlation with the upstream insertion of a retrotransposon, allowing early flowering after a vernalization treatment, thus conferring Spring growth habit (Yan *et al.*, 2006). In tetraploid durum wheat, the exact same phenotype is controlled by the upstream insertion of a different retrotransposon in the homoeologous *Vrn1* gene (Chu *et al.*, 2011). Aluminium tolerance, in wheat, sorghum and barley involve TEs. Aluminium tolerance is associated with upstream insertions of different TEs that mediate up-regulation and redirection to the root apex, of the expression of citrate transporter genes (Delhaize, Ma and Ryan, 2012).

There is evidence that TEs can not only respond to stress, but also confer stress tolerance. For instance, the retro-element insertion that causes the colour change in blood orange also confers responsiveness to cold temperatures. Similarly, insertion of the miniature inverted-repeat transposable element (MITE) in rice can confer tolerance to cold and salt stress (Naito *et al.*, 2009). The same is likely true of a wide range of other biotic and abiotic stresses, which is of interest in adapting crops to environment change.

TEs are normally kept in check via a sophisticated system of epigenetic silencing. One of the most interesting aspects of the responsiveness of TEs to stress is that it can result in a reversal of that silencing, which makes the TEs available as a source of new variation. *Tos17*, for instance, is a retro-element in rice that is transiently reactivated in tissue culture and has been used to generate tens of thousands of new mutant alleles (Piffanelli *et al.*, 2007). Similarly, the *En/Spm* element in maize was released from silencing due to exposure to radiation in the 1950s (Peterson, 1991). Recent evidence suggests that UV-B and perhaps ion beam irradiation also trigger TE activation (Yan *et al.*, 2006; Huiru *et al.*, 2009; Ya *et al.*, 2011; Qüesta, Fina and Casati, 2013).

Text Box 4.2.

Other examples of TE-induced mutation include the much prized "hose-in-hose" (Figure 4.5) primrose flower phenotype, which shows a conversion of sepals to petals, resulting from changes in tissue-specificity of a MADS box gene that is associated with a gypsy retrotransposon insertion in its promoter (Li et al., 2010), and the hose continuous flowering phenotype (blooming in all seasons) is caused by an intronic insertion that results in splicing failure of a gene controlling flower transition, a characteristic under photoperiodic and thermal control (Iwata et al., 2012). Recombination of the retrotransposon leaving a solo-LTR, restores normal splicing, but the resulting phenotype is not the wild-type phenotype (Spring blooming), but a climbing phenotype (occasionally re-blooming in Autumn).

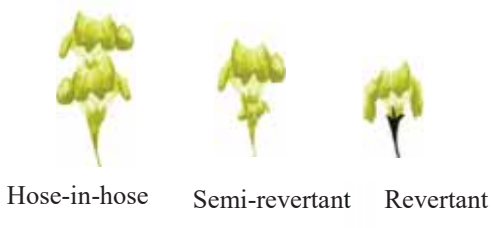


Figure 4.5 Hose-in-hose mutant of primrose, Primula vulgaris

TE activity is far less deleterious, on average, than random mutagenesis because TEs have been selected through evolution to minimize negative effects (Naito *et al.*, 2009). Furthermore, if a specific TE family is known to be activated in a particular species, new mutations caused by this TE family are thus tagged, which could make molecular identification of the cause of a new phenotype relatively simple (compared to DNA walking to a point mutation). An additional potential advantage of TE-enhanced radiation-induced mutagenesis is that the level of radiation needed to activate TEs may be far lower than that required to produce large numbers of mutations directly from irradiation exposure, thus minimising mutational load. Presumably, this would lead to fewer gross chromosomal rearrangements that could hinder, for instance, subsequent introgression experiments. Finally, TEs can be exploited as polymorphic DNA markers in measuring biodiversity studies and genotypic constitution in plant breeding germplasm.

Acknowledgement: this section was drafted as an outcome of the FAO/IAEA consultants meeting on “Enhancement of the efficiency of mutation induction by physical and combined mutagenic treatments”, held 2 – 6 June 2014 in Vienna, Austria, for which we are grateful.

5. MUTATION BREEDING IN SEED PROPAGATED CROPS: PARENTAL SELECTION, MUTANT GENERATION DEVELOPMENT, MUTATION DETECTION, MUTANT EVALUATION AND FACTORS INFLUENCING SUCCESS

This chapter is an adaptation and up-date of the Chapter 7: "Induced mutation techniques in breeding seed propagated species" by Konzak *et al*. 1977 in the 2nd edition of the Manual on Mutation Breeding. It deals with the practicalities of mutation breeding in seed propagated crops and uses annual cereal and legume species as examples. The processes of developing mutant populations, screening and selection of mutant plants and populations are described starting with the selection of the target/parent genotype to be mutated, i.e. the production of M_0 seeds for treatment, irradiation to produce M_1 seeds and the handling of mutant generations, with emphasis on M_1 to M_3 generations. The chapter introduces the main factors affecting the success of mutation breeding such as population size, propagation and isolation of mutant plants and examples of screening for desired mutants. Schematic diagrams and photographs illustrate the various steps and assist in the understanding of the practical application for mutation breeding.

5.1. SELECTING PARENTS AND HANDLING M_1 TO M_3 GENERATIONS FOR MUTANT SELECTION

A successful mutation breeding programme starts with well-defined objectives for improvement of a defined plant phenotype/genotype. Common targets are:

a) to improve one or a few specific traits of a preferred variety or an elite line (see Chapter 7);

b) to induce a morphological marker (colour, awns, bracts, hairiness, etc.) in order to establish distinctness in a promising line to make it easy to identify and meet the requirement for variety registration;

c) and/or to induce male sterility or fertility restoration making a line useful as a component for hybrid variety production.

5.1.1. Selection criteria for parent genotype

The parent genotype in which mutation is to be induced should be either: 1) a recently released cultivar, 2) an advanced promising line about to be released, or 3) a promising advanced line or introduced variety restricted from release by specific limitations, e.g. susceptibility to lodging, to a specific disease or pest, and to shattering, etc.

A primary consideration in selecting the parental variety seed source is its lack of natural variability for the trait(s) to be introduced by mutation. The chosen variety should have sufficient uniformity in most of the important agronomic traits. Usually, a stock of breeder or foundation seed is available from a recently released cultivar, and in accordance with most pure seed programmes; the genetic variability for agronomically important features is restricted to the levels outlined in local registration schemes, e.g. > 98 percent true to type. Commonly, however, to fully exploit an advance made by cross breeding, the mutation breeder may need to initiate his experiments bearing in mind that the parental material may carry some contamination via admixtures or outcrossing. However, for plant-breeding purposes this is of minor consequence and the non-availability of a conclusively uniform and pure stock need not deter the use of mutation induction. In such cases, to increase the chances for success it is especially important that the breeder: 1) limits his/her objectives, 2) restricts further introgression or genetic variability, 3) grows sufficient untreated M_0 (control) material to establish a concept of the genetic variation present in the stock and for increasing the purity of the parent stock selected for back-up experiments and, 4) develops a detailed information sheet on the characteristics of an individual or line as improved by the type of mutation sought. For some crops, genetic pure lines may be available in the form of doubled haploid populations.

5.1.2. **Planning for the M_1 generation**

5.1.2.1.Radio-Sensitivity

Once the decision on the genotype to be mutated is made and a homogenous seed stock is available, the next step is mutagenesis. The seeds before treatment are known as the M_0 generation. As explained in Chapter 1, radio-sensitivity testing should precede the bulk treatment to determine the optimum dose levels for mutation induction. Radio-sensitivity tests are normally performed in the greenhouse, but fields can also be used. Responses to irradiation vary between species and among varieties of the same species. Figure 5.1 shows typical radio-sensitivity test settings in a bean (cowpea) and a cereal (maize) after seed irradiation.

5.1.2.2.Control population

A control (untreated) population should always be grown to serve three purposes:

a. provide a comparison of the treatment effects on germination, growth, survival, M_1 injury and sterility;

b. assess the phenotypic variability of the parent genotype stock used to produce M_1 and;

c. provide a 're-purified' parent genotype as a back-up for initiating a new M_1 generation to be grown during the same season with the M_2 generated from the first M_1 if needed.

5.1.2.3.Mutagen and dose treatment

It is advisable to use three doses of the chosen mutagens, which should be ± 20 per cent of the optimal dose found through the radio-sensitivity tests. Normally the selected doses to be applied on cereals cause between 30 to 50 per cent reduction in seedling growth in laboratory tests. In practice, at least two replicates should be made, each with half the quantity of seeds selected for use with each dose, as an insurance measure against failure and against errors. When rather large numbers of seeds are treated, i.e. 5000 to 10 000 seeds per treatment, subdivision into several treatment replicates will improve the uniformity of the treatments and this is usually convenient because of the physical limitations of the irradiation chamber size.

5.1.2.4.Population size of M_1

Assuming a 90 per cent probability of success in recovering a mutant occurring at a frequency of 1×10^{-3} per test unit (e.g. per spike, fruit, or branch), and that each plant grown is expected to produce three units, the amount of seeds to be treated, if the M_1 has 80 per cent survival rate, would be about 600 seeds. However, as described below, because of the errors in estimating the frequency of the desired mutation, uncertainties in predicting M_1 survival, variation in treatment severity, etc., it is recommended that as much as ten times the estimated required

quantities of seeds be treated to ensure that an adequate population will be available for screening. Thus, in the above example treating about 6000 seeds might yield the breeder as many as 10 mutations in the desired direction.

While calculations to estimate the size of a treated population needed for isolating a desired mutant type are recommendable, such calculations may be of limited value in practice for several reasons, among which:

a) even the best estimates of mutation frequencies may involve considerable errors;

b) the optimum mutagen treatment for obtaining a desired frequency of mutations cannot yet be ensured since many unforeseeable physical and biological factors may influence the mutagenic effectiveness of a treatment;

c) and the breeder can hardly achieve his goal with a single desired mutant, thus it is preferable to have several mutants with similar phenotype which will provide option for further evaluation and selection of the best mutant.

In gamma irradiation the activity of the source affects mutagenesis and this declines over time. The M_1 generation normally requires the least space and effort. Therefore, growing extra M_1 material has little effect on cost, but adds to the assurance that a sufficiently large population of M_2 will be available for screening to obtain the desired mutant(s).

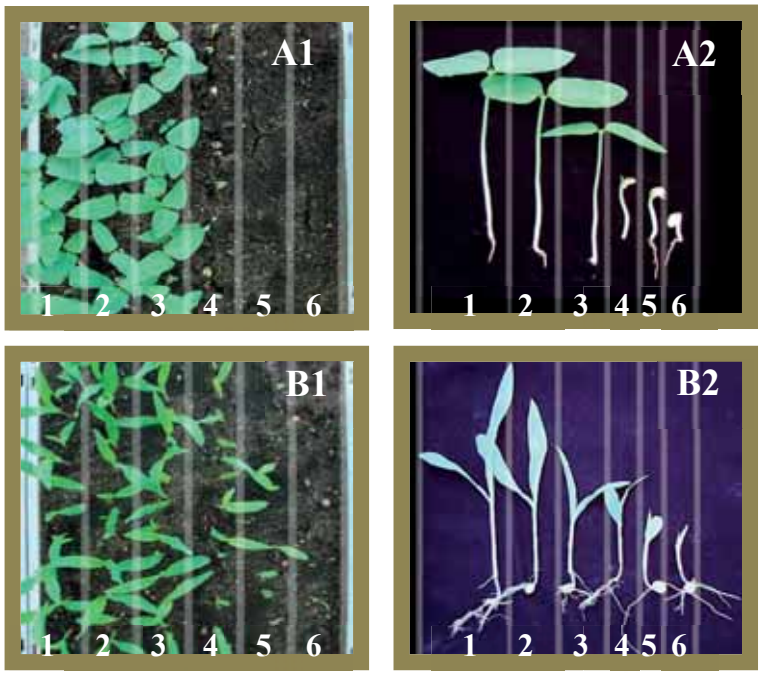


Figure 5.1. Photographs of radio-sensitivity: A) in cowpea (beans) and B) maize (cereals) showing survival rates and reduced growth in two-week-old seedlings after gamma irradiation of seed at: 1) 0 (control), 2) 75, 3) 150, 4) 300, 5) 450 and 6) 600 Gy. The source of gamma rays was a cobalt 60 gamma cell producing150 Gy/min. Courtesy of A. Mukhtar Ali Ghanim.

5.1.3. **Sowing of M_1 seeds**

Considering the detrimental effects of mutagens on seed viability (see Chapter 4), the M_1 must be handled with more care than untreated controls. The M_1 should therefore be grown in benign conditions.

5.1.3.1.Greenhouse conditions

If possible the M_1 should be grown in a greenhouse where careful attention can be paid with respect to watering, fertiliser provision, lighting and temperature, weed, pest and disease control to maximise plant survival and production of the next generation. Isolation of the M_1 population is also easier in the greenhouse, thus limiting pollination from external sources, which could bring in unwanted (non-mutant) variation. Normally only a few (M_2) seeds are needed per M_1 plant and multiple flowering units (e.g. tillers) may be reduced by growing individual plants in small pots or collectively in trays, which also maximises greenhouse space. However, it should be noted that the use of greenhouses is relatively expensive compared to the field.

5.1.3.2.Field conditions

If greenhouse conditions are not available or are not affordable, then field conditions can be used. It is particularly important to ascertain that the moisture and the physical conditions of the seed bed prepared for sowing the M_1 are optimal for seedling growth and development. Nitrogen fertility of the soil should be normal or slightly lower to limit excessive tillering (as normally only one seed head is harvested), but other nutrients should be at optimum levels. A restriction in field cultivation is that it is restricted to the selected variety's cropping season.

5.1.3.3.Time of sowing M_1

The M_1 material will develop optimally if sown during the season when the climate is best for early seedling and plant development and weed control is less of a problem. However, slightly later sowing (2 or 3 weeks) may help reduce tillering and may improve the conditions for isolation against cross-pollination. The delay should not be so much as to encourage weed types that are more difficult to control, alter the maturity in response to day length or temperature factors, or increase susceptibility of the crop to other hazards. Because of the induced injuries, crops requiring vernalization should be vernalized in the laboratory and transplanted or sown in the very early season when vernalization would normally occur from low temperatures. Where possible isolated facilities (greenhouse, protected nursery, etc.) can be used to propagate the M_1 population and minimize the losses.

5.1.3.4.Condition of the treated M_1 seeds

Dry seeds are easier to plant with machinery or by hand, and a more uniform growth can be obtained without extra care. If adequately low in moisture, dry seeds can be stored for some time prior to sowing. Vacuum packing is the best option, if available,

to store treated seeds for longer time in order to match with the normal growing season.

5.1.3.5.Density of sowing

In general, the spacing of M_1 seeds within and between rows should be such as to restrict development to primary tillers; to 2 – 3 in cereals, and the primary branching in grain legumes and other dicotyledonous species. This can also be adjusted based on the space available, the number of treated M_1 seeds, the expected survival rate based on the dose effect and the expected M_2 population size.

5.1.3.6.Weed control

Normally, a relatively weed-free seed bed should be prepared just prior to planting of the M_1 seeds. Pre-emergence herbicides recommended for the area and crop may be used successfully. Contact types of post-emergence herbicides may be useful for weed control if the M_1 plot is too large or the weeds are too numerous for manual control. Systemic herbicides, such as 2, 4-D should normally not be used with cereals because they tend to cause side-effects and may more severely affect one portion of the population relative to another, often resulting in sterility, distortion of plant phenotypes and reduced production of seed on the primary tillers of the M_1 plants. Other measures such as mulching can also be followed based on the crop and the area of plantation.

5.1.4. Isolation of M_1 material

Generally, it can be assumed that some level of genetic heterogeneity is always present even in populations of parent material from self-pollinating plants. Cultivars often encompass a group of related lines and advanced lines in a bulk population derived from F_5 or later generation. In addition, the probability is high that there will be some limited contamination through mechanical mixing or by outcrossing. Therefore, several disrupting hazards may be expected, especially in field plantings, which may affect the certainty of the origin of the variability observed in a mutation breeding programme as presented below.

a. Outcrossing – distant pollen grains may be transmitted by wind or insects from varieties of the same species growing nearby; the extent of the contamination varies with crop variety, the treatment, the mating system of the plant species and the distance from the mutated crop field in relation to wind directions and potential sources of contamination. Appropriate selfing method, such as bagging to shield flowers before anthesis, should be applied at the right time to prevent outcrossing (Figure 5.2). In cross pollinated species, where male and female flowers are separated, selfing can be

accomplished by hand pollination of the female flower. The female flower should be covered before opening to present cross pollination.

b. 'Volunteer' crop – M_1 should not be sown on land previously used for the same species this is particularly important for hand-seeded legumes.

c. Bird damage – the risk of loss of M_1 material to birds is often greater than with non-mutated plant materials because the range of maturity variation of in treated materials may be greater. In practice, the M_1 plots should be planted at some distance from bird populations. In some cases, an extra plot area might be sown on different dates to divert birds away from the mutagen-treated plots.

d. When the M_1 population must be planted where bird damage is possible, the plants might be covered with bird-proof nylon or metal netting, which is relatively inexpensive or the selfing bag should be left until maturity to serve as protection from bird damage (Figure 5.2).

e. Soil borne toxicity, disease or in some cases, parasitic weeds, such as *Striga* spp. may cause complete loss of the M_1 population and therefore, extreme care should be exercised to avoid planting the M_1 seeds on soil having such problematic history.

5.1.5. Care during cultivation and data recording

As stated previously, the M_1 population should receive optimum cultivation practices in either greenhouse or field cultivation for the selected crop, including supplemental irrigation, weed control by herbicide or by mechanical means, prevention of severe disease levels if necessary. In addition, records on the condition of the M_1 at various developmental stages are useful and need to be recorded.

a. Emergence – because the application of a mutagen commonly induces some delay in emergence for treated seeds, recording the estimates of emergence percentage at a time when the control population can be considered 50 – 90 percent emerged is useful. If emergence of treated populations is poor, either the treatments are too severe, or the cultural conditions are poor, and adjustments must be made for the next M_1 planting.

b. Seedling survival – estimates of seedling survival recorded at the tillering or branching stages provide data on delayed effects of the treatments. If seedling survival of treated population is low relative to emergence, the mutagen treatments could be considered too severe for the cultural conditions.

c. M_1 chimera induction – even crude data on the occurrence of sectors of chlorophyll deficiency or other morphological changes in the appearance of M_1 plants may be useful data for estimating the effectiveness and intensity of the treatments.

d. Delayed development – the retarded growth of some mutagen-treated materials can often be estimated in relation to seedling establishment, the time of flowering or maturation of the plants as well as by the variability in plant development within treatments.

e. Survival to maturity – estimates of the number of surviving plants in each treatment at the time of maturity provide information on the severity of the injury induced by the mutagen when compared with the number of seeds planted.

f. Sterility in M_1 – useful estimates of M_1 sterility can be obtained in various ways, or detailed counts may be made on appropriate samples of the population. These estimates may sometimes be made by visual inspection (Figure 5.3), or via M_1 seed yield (weight) corrected for differences in survival relative to the control population.

Figure 5.2. Isolation of sorghum M_1 plants by protecting heads before flowering with paper bags to prevent outcrossing and ensure self-pollination. Bags can also serve to protect the seeds from bird damage if maintained until harvest. Courtesy of A. Mukhtar Ali Ghanim.

Figure 5.3. Example of reduced fertility (seed set) in M_1 plants of barley with increased gamma irradiation dose rate from 0 (control) to 300 Gy. Courtesy of A. Mukhtar Ali Ghanim.

5.1.6. **Harvest of M_1**

Methods of harvesting the M_1 populations will depend on the pattern of ontogenetic development in the species, the methods of screening and the foreseen generation to be screened for desired mutants. In most instances, the genetic changes induced by mutagen treatments occur as chimeras in the somatic tissue of the M_1 plant (see Chapter 4), and the ontogenetic pattern is a prime factor affecting the expression of observed mutant-tissue chimeras in generative (reproductive) tissue. However, both the ontogenetic pattern of development and the seed yield of each M_1 inflorescence may have a bearing on the efficiency of the methods for analysing the M_1 generation. As a rule, monocots have a different developmental pattern from dicot species, but genera, species and even varieties may also differ in developmental pattern even though, the latter may be minor. In cross pollinated plants; male and female flowers, located at different position on the same plant (allogamous), might originate from different initial cells of the treated seeds and hence the M_2 progenies are likely to be heterozygous for mutant genes and might then require further controlled mating (selfing) to segregate for homozygous mutant plants in M_3 progenies.

A consideration of the relation of these factors to the methods of managing mutagen-treated populations of different plant forms is presented below.

5.1.6.1. Tiller, branch or plant progeny methods

With monocot species like cereals and grasses the maximum potential for induced genetic variability is in the primary tillers, which arise from the already differentiated primordial meristem present in the treated seed embryos. Some secondary tillers may individually yield higher frequencies of M_2 mutants but the same mutations would, generally, be also present in the progeny from primary tillers. The primary tillers are often the first ones to show signs of maturity, which is a useful guide especially if M_1 survival is low and the density of planting has not been effective in reducing tillers of all M_1 plants. With dicots largely self-pollinated seed plants, like beans, peas, tomato, etc., the methods applicable to M_1 analyses may be similar to those used for cereals except that each 'primary tiller' is equivalent here to a main branch on the M_1 plant, but in some studies even secondary branches might be analysed. However, as already stated, the approach to be followed with any particular species should be based on the knowledge of the ontogenetic pattern of that species development, since the nature of the primordia, including the number of preformed buds, degree of apical dominance, as well as other factors may influence the pattern of chimera formation.

When the seed yield from each branch or from the whole plant is relatively low, as it might be with crops like lentil *(Lens culinary),* pea *(Pisum sativum*) and chickpea (*Cicer arietinum*) whole branches, if not whole plant progeny bulks, have been widely used. In some other species there may be many branches, many inflorescences and

many seeds per fruit or aggregates of fruits on each plant. Thus, it would seem logical that a sample taken from each primary branch of each M_1 plant would be adequate.

The number of M_1 seeds sown, must be adjusted to render the expected levels of germination and survival. Where the number of seeds per fruit is not large enough and not many fruits are produced per branch, it is reasonable to suggest that each main branch be sampled, – in perhaps a somewhat large sample depending on the space requirements of each plant and the availability of resources.

5.1.6.2.Single or multiple seeds bulk methods

The single-seed bulk method, in some form, is applicable to both monocots and dicots and to all experimental situations. Its usefulness is based on the fact that the probability of occurrence of a single mutant offspring within the progeny of a fruit developed from mutated tissue is higher than the frequency of the particular mutation in the total population of plants (or branches) sampled. However, the method requires that the same number of seeds (one, or more in modified form) is taken from each fruiting structure (spike, panicle, etc.).

A modified single-seed bulk method has been successfully used for mutation studies with *Medicago polymorpha* (Brock *et al.*, 1971). The authors harvested pods from M_1 plants in bulks and in the following generation grew one M_2 plant from each of a randomly selected sample of pods.

5.1.6.3.Mass bulk methods

Mass population management also is applicable if the land and resources for mechanization are less costly than the labour required for other operations. However, in this case, with both monocot and dicot species some mechanism to limit the seed yield on each M_1 plant should be devised, even if only the sample for bulk handling is taken by hand at harvest.

5.1.7. **Management of M_2 population**

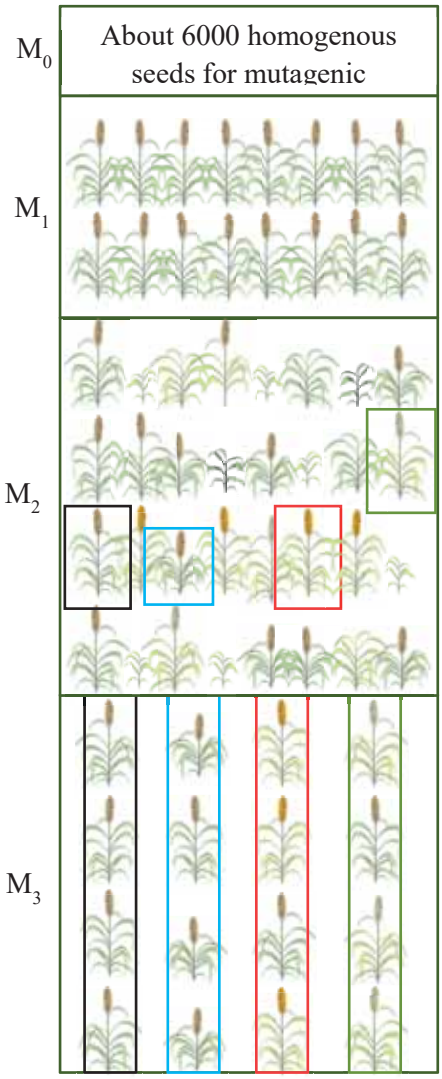
In practical mutation breeding, the nature of the trait sought, the availability of space in the field, greenhouse or laboratory, the labour needed, the possibility of mechanization, and other resources will have an important bearing on the harvest methods to be chosen and the precision and selection efficiency. A scheme for the development of mutant generations from M_0 to M_3 is given in Figure 5.4.

Selection of mutant traits is usually practiced for qualitative traits, in self-pollinated crop plants, in the M_2 generation as most of the mutants are – by then – recessive, the mutant phenotype can thus, only be seen in the M_2 generation at the earliest

(Figure 5.5). However, in cross-pollinated plants mutant genes are likely to be heterozygous in M_2 where further selfing should be practiced for producing M_3 progenies in which homozygous individuals for the mutant genes will segregate and selection can be applied. However, a useful strategy on outcrossing species is to knock out the dominant allele at heterozygous loci to unveil the recessive phenotype.

5.1.7.1.Systems of handling M_2 populations

All methods for the isolation of mutant genotypes in sexually reproduced plants are based on the pedigree method, modified to account for the chimeric structure of the M_1 plants. Furthermore, the applicable methods are based on population genetics procedures since the induced frequency of any specific mutant gene or desired mutant phenotype is appreciably lower in the M_1 population than that of a specific gene introduced into an F_1 population by hybridization. In addition, because a mutated tissue in an M_1 plant may appear only in part of the spike, pod or fruit, the segregation ratio of mutants in the progeny of seed units (pods, fruits, spikes, etc.) will usually be lower than in normally monogenic heterozygous material. The mutation breeder in such case must choose the method of screening most adaptable to his or her own circumstances. He/she should consider the merits, requirements and other aspects of alternative methods for managing M_2 populations in screening for mutants – the methods described below are primarily used for self-pollinated cereals, but with the appropriate minor modifications they could be applicable to other self-or largely self-pollinated plants.



- Initial parental germplasm: highly homogenous seeds before treatment.

- 1st mutant generation, treated seeds grown with narrow spacing to maintain few tillers/branches.
- Ensure protection from out crossing.
- Harvest each plant separately or as appropriate bulking.

- 2nd mutant generation, apply the screening method: plant in head to row, and select individual mutant plant separately (in case of qualitative traits).
- Control of untreated seeds should be planted simultaneously to verify the identity of the mutant and exclude contaminations.
- For cross-pollinated plant self M_2 as appropriate to produce M_3.

- 3rd mutant generation, seeds of selected mutants are planted for further selection and verification.
- Selection for quantitative trait on row bases may start at M_3.
- Ensure homogeneity of the mutant seeds by appropriate measures of isolation.
- Start selection in cross-pollinated plants in M_3.

Figure 5.4. Scheme for mutant population development, identification, selection and advancement of mutants from M_0 to M_3 generation.

Figure 5.5. Segregation of M_2 tomato plants for chlorotic mutant seedlings (yellow arrows) in tomato after seed (M_0) treatment with gamma irradiation at 300 Gy. Courtesy of A. Mukhtar Ali Ghanim.

1. *M_1 population bulk* - If the parental material is quite homogeneous, the tillering of M_1 well controlled by close spacing, and the M_1 grown in isolation, the bulk method of M_2 population management can be very efficient, especially for the selection of relatively divergent mutant phenotypes. Here, the whole M_1 population is harvested as one bulk and the M_2 population is grown out as a population of single plants that are screened for mutant phenotypes. The method is adaptable to mechanization at virtually all phases including the selection of variants, e.g. mechanical screening for seed size, weight, shape, etc. In some instances, particularly when mutants are better identifiable in M_3 than in M_2 a combination of methods may be desirable:

    a. M_1 population bulk to M_2 single seed bulk to M_3 ear to row progenies;

    b. M_1 population bulk to M_2 ear to row progenies.

2. *M_1 ear to row bulk* - This method, based on randomly harvested M_1 ears, is similar to method 5 below, but differs in that the relation of the ears, branches, fruit, etc., to one another is not maintained, permitting a type of bulk processing comparable to that obtained with method 4 but requiring smaller M_2 progenies (perhaps 25 – 30) and adaptable to semi-bulk harvesting of units from M_1 plants.

    As with methods 1 and 5, planting of the M_1 in a way to limit the production of primary tillers remains especially important. The method is intermediate in cost of operation and about as precise as method 4 but less precise than method 5.

3. *M_1 single-seed or multiple-seeds bulk* - This method involves selecting a single seed at random from each M_1 spike (or fruit, branch, etc.) of M_1 plant to constitute an M_2 population of single plants from the resultant bulk. M_2 single plants can be selected for mutant phenotypes that can be further progeny-tested in the M_3.

   Alternatively, single M_2 ears can be harvested for selecting within M_3 ear-row progenies for new mutant phenotypes. It is perhaps the most efficient method in terms of cost and space utilization, but its effectiveness depends on the ability to identify a single individual mutant in M_2 as is the case with method 1. It should be noted, especially for the single-seed method, that the greater efficiency of the method is achievable only by growing the same quantity of M_2 (in terms of number of M_2 plants) as with other methods. This requires treating a larger amount of parent material (M_0) for production of a larger M_1 population. If the method is followed through M_3 or a later generation, it has the further advantage that there is no increase in population with each generation. However, a drawback is the greater labour requirement.

4. *M_1 plant to row* - In this method, all seeds or a sample of the seeds produced from a given M_1 plant are grown to produce the M_2 generation, which is then screened for mutant phenotypes. The success of its use will depend to a large extent on how well the secondary tilling or branching has been controlled because the secondary tillers tend to dilute the yield of M_1 mutants. This method is preferred when seeds produced per plant are relatively low as in common bean *(Vicia faba),* pea *(Pisum sativum*), lentil (*Lens esculenta*), etc. Successful use in cereals will depend on the screening efficiency, targeted trait and land availability since generally a somewhat larger number of M_2 is needed. The overall cost for land, labour, etc., is intermediate between the spike progeny and the bulk methods.

5. *M_1 ear, branch, pod, and fruit (within plant) to row.* Here, each ear taken from the M_1 is processed as a separate entity and sown out as an ear-row progeny, which is then screened for mutant phenotypes. This method offers the greatest precision with regard to the origin of a mutant when the material treated is genetically homogeneous as regards the non-mutant allele, and when outcrossing is controlled. This is because the progeny of several ears from a given plant will rarely all carry the same mutant phenotype, and virtually never in the same ratio. The method is, however, the costliest in terms of space, labour, equipment and materials (Figure 5.4).

All these management methods assume some control over outcrossing, and the ability of the breeder to distinguish features of the parent genotype in any mutant type induced.

5.1.7.2.Size of M_2 population

The M_2 population size will, to some extent, be a function of the available space and the screening methods to be used. The size of the population may be assessed by either sampling a few seeds from many M_1 plants or more seeds from fewer M_1 plants. If the number of M_1 plants is low, but with relative high fertility then 20 – 25 seeds may be harvested from 2 – 3 spikes per M_1 plant.

However, if the population of the M_1 is large and with low fertility then 1 – 5 seeds may be sampled per spike. In principle, the greater the number of individuals, the higher the chances for selecting the desired mutant. With the M_1 bulk progeny method (1) of analysis, the estimated M_2 population size should be about twice that of the M_1 spike or M_1 plant progeny populations (M_2) as an offset to the usually lower selection efficiency.

With the M_1 single-seed bulk method (3), management efficiency is achieved only when taking as large an M_2 as for the M_1 spike progeny method, i.e. if you have 5000 M_1 plants each represented by 3 spikes and each spike is represented by 30 seeds, then the total seeds will be 30 seeds × (3 × 5000 spikes), that is about 450 000 seeds. In practice this number of seeds each from a single main branch or spike might be somewhat difficult to obtain since a very large M_1 (450 000 plants) would need to be grown for that. A modified bulk approach might involve making several (2 or 3) different single-seed bulks from the same M_1 population. The prime consideration is that each test unit would be equally represented in the M_2 population.

The successful use of the mutation breeding method depends on the choices the breeder can make among the selected phenotypes, since many of them will carry mutations in other traits as well. Therefore, the population grown should be large enough to ensure an opportunity to select more than one instance of a mutant with the desired phenotype. There is also evidence that certain kinds of mutations appear more rarely than others as discussed later in this chapter.

Mutations influencing, e.g. fertility, flowering time, flower morphology, plant height and pale green, are rather common; some other types of mutations are much more infrequent. Dominant mutations are rare, but can still be obtained and might in fact be sought for specific purposes. Disease and insect resistance mutations are relatively rare, but major differences in the frequencies of particular kinds of mutations may also depend on the parent genotype (especially in polyploid plants) as well as on the ontogenetic development pattern of meristematic tissues following treatment. With

those plants in which the M_1 plant develops from a single cell or adventitious bud, i.e. for vegetatively propagated crops (VPCs), the frequency of mutations could be higher than in plants with multi-cellular meristem initials. However, in general, mutagen treatments of pollen have so far yielded much lower frequencies of mutations compared to mutagen treatment of seeds.

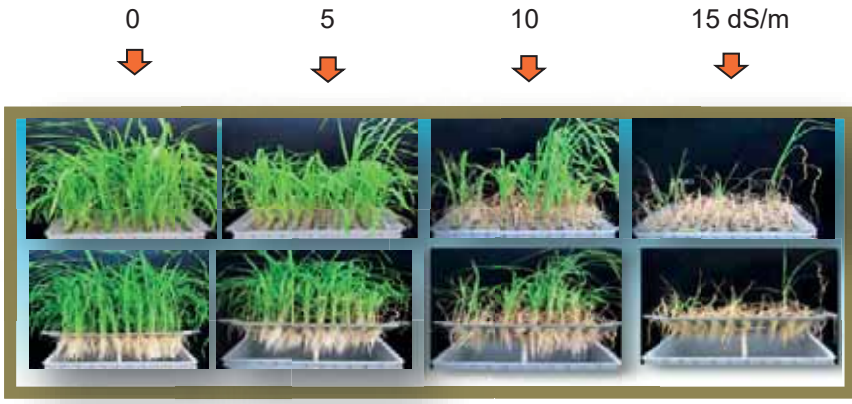


Figure 5.6. Screening for salinity tolerance, in hydroponic nutrient solution, of rice mutants at four levels of salt ($NaCl_2$) (0, 5, 10 and 15 dS/m) showing variation in shoot and root growth after 14 days of applied stress (The Joint FAO/IAEA Plant Breeding and Genetics (PBGL), Seibersdorf, protocols 2014). Top row depicting show and lower row for roots. Courtesy of A. Mukhtar Ali Ghanim.

Figure 5.7. Screening lentil mutants, in hydroponic solution, for drought tolerance using PEG6000 at four levels of concentrations (A, B, C and D), respectively: 0, 10, 15 and 20%. Photos were taken 6 weeks applying stress pressure protocol optimization experiment at PBGL, Seibersdorf, Austria in 2014. Courtesy of A. Mukhtar Ali Ghanim.

5.1.7.3.Screening methods and mutant selection techniques

Various screening methods to identify and select desired mutants have been proposed and tested. The efficiency of each method is dependent on factors more or less under the control of the breeder.

1. Visual methods of selection for identifying mutant phenotypes are common and can be very efficient. However, when breeding for specific traits it is important that the breeder ignores all other deviants. Unusual genetic variants may however, be of value for fundamental studies and in developing a mutant germplasm base for future breeding purpose. As a precautionary measure seeds from variants should be preserved. Visual selection is often the prime basis for selecting for disease resistance, earliness plant height, colour changes, non-shattering, adaptation to soil, climate, growing period, etc. The procedures involved are essentially the same as for variation introduced by cross breeding. However, with mutation breeding, ancillary techniques can be used as aids to visual selection and must be stringent and efficient for concentrating the breeder's attention on a few specific individuals from relatively large populations. Thus, mass-screening techniques (phenotypic and genotypic) are particularly suited to mutation breeding. Advances in screening methods such as laboratory and greenhouses techniques (Figures 5.6 and 5.7) enhance efficiency of visual selection of mutants.

2. Mechanical or physical methods of selection can also be used very efficiently in screening for seed size, shape, weight, density. etc., using appropriate sieving machinery as they are readily adaptable for processing of large quantities of seeds. These would normally be applied to seed produced from M_2 plants and are perhaps most adapted to the M_1 bulk population method of managing M_2 material. Processing individual M_2 lines or plants would also be feasible, but costlier.

3. Other selection methods, such as chemical, biochemical, physiological, physio-chemical, and various specific methods may be needed for selecting certain types of mutants. However, virtually all employ visual parameter to expedite detection. Low alkaloid content mutants, for example, maybe sought using colorimetric tests on M_2 seeds or plants or even M_3 seeds; protein analyses by colorimetric, chromatographic or electrophoresis techniques may be conducted on individual seeds from M_1 plants, on seeds from M_2 plants or on the bulk from M_2 plant progenies, the efficiency depending on the degree to which such techniques can be mechanized. When searching for resistance to herbicides or fungicides in a susceptible variety the chemical products might be applied to M_2 seedlings in the laboratory or

even in the field using slightly higher concentrations. Repeated application of the chemical to plants showing evidence of tolerance or resistance should then be made to confirm the response, but progeny tests of selected individuals are also necessary to confirm the genetic basis. For example, screening for herbicide tolerance can easily be conducted by thinly sowing a large M_2 population and then applying an herbicide to the entire population and retaining any survivors for further testing. Whilst this provides a very efficient screen, it does mean that the rest of the population is sacrificed in order to identify the desired mutants. It has been successfully used to produce the imidazolinone tolerant barley 'Scope' in Australia (Moody, 2015). In germination and seed viability testing, changes in phenol reaction of seed coat tissue may simply involve exposure of seeds from M_2 plants (or an M_3 seeds population bulk) to a given concentration of phenol, then visually selecting the individual seeds for appropriate changes. Insensitivity to gibberellin (which can induce short stature) can be identified by spraying gibberellin solution onto seedlings or soaking seeds in a gibberellin solution, then searching for seedlings with little or no response. When an enzyme involved in a particular pathway is known to be altered by mutation, methods to identify presence or absence of this particular enzyme, its precursors or its derivatives might be devised for use in rapid screening for desired mutants.

4. Screening for abiotic stresses such as drought, salinity, heat etc., requires setting up of the selection pressure and maintaining a uniform stress over the M_2 and M_3 populations. Recent advances in hydroponics and laboratory techniques have led to the development of different screening methods for abiotic stresses for conventional breeding programmes that can readily be adapted to handle mutant population of larger size with greater efficiency to identify phenotypic mutants in M_2 or M_3 generations (Sarsu, *et al.,* 2017 in press; Bado *et al.*, 2016). The choice of the mutant generation on which to apply the selection protocols will depend largely on the nature of the trait – qualitative or quantitative. For qualitative traits the protocol should be further expanded to provide for quantification of the effect of the trait (e.g. yield) and testing in some multiple environments to ensure stability and heritability of the trait and ensure its wide use among breeders. In recent years there have been huge advances in high-throughput phenotyping, now known as phenomics. Phenomics screening often involves optical imagining, including red-green-blue (RGB) colour model and multi-spectral cameras, coupled with bespoke soft-ware imaging analysis programmes (Tardieu *et al.*, 2017). Advanced phenomics can involve automated, robotised greenhouse, nursery and field platforms. Field trials can be imaged remotely at multiple levels (by hand, by drone or from outer-space). These methods offer unprecedented throughputs which have huge potential in increasing the efficiency of

mutation detection both in terms of accuracy and the increased numbers of mutants that can be screened. The downside is that these are very expensive, especially the more sophisticated systems, however, simple, cheap systems such as hand-held imaging cameras are also available.

5.1.8. **Management of the M_3 generation**

Progeny tests are essential for the identification of all mutant lines useful for plant improvement and re-selection from M_3; this is done to establish that the trait is heritable. Further progeny tests, may be necessary to stabilize a potentially useful variant. Furthermore, it is not uncommon that a mutant may be homozygous for the desired character but segregate for other undesirable ones, they still can be chosen when their selection might be useful for improving the genetic background of the desirable mutant. In rare cases a mutant may be the result of a modification in epistatic relations of more than one modified locus; in such a case the M_2 mutant phenotype may not re-appear among M_3 progeny. If the phenotype is the result of an interaction involving a heterozygous locus then it cannot be fixed in an inbred line, which may explain the disappearance of the phenotype between the M_2 and M_3 generations. If this is the result of interactions between independent loci that was lost due to independent assortment of gametes, then going back to the M_2 and sampling a larger M_3 should facilitate the detection of the phenotype and eventual fixation.

In several situations, M_3 progeny tests may be essential for the detection of mutants particularly of those not easily discernible from single (M_2) plants. This may be particularly true for traits that are influenced by environment, e.g. pigmentation and some biochemical or physiological mechanisms. When the number of seeds per plant, pod, fruit, spike, etc., in the M_1 is low, it is desirable to grow out an M_3 population from all M_2 plants and screen those plants, since in some practical cases as much as 60 percent of the total mutants are observed first in the M_3. The frequency of mutant individuals in the unselected population is normally higher in M_2 than in M_3 but space requirements and other considerations make it generally more cost-effective to screen for mutants only in M_2. Genotypic screening (e.g. for a mutation in a specific gene) in the M_2 is more efficient than phenotypic screening which may be influenced by the environment (see Chapter 8-C), but obviously requires knowledge of the gene in question and an efficient system to detect mutations that are likely to result in phenotypic changes.

In some other cases, especially in polyploids like durum wheat, *Triticum durum* and bread wheat, *Triticum aestivum* it may be necessary to reselect even an M_3 line and grow the M_4 progeny of few selected individuals with the mutant phenotype to be reasonably sure that the trait in question is due to mutation. In cases where one wants to test the multi-variate phenotype of a specific mutation seeds harvested from M_3

families can be submitted for field trialling in comparison with the parent line and suitable local controls. In some rare cases, new varieties can be developed from especially useful M_3 mutants, but it is more likely that further crossing and selection is necessary to produce a marketable product. The breeding of Kebari, the ultra-low gluten barley, is a good illustration of this effect (Tanner *et al.*, 2016). Here 3 null mutants at hordein loci were combined to create a triple null, which was then selected for acceptable phenotypes whilst maintaining the mutants to result in the release of a line that is well below the maximum gluten levels that coeliac sufferers can tolerate.

5.1.9. Contamination in self-pollinated mutant crops

Sometime, authenticating the genetic origin of the variation in mutagen-treated plant material may be of interest and concern not only to geneticists but also to breeders. Contamination is a common problem in any plant-breeding programme and causes even greater problems in mutation breeding. This is potentially a bigger problem for a non-breeding organisation that must purchase seeds of a commercially available cultivar rather than a breeding company utilising its own pure stock seeds. Commercially available seed stock must reach strict levels of purity but even if this is 99 percent, it means that 1 in every 100 seeds could be a contaminant. Contamination is manageable when it affects only a very small proportion of the treated population, and when the characteristics of the contaminants can be recognized easily (Figure 5.8), but the differences may be much subtler in practice as obvious differences should have been eliminated in the stock production process. However, it should be stressed that, neither the risk of contamination nor the fear of uncertainty over the origin of the variation isolated from mutagen-treated populations should deter the breeder from using the mutation approach when that approach can be more efficient and expedient to attain a specific objective. Contamination can be reduced to manageable levels by taking certain simple precaution measures. Moreover, contaminations (depending on the source) are often distinguishable by characteristic phenomena, and a number of analyses can be made that will usually lead to a conclusion about the origin of genetic variants isolated from mutagen-treated populations. A whole genome profile can now be generated for many crops at a relatively low cost and a pool of potential mutants can then be checked against the parent genotype to rapidly distinguish between genuine mutations (identical profile to parent) and contaminants (different profile).

5.1.9.1. Basis for and sources of contamination

The prime sources of contamination in mutant populations are described below.

1. Mechanical mixtures sometimes occur during the harvest of the immediate or previous generations of the parent genotype seeds from: inadequately cleaned harvesting machinery or inter-mingling of plants from different plots

during harvest, or ratoon of the same species from seeds of the previous plantings, or movement of seeds (manually, by machine, water, or animals, etc.). Ideally, the mutants should be grown in isolation to prevent uncontrolled outcrossing with other varieties of the same crop grown nearby. Contamination can also occur in seed stocks in any generation prior to or following the mutagen treatment but is less important after selections and isolation into progeny lines because deviants can be removed easily. The chances for outcrossing are increased with increasing mutagen dose, because of increased male sterility in the mutated population.

2. It is advisable to identify, purify, ensure homogeneity through selfing and progeny test of the targeted parent genotype material. Rouging-out of off-types prior to flowering will reduce cross contamination. Reducing genetic variability in the parent material before treatment is the most efficient precautions against contamination.

5.1.9.2.Criteria for distinguishing contaminants from mutants

It is impossible to prevent all and every condition that could lead to contaminations, due to costs, expediency and availability of proper facilities. However, there are tests by which the breeder can categorize or classify variations into contaminant or possible contaminant; mutant or possible mutant origin.

Figure 5.8. Outcrossing in the pale green mutant rice fields. The dark tall green plants are likely a result from outcrossing of the wild type with the mutant. Outcrossing rates are often higher than mutation rates. Courtesy of Q. Shu.

Contamination

1. Genetic variation from contamination due to admixture

The variation is likely to be of contaminant origin as a mechanical mixture present in the parent variety population, in any of the following situations.

i. Variant individuals having essentially the same phenotype(s) appear repeatedly in both M_2 and control populations.

ii. When no segregation is observed for a certain phenotype in the M_2, i.e. it is uniform.

iii. Specific variant individuals segregate in an approximately 3:1 ratio in an M_2 plant progeny line.

iv. Variant individuals are found in an M_2 branch, spike or plant progeny line that also carries a wide range of variation in other traits.

2. Genetic variation from contamination due to outcrossing

Although not always conclusive, the variation may be suspected of contaminant origin as an out-cross between the M_1 and an outside source – or mechanical mixture – in the following situations.

i. An M_2 variant individual has been changed in many ways and/or segregation in M_3 of the selected variant shows the variation involving several independently varying traits; conversely a variant with only one or only a few characters changed is likely to be a mutant.

ii. Variant individuals of the same phenotypes appear unusually frequently in M_2 populations.

A similar variant does not appear in the progeny of one or more sibling M_2 plants from an M_1 branch, spike, and panicle or plant progeny. This test is not critical, however, since mutants can originate even in rather small chimeras of a single plant, and possibly also as recombinants from an altered base sequence, which would be not likely to occur in siblings.

iii. A possible 'donor' parent for the trait(s) in question can be identified;

iv. Partial pollen sterility occurs in some M_2 or M_3 plants of a questioned progeny line, suggesting that the M_1 was partially sterile and more receptive to cross-pollination.

Mutation

1. Genetic variation of mutation origin

Due to chimerical nature of the induced variation in seed primordia, several types of variations may be directly associated with the mutation event, when the following observations can be made.

i. Variants of the same phenotype will commonly appear at a very low frequency even in a bulk M_1.

ii. Variants will appear as one to a few individuals in a branch, a spike or plant progeny, virtually never appearing in a 3:1 ratio in more than one or a few of several branches or spike progenies, but usually at a far lower ratio in one or two of a group of sub-lines and be absent from the remaining sub-lines derived from branches or spikes of a single M_1 plant. The ratio of mutant to normal should be appreciably less than 1:3 or 3:1, especially if an M_1 plant progeny consists of a sufficient number of individuals (about 40 – 50).

iii. Genetic variation of induced mutation origin.

A mutant is most likely to have been induced if in addition to meeting the criteria of mutation origin there have been similar variants isolated from several different plant progenies in a treated population at a statistically significant higher frequency than what could be observed in the M_0 control population. The number of spontaneous mutations occurring at the same time is extremely low. Spontaneous mutations can, of course, occur at any time during the life cycle, but can be determined to some extent by the procedures mentioned above.

5.1.9.3.Genotypic and phenotypic analyses

In general, for practical purposes, the criteria described below are the most critical, even though they can seldom be performed for large numbers of variants. However, tests of large numbers of variants are rarely necessary as, in most instances; the breeder finally uses relatively few of the variants isolated. The following analyses will usually permit a conclusion relative to the origin of any specific genetic variant.

1. Genotypic

   i. Progeny testing: selected variants should breed true in progeny grown from selected M_3, but might not do so in M_2 progeny tests. If segregation does occur in progenies from M_2 single plants, the variation should be limited to the selected trait although it may sometimes occur for a few (1 or 2) others.

   ii. Backcross and other crossings: selected variants should show a relatively simple genetic segregation in backcrosses to the parental genotype and to some other strains (1 or at most 2 gene segregations).

2. Phenotypic

   i. Morphological characteristics: in general mutants should show a measure of similarity to the parent genotype except for modifications related to the mutation involved. Often a whole complex of changes in phenotype could be caused by a simply inherited mutant, but a similarity in many other features should still remain.

   ii. Physiological, phyto-pathological and biochemical characteristics: the variant should show similarity to the parent genotype in a large number of measured traits, especially in traits governed by several different genes or complexes, such as quality characteristics, disease and pest resistance, and biochemical traits.

5.2.THE DETECTION OF INDUCED MUTATIONS

Mutation induction and mutation detection are two independent processes. The extent to which an induced mutation may survive and give rise to a mutant at the level of the organism is controlled by many factors as listed below.

- Uni-cellular or multi-cellular initial structures (bud and embryo meristems).
- Mode of reproduction: vegetative or seed propagated; self-pollinated or cross-pollinated.
- Stage of differentiation of the primordial cells from which inflorescences arise (whether they are already present in the dormant embryo or arise later after the mutation event).
- Number of primordial cells involved in the origin of each inflorescence.
- Time in the life cycle of the plant when such primordia arise.

- Genetic architecture of the organism (whether primarily diploid or polyploid).
- Characteristics of the locus involved in the mutation process – single or multiple gene(s).

Other genetic factors, such as polygenic inheritance, linkage, gene interaction and previous selection history of the character under study, may reduce the rates of phenotypic detection of an induced mutation. Variables relating to the mutagen used, treatment conditions, and pre-and post-treatment modifying factors also influence the manifestation, transmission and recovery of an induced mutation (van Harten, 1998; Toker, Yadav and Solanki, 2007, see also Chapters 1, 2 and 3).

Induced mutation can be accomplished easily, but such mutations will be of no use to the breeder unless they are manifested at the level of the organism and transmitted to subsequent generations, and this will depend on several principles. Thus, great attention should be given to the adoption of suitable screening procedures as described earlier.

5.2.1. **Inter- and intra-somatic selection**

Unicellular organisms are quite easy to screen for mutations and do not present the same problems for mutant recovery as described in the multicellular organisms (Brunner, 1995;). On the other hand, crop plants are all multicellular; hence the cells carrying any mutation will have to compete with normal cells regarding growth and survival. While, in asexually propagated plants, such competition can be overcome *in vitro*, for example, by raising whole plants from single cells as in *Saintpaulia* spp. or other ornamentals (Broertjes C, & van Harten, 2013), in sexually propagated plants, this competition leads to two types of endogenous selection, which will intervene before any mutation induced in a treated seed may be expressed in the M_2 generation.

The first process occurs in the M_I somatic tissue and is termed ‘diplontic selection’ defined as a “competition between cells within a meristem” (Klekowski, 2011 and Rajarajan *et al.*, 2014). The second process of selection happens in the gametes of the M_1 plants and is hence referred to as ‘haplontic selection’, which can be defined as ‘the competition occurring during haplophase, *i.e.* between gametes’ for the production and transmission of the mutation to the zygote (Figure 5.9). Haplontic selection operates more rigorously in the pollen than in the ovules. Only a mutation that passes through both the somatic and gametic sieves will show phenotypic expression in the M_2 and subsequent generations. Among sexually propagated plants the handling procedures are simpler for those that are hermaphroditic or monoecious than for dioecious plants. On the other hand, in asexually or apomictically propagated plants the somatic sieve is the only important one since the gametic sieve does not

operate. Again, among apomicts there is a difference between those that are solely propagated through vegetative parts and those that are seed propagated without there having been through any meiosis in the cells that give rise to the functional megaspores. The sexually and the apomictically propagated plants will have to be considered separately, since the problems of recovery are quite unique to each of these groups.

In rice and wheat, the number of initials in a meristem may vary from one to two or more. When only a single cell is involved in the mutation process, then the entire inflorescence will carry the mutation. Alternatively, if several cells are involved, the inflorescence could appear as chimeric. M_1 segregation ratios will help estimate the number of inflorescence initials. The expected segregation ratio of normal to mutant phenotypes will be 25 per cent (3: 1) if a single cell initial alone is involved; when more than one initial exists, since all of them may not mutate simultaneously, the segregation ratio will be less than 25 per cent in the M_2 generation with a deficiency of recessives (Cheema and Atta, 2003).

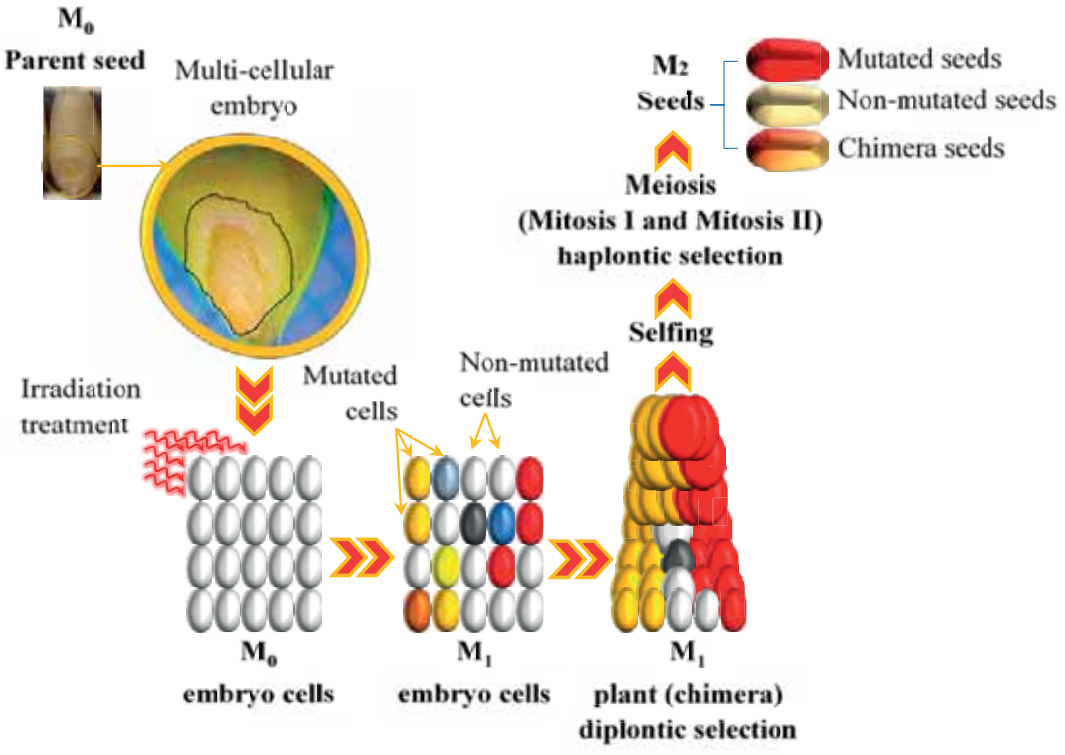


Figure 5.9. Schematic diagram illustrating the effect of diplontic (somatic) and haplontic (meiotic) selection of mutated cells, after irradiation treatment of the multi-cellar embryo, on the mutants M_2 seeds.

When a plant produces several tillers, as in barley or wheat, the first-formed tillers may carry more different mutations than later-formed ones. This is because of the greater probability that the first-formed tillers arise from several initials already present in the dormant embryo subjected to treatment. Thus, a reduction in tilling achieved through close planting can increase the frequency of mutants recovered in M_2. It has been found that close spacing of barley plants raised from irradiated seeds reduced the average number of initial cells involved in spike organization, thereby increasing the area of mutated sectors (Singh, 2016). However, diplontic selection does not seem to play the same role under all experimental conditions and in all plants. Frydenberg and Jacobsen, (1966) established that the spikes of secondary shoots in barley carried even more mutations than the main spike. The situation is even more complex in dicotyledonous plants. Scheibe and Micke, (1967) also demonstrated that the mutation frequency in sweet clover after seed irradiation was higher in basic secondary shoots but diluted in the main shoot by extensive branching before the formation of the flowers.

Corresponding observations were made in mutation experiments with perennial grasses like *Alopecurus,* where the yield of mutations was highest in the first seed harvest from M_1 plants but dropped sharply to the second harvest in the next year. For these reasons, it is always preferable to keep the seeds of each inflorescence separately and carry them forward to the M_2 generation as ear or inflorescence progenies rather than whole plant progenies. This will help avoid a numerical dilution of the mutants occurring in the population and thereby facilitate their detection.

5.2.2. **Genetic structure**

The genetic structure of the plant greatly influences the rigour of both the somatic and haplontic sieves. Where duplicate loci are present for the basic metabolic factors, as in many polyploids, both mutations and chromosome aberrations survive to a greater extent than in plants with a strictly disomic genetic constitution. Thus, polyploidy may facilitate the later recovery of induced mutations. On the other hand, the phenotypic expression of an induced mutation may be masked because of the buffering effect of the duplicate factors. Therefore, in many polyploids chlorophyll mutations are rare in the M_2 generation. However, while chlorophyll mutations may be rare or absent, viable morphological mutations may occur at a high frequency in polyploids like bread wheat (Hancock, 2012).

The nature of polyploidy is also important in relation to both the discovery and the expression of an induced mutation. An understanding of the genetic constitution would help to formulate suitable handling procedures. For example, in an autotetraploid with a genotype *AAAA* at a locus the M_1 plant may become *AAAa* if the mutation *A* to *a* occurs. In the M_2 only plants with the genotypes *AAAA*, *AAAa* and

AAaa will occur (unless there is random chromatid segregation) and hence, the population will be phenotypically homozygous. A recessive phenotype may only arise in M_3, therefore in such cases the screening should be carried on to M_3 and later generations.

5.2.3. **Function of locus**

The function of the locus concerned and its neighbouring genes will also determine the viability and frequency of the transmission of mutations. It is obvious that a mutation in a locus with an important metabolic function will be less likely to survive than one not concerned with growth and survival of the plant.

5.2.4. **Mutability**

It is important to note that different genes are not equally mutable. For example, when studying the rate of spontaneous mutations in maize found it was that the gene *r* (for colour) is relatively mutable, whereas the genes for waxy *(wx)* and shrunken *(sh)* endosperm are relatively stable (Bennetzen and Hake, 2009). Cox, (1972) suggested that mutation rates at different loci in the same cell may differ considerably and that these rates are genetically controlled. To ascertain this assumption, the author argued that genes in higher organisms may be organized not according to their function, but into different segments of the genome that have different intrinsic mutation rates.

5.3.IDENTIFICATION, EVALUATION AND DOCUMENTATION OF MUTANTS

5.3.1. **Identification of mutants**

For practical plant-breeding purposes, the question of whether a useful variant selected after mutagenic treatment represents an induced mutant, a spontaneous mutant, a line already intrinsic in the untreated material or a product of segregation after selfing or outcrossing, is of minor concern. However, for mutation research and for the evaluation of the efficiency of mutation breeding as compared with other breeding methods this question is of great importance.

Although it appears impossible to state with absolute certainty that a variant has been induced by the mutagen used in the particular experiment, typical macro-mutants of self-pollinating crops such as: dwarf and semi-dwarf, spelt, early maturing, disease resistant types and other major changes may be considered as being most probably due to an induced mutation, mainly if the same variants are not found in comparable control populations. Various precautions and procedures may help to verify the identity of recognized mutant.

- Mutagenic treatment of highly inbred material 'pure line' and, if possible, of a single plant progeny selfed repeatedly under controlled conditions (by field isolation or bagging).
- Mutagenic treatment of a 'pure line' marked by alleles not occurring in the other breeding materials.
- Strict isolation of the M_1 generation.
- Pedigree breeding from M_2 to M_3 or M_4.
- Re-selection within the selected variant.
- Comparison of the variant with similar strains and varieties.
- Testing the race spectrum of disease resistance.
- Analysis of reciprocal crosses between variant and mother strain, if possible combined with fingerprinting and/or DNA and RNA sequencing.
- Segregation analysis up to the M_3, M_4.
- Test crosses of the variant with other genotypes showing the variant character.
- Mapping the mutant locus.
- Cytological investigations to reveal induced chromosomal rearrangements.
- DNA testing/sequencing of candidate genes when the causal gene is deliberately targeted or can be predicted from the phenotype.

5.3.2. **Propagation and evaluation of useful mutants**

When a mutant appears promising it will take one or both of the two courses illustrated in Figure 10; (1) direct increase by repeated cycles of selfing to produce sufficient seeds for evaluation in replicated trials, or (2) use in crossing schemes, either back to the parental line or to other elite lines to transfer the mutant trait into contemporary breeding material. In many species, the rate of genetic progress means that the parent cultivar will be inferior to more contemporary elite cultivars, even if the parent cultivar was the best available at the time of planning the mutation experiment. The isolated mutant is also likely to carry other undetected mutations that may result in an inferior phenotype in other locations so some form of transfer by hybridisation and selection is generally desirable. The seeds of the mutant needs to be multiplied prior to proceeding to multi-location trials. The mutant, the mother genotype and other prevailing varieties with which it is intended to be compared, should be propagated under the same conditions to produce comparable seed properties for field testing.

The methods of testing seed propagated mutants in comparative trials are essentially the same as for any other newly developed genotype (Hertel and Lobell, 2014; Johnson *et al.*, 2017). Mutants exhibiting variations in vernalization requirement, photoperiod response, growth habit, stature, resistance to biotic and abiotic stresses and yield components should be tested in a wide range of environments, i.e. various locations, soils, water and nutrient conditions, seed rates, planting distances, sowing dates, etc. For the first trials the number of replications per mutant is often reduced in favour of a higher number of treatments and locations. Inter-varietal competition is expected when neighbouring plots differ widely in stature and/or growth pattern and the mutant growing type is rare among the varieties tested. It is not predictable whether, for example, the taller or the shorter varieties have an advantage in a particular trial. Competition effects can be minimised by an appropriate shape and size of the plots and by excluding border rows from plot evaluation.

Important details for documentation include: origin (pedigree) and descendance of the treated material (number of generations after a cross), material mutagenized, pre- and/or post-treatments applied, mutagen type and dose used, mutant generation of the first selection and the morphological and physiological differences between the mutant and its mother genotype It should be noted that in most national variety registration and release systems there are established guidelines for evaluation of new genotypes for the purpose of release, including mutant varieties.

5.3.3. **Recording of experiments and documentation of mutants**

5.3.3.1.Recording of experiments

All relevant facts and information of the mutation breeding experiments should be stated when reports are written, and the results should always be presented in a clear, understandable way and should include all important details. It is especially important that careful consideration be given to the protocols formulated for conducting the experiments that could then be reported in publications, according to a standardized format. There are many excellent examples, with variable layout, that illustrate experiment documentation and publication. As an illustration, a general outline is given below.

I. Experiment title

II. Experiment identification: location, main investigators, experiment number, date, etc.

III. Specific objectives

IV. Material and methods

A. Constants in experiment

1. Material

a. Biological parent material (name, identification number and pedigree if appropriate); indicate source, composition (bulk or lines), etc.

b. Ideally, a sub-sample of the parental material should be logged in a gene-bank.

c. Mutagen(s), source, energy, dose rate, tests to ensure purity, etc.

2. Methods

a. Pre-treatment(s), including preparation for treatments.

b. Treatment(s)

- Mutagen dose(s): (i) Radiations, rate and time(s), distance from source; (ii) Chemicals, concentration(s), time(s), composition and amount of treatment solution, etc.
- Conditions of treatment(s).

c. Post-treatments; handling of treated material, storage, planting, etc.

B. Experimental variables: Record treatment and replicate numbers in sequence depending on the specific experimental variables, presenting the detail of experimental design relative to specific objectives; arrange variables (whether materials and/or methods, treatments and replicates) as per the above listing.

V. Results

VI. Summary and conclusions

VII. References

5.4.FACTORS INFLUENCING THE SUCCESS OF MUTATION BREEDING

The success of the mutation breeding programme is measured mainly by the production of superior varieties, but also by the spectrum and quality of mutants

induced, identified and recovered from a segregating mutant population. Even with a full consideration of the requirements for the mutation experiments there are other factors which could limit the success in recovering the targeted mutant trait. These mainly include, the situations described below.

5.4.1. **Differences due to the genotype**

Much evidence exists that genetic differences, even when they are as small as single gene differences, can induce significant changes in radio-sensitivity, which in turn influences not only the total rate but also the spectrum of recoverable mutations and the degree of background (Zaman *et al.*, 2007). Although nobody is able to predict the influence of a particular genotype on the mutation spectrum, the choice of the parent material is a key factor of any programme in mutation breeding (Bradshaw, 2016).

More definite information is available with regard to the influence of the ploidy level on the mutation spectrum. In diploid species the great majority of mutations occur in single recessive genes. However, deviation from the normal 3:1 ratio due to deficiency of recessives has been very frequently observed. Dominant vital mutations hardly occur, in fact that they are mostly lethal or semi-lethal in the homozygous condition, in contrast to diploid organisms, as the dose required to produce them is unlikely to result in viable plants. Many genes are re-duplicated in polyploids, which increases their ability to bear a high mutational load, including gross chromosome aberrations, with no apparent negative effects. This results in the more frequent discovery of dominant and semi-dominant mutations amongst such species.

Phenotypic buffering is another property of polyploids that restricts mutability of many characters, especially those essential for the whole life of the plant: e.g. the process of chlorophyll formation. Thus, chlorophyll mutations decrease with the increasing level of ploidy Stadler, 1929 cited by Jankowicz-Cieslak, Mba and Till, (2017); however, the total rate of mutation increases. For example, in *Triticum* spp. the total mutation rate was about three times higher in hexaploid wheat than in the tetra and diploid genotypes (Rajarajan *et al.*, 2014). Differences in mutagenic response exist also between species of the same level of ploidy and between varieties within the same species. Different monosomics of *Triticum aestivum* showed differences in mutation frequency owing to factors controlling the chlorophyll development (Lundqvist, 2014; Umavathi and Mullainathan, 2016; Protić *et al.*, 2013). In a review on mutation breeding in plants Gottschalk and Wolff, (2012) reported results confirming that the closer the varieties are in their genotypes, the greater is the similarity in their spectra and frequency of mutation.

In studies of induced mutations in quantitative characters differences in the ploidy level were not as important as the genotype at the same ploidy level (Bharathi

Veeramani *et al.*, 2005). Thus, it seems that genetic variability in the background of a genotype is an important factor. According to several authors those characters that showed greater variability in the background could be improved more easily and give better expectation of mutant improvement.

In biennial or perennial plant species it seems preferable to use early flowering genotypes for mutagenic treatment and to harvest seeds from the M_1 plants in the first season. Any valuable mutant characters can be transferred later quite easily to late maturing genotypes, if they are of particular breeding value (Wani *et al.*, 2014).

Heterozygosity as a genotypic property can also influence the type and frequency of mutation. Many polyploids are less sensitive to chromosome aberrations if they are in heterozygous condition (Bradshaw, 2016). As Gregory (1960) stated "*The chief limiting factor in mutation production and mutant recovery is the genetic constitution of the experimental organism and not the type of mutagen used. Thus, for the plant breeder, knowledge of what might be called mutant expectations in his material may be more important than a resolution of the mechanism of mutational change at the sub-microscopic level*".

5.4.2. **Type of mutagen and dose**

The difference in mutation spectrum among different sources of irradiation is obvious in the spectrum of induced flower colour changes following mutagen treatment (Jain and others, 2010). For instance, densely ionizing radiations such as different sources of ion beam produce relatively more chlorophyll mutations of the albina, striata, and xantha type (Figure 5.11), whereas the frequency of the viridis type is highest following gamma-ray treatment. Thus, the chance of selecting desired mutants might be considerably increased by broadening the choice of mutagens. However, as discussed earlier, besides the mutagen, other factors also affect the mutations spectrum and the quality of induced mutants.

Another problem in the mutant quality is the number of mutation events that occur in the same meristematic cell at the time of treatment that are transmitted to later generations. The number of desirable event is far less than the undesirable ones and consequently the number of mutant plants that carry only desirable changes will further decrease if more than one mutation per cell is induced. Several measures can be taken to avoid this undesirable result. Firstly, one should not apply too high a dose of any mutagen. Secondly, one should seriously consider that super-mutagens, which give mutation rates of at least 50 percent on the basis of plant or spike progenies, may not be at all advantageous for mutation-breeding purposes. Thirdly, if high mutation rates have been induced, they should be allowed to segregate, and selection for useful types should be conducted in M_3 or later generations (Hansel, Simon and Ehrendorfer, 1972). However, one should realize that the latter technique will not eliminate those

undesired mutations that are closely linked with a desired character in the same chromosome.

In recurrent irradiation experiments the number of recoverable mutants can be increased, but since the number of multiple mutations also increases, the mutant quality from the breeding point of view may decrease (Micke, 1969). It would seem wiser to allow segregation of mutation material, or to put the same effort as for repeated mutagenic treatments into crosses of deviating plants selected in the M_1 generation for purification of the mutant character, or transfer of the mutated locus into another genetic background. Both actions might change the quality of a mutant in the desired direction.

5.4.3. **Pleiotropy and linkage**

Generally, it seems to be nearly impossible to find a mutation in an organism that results in only one single divergent phenotype compared with its initial wild genotype. For instance, mutations resulting in pale green plants also result in reduction in general plant growth and delayed maturity and, in most cases a group of distinct variants can be observed and this group as a whole is transferred from one mutant generation to the next showing mostly a 3:1 segregation ratio. Theoretically, there are three possible interpretations for this behaviour:

a. a single mutant gene is responsible for the whole complex of deviating characters;

b. a tiny portion of a chromosome has been lost containing several genes;

c. and several closely linked or neighbouring genes have mutated.

Monohybrid segregation will occur in all these cases but only the first one is a true example of a pleiotropic gene action. The other two events simulate a pleiotropic effect of one gene, although several genes are lost or altered. It is practically impossible, in most cases, to state which of these possibilities is realized; therefore, the term 'pleiotropic gene action' is commonly used in the literature for the whole group of these phenomena.

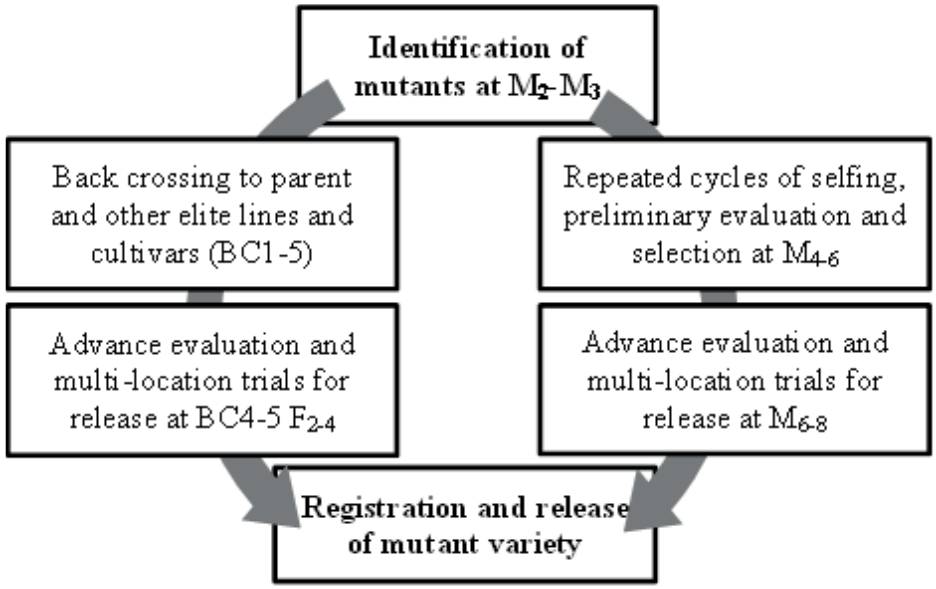


Figure 5.10. Schematic diagram showing options for advancing identified mutant in M_2 or M_3 through either direct selfing and evaluation (right course) or backcrossing to parent or elite lines and cultivars (left course) to multi-location trials, registration and release to farmers.

Figure 5.11. Examples of different types of irradiation-induced mutations on spikes (A-Hood spikes) and leaves (B-Albino, C- Striata, and D- Xantha) in barley (Hordeum vulgare). Courtesy of L. Gomez-Pando.

Quite often, true pleiotropism may be realized but there is no doubt that not only minute deficiencies but also a more or less simultaneous mutation of closely linked genes frequently arises by application of mutagenic agents (Gottschalk and Wolff, 2012). Although there is plenty of evidence for a differential chromosome-breaking ability of different mutagens, there are so far, no systematic studies regarding the extent to which pleiotropic effects are associated with mutations induced by a particular mutagen. Advances in whole genome sequence of the mutants and their parents will enable precise identification of the genes affected by mutation and associate them with the expressed mutant phenotype (Caldwell *et al.*, 2004; Jannink, Lorenz and Iwata, 2010, see also Chapter 8.3).

This 'pleiotropic gene action' is a serious handicap in the practical performance of mutation breeding. A large number of progressive mutants of different cultivated plants cannot be used for practical breeding purposes because some negative features are combined with the useful character. If the whole spectrum of phenotypic alterations is really due to the action of one single gene, there may be no hope of using the positive feature for practical breeding, because it cannot be separated from the negative ones.

However, there is some evidence from experiments that specific detail of such a true pleiotropic spectrum can be altered by transferring the mutated gene into a different genetic background including the parent genotype (Gottschalk and Wolff, 2012). Therefore, if a really important and valuable new character is part of a pleiotropic spectrum, the mutant should be crossed with a large number of different varieties or genotypes of the respective species in order to reduce the intensity or the strength of the negative characters of the spectrum in a particular genotypic composition.

If 'pleiotropic mutation' is due to a deficiency, there is no possibility of a partial repair. But if such a complex is caused by two or more independently functioning neighbouring genes it is, in principle, possible to separate the positive and negative characters by means of crossing and target selection. Some examples are known in which such a separation could be achieved (Gottschalk and Wolff, 2012). However, the frequency of such a recombination event will be extremely low because of the very close linkage of the genes in question. It may therefore be only worthwhile searching for such rare recombination event for particular important breeding goals that cannot be achieved by other means.

Finally, it should be mentioned that many characters of a plant organism are controlled by polygenic systems and that different genes of such a system often develop distinguishing pleiotropic spectra, as could be shown, for instance, for the numerous *erectoides* loci in barley (Kuczyńska *et al.*, 2013; Lundqvist, 2014).

6. MUTATION BREEDING FOR VEGETATIVELY PROPAGATED CROPS

6.1.APPLICATION OF MUTATION TECHNIQUES

A wide range of crops are not propagated through seeds, these are mostly plants grown for their edible roots, tubers, leaves, and fruits, the propagation is obtained by multiplying stem, root, stolon, tuber, and fruit cuttings, and even leaf fragments. Multiplication is thus asexual, and such crops are known as vegetatively propagated crops (VPCs). These plants include many economically important crops such as banana, cassava, potato, sugarcane, many ornamentals, fruit trees and other commodity crops such as tea and rubber. As an example, most edible bananas are triploid and sterile, thus seedless, hence conventional breeding methods are difficult if not impossible to implement. Mutation induction offers a useful tool for the improvement of many VPCs, including banana using *in vitro* together with greenhouse propagation techniques, i.e. cuttings and/or grafting.

Conventional plant breeding depends on the availability of utilizable genetic variation for crossing and selection of desired genotypes. Genetic variability utilised by conventional breeders is either found within the elite breeding pool (preferred breeding material), exotic germplasm (second choice) or wild species (third and most difficult breeding option) and transferred into advanced lines through hybridization and the natural meiotic processes of recombination. The desired recombinant genotype is later screened from segregating populations, and then through trials and selection procedures, a selected potential mutant-line may be released as a new variety. However, these procedures are restricted to sexually propagated crops (see Chapter 4). Most VPCs are not suitable for such methods and even when feasible, the application of cross breeding to VPCs is often too time consuming and remains difficult since most VPCs exhibit a high level of heterozygosity, aneuploidy and/or polyploidy and complicated biology (dormancy, long seasonal cycles, long juvenile stage, fertilization incompatibility, etc.). In addition, it is often impracticable to grow out large populations that exhibit sufficient genetic variation for screening due to space, time and cost considerations.

Thus, for VPCs mutation breeding offers an alternative approach in widening the genetic variability and producing novel traits by increasing the frequency of mutations (over the spontaneous rates) and inducing desired genetic mutations (such as unmasking recessive traits) that lead to superior performance and the development of a new variety. As stated by Donini and Sonnino, (1998), in most of the vegetatively

propagated species, hybridization is not an easy option, and induction of mutation is the only method for improvement.

VPCs are more complicated to handle as they present a wide range of targets (plant parts), each of which requires specialised methods for the induction of mutation. However, in recent years there has been new developments in enabling biotechnologies, particularly in tissue culture, that provide efficient methods for mutation breeding in VPCs. These include *in vitro* micro-propagation to raise populations for mutation induction, mutant screening and mutant line development, and have been reviewed recently by (Bado *et al.*, 2016).

6.2.SELECTION OF PARENTAL LINES AND MUTAGENIC TREATMENTS

6.2.1. **Selection of parental lines**

Breeders of all crops need to carefully choose the variety/genotype to be mutagenized; this involves consideration of its genetic characteristics and uniformity, and the possible availability of the desired traits in the genetic pool of available germplasm. In practice, the chosen variety/genotype should be well adapted, have a superior agronomic performance and require the least number of genetic modifications, e.g. resistance to a specific disease, resistance to lodging, short and stiff straw, etc. The parental material is important because the genetic background of the plant generally defines the range of mutant traits that can be obtained (Suprasanna and Nakagawa, 2012a). It is especially important in VPCs and the methods used often involve tissue culture and therefore the selected target genotype must be amenable to *in vitro* techniques. Most VPCs are highly heterozygous (*Aa* in case of diploids, *Aaa* for triploid and *Aaaa* for tetraploids) and a major aim of mutation breeding is to knock-out the dominant allele to un-mask recessive traits. It should be noted that, albeit rare dominant mutations can also be produced. There has been much discussion on the status of ploidy of parent plant material to be used for mutagenesis (van Harten, 1998). In some cases, cultivars with higher ploidy gave higher number of mutants as in the case of octoploid *Dahlia*, hexaploid *Chrysanthemu*m and tetraploid *Begonia* (Broertjes and van Harten, 1987) . However, it was also observed that in some polyploids e.g. tetraploid *Freesia*, no mutants could be recovered. Optimal treatment conditions will have to be considered carefully on a case-to-case basis and from other related relevant reports before designing a mutation induction experiment for a VPC.

Plant material for mutagenic treatment should be disease-free (including virus-free), the plant parts should also be uniform in terms of developmental stage and origin, and be representative of the chosen clone, variety or genotype. Preferably the materials should come from or be derived from the same ortet, otherwise materials may need further cloning in the field, greenhouse or *in vitro*.

There are several plant targets for mutagenic treatment, these include:

- shoot tip or meristem tip from *in vivo* vegetative buds, stem cuttings, leaves, petioles etc.;
- adventitious buds from explants of root cuttings, stem cuttings, leaves, petioles, pedicel or somatic tissues; and
- cuttings from roots, stems, leaves, petioles, pedicels, etc.

In general, freshly induced/formed buds are highly appreciated for mutation induction of vegetatively propagated crops. Both apical and axillary buds in fixed positions on the plant and newly formed adventitious buds may be submitted to mutation induction under specific *in vitro* or *in vivo* culture conditions. In all cases, the plant materials to be treated should be selected according to the aim of the breeding programme, which is usually to develop a genotype differing by only one single trait from the parental germplasm (van Harten, 1998).

As stated in the previous chapters mutations induced on meristems, often leads to chimeric structures, thus it is mandatory when considering vegetatively propagated crops to take into account the handling of chimeras. This is also closely related to the preferred mode of propagation, e.g. budding or grafting and the degree of heterozygosity level of the selected crop. Only uniform and representative material of the clone or variety should be selected for mutation induction purpose as the aim is to produce a stable (non-chimeric) mutant.

6.2.2. **Population size**

In any breeding process the success in obtaining a desired mutant depends on the ability to develop a sizable population that can be mutagenized in order to allow the screening for desired traits. Here, the randomness and low occurrence of mutations in a plant genome needs some consideration. Furthermore, the frequency of mutations in M_1V_2 and M_1V_3 generations is often related to the position of the axillary buds taken from the M_1V_1 shoot. It could also vary depending on whether the newly formed bud was pre-existing on the plant material, prior to irradiation or it arose after irradiation. Thereby, it could be expected that, in each species or variety, a given portion of M_1V_1 shoots will have a higher or a lower chance of recovery of somatic mutations. Once the most suitable axillary buds have been located preemptive action may be taken to force their growth either by cutting back the other buds on the shoot cutting (or by using these axillary buds directly for in propagation through *in vitro* culture and grafting – this action is often used for mutagenesis of woody species, e.g. fruit trees – for further details see pictures 3 and 4.

The success in obtaining desired mutants depends on the mutated population size, by increasing the number of treated plant materials one may expect a higher probability of success. A population size of 800 treated buds, which could give rise after vegetative propagation to a population of about 4000 shoots in the M_1V_2 generation, is considered adequate. For each dose 50 scions (with two or three buds) are needed, the same number should be used as a control population, e.g. for apple and cherry fruit trees at least 2000 M_1V_2 generation are needed (Donini and Sonnino, 1998; Micke and Donini, 1993). In another example on chrysanthemum, considering that a stem cutting of 10cm has about 10 axillary buds, it was therefore defined that 80 – 100 cuttings would be needed for inducing positive mutations (http://www.fnca.mext.go.jp).

A common induced mutation frequency is in the order of 0.5 percent, i.e. 5 desired mutants in a population of 1000 plants (Predieri and Di Virgilio, 2007). Theoretically, one should aim to induce mutations in a minimum of 500 plant propagules, but in practice at least 800 plant targets appear to be the minimum starting population to provide a realistic probability for selection, as stated above. This will be followed by deriving useful information on the number of propagules that can be generated through vegetative means or regeneration/proliferation rate (in case of *in vitro* cultures), number of vegetative multiplication cycles or sub-cultures after irradiation, probability of well-developed rooting and high frequency plant survival. The number of vegetative multiplication cycles or sub-cultures after irradiation may vary from a minimum of 3 to 5 or more, depending on how many chimeric structures remain after each round. The rooting rate may be only 80 percent of the regular frequency obtained for control (non-treated) plants. Plant survival can be calculated on the basis of experience with the specific plant material. Predieri and Di Virgilio, (2007) proposed the following formula to calculate the number of shoots (X) to be submitted to mutation induction treatment:

$$X = \frac{P}{[(a \times b) \times c) \times d]}$$

Where: P is the estimated number of plants to be planted in the field; a) the expected proliferation rate; b) the number of subcultures, c) the expecting rooting percentage, and d) the expected survival rate.

In another study Danso *et al.*, (1990) applied gamma irradiation at 25 and 30 Gy to 1425 cassava (*Manihot esculentus*) cuttings, selection at the M_1V_4 stage produced a mutant variety named “Tekbankye” which had good poundability, high dry matter content (40 percent) and low incidence of Cassava Mosaic Virus (CMDV). Vegetative buds of cocoa (*Theobroma cacao*) were also irradiated with 15, 20 and 25 Gy and subsequently grafted onto rootstocks to generate M_1V_1 shoots. At M_1V_3 the shoots were screened for Cocoa Swollen Shoot Virus (CSSV)-resistance, stable mutant lines

were selected in the M_1V_5 generation with high yield and bean quality. These were then tested in multi-locational on-farm trials in Ghana for over 10 years with no symptoms of the CSSV virus disease (Danso *et al.*, 1990).

6.2.3. **Mutagenic treatments**

The choice of mutagenic treatment is related to its effectiveness and efficiency in terms of mutation frequency, the availability of mutagenic treatments/facilities and he population size and physical size of the material to be treated. More than 90 percent of released VPC mutant varieties have been developed using physical mutagens (http://mvd.iaea.org). Acute, semi-chronic or chronic, and recurrent exposure may be applied to induce somatic mutations, however chronic irradiation has proven to be more useful for mutation breeding in vegetatively propagated crops because the mutants obtained undergo less radiation damaged and may be used directly as a new cultivar. All types of ionizing radiations have been used, including sparsely ionizing radiation such as X- and gamma-rays, which can easily penetrate plant tissue, and in contrast UV light, which has low penetration power is normally used only for small and sensitive plant parts (e.g. in single cells and thin tissue layers irradiation). Densely ionizing radiation such as thermal or fast neutrons, usually cause drastic changes (large deletions and gross chromosomal aberrations), which are frequently deleterious.

Chemical mutagens, on the other hand, are known to favour micro-mutations i.e. point mutations, which may be preferred as they are most likely to generate changes in the DNA structure and function and thus, lead to inheritable mutations. However, chemical mutagenesis, unlike physical mutagenesis, usually produces wide spread mutation events, and therefore background mutational load can be an issue. Chemical mutagens commonly used in VPCs include ethylmethanesulphonate (EMS), N-(nitrosomethyl) urea (NMU) and N-Nitroso-N-ethylurea (ENU). These alkylating agents are efficient but are light-sensitive and additional precautionary measures need to be taken because of their higher volatility. Bulky plant materials, such as bulbs, stolon fragments, scions etc., are difficult to mutagenize in a reproducible way using chemical mutagens (Broertjes and van Harten, 1987) Chemical mutagens usually have low penetration capability into target plant parts and this could explain their low efficiency in the *in vivo* mutation systems of VPCs. As stated in Chapter 2, strict health and safety precautions need to be observed when dealing with chemical mutagens.

6.2.4. **Selection of optimal mutagenic dose**

One of most critical prerequisites for successful mutation breeding is the determination of the optimal mutagen dose. The dose required for a particular experiment depends on the desired effects but may be restricted by undesirable effects of the mutagenic treatment, which could lead to sterility and/or lethality. There is a

strong correlation between the genotype and the sensitivity of the plant material to the mutagenic treatments in plants. The dose increase causes drastic mutations, such as chromosomal aberrations, and can cause cell damage in apical meristem thereby lower doses are usually preferred. So, it is recommended to conduct preliminary assays to determine the appropriate doses for each plant material. Radiation and chemical sensitivity tests should always be carried out to determine the mutagen dose that results in a 50 percent reduction in plant height, root initiation, etc., this value is known as RD50 or EMD (Efficient Mutation Dose) and is widely used to predict the most effective and most efficient mutagen dose (Figures 6.1a and 6.1b). In practice, a breeder applying irradiation treatment on vegetatively propagated crops may decide to settle for a growth reduction of 30 – 50 percent (RD30 – 50) for M_1V_1 plants or a survival rate of 40 – 60 percent (LD40 – 60) depending on the sensitivity of the plant material.

It is generally advised to use the doses which correspond to 60 percent (higher dose) and 40 percent (lower dose) of the determined LD50 and for each dose and to treat 30 – 50 shoot meristems bud-scions, stem cuttings, or rooted scions, etc., at a time (Donini and Sonnino, 1998). An equal number of control materials for the comparison should be planted as the same time. Measurement on shoot length reduction, of *in vivo* treated material should be recorded 30 – 60 days after the treatments (Bado *et al.*, 2015), depending on the species, for example 60 days are needed for the evaluation of the shoot length reduction for sweet cherry (Kunter *et al.*, 2012). An extensive table showing radio-sensitivity doses and estimated of LD50 or RD50, of various plant species submitted to fast neutrons and to both acute and chronic gamma irradiation can be found in (Shu *et al.*, 2012). Table 6.1 below presents some chemical mutagens and the recommended concentrations and table 6.2 summarises the recommended doses for gamma-rays treatment of vegetative propagules used for VPS mutation induction (Suprasanna *et al.,* 2012; Bado *et al.,* 2015).

Figure 6.1a. Cassava stem cuttings of the variety Sepang 1 (S-1) readied for irradiation using gamma-rays at different doses; 0, 10, 20, 30, 40, 50, 60, and 70 Gy. Courtesy of F. Ahmad, M. Akil, N. Talib and R. Ibrahim.

Figure 6.1b. Effects of gamma irradiation on budding, shoot length, and leaf size on cassava var. Sepang 1 (S-1) stem cuttings with different doses of gamma rays 20 days after planting. Courtesy of F. Ahmad, M. Akil, N. Talib and R. Ibrahim.

TABLE 6.1. EXAMPLES OF CHEMICAL MUTAGENS AND CONCENTRATIONS USED FOR MUTATION INDUCTION IN VPCS (adapted from Donini and Sonnino, 1998)

	Recommended Treatment		
Species	Plant material	Mutagen	Concentration
Potato (*Solanum tuberosum*)	Buds on Tubers	EMS	100 – 500 ppm
Grape (*Vitis vinifera*)	Dormant buds	EMS	0.15 – 0.20%
Sweet potato (*Ipomea batatas*)	Shoot tips	EMS	0.5%
Apple (*Malus domestica*)	Growing shoots	EMS	1%
Carnation (*Dianthis caryophyllus*)	Rooted cuttings	EMS	2.5%
Rose (*Rosa* spp.)	Budwood	EMS	2.5%

TABLE 6.2. ORNAMENTAL PLANTS IN WHICH VEGETATIVE PROPAGULES ARE USED FOR MUTATION INDUCTION (Donini and Sonnino, 1998).

Plant	Vegetative propagules	Mutagen - Gamma-rays
Amaryllis	Bulb	250 rad* – 5 Krad
Bougainvillea	Stem cuttings	250 – 1250 rad
Canna	Rhizome	2 and 4 Krad
Gerbera	Rooted plantlet	1 and 2 Krad
Gladiolus	Bulb	250 rad – 5 Krad
Hibiscus	Stem cuttings	1 – 2 Krad
Narcissus tazetta	Bulb	250, 500, 750 rad
Perennial portulaca	Stem cuttings	250 – 1250 rad
Polianthus tuberosa	Bulb	250 rad – 8 Krad
Rosa spp.	Stem with budding eyes	2 – 6 Krad
Tagetes erecta	Rooted cuttings	500 rad – 2 Krad
Lantana depressa	Stem cutting	1 – 4 Krad
Chrysanthemum sp.	Rooted cuttings	15, 20, 25 Gy

*1 Gy=100 rad

6.3.CHIMERAS

In previous chapters the origin and structure of chimeras have been largely described and discussed. Thus, this chapter will focus on the handling of chimeric structures in vegetatively propagated crops (VPCs).

Both solid mutants and periclinal chimeras can be used as new varieties in VPCs (Suprasanna and Nakagawa, 2012a). Experiments using appropriate materials must be carried out to clarify (disassociated) the chimeric situation. For VPCs, various methods have been developed which involve tissue isolation and dissection during post-mutagenesis aiming at reducing the genotypic complexity of the resulting plants.

In banana, to dissociate chimeras, Roux *et al.*, (2001) evaluated three different *in vitro* propagation systems (shoot-tip culture technique, multi-apexing culture technique and corm slice culture technique). The average percentage of cyto-chimeras was reduced from 100 percent to 36 percent after three sub-cultures using shoot tip culture, from 100 percent to 24 percent when propagating by the corm slice culture technique, and from 100 percent to 8 percent after the same number of sub-cultures using the multi-apexing technique. Although none of the systems studied eliminated chimerism completely, the study showed the possibilities to reduce chimeras depending on the type of shoot produced (axillary or adventitious) and the multiplication rate (number of new shoots produced per subculture). Nevertheless, in all cases after three subcultures the proportion of the number of chimeras tends to stabilize (see Chapter 2).

6.4.HANDLING MUTANT POPULATIONS AND RELEASE OF NEW VARIETIES

Since mutagen application causes some physiological and genetic injury to the plants, M_1V_1 plants should be grown in non-stressed conditions otherwise the M_1V_2 population obtained may be insufficient for selection and mutant line development. Care should be taken to ensure optimal watering, temperature; lighting and fertiliser (particularly nitrogen) should also be optimised as described for growing the first M_1 generation of seed propagated crop mutation induction.

In general the development of VPC mutant populations is done entirely by vegetative propagation and flowering, meiosis and seed production are usually avoided, so that the mutation will be inherited only by the lineage of the cells which have been mutated with no other change for the rest of the plant itself (Suprasanna and Nakagawa, 2012). The entire process is summarized in Figure 2 and described below

First year (M_1V_1): M_1V_1 generation is often not suitable for mutant selection since it is not possible to detect homohistant mutants due to chimerism, which then need to be dissolved (see Chapter 8-A). As the detection and isolation of induced somatic mutations may present considerable difficulties, appropriate selection methods must be used prior to clonal propagation.

Second year (M_1V_2): Chimeric structures may continue in the M_1V_2 generation so this generation must be closely monitored in identifying any 'deviant' from the standard material. Further visual selection and measurements are needed to identify mutants such as plant growth habit, internode length, branching type, fruit characteristics and number of buds. Selected individual M_1V_2 mutated shoots are propagated for further observation to confirm their characteristics and to assess uniformity and stability in M_1V_3 generation (Drake *et al.*, 1998).

Third year (M_1V_3): Preliminary evaluation can start here, since this generation should have solid mutants whose uniformity must be assessed. Non-uniform mutant clones must undergo a further round of propagation to reach uniformity. In this generation the evaluation of main desired traits, which may include yield, quality, biochemical/mineral content, seed/fruit size, flower traits, weight could be undertaken or delayed until more advanced generations.

Fourth to Ninth year (M_1V_4 to M_1V_9): Uniform clones may be propagated and planted in experimental trials to test their performance for desired traits such as biotic/abiotic stress. As early as in M_1V_4 generation, replicated trials of selected mutants may be conducted using parental or local varieties as checks. The M_1V_5 and M_1V_6 generations can be used in multi-locational trials and tested for performance in a range of environments and agronomic traits.

Final assessment can be made in M_1V_9 to M_1V_{10} generations depending on plant species, the desired mutant clone or clones will be released as a new improved mutant variety. With ornamental plants, selection of new mutant varieties can be quicker, depending on plant type, mutagen and explants treated as the desired changes (different flower colours and shapes and growth habit) may be reached at any time during the process. Many mutant varieties of ornamentals such as achimenes, chrysanthemum, carnation and rose have been reported (http://mvgs.iaea.org/Search.aspx). Most of these mutant varieties were derived from irradiating rooted stem cuttings, detached leaves and dormant plants (Ahloowalia, 1998).

In fact, in the case of flowers, chimeras might also be used for commercial purposes due to their peculiar phenotypes. Many mutant varieties had been developed in vegetatively propagated fruit trees (see example on sweet cherry below). One of the specific and classic success is seedless fruit in citrus, grapefruit, lemon, mandarin and oranges, which were obtained by exposing bud wood to gamma rays, thermal neutrons, or X-rays (Bado *et al.*, 2016). Figure 6.4 shows the use of budding and grafting in developing mutant lines.

Mutation breeding programmes aimed at improving abiotic and biotic stress tolerance require appropriate screening methods. It is also important to note that such methods

have to be rapid and economical, considering a large number of individual plants to be screened in the post irradiation-handling from M_1V_2 or later stages. Any valuable pre-screening techniques which can reduce the number of potential mutants to reasonable levels prior to replicated field trialling may be considered.

6.5.SCREENING TECHNIQUES FOR ABIOTIC STRESS

Major stresses include salinity, drought, alkalinity, heavy metals and high/low temperature. Screening methods need to be devised for rapid, sensitive, efficient and preferably non-destructive testing. Screening for abiotic stress tolerance can be evaluated by assessing variations in chlorophyll fluorescence, net photosynthetic rate, transpiration rate, stomatal conductance, water-use efficiency, free proline content, etc. Besides, physiological parameters, biochemical and molecular markers may, also, be developed (see Chapter 8-C). Two approaches are presented below.

Indirect methods of screening for abiotic stress tolerance can also be conducted *in vitro*, in the greenhouse or in field conditions. Various physiological and biochemical indicators are suggested, for example photosynthesis rate, stomatal conductance, chlorophyll fluorescence, lipid peroxidation, electrolyte leakage and relative water content. Chlorophyll fluorescence and thermal imaging are well-established, powerful, non-destructive, and rapid techniques for detecting and diagnosing plant stresses in the field by providing information on both stomatal and photosynthesis-related parameters, which are the key factors that determine plant yield (Li, Zhang and Huang, 2014).

In comparison to screening for stress tolerance under *in vivo* conditions (field or greenhouse), screening under controlled conditions can be advantageous by using stress inducing agents. Pre-field screening to reduce the number of potential candidates to reasonable numbers prior to replicated field trialling is also valuable (for practical examples, which may be applied to VPCs see, (Bado *et al.*, 2016).

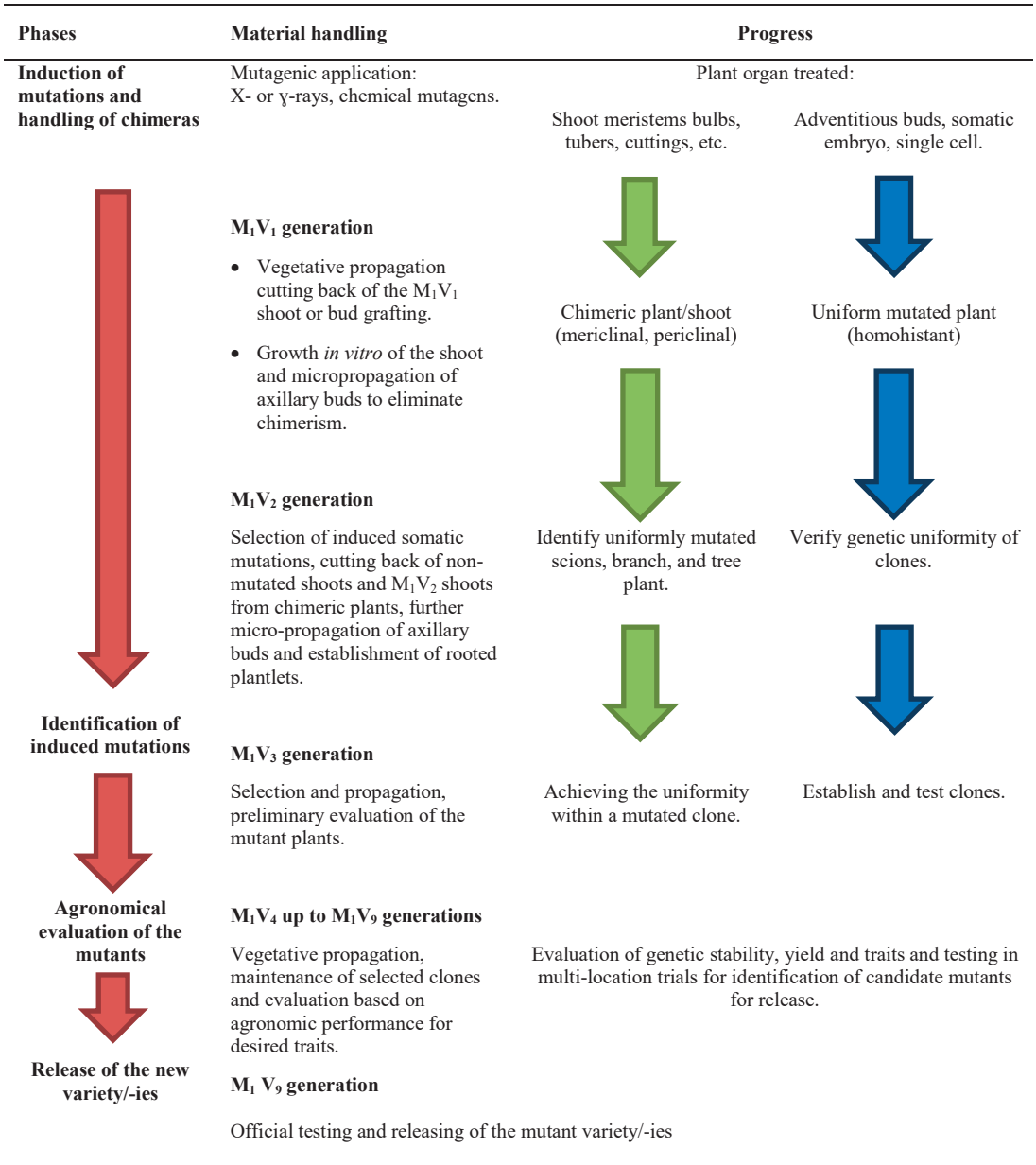
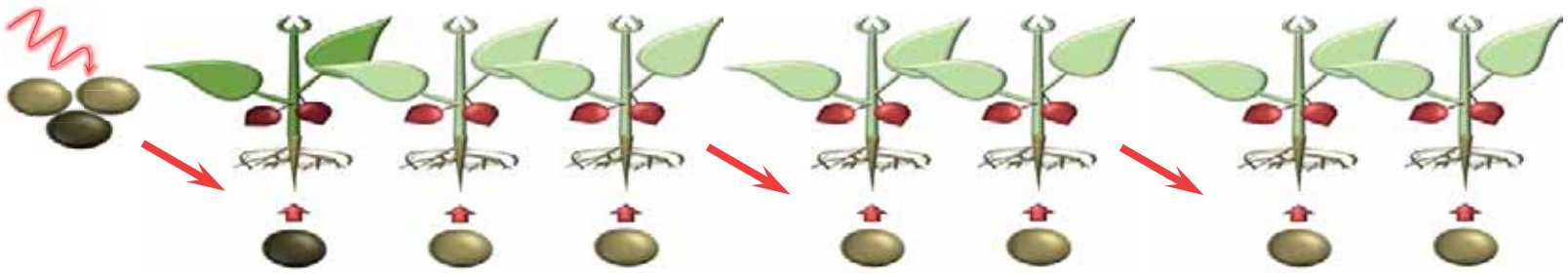


Figure 6.2. Mutation breeding scheme for the improvement of VPCs (as modified from Donini and Sonnino, 1998).

Irradiation of M_0 seeds with optimal dose



Irradiated seeds advanced as M_1 population

Seeds from the M_1 plants are sown again as M_2 population

The procedure is repeated for M_3, M_4 populations to stabilise any putative mutation

a) seed propagated crops (ex: ^{60}Co gamma ray irradation)

Irradiation of cuttings

Development of irradiated axillary bud into M_1V_1 shoot

Development of the new axillary bud into M_1V_2 shoot

The procedure is repeated several times to M_1V_3, M_1V_4, etc…

b) Vegetatively propagated crops

Figure 6.3(a,b). General mutation induction principles for seed versus VPCs. (as modified from Broertjes and van Harten, 1987).

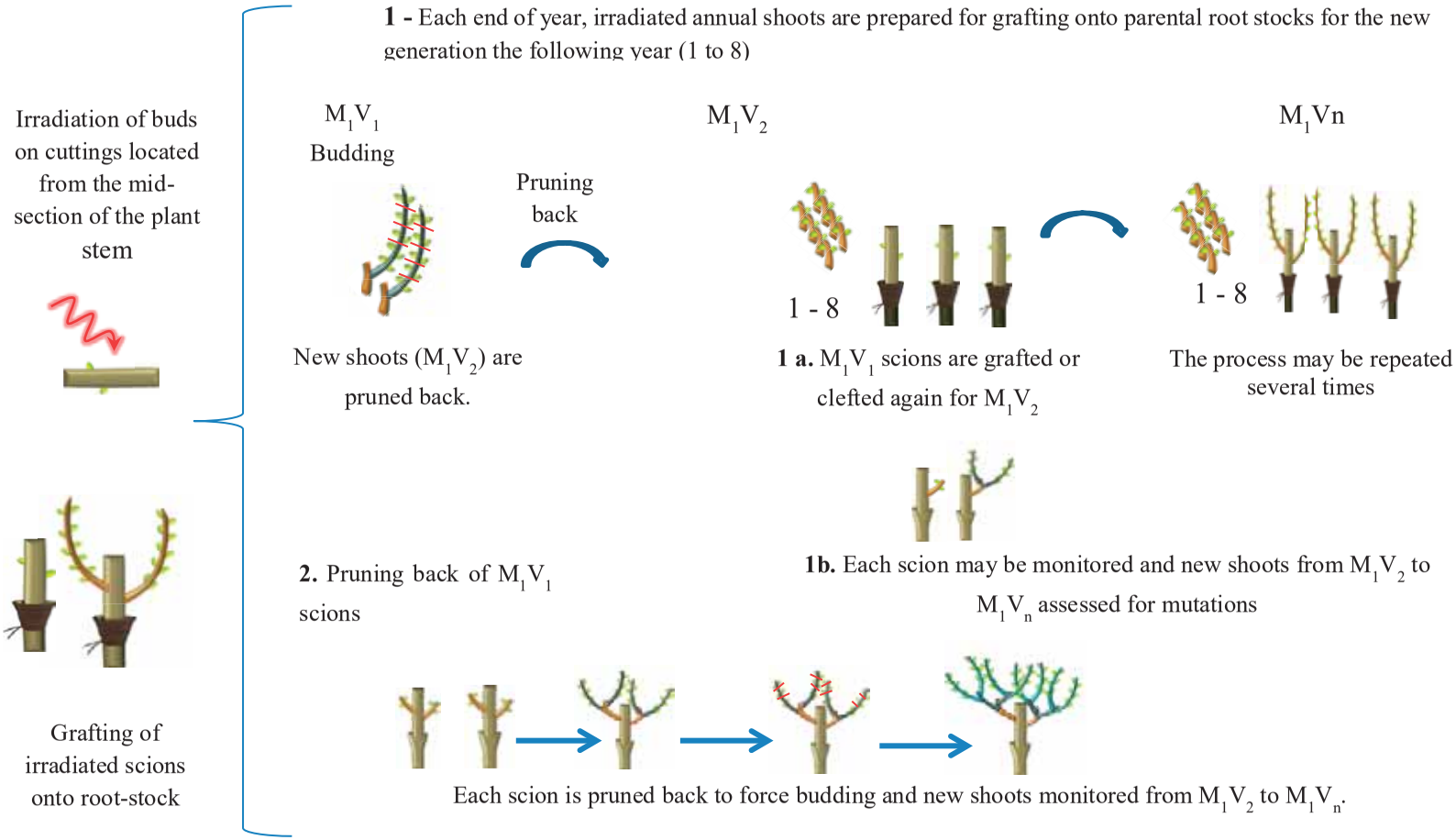


Figure 6.4. Isolation of somatic mutations in vegetatively propagated fruit trees (as modified from Broertjes and van Harten, 1987).

6.6.SCREENING TECHNIQUES FOR BIOTIC STRESS

Vegetatively propagated crops such as banana pose unique problems compared to cereals because they have a reduced genetic diversity as they have been grown in monoculture for years and cannot be selfed easily nor cross pollinated to enhance variation. Furthermore, because of its triploid parthenocarpic nature, bananas do not produce seeds and conventional breeding is thus a long process that often results in varieties not accepted by consumers.

Fusarium oxysporum f. *sp.* cubense is a fungus, which threatens the Cavendish banana production worldwide. A mutation breeding experiment for resistance to *Fusarium* wilt in banana clones was carried out by Mak *et al.*, (2004) using the double tray technique to screen banana plantlets in greenhouse conditions (Figure 5). The technique consists of a perforated upper tray which contains sterilized sand media to grow banana plantlets and a lower tray to accumulate nutrient solution and pathogen derived wash. Meristem derived from two-months old plantlets (10-15cm long) of mutant banana clones 'Intan' (Pisang Berangan, AAA), 'Gold Finger' (AAAB), 'Novaria' (Cavendish, AAA), and 'Mutiara' (an improved Pisang Rastali, AAB) were tested against *Fusarium oxysproum* f. sp. *cubense* (FOC) race 4 under greenhouse conditions. Susceptible plants showed both foliage and rhizome a symptom within 10 – 30 days, this technique is therefore accepted as a rapid method for early screening against *Fusarium* wilt disease.

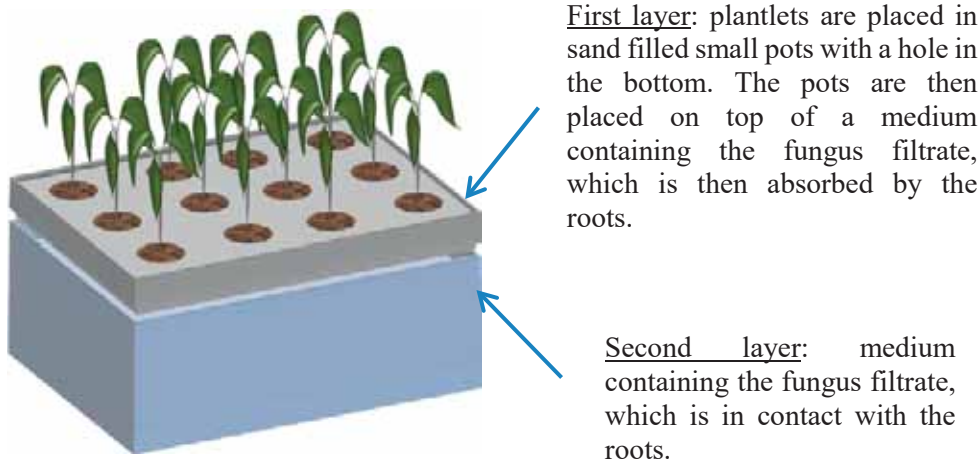


Figure 6.5. Double layer method modified from Mak et al., (2004).

6.7.PRACTICAL EXAMPLES OF THE APPLICATIONS OF MUTATION BREEDING IN VPCS

There are many varied methods for mutation breeding in VPCs. Several of these involve *in vitro* techniques and protocols are presented for banana, sugarcane and melon in Chapter 8-A. Here we show an example in a fruit crop; sweet cherry (Figures 6.6a,b) and a novel method recently developed using micro-tubers in potato (Figure 6.7a,b).

6.7.1. **Practical example of mutation breeding in Cherry**

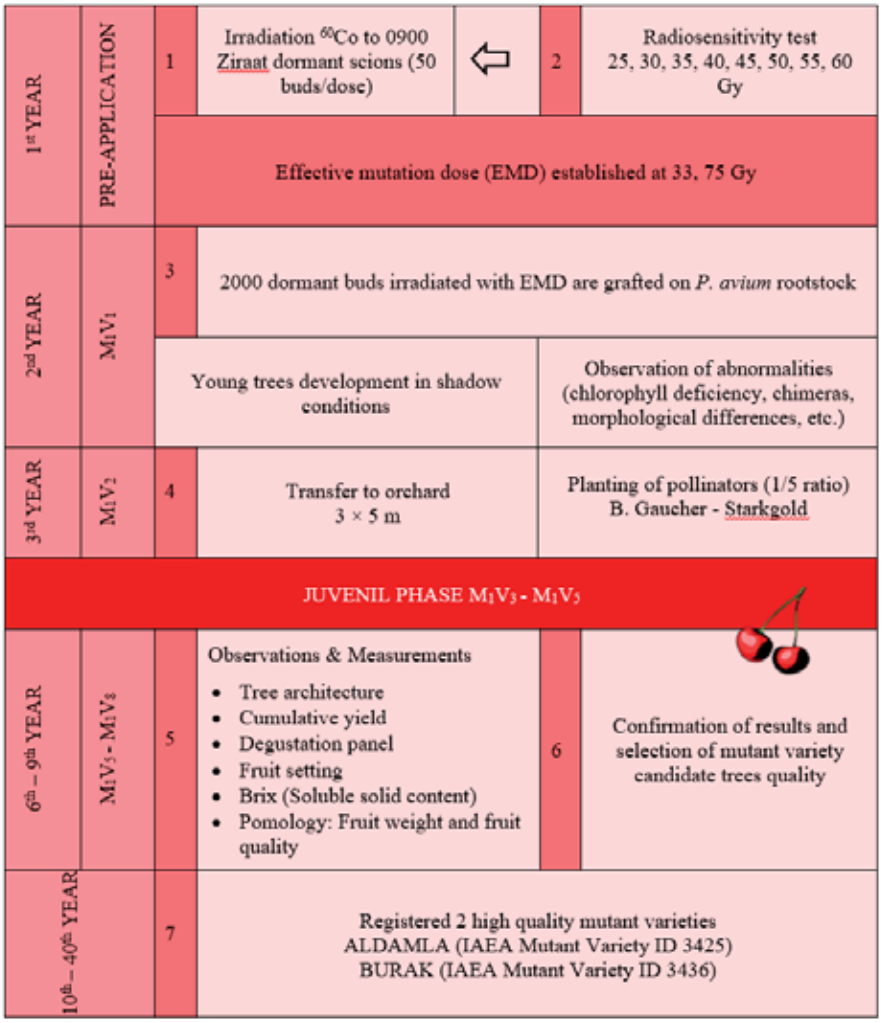


Figure 6.6a. New mutant sweet cherry (Prumus avium) varieties: ALDAMLA and BURAK, Turkey - Turkish Atomic Energy Authority and Atatürk Horticultural Research Center. B. Kunter, M. Baş, Y. Kantoğlu, and M. Burak, 2013.

Figure 6.6b. summary of the procedures for sweet cherry mutation induction.

6.7.2. **Practical example of mutation breeding in potato**

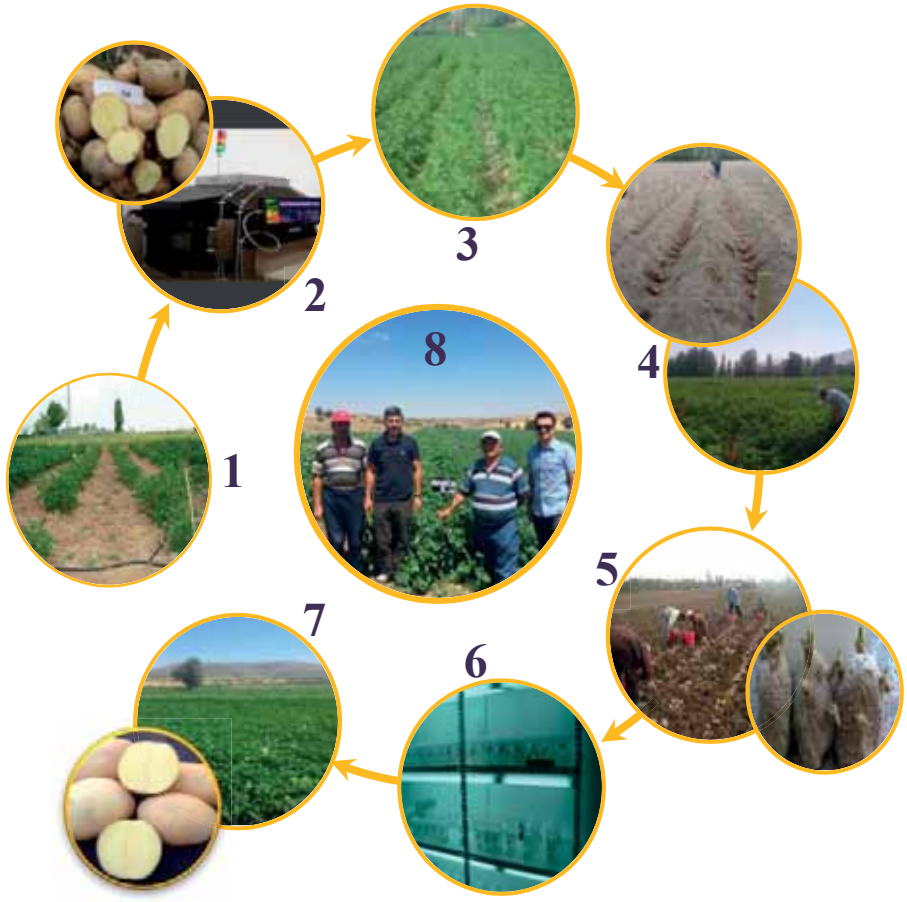


Figure 6.7a. Mutation Breeding for Potato (Solanum tuberasum L.) variety NAHITA improvement using gamma ray irradiation. ***1****. Radiosensitivity test conducted directly in the field (0, 25, 35, 45 Gy) and the Effective dose determined;* ***2.*** *The parental potato variety: Marfona is then irradiated with the selected effective dose: 35 Gy;* ***3.*** *The M_1 V_1 Generation was planted together with the parental variety in the field and visual observations for abnormalities conducted: changes in flower types and colours, in tubers sizes, shapes and numbers recorded;* ***4.*** *All tubers from an individual M_1V_1 plant were planted in row as M_1V_2 for further observation of uniformity and yield increase potential;* ***5.*** *The propagation is pursued following the same process from M_1V_3 to M_1V_8 while the evaluation of the quality of the mutants is thoroughly conducted and, for example, all early yellow sprouting lines discarded;* ***6.*** *Some outperforming mutant clones were then micropropagated in vitro for population increase and transferred to the greenhouse;* ***7.*** *The selected mutants with excellent yield and tuber quality are confirmed and the application for release placed in M_1V_9;* ***8.*** *Released of the new potato mutant variety: Nahita. (Sekerci et al 2016-Turkey).*

<table>
<tr><td rowspan="2">1st YEAR</td><td rowspan="2">PRE-APPLICATION</td><td>1</td><td>Irradiation ^{60}Co to Marfona potato variety tubers</td><td>⇦</td><td>2</td><td>Radio sensitivity test 15, 30, 40, 50, 70, 100 Gy (90 tubers dose)</td></tr>
<tr><td colspan="5">Effective mutation dose (EMD) established at 35 Gy</td></tr>
<tr><td>2nd YEAR</td><td>M_1V_1</td><td>3</td><td colspan="4">M_1V_1 irradiated tubers were planted in field together with parental control for visual observation of abnormalities (leaf chlorophyll deficiency, tuber morphological differences, etc.)</td></tr>
<tr><td>3rd YEAR</td><td>M_1V_2</td><td>4</td><td colspan="4">M_1V_2 lines were planted in rows with parent for selection for uniformity, flower types and tubers (number, size, etc.)</td></tr>
<tr><td colspan="6">M_1V_3 - M_1V_8</td></tr>
<tr><td rowspan="2">4th – 8th YEAR</td><td rowspan="2">M_1V_3 - M_1V_8</td><td rowspan="2">5</td><td rowspan="2">Sub-sequent planting M_1V_3 to M_1V_8 and observations & Measurements
Yield and yield components
Light sprout
Tuber numbers per plant
Biotic/Abiotic stress screening
Tuber characteristics (color, shape, etc.) and quality
Storage duration</td><td rowspan="2">6</td><td rowspan="2">Evaluation of mutants, propagation selected lines in laboratory and field, confirmation of results and selection of candidates for mutant variety(ies)</td></tr>
<tr></tr>
<tr><td>9th – 10th YEAR</td><td>7</td><td colspan="4">Registration of 1 high yield quality mutant variety NAHITA / ID 4463</td></tr>
</table>

Figure 6.7b. Summary of the procedures for potato mutation breeding. Şekerci et al 2016.

7. MAJOR TRAITS TO BE IMPROVED BY MUTATION BREEDING

The strategies utilized to select and improve desirable and specific characteristics in a plant breeding vary with plant species, the environment where the plant is to be cultivated, the farmers' cultivation methods, and the utility and demand of the end-product. The objectives of a mutation breeding programme are basically the same as those of any other breeding methods, the advantages are that mutation breeding aims to improve and already elite genotype usually for a single trait (improved yield, quality, stress resistance, agronomy, etc.). Mutation induction and detection of desired mutants represents an accelerated means of reaching the desired superior variety (Bado *et al.*, 2015) . In theory all genetic traits can be targeted by mutation breeding and in this chapter, we will attempt to display the wide range of mutant varieties that have been produced in a wide range of crops for a wide range of traits, world-wide.

7.1.YIELD IMPROVEMENT

7.1.1. **Yield and yield components**

Stable and high yield potential over a range of environmental conditions is probably the most important objective of most plant breeding programmes. Yield is a complex trait strongly influenced by other breeding objectives, such as, plant architecture, maturity, nitrogen utilization efficiency, resistance to biotic and abiotic stresses, etc. It is difficult to use mutation breeding to improve the yield potential of crops that are well established and which have been subject to intense and refined breeding over long periods of time.

Despite the difficulty of detecting yield mutants there is no doubt about their existence and a number of mutant varieties with increased yield have been released (Table 7.1). Positive yield mutations are formed at a low frequency, perhaps 1/1000 to 1/500 plants in an M_2 population (Saeed and Hassan, 2009). Thus, when planning mutation induction for yield improvement large populations are needed in order to increase the probability of finding yield mutants.

Since yield is so highly influenced by environmental fluctuations, one cannot expect to recognize mutants in yield performance from observations on a single-plant basis. Selection methods, therefore, deserve more attention than do the selection methods for isolating mutants for qualitative characters. The selection method for a character like yield is further complicated by the fact that spontaneous or induced mutants can react differently from the mother genotype to environmental changes. However, changes in genotype × environment interactions may be utilized in practical cultivation of the crops. If, for instance, the environmental changes are represented by

placement or level of nitrogen dressing, the problem is how to pick out those mutants that compete best at a given placement or at a particular level of fertilizer. Another important point is that of competition between plants; most cultivars are grown as a homogeneous genotype and do not compete with other genotypes; therefore, screening must be done with this in mind. For example, if an assortment of wild type tall plants grown together with induced semi-dwarf plants, the latter will be shaded out, but this interaction will never occur in normal agricultural conditions where often a semi-dwarf cultivar has a yield advantage.

There are two different selection methods for mutants with improved yield. In the first method, mutants for qualitative characters are selected and isolated. Their yielding ability is then tested in subsequent generations. The idea behind this method is that some genes for qualitative characters may exhibit a positive pleiotropic effect on yield, or that changes in such genes may be linked with other mutations affecting yield. The method has led to positive results in many cases. As an example, the *erectoides* mutants in barley should be mentioned. The *erectoides* character is associated with high yield in some cases, especially under heavy nitrogen fertilization, however, the method has clear limitations. This *erectoides* character is governed by many loci in which mutations can occur, and not all of them are associated with high productivity. From general reasoning it should be expected that yield is governed by many loci, each of them having a relatively small effect. Not all of them, however, can be expected to be associated with visible qualitative changes in the phenotype.

The second selection method is direct, and thereby considers the limitations mentioned above. It is analogous with selection methods applied in populations derived from cross breeding; the selection procedure starts with progeny testing of individual plants. Since groups of plants are needed to detect yield mutants, the progeny testing can only start in the M_3 families at the earliest.

In practical breeding work on self-pollinating crops, it may sometimes, be advisable to delay the progeny testing until later generations, M_5 or M_6. By that stage a fairly high degree of homozygosity (uniformity) is attained, and selected families need not be re-selected before they are handed over for testing on a large scale in field trials.

Since the influence of the environment on yield is so high, efforts should be made to minimize this effect as much as possible, particularly in experiments with single-plant progenies. By large spacing of the population plants to be tested sufficient seeds can be obtained for replications in the experiment with single-plant progenies. Experiments of this type will, in general, have a great number of families. To control the experimental error special designs are required, such as the widely used split-plot design introduced by Gaul (1964). In this case each plot includes a control row and test-rows from single plants of untreated and treated material of the same genotype.

All measurements are taken on a split-plot basis. Data from such an experiment can be used to construct distribution curves for continuously variable characters (Kusaksiz and Dere, 2010).

As far as yield is concerned, the breeding aim is almost always to increase the mean (directional selection). The left-hand half of the distribution curve is therefore of little or no interest. If yield ability is considered alone, only those mutant families that yield more than the mother genotype are of interest. The practical value of a variety does, however, depend on the performance in several characters, all of which should be considered in a selection experiment. This and other problems related to selection intensity are exactly the same in mutation trials as they are in cross breeding.

When selection is continued in the following generations, more seeds are available from each family. The plot size can therefore be increased and replication including multi-location trials can begin, and the screening between families can thereby be more precise. Even if an experiment with M_4 families can be made quite precisely, one should not place too much emphasis on the results from single experiments. The reason for this is, again, genotype × environment interaction. Genotype × year interactions are also common among families derived from mutagen-treated material. This type of interaction cannot be utilized in practice and must be treated as experimental error. For later selection, e.g. in M_4 and M_5, it is better to base the selection on the mean performance over two or more years.

Statements presented so far, apply mainly to the self-pollinating annual crop species. At present there are far less experimental results and practical experiences with induction of mutational changes in yield ability and other quantitative characters for cross-pollinating, perennial and vegetatively propagated crops. For vegetatively propagated species, e.g. potato, variability experiments of the type described should also be a suitable basis for the initiation of selection among clones (for further information see Chapter 6).

7.1.2. **Examples of mutants with improved yield**

TABLE 7.1. EXAMPLES OF MUTANTS WITH IMPROVED YIELD

Crop	Trait	Method	Landmark mutants/cultivars (country)	Reference
Rice *Oryza sativa*	High yielding	gamma rays	Zhefu – 8 cultivars: 1985 – 2005 (China)	MBNL* Nos. 25 and 26, 1985
Bread wheat *Triticum aestivum*	High yielding		Jauhar – 78. 1979 (Pakistan)	Ahloowalia *et al.,* 2004
Barley *Hordeum vulgare*	Semi-dwarf (*GPert*), malting quality	Gamma	Golden Promise (UK)	Sigurbjörnsson and Micke, 1974
Barley *Hordeum vulgare*	Semi-dwarf (*sd1*)	X-ray	Diamant (Czech Republic)	Ahloowalia, Maluszynski and Nichterlein, 2004
Banana *Musa sp.*	High yielding	Gamma	Al Beely (Sudan)	PBGNL** Nos. 16 and 17, 2006
Groundnut *Arachis hypogea*	High yielding	Gamma ray	TAG24 (India)	Kale, Badigannavar and Murty, 1999
Blackgram *Vigna mundo* L.	High yielding	Gamma ray	TAU-1 (India)	(Ahloowalia, Maluszynski and Nichterlein, 2004)
Cotton *Gossypium sp.*	High yielding	Gamma ray	NIAB 78 (Pakistan)	Ahloowalia, Maluszynski and Nichterlein, 2004

*MBNL: Mutation Breeding Newsletter; **PBGNL: Plant Breeding and Genetics Newsletter

7.2.TOLERANCE TO ABIOTIC STRESS

Abiotic stresses encompass several unfavourable environmental conditions such as soil salinity, drought, extreme pH, flooding and harsh weather. The approaches utilized in plant mutation breeding for such traits are often quite simple. Recently, two candidate salt tolerant rice lines induced using carbon and neon ion beams have been reported (Abe *et al.*, 2007).

Though many of the physiological mechanisms of abiotic tolerance are unknown, the threat of global warming and climate change forces new approaches in adapting crops to fluctuating environments. Huge numbers of potentially useful genotypes have been

generated from mutation breeding research and breeding programmes and are available in germplasm collections, e.g. tolerance to cold, heat, day length and drought, available in China, Japan, USA, etc., (http://mvd.iaea.org).

The methods of mutation breeding have hitherto been used only to a very small degree for developing varieties with tolerance to low temperature, heat, drought and salinity of the soil. Therefore, a brief survey of the results of conventional breeding methods and some information on the genetic bases of the tolerance are given.

7.2.1. **Drought**

Climate change is responsible not only for global temperature increase but also for region-specific increases or decreases in rainfall. Water shortage has a negative impact on agricultural production and this is particularly acute in developing countries. Crop plants cannot grow without water, it is essential for all stages of crop development from germination, to vegetative growth and reproductive periods (fruit and seed development). Changes in rainfall (duration and timing) can impact all stages in crop production and crops of all climatic regions, arid, temperate, tropical etc. Table 7.2 provides some examples of released crop mutant varieties with drought tolerance.

7.2.2. **Salinity**

There are two ways to develop new mutant varieties with salinity tolerance, one is to mutate a variety that is high yielding but susceptible to salinity, the other is to mutate a variety that is low yielding but tolerant to salinity, such as a traditional local variety grown in the affected area. In such a case, because it is much easier to improve agronomic performance than to enhance salinity tolerance, the latter is the better option. However, it is important in such situation to carefully choose the most suitable mutagen and technology to achieve this specific breeding goal. The final step is to deploy an efficient method for mutant screening and validation. As in all mutation breeding programmes, following selection, it is important to verify and confirm if the selected mutated trait is heritable.

7.2.3. **Temperature**

Much work has been done during the past decades on tolerance to low temperature, particularly in cereals, potato, fruit and forest plants. The genetic basis of frost resistance is very complicated and evidently not uniform considering the different types of cultivated plants. In barley winter hardiness is controlled not only by dominant but also by recessive genes and there is a correlation between the degree of dominance and the average winter hardiness. According to some authors, the action of a polygenic system must be assumed, probably supplemented by series of multiple alleles. Also, in bread wheat whole groups of different genes are effective and show

transgressive inheritance. Winter hardiness seems to be dominant in wheat and there is obviously a variance in the degree of dominance in relation to the parents used for hybridizations. The action of seven dominant genes influencing the character 'tolerance to low temperature' in rice (*Oryza sativa)* has been identified (Cruz *et al.*, 2013).

Further indications of a broad genetically conditioned variance in respect of this character were observed in cabbage (*Brassica olerocea),* radish (*Raphanus sativus*), buckwheat (*Fagopyrum* spp.)*,* pea (*Pisum sativum*), alfalfa (*Medicago sativa*) and in different species of lupin (*Lupinus* spp.). Moreover, it should be mentioned that certain wild potatoes and some primitive cultivated potatoes are characterized by a considerably high degree of winter hardiness. A polygenic system could also be found in these species and the feature 'winter hardiness' is inherited partly in a dominant, partly in an intermediate way. Furthermore, it could be shown that genes causing a certain tolerance to low temperature are even present in the genomes of cultivated plants that grow in relatively warm zones of the earth. This may be valid with regard to cotton (*Gossypium sp.*), tobacco (*Nicotiana sp.)*, maize (*Zea mays*) and to some wild-growing tomato species such as tomato (*Lycopersicon hirsutum* and *L. peruvianum*).

The first results, using methods of mutation breeding for abiotic stress were for resistance to cold in barley. Winter varieties of barley were produced by the application of X-rays to summer varieties during the early epoch of mutation research (van Harten, 1998): winter hardiness was found to be a recessive character in this plant material. There are also winter-hardy mutants of oat (*Avena sativa*) displaying more ascorbic acid compared with their initial parental lines, which were susceptible to low temperatures.

A gamma-ray induced mutant strain of soybean (*Glycine max*) was obtained that can germinate at a temperature of 4°C, while the germinating temperature of normal soybean varieties is at about 8°C (Khan and Tyagi, 2013). There is no doubt that this mutant will represent a valuable basic material for developing strains and varieties that may be cultivated in cooler regions.

A comparable situation exists in the field of tolerance to heat. Heat tolerant genotypes have been selected in several crops. In fact, this type of tolerance is being looked at with enhanced scrutiny in the wake of climate change and its probable effects on agriculture.

TABLE 7.2. EXAMPLES OF MUTANTS WITH IMPROVED ABIOTIC STRESS TOLERANCE

Crop	Trait	Method	Landmark mutant/cultivar (country)	Reference
Rice *Oryza sativa*	Salt tolerance	Gamma ray	NIAB-IRRI-9 (India)	MBNL No. 45, 2001
Rice *Oryza sativa*	Salt tolerance	Ion beam irradiation	Japan	Abe *et al.*, 2007
Rice *Oryza sativa*	Salt tolerance	Gamma	VND95-20 (Viet Nam)	Do *et al.*, 2009
Bread wheat *Triticum aestivum*	Drought tolerance	Gamma	Njoro BW1 (Kenya)	IAEA Bulletin, 50 – 1
Maize *Zea mays*	Drought tolerance	Gamma ray	Kneja 698W (Bulgaria)	PMR*, 2012
Barley *Hordeum vulgare*	High altitude (harsh weather), early maturity	Gamma	UNA La Molina (Peru)	MBNL No. 43, 1997 Gomez-Pando *et al*., 2009
Amaranth *Amaranthus cuadatus* L.	High altitude (harsh weather)	Gamma	Centenario (Peru)	Gómez-Pando *et al.*, 2009
Rice *Oryza sativa*	Tolerance to cold	Gamma ray	Kahmir Basmati (Pakistan)	Ahloowalia, Maluszynski and Nichterlein, 2004
Rice *Oryza sativa*	Tolerance to heat	Gamma ray	Nagina 22 (India)	Poli *et al.*, 2013
Soybean *Glycine max*	Tolerance to cold, drought and water logging	Gamma ray	Heinong 26 (China)	Khan and Tyagi, 2013

*PMR: Plant Mutation Report

7.3.TOLERANCE/RESISTANCE TO BIOTIC STRESS

7.3.1. **Resistance to disease**

Biotic stresses are primarily diseases caused by fungi, bacteria, and viruses, and the damages induced by insects, animals, nematodes, weeds, and any other biological causes. Mutation breeding has been successful in improving disease resistance; the success in tackling pest resistance has yet to be taken to such level. Diseases involve a complex inter-play between a host plant and a pathogen. Therefore, the resistance/susceptibility response can involve several components; this implies that there are many targets for mutation improvement. For example, induced mutations

may change the interaction and inhibit certain steps in the mechanism of infection. Numerous mutants have been developed through mutation induction, showing enhanced resistance to various diseases: virus, bacterial, and to some extent fungi (http://mvd.iaea.org/ ; Lebeda and Svabova, 2010).

Unlike diseases, there is little interaction between host plants and their insect pests, as one pest may attack other plant species or even different plant genera. This predator-to-host interaction may explain why there are fewer examples of mutant varieties carrying induced pest resistance. Tolerance towards insects is a quantitative reaction and may include characteristics such as plant vigour, the ability to produce many shoots and many roots, as well as strength of stem tissue and avoidance (little or no vegetative growth when insect pests are prevalent). Recently, there has been some progress in identifying resistance gene(s) to brown plant hopper in rice (Fujita, Kohli and Horgan, 2013), as such, it may be possible to induce resistant rice mutants efficiently by targeting the identified gene(s) related to the resistance mechanism. In fact, this aspect was the foundation for transgenic breeding for pest resistance success, e.g. GMO crops carrying the *Bt* toxin gene.

The constant challenge in breeding for disease and pest resistance is to deal with and overcome the development of new aggressive strains of the pathogens. The advances in molecular technology and recent findings in cloning of disease resistance (R) genes should allow the improvement of crop disease resistance by applying a holistic approach including traditional breeding, genomics, transgenic deployment, and/or mutagenesis tools. The discovery of genetic resistance in plants is attributed to Orton who at the end of the last century selected cotton for resistance to wilt caused by *Fusarium oxysporum* f. sp. *vasinfectum* as cited by Epstein *et al.*, (2017). For recent reviews on the genetics of resistance (host) and pathogenicity (disease organism) see Boyd *et al.*, 2013; and Servin *et al.*, 2015.

But as the disease is the product of an interaction between the host organism and the pathogenic organism, genetic variability can be expressed in the former as well as in the latter. In the pathogen the variability is expressed by means of genes for virulence or avirulence whose differentiation on a set of differential varieties allows the identification of the phenotype known as 'physiologic' or 'pathogenic races'. This kind of interaction is identified in the host as specific resistance, which in many instances, has proven to be of a temporary value in breeding because the pathogenic organism creates new virulent races even while the plant breeder is developing novel resistant varieties, hence creating a dynamic equilibrium. Thus, when dealing with plant disease resistance one has to also consider both partners, and have a holistic approach including plant pathology and other related fields. Barah and Bones, (2015) in an extensive review of studies on plant-insect interactions using different biological tools stated that most probably, in the near future, the combination of high-throughput

profiling techniques, bioinformatics tools, and published data from ecological studies will provide ways by which to achieve a comprehensive systems-level understanding of various aspects related to plant defence responses, which in turn can be used to develop better crop varieties using all breeding tools currently available including mutation induction.

7.3.1.1.Induction of mutations and mutant selection

The first report on the induction of mutations for disease resistance is attributed to Freis and Lein, 1942 as cited by Gupta (1998). These authors isolated a mutant in Haisa barley simultaneously resistant to three races of powdery mildew in Germany, as a result of a treatment with X-rays, the survey mentioned about 12 000 progenies.

As in other characters, the success in induced mutagenesis programmes for resistance to diseases depends upon careful selection of the parent variety, an effective mutagen treatment and a specific and discriminating screening method. The latter should be the most reliable and efficient manner of separating susceptible from resistant plants. For this purpose, it is essential to know the genetic sources of variation of the pathogen, thus permitting the choice of the most adequate race or races of the pathogen for inoculation.

From the host-parasite interactions it is evident that a mutation induced in a susceptible host can be expressed or may remain cryptic, according to the type of inoculation employed in the screening process. As a result, the mutation frequency observed will not be identical with that actually induced. By working with a range of disease races the proportion of cryptic mutations increases and a wider spectrum of resistance will be detectable. However, later generations will give a higher probability for selecting a desired resistance mutation since it will appear in more plants, particularly as the mutations are likely to be recessive.

Both physical and chemical agents have been employed successfully for inducing disease resistant mutants, although irradiation has been used more frequently (Table 7.3).

In 2010, the FAO/IAEA Joint Division published a compilation of studies realized under CRPs and/or TCPs relating to screening mutant and non-mutant crops for tolerance/resistance to disease, with an emphasis on fungus derived disease (Lebeda and Svabova, 2010).

7.3.2. **Resistance to pests**

Since the dawn of agriculture breeders and farmers have succeeded in selecting and growing crops with various types of resistance in order to ensure thriving crop growth

and productivity. The contribution of agronomists and soils scientists has provided enhanced protection with the use of herbicides and/or insecticides. However, the long use of these products has weighed on the environment and subsequently on the human health, thus the development of plant resistant to insects and to herbicides has become of significance in protecting the environment and human health. Oerke, (2006) in an extensive review stated that among crops, the total global potential loss due to pests varied from about 50 percent in wheat to more than 80 percent in cotton production, 26 – 29 percent for soybean, wheat and cotton, and 31, 37 and 40 percent for maize, rice and potatoes, respectively. The author also noted that overall; weeds produced the highest potential loss (34 percent), with animal pests and pathogens being less important (losses of 18 and 16 percent). Thus, enhanced efforts are being assigned in mutation induction to address that situation. Several mutants resistant to insect have been registered in the IAEA - MVD database: rice, maize, black gram, white lupine, fodders beet, rapeseed, hybrid maize and bitter gourd in China, the Russian Federation, Vietnam and India respectively (http://mvd.iaea.org/).

7.3.3. **Examples of mutants with improved biotic tolerance/resistance**

TABLE 7.3. EXAMPLES OF MUTANTS WITH IMPROVED BIOTIC STRESS TOLERANCE

Crop	Trait	Method	Landmark mutant/cultivar (country)	Reference
Rice *Oryza sativa*	Resistance to blast and virus diseases	Gamma ray	Camago 8 (Costa Rica)	MBNL No. 43, 1997
Bread wheat *Triticum aestivum*	Resistance to black stem rust (Ug99)	Gamma	EldoNgano-I (Kenya)	PBGNL Nos. 32 and 33, 2014
Chick pea *Cicer arietinum*	Blight resistance	Gamma	Hassan-2K Pakistan	Hassan *et al.* 2001
Lentil *Lens culinaris Medik.*	Blight resistance	Gamma	NIAB MASOOR 2006 Pakistan	Sadiq *et al.*, 2008
Barley *Hordeum vulgare*	Powdery mildew	X-ray	Comtesse (Germany)	MBNL No. 33, 1989 and No. 36, 1990
Barley *Hordeum vulgare*	Mildew resistance	EMS	Betina (France)	Sigurbjornsson and Micke, 1974
Japanese pear *Pyrus pyrifolia* Nakai	Black spot resistance	Gamma	Gold Nijisseiki (Japan)	Saito, 2016
Pepermint *Menta × piperita* L.	Wilt disease resistance	Neutron irradiation	Murray Mitcham; (USA)	Todd, Green and Horner, 1977

7.4.QUALITY

7.4.1. **Quality, nutrition and functionality**

With respect to food, quality usually refers to the composition of organic compounds produced and stored by plants, such as starch, protein, fatty acid, vitamins, and other nutrients (Table 7.4). Enhancing the nutritious value of harvestable products is

therefore an important objective for plant breeding. The simplest route for modifying quality traits using mutation is by inducing knock-outs in genes involved in the metabolic pathways; thus, increasing the synthesis of upstream substances and decreasing the production and concentration of downstream substances or producing novel gene products, e.g. altered fatty acid composition of many oil crops *via* mutating genes. Apart from oil palm, all oil crops have been improved by mutation (Vollman and Rajcan, 2009). Canola and others oil producing brassicas have been mutagenized using either chemical or physical mutagens for the improvement of several important traits including high quality edible oil, obtained through the reduction of the levels of toxins (glucosinolates) and of the erucic acid (Cheng, 2014; Singh and Verma, 2015). Recently, mutation breeding has been used for enhancing bio-availability of important nutrients in certain crops. For example, crops with low phytic acid content are preferred because the bioavailability of mineral elements and phosphorus can be significantly increased. In this regard, two barley mutant varieties have recently been released for commercial production for additional information see review by (Raboy, 2009).

Mutation breeding has also been used for breeding crops with special functionality. In Japan, for example, rice mutant varieties with low glutelin content, such as LGC-1 and its derivatives, have been developed for people who must restrict protein intake, as it is the case of patients with kidney disease. In Indonesia a mutation induction programme on sorghum improvements showed that mutated genotypes presented high nutrition values in terms of protein and starch contents so that it could be used an alternative food source (Soeranto *et al.,* 2001).

In addition to nutritional traits, quality also relates to medical (e.g. drugs) and industrial traits (e.g. starches and oils). The first important problem the breeder is faced with, in connection to quality is to define precisely the desired quality parameters and to establish their order of priority in his/her particular situation. This may be challenging as in most breeding programmes yield, disease resistance, and climatic adaptations are higher on the agenda. This is mostly a matter of importance, but in other cases it may simply come from the recognition of the fact that it is more convenient for the breeder to select first for the field characteristics. This will have also a positive economic effect, as it reduces the number of samples that have to undergo time-consuming and costly laboratory tests. Restricting the population before screening for quality traits may, of course, also reduce the probability of identifying genotypes with the quality factors desired.

The next critical problem is to develop simple, speedy and cheap screening techniques for the characters to be improved. Such techniques should be simple enough and require only little material so that primary screening in the early generations can be done in the breeder's facilities (greenhouses and fields). In some instances, screening

may best be executed by a series of steps, beginning with a coarse, rapid and sometimes only suggestive analysis, which must be followed by a more vigorous chemical analysis once more material is available, and ultimately by testing in production environments (glasshouses or fields) on a larger scale and with methods close to the end use of the product (e.g. milling, baking tests, brewing, feeding trials, etc.). The latter tests will almost always be performed at specialized laboratories.

As a first step to improve the quality factors of cultivars, the breeder normally screens available varieties, breeding lines and germplasm collections. After detecting the desired traits, the breeder has to choose the most economical way to incorporate such traits into top varieties and to combine these traits with other desired traits.

As far as screening for nutritional quality of cereals is concerned, the results of analysing large germplasm collections have not been, to date, very satisfactory. In particular, the search for genotypes with significantly increased levels of particular amino acids, such as lysine, has been somewhat disappointing as there is a large yield penalty. This is understandable since from the standpoint of natural evolution a high percentage of lysine seems to carry no beneficial fitness value. However, a few genotypes with higher lysine content in the grains have been found in collections of maize, barley and sorghum. Induced mutations for quality characters may have an advantage if they are produced in a good genetic background, suitable for modern agricultural production and easy to utilize in cross breeding. Seed quality mutants may be pre-screened rapidly using soft X-ray imaging and near infrared spectroscopy as demonstrated in barley and rice mutants by Jankowicz-Cieslak *et al.*, (2013).

Quality traits may be subject to modification by environment, for example, quantitative characters such as protein content are highly susceptible to the influence of soil, moisture, fertilizer, temperature, and light conditions. This interaction makes selection difficult where replicated trials cannot yet be carried out. Therefore, caution is advised in drawing conclusions about the success of selection and results should only be accepted after robust testing.

7.4.2. **Starch**

The polysaccharides, of which starch is the principal one, constitute the major component of cereal grains and are probably the most important compound for energy storage. Starches as a class of compounds have considerable diversity in both their starch grain structure and their chemical composition. The classification of carbohydrates in crops includes sugars (monosaccharides and disaccharides), oligosaccharides, starch (amylose and amylopectin) and non-starch polysaccharides usually found in the cell walls, such as pentosans. Most of this diversity is reflected in nutritional or technological characteristics, such as digestibility, suitability for bread making and malting, and cooking characteristics. This is important as a quality

parameter with regard to both human and animal nutrition. For example, cell wall polysaccharides of cereal grains contribute to dietary fibre intake and are thus an important health factor (Lafiandra, Riccardi and Shewry, 2014). In recent years there has been a renewed interest in the improvement of crops, mainly cereals with enhanced polysaccharides nutritional qualities (Lafiandra, Riccardi and Shewry, 2014).

In maize great variation is found in the content of starch, varying from 9 – 74 percent. Rice varieties with high content of resistant starch are being developed in China for dietary therapy of patients with type 2 diabetes. These varieties have about 10 times higher resistant starch than normal rice varieties and preliminary tests have shown they are effective for controlling the glycemic index when added into diets, though more studies are needed (Shu and others, 2009). It is expected that more foods with such novel functions will be developed through mutation breeding.

7.4.3. **Protein**

In many parts of the world, especially in the lower income groups, there is a dietary shortage of protein and/or certain essential amino acids. Plant products, particularly cereal grains are the basic food of most of the world's population living in the regions with large vulnerable populations; they are the main source of protein as well. Cereal grains are relatively low in protein content and their amino acid composition in general does not suit human requirements. Therefore, where cereal grains constitute the major part of a human diet, the supply of protein and essential amino acids will be inadequate. Increasing the quantity and nutritional quality of grain protein, e.g. to include essential amino acids such as lysine, is a potential means of combating malnutrition.

To estimate the protein quantity, a number of methods are available based upon nitrogen determination including Kjeldahl and Biuret techniques, etc., (De Mey *et al.*, 2008). Some of them have been more or less automated. In general, however, due to the cost of the equipment needed the use of these methods has been a limiting factor for an ambitious programme for protein improvement in crops. Therefore, more rapid methods have been sought and in recent years many researchers have used the protein dye binding property as a means of measuring the protein amount. The dye most often used is acrilan orange, which is specific for the basic amino acids (lysine. histidine, arginine). The use of this method implies the assumption that the proportion of basic amino acids to the total protein content is constant. Such an assumption can only be correct if one deals with material that does not genetically deviate in its proportion of basic amino acids.

Micro-Kjeldahl, Lowry and Bradford procedures were compared for determining the protein content of common bean (*Phaseolus vulgaris*) seeds during their development

in order to establish their respective efficiency. Several other nuclear technologies have been developed for determining the total content as well as the amino acid distribution of proteins in seeds and general quality, for example in dry beans (Vakali *et al.*, 2017).

7.4.4. Fats, oils and fatty acids

Vegetable oils are one of the most valuable agricultural products in terms of energy source, provision of essential fatty acids, carriers of fat soluble vitamins and as resource for many industrial products. Their value and utility primarily depends upon their fatty acid composition (Kramer, 2012). Therefore, breeding plant for quantity and quality of vegetable oils is likewise important. As for other quality traits, rapid, precise and cheap analytical methods are a requirement. Traditional oil extraction methods, e.g. Soxhlet method, need to be scaled up and are generally too time-consuming for screening a larger number of samples. The resonances associated with the protons of the oil component may be selected from the nuclear magnetic resonance (NMR) spectrum so that the instrument's 'signal' is a function of seed oil content. Digital readout of the NMR signal permits rapid screening of small seed samples (even single seeds) with a relative error in the oil determination of less than 1 percent, in seeds like soybean, with a very high negative correlation between oil and protein content, NMR also offers an indirect means of selecting for protein content (Weir *et al.*, 2005). Liquid chromatography is also used for both quantitative and qualitative determination of fatty acids. The method is so sensitive that the fatty acids of as little as half a rapeseed may be determined and thus, has been developed into a rapid and safe serial test and more rapid screening method (Bromke *et al.*, 2015).

So far, mutation induction for altering fatty acid composition has been the most frequently carried out and the mutants obtained had increased or decreased values of linolenic acid without any change in linoleic acid (IAEA-TECDOC-781, 1994).

7.4.5. Toxins and anti-nutritional factors

Many plant species produce toxic, noxious or bad tasting substances and store them in their tissues in such high concentrations that animals are repelled from eating leaves, seeds or other organs. However, careful investigations reveal that some variability always exists within and between species with regard to the content and genotypes can be found that are practically free from noxious substances. A rather reasonable theory assumes that the wild-type plants originally contained only negligible amounts of bitter or toxic substances but that spontaneous mutations induced a metabolic block which led to enrichment and storage of such compounds.

Breeding has been used in an attempt to revert to low 'wild-type' toxin types, but it has so far been successful only in a few cases. A more serious problem than the

induction process itself, is the lack of efficient methods for the screening of large plant populations for the desired character. Furthermore, the breeder may be faced with the problem that plants that show the desired chemical change suffer from other metabolic alterations and are low yielding.

The cruciferous and the leguminous families often contain noxious substances in the form of glucosinolates, alkaloids and glucosides. *Lupinus* species produce several different alkaloids and the utilization of these protein-rich plants was nearly impossible until successful screening experiments for spontaneous mutants free of alkaloids were performed (von Sengbusch, 1938 as cited by Boersma (2007). *MeliIotus albus,* the white sweet clover contains a glucoside of the o-oxycinnamic acid class, which converts into coumarin and the toxic dicoumarol. Large-scale screening experiments for non-bitter plants failed for a long time, but this was finally achieved in selecting non-bitter mutants after treatment with mutagenic chemicals or ionizing radiation. A prerequisite for this successful mutation experiment was the development of a rather simple method for mass screening, which made the investigation of 1000 plants per day feasible on the basis of a half leaflet per plant. While all non-bitter mutants exhibited a lower vitality, it was possible to increase their vitality even above the original strain by heterosis breeding.

Rapeseed (*Brassica napus*)*,* mustard (*B. campestris*), Indian mustard (*B. juncea*) and rocket (*Eruca sativa)* are widely cultivated oil crops, but their seed meal and green matter are also valuable protein sources for which they have, so far, only been partially exploited. One of the main reasons for this is their content of glucosinolates, which yield degradation products with toxic effects on animals and through the milk, also on children. The first attempts to lower the glucosinolates content by conventional breeding have produced interesting results (Röbbelen, 1973, cited by Jambhulkar, 2015) and the process has been pursued with mutation supported by more precise and sophisticated screening methods (Bjerg, Sørensen and Wathelet, 1987).

Pea *(Lathyrus sativus)* is used in some parts of India for human consumption but contains a neuro-toxic component that seriously injures children, causing the disease known as: Lathyrism. The toxic factors have been studied and success in isolating mutants practically devoid of the neurotoxic principle ß-N-oxalyamino alanine have been reported (van Harten, 1998). The use of cassava (*Manihot esculentus*) as an important item in human and livestock nutrition in the tropics is hampered by chronic toxicity caused by the build-up of cyanogenic glucosides after harvest. Screening techniques to check large numbers of samples are available (Tivana *et al.*, 2014). However, vegetative propagation practised throughout centuries has notably limited the genetic variability of cassava and mutation induction is forcibly being applied as it appears as a worthwhile approach when combing *in vitro* techniques and more precise screening methods.

7.4.6. **Examples of mutants with improved quality**

TABLE 7.4. EXAMPLES OF MUTANTS WITH IMPROVED QUALITY

Crop	Trait	Method	Landmark mutant/cultivar (country)	Reference
Rice *Oryza sativa*	Grain quality	Gamma ray	VND95-20 (Viet Nam)	Do *et al.,* 2009
Rice *Oryza sativa*	Grain quality	Gamma ray	Shwewartun (Myanmar)	MBNL Nos. 11 and 12, 1978 Ahloowalia, Maluszynski and Nichterlein, 2004
Rice *Oryza sativa*	Glutinous endosperm	Gamma ray	RD6 (Thailand)	Ahloowalia, Maluszynski and Nichterlein, 2004
Bread wheat *Triticum aestivum*	Grain colour		Jauhar 78 (Pakistan)	MBNL No. 2, 1973
Cassava *Manihot esculentus*	Cooking quality	Gamma ray	Tekbankye (Ghana)	MBNL No. 44, 1999
Sorghum *Sorghum sp.*	Grain colour	Gamma ray	Djeman (Mali)	MBNL No. 44, 1999
Sunflower *Helianthus annuus*	High oleic acid	Gamma ray	NuSun (USA)	Ahloowalia, Maluszynski and Nichterlein, 2004
Tobacco *Nicotiana tabacum*	Pale green	X-ray	Chlorina F_1 (Indonesia)	Sigurbjornsson and Micke, 1974
Grapefruit *Citrus paradisi Macf.*	Red fruit flesh and juice	Retrotransposon	Rio Star (USA)	MBNL No. 37, 1991
Chrysanthemum *Chrysanthemum sp.*	Reduced axillary buds	Ion beam	Aladdin 2 (Japan)	Shirao *et al.*, 2013
Osteospermum *Osteospermum ecklonis*	Flower colour	Ion beam	Vient flamingo ; Vient labios (Japan)	Sekiguchi, Hase and Tanaka, 2009
Various flowers, i.e. Canna lilies, Moss-rose	New colours and forms	Gamma ray	Golden Creman; Cream Prapanpong; Orange Siranut; Pink peeranuch; Yellow arunee (Thailand)	MBNL Nos. 33 and 34, 1989, Wongpiyasatid, Hormchan *et al.*, 2000

7.5.AGRONOMIC TRAITS

7.5.1. **Flowering and ripening time**

Early and late maturing mutants are frequently induced through mutagenesis and are easily identified. Early maturity in cereal and legume crops is one of the most useful characteristics for cultivation in cool temperate regions, offering the opportunity to flower in frost-free conditions, harvest prior to frost, and, in drought-prone regions the ability to produce a viable crop prior to drought conditions. The flowering time and maturity of cereals and other crops are controlled by the plants' ability to sense season temperature and day-length signals controlled by vernalisation and photoperiodic sensitivity genes. Sweden was home to pioneering work on mutation breeding in barley where the barley variety Mari, a mutant variety exhibiting early maturity and semi-dwarfism was developed. Mari is a direct mutant resulting from irradiation of Bonus Barley in 1960 (Lundqvist, 2014) from which many valuable indirect-mutant varieties were bred. Early flowering and early maturity mutants have been induced in a number of other crops; notable examples include banana, cotton, pearl millet, rice, and soybean.

Quite often several additional characters are changed in early maturing mutants. The yielding capacity of early maturing mutants is generally reduced. However, early maturing mutants, especially slightly early maturing ones, with yielding capacities equivalent to or higher than their original varieties have been induced in several crops. It has been reported that correlations between total yield (grain + straw) and ripening time are positive, but correlation between grain yield and total yield is negative in early ripening barley mutants. Thus, in the evaluation of the practical value of early maturing mutations, data on yield per day during their growth period should be collected.

Plant height can also change in early maturing mutants and significant positive correlations between the two characters have been reported. Reductions of internode number, short basal internode or increase of upper internode length were observed in early maturing barley mutants. Furthermore, other characters, such as panicle length, 1000 grain weight, panicle number, straw-stiffness and protein content, were changed in early maturing mutants according to Gottschalk and Wolff, 1983 as cited by (Datta, 2014).

Early heading mutants can be identified by simple observation or measurement, and their screening and isolation can be conducted rather effectively because of relatively high heritability (in the broad sense) of heading date. It is acknowledged that, the degree of dominance of induced mutations influences efficiency of screening and isolation of the mutations. Early heading macro-mutations are, in most cases, recessive to the original genes. However, dominant or partially dominant early

heading mutations have also been isolated in barley, oat and other crops (Dumlupinar *et al.*, 2015). Differential responses of early heading mutants of spring barley to light (quality and quantity) and temperature have also been reported. Studies with *Arabidopsis* gave evidence for the occurrence of mutations that had lost the light requirement for germination ability (Franklin and Quail, 2010).

7.5.2. **Adaptability**

Wide geographical adaptability has become an important character for crop varieties in recent years, mostly in relation to the foreseen climate change. An FAO study (Burke, Lobell and Guarino, 2009) on the shift in African climates by 2050 and the implications for crop improvement, extensively examined the variation both in the possible shift of isohyets and the difficulty to identify "analogue crops" to be used for better adaptability in different regions.

Photoperiod insensitivity is a prerequisite for wide adaptability when cultivars are to be grown in different localities at different latitudes, and mutants insensitive to photoperiodic differences have been reported. Wide adaptability is achieved, however, not only by photoperiodic response, but requires the variety to respond or remain neutral to a number of different other environmental conditions. It is therefore related to many physiological characters including those involve in the reproductive biology. Durum wheat mutant varieties showed wider adaptability than their mother variety, especially in regions where both soil fertility and water resources were not limiting factors (Donini and Sonnino, 1998) . Mutants in practical breeding projects should be tested at different locations under different environmental conditions. One of the most promising potential uses of induced mutations is to break through barriers at the outer limits of the adaptability of a species. A number of practical examples of this already exist, e.g. Mari a barley mutant variety (Sigurbjörnsson, 1975; Xia *et al.*, 2017). Thus, barley can now be grown under short season in Scandinavia and in equatorial regions with short day lengths. It also spans from sub-sea level land locations to the high Andes.

7.5.3. **Plant type, growth habit and architecture**

Mutation breeding is frequently used to alter plant architecture. Plant architecture includes phenotypic traits such as plant height (i.e. culm length of cereals), plant-type, branching habit (e.g. number of tillers), size, number, shape and orientation of leaves, stolon characters, size and number of flowers. Prime examples are mutants conferring reduced plant height, which generally have positive effects on increasing yield by reducing lodging and increasing tillering. One of the most successful utilization of plant height mutations is the deployment of semi-dwarfism in annual cereals crops, e.g. *rht* (reduced height) genes in wheat, *sdw* in barley and *sd1* in rice. The *sd1* mutant gene of rice produces short and stiff stems, which also confers increased grain yields

through a reduction in lodging and greater mobilization of resources to grain. Semi-dwarf rice and wheat varieties were leading examples of breeding progress as defined by the "Green revolution", for which Norman Borlaug won the 1970 Nobel Peace Prize. The first semi-dwarf mutant variety of rice var. Reimei was induced through gamma irradiation Futsuhara, 1968 as cited by Kikuchi and Ikehashi, (1984), the height was reduced by at least 15cm when compared to the original mother variety: Fujiminori. The variety Reimei carries the same *sd1* (semi-dwarf) allele as do the spontaneous mutant var. Dee-Geo-Woo-Gen and the induced mutant variety Calrose 76. All these varieties are widely utilized across Asia and America (Lestari, 2016). Since the identification of the *sd1* gene, in Japan, 80 of the 229 registered rice varieties represent descendants of Reimei. In the USA, greater use has been made of the semi-dwarf trait from cv. Dee-Geo-Woo-Gen derivatives; however, additional induced or identified spontaneous mutations have also been successful (Rutger and Mackill, 2001). Similar achievements have also been made in wheat, oat, barley, but also in some fruit trees such as apple, peach, cherries.

Barley mutants have been used to study the basic architectural building blocks of plants, in about 100 phytomer mutants (Forster *et al.*, 2007). They can be used to study and predict the type of organ to be formed by studying the ontogeny of meristems at various positions (apical or branch) and at various stages in the plant life cycle. The most basic phytomer unit consists of a stem segment with a leaf and/or root attachment which can be replicated in an apical or side direction. Studies of phytomer mutants can also be used in taxonomical studies in defining what type of structures is developed from a meristem in a given species. For example, a classic taxonomic difference between wheat (*Triticum*) and barley (*Hordeum*) genera is that spikelet production in wheat is determinate whereas it is indeterminate in barley, and conversely floret production in wheat is indeterminate, but determinate in barley. This leads to wheat having multiple florets per spikelet whereas barley is restricted to one. Wheat therefore produces far more seeds per spike (ear) than barley. However, one phytomer mutant in barley, known as the "wheat mutant" exhibits indeterminate floret production and therefore of interest in increasing yield (Forster *et al.*, 2007); it is also of taxonomic interest as it indicates that this particular taxonomic descriptor is governed by a single gene.

7.5.4. **Resistance to lodging and stem breakage**

Lodging susceptibility and stem weakness are serious problems in many crops including: wheat, oats barley, rice, corn, sugar cane, sorghum, flax, cotton, soya, broad beans, etc. Lodging has been analysed by several investigators especially in cereals. As a general rule, lodging is caused by either (1) uprooting of the plants; (2) breakage of the stems near the ground level; and/or (3) leaning and bending of the stems: the last cause being the most common one and often caused by harsh weather such as high

wind and heavy rain. Lodging is a complex event bound to the concomitance of several external and internal factors. Apart from external factors, such as environmental conditions, agronomic procedures, diseases, the most important plant characters that can influence lodging are: strength and elasticity of the stem, structure and development of the root system, and stem length. Although lodging resistance and stem-length reduction are not always associated, the shortening of the stem is a characteristic that is worth selecting for. Additionally, short cultivars are usually better adapted to mechanical (e.g. combine) harvesting.

The general selection procedure for lodging-resistant mutants can be outlined as described in this paragraph. Owing to the close association between stem length and lodging resistance, short-stem plants can be isolated visually in the M_2 or M_3 generation. Once the mutations for stem shortening are confirmed and, if they have a potential interest, in the following generation they can be multiplied in a single plot or in small plots in order to derive useful information on agronomic value. Field trials can then take place, generally starting from the M_5 generation, possibly in different locations and, for cereals, under high nitrogen dressing levels, which can be expected to exercise a strong selection pressure. In this way both the lodging resistance and the yielding ability and other important characters (earliness, quality, etc.) associated with outstanding varieties can be tested in the M_5 and subsequent generations.

Numerous lodging-resistant mutant lines have been developed from plant material treated with chemical or physical mutagens (http://mvd.iaea.org).

From a general review of the results so far available the following remarks can be made.

1. Mutations for lodging resistance are relatively easily induced in cereal crops, and often induced in many other crops.

2. The increase in standing ability is mostly due to reduced height of the stem; other changes can occur, parallel with stem shortening, namely changes in the number of internodes, modifications in the relative length of internodes; modifications of the root system can also take place. Further work will be needed to evaluate the relative importance of these factors in determining the behaviour of the selected lines.

3. The morphological and anatomical changes mentioned above can occur without evident changes in important agronomic characteristics such as inflorescence characters, yield potential, seed quality, date of maturity, disease resistance etc.

4. Parallel to the increase in standing ability the capacity of the selected mutant line to endure higher amounts of nitrogen fertilizers with positive influence on yielding ability is generally ascertained.

5. Numerous progressive stiff-straw mutants have so far been isolated in several cereal species and have performed better than their mother variety in terms of yield. This is the case, for instance, of mutants obtained in oat, barley, rice, and in bread and durum wheats (Kato, 2008). New varieties have been bred from some of these foundation mutants.

6. On the other hand, the utilization of lodging-resistant mutants in cross breeding is also interesting because of the possibility of removing undesirable pleiotropic effects (e.g. excessive spike density) possibly carried by the original mutant.

7. The difficulty inherent in the genetic pattern of lodging and stem breakage, which is also very complex in a diploid species such as barley, has prevented any real advance in the knowledge of the genetic nature of the mutation for lodging resistance.

8. The appearance as early as M_2 of such complex phenotypes, which breed true in the following generations, may indicate that simple heritable changes may be responsible for the control of important traits.

7.5.5. **Shattering and shedding resistance**

A great amount of potential harvested products (fruits or seeds) are lost every year due to wind and rain prior to harvest. Specific anatomical and histological features of crops are responsible for these losses, which occur in one of the following ways:

- by shattering of the fruits (in legumes, crucifers, but also in sesame, buckwheat and other species),
- by shedding of seeds, fruits or whole inflorescences (in cereals, legumes, grasses and others).

Mutation breeding has not been exploited much in developing shattering and shedding resistant varieties, but several resistant types have been selected from natural populations during the past decades. Shattering-resistant spontaneous mutants of lupin (*Lupinus lure us*) have been known since 1935; they were used as valuable partners in lupin cross breeding. Analogous findings were obtained in *Lupinus lutescens*, *L. polyphyllus*, *L. mutabilis*, and *L. angustifolius*, and also in beans (*Vicia faba)*. In lupin, the resistance to shattering is associated with the formation of a very thin layer

of fibres in the wall of the pod. Recessiveness is assumed, but the character in question is very complex and it is not clear whether the resistance is really due to one single gene (Maluszynski and Kasha, 2002).

Shattering resistance is an important breeding aim in crucifers' oil plants, large differences could be found with regard to this character when comparing different species and varieties of this group of cultivated plants. Shattering resistant rapeseed varieties possess a large number of specific thin-walled and lignified cells in their fruit walls. Corresponding results were obtained in mustard (*Brassica juncea*); after X-rays treatments a shattering-resistant mutant was produced with thick fruit walls. Shattering resistant plants were also selected in *Sesamum indicum* (Ji *et al.*, 2006; Boureima *et al.*, 2012).

Two genes have been found responsible for seed shedding in sorghum (*Sorghum virgarum*) and resistance is triggered by recessivity of both these genes. It would be of considerable importance if wild grasses and wild legumes such as lupin (*Lupinus perennis*) and many bean species (*Vicia spp*) could acquire such characters, which are considered standard for cultivated plants. The first results in this direction were obtained in forage grasses (*Phalaris*) and blackgrass (*Alopecurus*) after X-ray treatments and in soybean after neutron irradiation (Khan and Tyagi, 2013).

The semi-dwarf trait is generally related to modern production systems. This has two major advantages: 1) it changes the source-sink relationship so that more energy is directed towards seed production and less to vegetative growth, and 2) semi-dwarf crops are amenable to mechanical (combine) harvesting. Some other characteristics of plant architecture, such as increased branch number in cereal and legume crops, more compact growth habit such as high plant density, shorter stolon length of potato etc., can also affect the efficiency of growth and harvest, particularly in modern, mechanized agriculture.

In the flower industry traits such as: reduced number of flowers, change in shape and flower colour etc., are usually welcome because they readily add value to the plant. One unique mutation in chrysanthemum with a lower number of axillary flower buds was induced by ion beams in Japan (Shirao *et al.*, 2013). This mutant is very useful for the flower industry because in non-mutant chrysanthemum large flowers can only be produced by removing (hand picking) axillary flower buds from the plant. This mutation negates the need for axillary bud removal. Notable successful chrysanthemum varieties carrying this mutation include var. Imajin (Imagine) and var. Alajin (Aladdin).

Another important plant character induced by mutagenesis is the non-shattering rice. This mutation, induced by gamma-ray irradiation utilizing a shattering indica variety, turned out to be to be useful for release of a forage rice variety: "Minami-yutaka" in

Japan (Hiroshi, 2008). The mutation, which may also be induced by gamma-ray irradiations in other gramineous species, is useful for improving other forage crops which currently have high levels of seed shattering in their inflorescences. To make agriculture greener, traits such as super-nodulation, enhanced water use efficiency, and healthier nutrients uptake from soils will become more and more important, in relation to the movement towards healthier and organic-based living style. The success in breeding and release of a super-nodulating soybean variety is a significant advance in this field (Takahashi *et al.*, 2005).

For species relatively new to agriculture, e.g. blueberries, jatropha and those that, up to now, have received little attention of plant breeders, e.g. medicinal plants and culinary herbs and spices, there is an urgent need to identify, develop and establish domestication traits. For species that have been in domestication for thousands of years many agronomic traits have been provided through spontaneous mutants and selection and have been incorporated into the crop as they arise. The small grain cereals, such as rice, wheat, barley, etc., provide good examples. The grassy wild progenitors of these crops possess natural seed dispersal mechanisms whereby the seed head shatters into pieces at maturity and individual units carrying seeds fall to the ground and are dispersed by hooking onto passing animals. Such dispersal mechanisms are inappropriate to agriculture and cereal crops were not established until mutants for non-shattering were found (Ji *et al.*, 2006). Interestingly the barbed awn trait useful for natural dispersal has persisted, however, barbs are now thought to be associated with the dust produced during mechanical harvesting that causes 'Farmers lung' disease, smooth awn mutants are therefore of interest. These respiratory diseases associated with occupational inhalation of dust during harvest and processing have been, for example, described in hop (*Humulus lupulus*) cultivation, and may then be addressed by developing non-shattering and/or awn-free mutants varieties (Reeb-Whitaker and Bonauto, 2014).

7.5.6. **Other agronomic traits**

Mutants are usually named after the most distinguishably changed character, and several hundreds of differently named mutants with common changes in growth habit and plant type appear in articles on induced mutations (see Chapter 3). The plant type must be considered an integration of variations in individual characters and must be described by discriminate selected criteria taking into account existing variations of other individual characters and their contribution to the characteristic feature. Different plant types result from different patterns of growth and differentiation of plants. Dwarf mutants, for example, are very frequently observed in various plant species. They are characterized by shortened height and at the same time in many instances reduced number plant organs, indicating a growth-rate reduction in many or all plant parts throughout their entire life. However, there are examples in which plant

parts or organs are disproportionately reduced in size, e.g. semi-dwarfs in barley: the *GPert* mutation in barley bestows gibberellin insensitivity that affects most tissues and cells to produce semi-dwarfism whereas the *sdw* mutation in barley mainly reduces the length of the first internode. Mutants in which the vertical axis is disproportionately shortened and called brachytic types have been reported. In such a 'stout' mutant of sorghum the plant height is reduced to three–fourths while the stem diameter is twice as large as the original form. Similar changes of relative size and length/width ratio in various organs have been observed frequently in other crops. Giant mutants have also been reported in pea, tomato and barley. In these cases, several or all plant parts were enlarged.

A distinct change of growth habit observed in mutants is ageotropism or laziness. Ageotropic or 'lazy' mutants have been found in maize, rice, barley, lupin, and peas in the late 1930s and described in a review by (Howard III *et al.*, 2014). These mutants have mostly been used for genomic studies including transposon-tagging in maize. In pear (*Pyrus communis* L.), Nashima *et al.*, (2013) worked on the identification of genes expressed differentially between the mutant: giant La France (GLaF) and the parental variety: 'La France' based on the increased size of fruit. One conclusion drawn from this observation was that, considering the bud mutation is a quite localized event leaving the rest of the genome untouched, this result might open the way to more investigation to identify key genes governing other important traits.

Dense organ formation in mutants is observed in various parts of the plant. Abundant tillering or branching, often accompanied with dwarfism, dense node or multi-nodal, fascinated, double leaf, dense compact or erect ear, increased row-number of ear (in barley) and multi-florous or multi-ovary types are mentioned as examples. Such plant-type mutants are classified or named by the most characteristic changes, but they may also show changes in other characters. Table 7.5. below present some valuable mutant varieties developed for their agronomic traits.

7.5.7. **Examples of mutants with improved agronomic traits**

TABLE 7.5. EXAMPLES OF MUTANTS WITH IMPROVED AGRONOMIC TRAITS

Crop	Trait	Method	Landmark mutant/variety (country)	Reference
Rice *Oryza sativa*	Semi-dwarf	Gamma ray	Reimei (Japan)	Das, Patra and Baek, 2017
Rice *Oryza sativa*	Short stature	Gamma ray	Calrose 76 (USA)	Rutger, Peterson and Hu, 1977
Rice *Oryza sativa*	Short stature, early maturity	Gamma ray	TNDB 100 (Viet Nam)	MBNL No. 45, 2001
Basmati rice *Oryza sativa*	Short stature	Gamma ray	CRM 2007-1 (India)	PMR No. 1 (1 and 2), 2006
Durum wheat *Triticum aestivum*	Short, resistant to lodging	X-rays	Creso (Italy)	MBNL No. 6, 1973
Durum wheat *Triticum aestivum*	Resistant to lodging	Gamma ray	Gergana (Bulgaria)	MBNL No. 37, 1991
Barley *Hordeum vulgare*	Early flowering (*Eam8*)	Gamma	Mari (Sweden)	Sigurbjönsson, 1975
Rice *Oryza sativa*	Semi-dwarf (*Sd*)	Gamma	Calrose-76 (USA)	Lestari, 2016
Barley *Hordeum vulgare*	Semi-dwarf (*Sdw*)	X-ray	Diamant (Czech Republic)	Ahloowalia, Maluszynski and Nichterlein, 2004
Basmati rice *Oryza sativa*	Non-lodging	Gamma	CRM 2007-1 (India)	PMR Nos. 1 and 2, 2006
Groundnut *Arachis hypogea*	Early maturity, semi-dwarf	Gamma ray	TAG24 (India)	Patil *et al.,* 1995
Chrysanthemum *Chrysanthemum sp.*	Reduced axillary buds	Ion beam	Allajin (Aladdin) (Japan)	Shirao *et al.*, 2013
Rice *Oryza sativa*	Non-shattering	Gamma ray	Mini-yutaku Japan	Hiroshi, 2008
Rye *Secale cereale*	Short life cycle	Gamma ray	Soron (Peru)	Gómez-Pando *et al.*, 2009
Banana (*Musa acuminata*)	Short stature	Gamma	Novaria (Malaysia)	MBNL No. 44, 1999, Mak *et al.*, 1996
Rice *Oryza sativa*	Herbicide resistance	Gamma ray	Rice (USA)	Maluszynski, Ahloowalia and Sigurbjörnsson, 1995
Corn *Zea mays*	Herbicide resistance	Gamma ray	Corn (USA)	Mabbett, 1992
Rice *Oryza sativa*	Herbicide resistance	Gamma ray	IRAT 239 (Guyana)	MBNL No. 33, 1981
Cherry *Prunus avium*	Compact growth	Gamma ray	Aldamla and Burak	Kunter *et al.*, 2012

7.6.MUTANTS TO FACILITATE PLANT BREEDING

Plant mutation breeding is already a form of accelerated breeding as it can induce required changes directly into elite germplasm with no or little penalty. However, there are mutant traits that could be exploited further in plant breeding.

- Increased recombination inter- and intra-specific, (see Chapter 3).
- Limited recombination – introduction of “alien” recombinants that do not recombine (see Chapter 3).
- Haploid inducer genes in various species, new work in Arabidopsis (see Chapter 8).
- Outcrossing, cytoplasmic male sterility, promotion of open pollination.
- Long lived pollen.
- Off-season flowering (e. g. cassava), not restricted to season.
- Elimination/overcoming self-incompatibility.
- Mutants for cultivability/tissue culture response, e.g. Golden Promise barley a good genotype for transformation in barley.
- Markers for traits (SNPs).

8. SPECIFIC TECHNIQUES FOR INCREASING EFFICIENCY OF MUTATION BREEDING

This chapter will consider the various technologies that may be used to enhance the out-come of mutation induction either by increasing the population size at any given level from M_0 to M_n, such as: **A)** *in vitro* techniques, or **B)** by more rapidly stabilising the mutations in a fully homozygous state, such as the haploid/doubled-haploid techniques and finally **C)** the application of molecular markers techniques for the identification and screening for mutations in the DNA sequence, which may be related to crop improvement.

A) *IN VITRO* METHODS IN PLANT MUTATION BREEDING CHANGE

8.1.BRIEF REVIEW OF PLANT TISSUE CULTURE

Plant tissue culture is generally defined as the process whereby small pieces of plant materials (explants) are isolated and grown aseptically *in vitro*. Plant cells have both plasticity and totipotency (Haberlandt, 1902), and are therefore suited to *in vitro* culture. The culture of plant parts can be done at various levels: cells, tissues, organs and whole plants. Each plant part (buds, leaves, flowers, fruits, roots etc.) is composed of cells from the same origin and thus any part of the plant may be sourced for culture purposes

In the early 1900s, the advent of *in vitro* techniques opened new avenues for examining and manipulating cells, tissues and organs grown in culture. It was observed that, when exposed to changes in culture conditions (culture media, temperature, light, etc.) different responses were achieved; of particular interest was regeneration, i.e. the production of new plants. One major break-through was the production embryos in culture from de-differentiated callus. These "somatic embryos" (as opposed to zygotic embryos) could be produced in large number and grown through maturity.

In vitro methods have been an important part of plant biology and crop breeding since the early 1960s. Plant tissue culture comprises a set of methods and strategies that involve: the transfer of *in situ* plant parts to sterile *in vitro* culture, manipulations under a laminar air flow (sterile air) and the culture on well-defined media (solid or liquid) which comprises carbohydrates, major and trace mineral elements, vitamins and growth regulators, (Murashige and Skoog, 1962; Grafi, 2004; Thorpe, 2006). Specialised methods have been developed the culture of protoplasts, anthers,

microspores, ovules and embryos. In addition to tissue culture being used for basic studies in cell biology and plant development it has been major tool in accelerating plant breeding. The ability to manipulate cells, tissues and organs in culture has allowed: the synchronous production of large plant populations, i.e. clonal production, the production of genetically pure lines (haploids and doubled haploids), the production of pathogen-free propagules, the genetic transformation and other new methods in mutation induction.

8.2.PLANT REGENERATION SYSTEMS

8.2.1. **Micro-propagation**

In vitro propagation mainly consists of the proliferation of apical or axillary buds into newly developed shoots, which in turn provide buds for subsequent rounds of culture and multiplication. The technique is often called micro-propagation and is a cloning strategy (Figure 8.1). Micro-propagation is used routinely in commercial production of true-to-type plants, which retain the exact same genetic background as the donor plant. Initially, the process was used for studies of plant development (anatomy, histology and cytology, for example), but it rapidly became an industrial tool for mass production of high price valued plants, mostly ornamentals, flowers and fruits trees as well as vegetatively propagated crops, e.g. potato. For ornamental plants the association of micro-propagation with mutation induction also proved to be of high economic interest (Ahloowalia, 1998; Jain and Spencer, 2006).

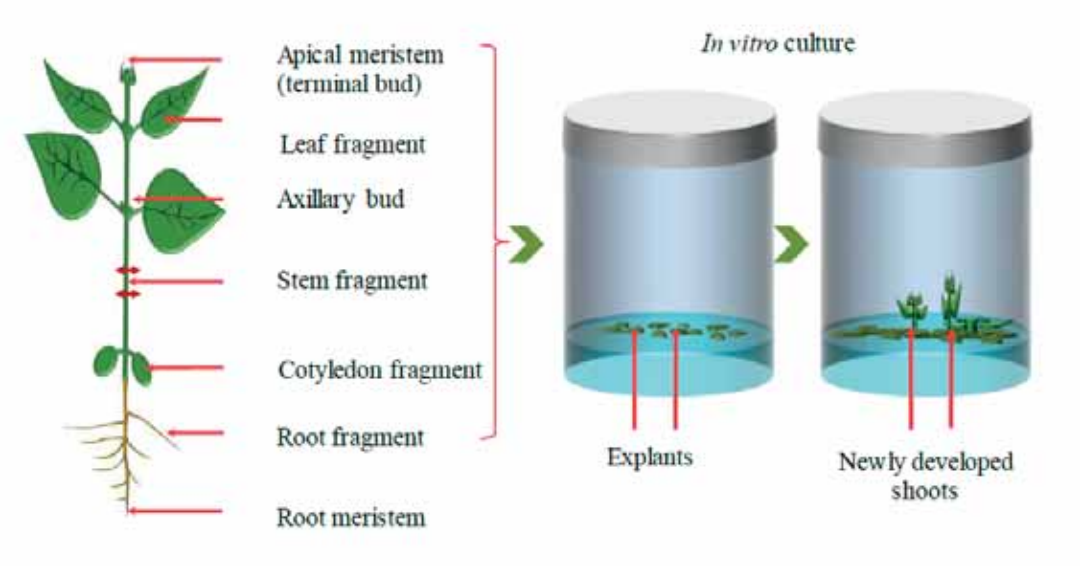


Figure 8.1 Different types of explants for plant micro-propagation.

8.2.2. **Meristem culture**

In the 1950s it was observed, that when a very small section of the apical dome in the meristematic zone – a few cells – is cultured, a good rate of clonal multiplication ensues and in addition, the propagules produced were virus-free; (Barba *et al.*, 1994) This finding found major application in commercial production of "virus-free plants" in many ornamental, food crops and even trees of commercial value. The apical meristematic dome, which is composed of non-differentiated cells, is not linked to the vascular systems and thus contains a few or no virus. Additionally these cells are genetically stable and responsive to culture, and thus, this approach is among the *in vitro* techniques that are also very useful for a rapid mass production of mutated plants (Ahloowalia and Maluszynski, 2001).

8.2.3. ***In vitro*** **morphogenesis**

Although micro-propagation is known to have the potential to produce hundreds, and even thousands, of plants, it is still limited by the number of pre-existing buds from the initial plant material. Morphogenesis is an efficient way of increasing the chances to get new buds and thus new plantlets. There are two types of morphogenetic processes. In "direct morphogenesis" the newly formed buds emerge directly from the tissue in culture: i.e. leaf, stem and root tissues – cambium, epidermis – or any other plant tissue (Figure 8.2a). Conversely, in "indirect morphogenesis" the buds usually arise from a transitional structure of de-differentiated cells into a callus (Figure 8.2b). The process might also result in a more sophisticated developmental process – somatic embryogenesis – leading to a newly formed embryos (Thorpe, 2012).

Progress continues to be made in our understanding of the genetic basis underlying tissue culture response (Phillips, 2004). Advances in *in vitro* systems are readily taken up and exploited in plant improvement schemes including mutation induction, mutation selection and mutant line development.

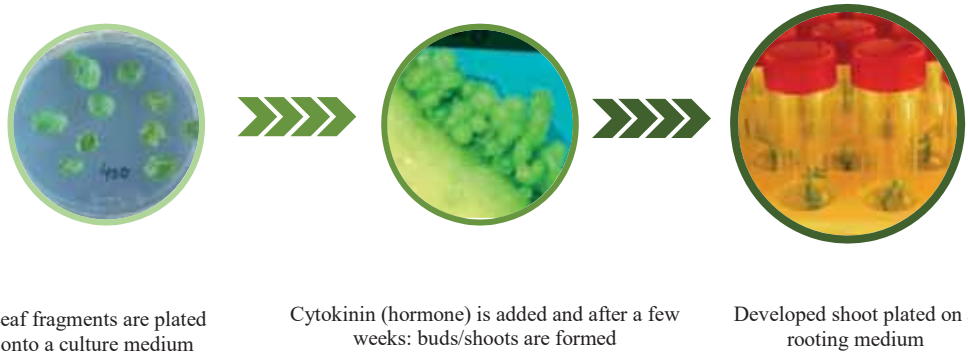


Figure 8.2a. Direct organogenesis and/or embryogenesis: Direct shoots/buds formation in cassava (Duclercq et al., (2011).

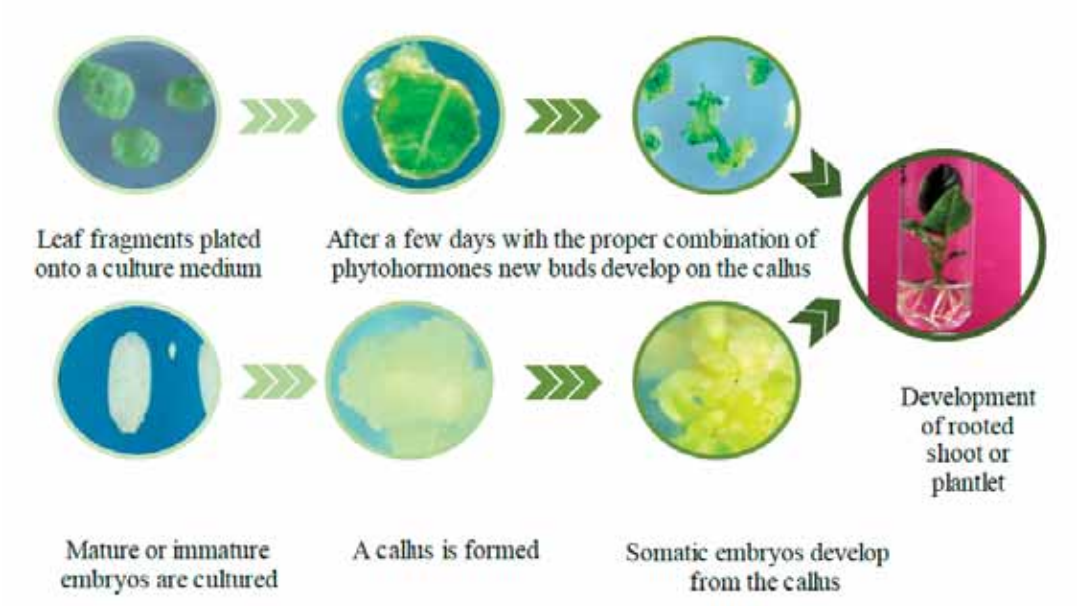


Figure 8.2b. Indirect organogenesis and/or embryogenesis: Callus formation on cassava leaf fragments; and immature embryo culture followed by bud and/or secondary embryo development. As modified from (Duclercq *et al.*, 2011).

8.2.4. **Haploid and doubled haploid production**

In some mutation induction experiments, the traits to be exploited are either difficult to identify or not noticeable in heterozygous genotypes, this is because the vast majority of mutations are recessive. The advent of haploidy and doubled haploidy has opened up a new area for mutation breeding as it provides a means for producing mutants in haploids and then fixing these in a homozygous state of doubled-haploid

mutant plants. The potential outcomes from haploid culture in mutation breeding are wide ranging from stabilization of homozygous recessive traits, immediate fixation of mutated genes, increase selection efficiency, the ability to screen for desired mutants *in vitro* and huge savings in experimental times. For further information see Chapter 8-B.

8.3.PLANT TISSUE CULTURE FOR MUTATION BREEDING

When applied in combination with mutation induction, plant tissue culture increases the overall efficiency of the mutagenic treatments. It enables the creation of new genetic variation, it facilitates the handling of large plant populations, it allows early *in vitro* selection, and it provides a means for easily cloning selected variants. Plant tissue culture also provides the ability to handle these large mutagenized populations in a laboratory scale, thus allowing the development and implementation of efficient and reliable methodologies to screen for biotic and abiotic stress responses (Pathirana, 2011).

8.3.1. **Micro-propagation of mutated plants**

Here selected putative or confirmed mutant plants are entered into a micro-propagation or regeneration programme. In the case of seed propagated crops, the process of *in vitro* culture can be considered, but only if it provides some additional advantages over normal field handling methods such as: developing pure genetic lines (doubled haploidy) or facilitating and speeding up the selection through the establishment of easy and reliable screening processes, or clonal production. In the case of vegetatively propagated crops, *in vitro* techniques offer an exceptional advantage over greenhouse and/or field experiments as large mutant populations can be handled in a relatively small area, i.e. in the laboratory.

8.3.2. ***In vitro* mutation induction**

Since the 1970s, *in vitro* mutagenesis has gained increased popularity as it has overcome major limitations of conventional mutagenesis, especially in the production and handling of large mutant populations and the establishment of reliable and easy to apply screening methods (Maluszynki *et al*. 1995; Suprasanna *et al*., 2012). In addition, direct adventitious buds formation and somatic embryogenesis allow for rapid dissolution of chimeras and facilitate the development of homohistant mutants (Geier, 2012; Jankowicz-Cieslak and Till, 2017).

In vitro mutagenesis is a powerful tool for rapid multiplication of any new and favourable trait obtained; however, there are also some constraints that should be taken into consideration, in order to maximize the benefits.

Advantages of *in vitro* mutagenesis

- High mutation frequency.
- Uniform and repeatable mutation treatments.
- Use of single cell systems, and application of selective agents to provide homogeneous and synchronised cultures.
- Less space to handle large population within short time frames and the production of disease-free plants.
- Multiplication via tissue culture is fast and independent of seasons.
- *In vitro* selection and rapid cloning of selected mutants.
- Tissue culture takes place in special conditioned areas making the whole process easier to control and monitor.
- Tissue culture provides high phytosanitary conditions, which is an excellent system for obtaining healthy starting material and is maintained throughout the plant regeneration process and clean plants are produced to satisfy quarantine regulations.
- Plant tissue culture allows a wide choice of plant materials for mutagenic treatment.

Some limitations and problems relating to *in vitro* mutation induction

- Specialized laboratory, equipment and trained staff is needed.
- Establishing cell cultures showing good regeneration may be difficult from a technical point of view.
- Some methods are genotype dependent and limit widespread use.
- Cultured cells and whole plants often express different sets of genes according to cultural medium and environments.
- Selection at the cellular level is not possible for many traits of agronomic importance.
- Not all observed variation is genetic in nature, which complicates the selection process.
- Effective selection of desired mutants is often hampered by inadequate knowledge about biochemical pathways and developmental processes.
- Ultimately plants produced and selected need to be tested in the field.

8.3.3. Types of explants for *in vitro* mutagenesis

In vitro mutagenesis can be applied immediately before, during and/or after the *in vitro* culture. The *in vitro* mutagenesis processes involve important prerequisite steps including the selection of the proper target material, the choice of explants and the deployment of appropriate culture medium. In addition, there is also a need to consider the genetic constitution and ploidy of the selected material which could seriously affect the success of the programme – some genotypes are recalcitrant to *in vitro* methods.

The choice of the mutagenic treatment and the determination of the optimal dose are similar to methods for seed propagated plants (see Chapter 5), with the important difference regarding the initial dose, which is usually significantly reduced due to the high-water content of *in vitro* plant materials.

8.3.4. Mutagens used *in vitro*

As stated in Chapters 1 and 2, physical as well as chemical mutagens have been applied successfully on *in vitro* derived plant materials. However, among the physical agents, gamma- and X-rays and also UV radiation are the most popular choices. EMS and sodium azide have been largely used as chemical mutagens in tissue culture (Suprasanna and Nakagawa, 2012; Oladosu *et al.*, 2016). Predieri and Di Virgilio (2007) for example, stated that X- and gamma- rays are the most convenient and easiest to use radiation types for inducing mutation in tissue culture, mostly in regards to safety, environmental issues and post treatment handling as compared to chemical mutagens. More than 90 percent of released *in vitro* mutant varieties are derived from physical irradiation (http://mvgs.iaea.org/Search.aspx).

8.3.5. *In vitro* radio-sensitivity testing

One of the first steps in mutagenic treatment is the estimation of the most appropriate mutagenic treatment. This involves the determination of radio-sensitivity and the dose that produces a 50 percent reduction of vegetative growth (RD50). The radio-sensitivity is usually estimated through the physiological response of the irradiated material (Figures 8.3a,b,c)). This estimation may be carried out in the same way as for *in vivo* mutagenesis. Usually at least 20-30 cultures are tested for each dose over a range that covers 50 percent lethality or 50 percent growth reduction, LD50 and RD50, respectively. However, it is sometimes necessary for *in vitro* mutagenesis of certain crops to lower this value to take into account the fragility of some tissue culture derived plant material, and in this case a LD30 may be selected (Patade and Suprasanna, 2008). The breeder has the final say in the dose choice as he/she knows his/her material best and how to handle the subsequent mutant populations. Radio-sensitivity may vary with the species, the cultivars and genotype, with the

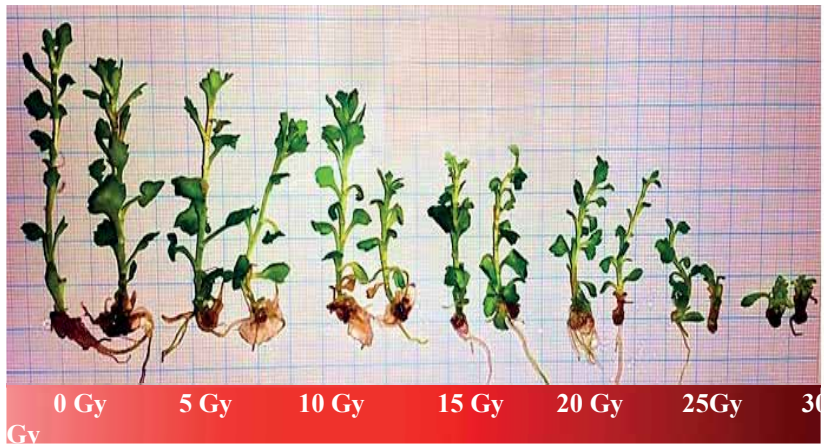
physiological conditions of the plants, organs, as well as with the types of mutagenic treatment to be used: physical or chemical, and finally with the conditions surrounding the experiment (Table 8.1).

TABLE 8.1. SOME EXAMPLES OF DOSE RANGE FOR RADIOSENSITIVITY TESTING OF IMPORTANT CROPS FOR *IN VITRO* MUTAGENESIS (Shu *et al.*, 2012)

Crop species	Treated material	Mutagen and dose (LD50 Gy)
Chrysanthemum	Rooted cuttings	γ-rays, 25
Banana	Shoot tips	Carbon ion beam, 0.5 to 128
Banana	Shoot tips	γ-rays, 60
Banana var. Lakatan Latundan	Shoot tips	γ-rays, 25 – 40
Banana *sp.*	Embryogenic cell suspension	γ rays, 10 – 40
Pineapple var. Queen	Crowns	γ rays, 0 – 45
Begonia	*In vitro* cultured leaflets	γ-rays, 100
Weigela	*In vitro* shoots	γ-rays, 40
Potato	Callus cultures	γ-rays, 30 – 50
Potato	Micro tubers	γ-rays, 10 – 30
Sugarcane	Buds / callus cultures	γ-rays, 20 – 25
Cassava	Somatic embryos	γ-rays
Cassava	*In vitro* stem cuttings with two nodes	γ-rays, 25 – 3
Peppermint	Stolons and rhizomes	γ-rays, 30 – 40
Sweet potato	Embryogenic suspensions	γ-rays, 80
Pear	*In vitro* shoots	γ-rays, 3.5
Yam	Stem cutting	γ-rays, 20 – 50
Yam	Microtuber	γ-rays, 40
Dendrobium orchids	Protocorm like bodies	γ-rays, 35

(a)

(b)



(c)

Figure 8.3. Radio-sensitivity testing, an example from chrysanthemum in vitro mutation induction: (a) 20 days old in vitro plants irradiated by ^{60}Co gamma irradiator;(b) Irradiated in vitro plantlets transferred onto fresh MS medium to mitigate against toxic effects of irradiation; (c) Radio-sensitivity tests: doses 0 (control), 5, 10, 20, 25 and 30 Gy on shoot growth and development applied on 30-days old plantlets. Courtesy of G. Haspolat, B. Kunter and Y. Kantoğlu.

8.3.6. **Chimeras**

Following an irradiation or a treatment with chemical mutagens of a plant tissue, usually only the cells hit by the mutagen agent will exhibit a mutation, i.e. changes in the DNA structure, consequently only cells derived from these mutated cells will inherit the DNA modifications. Chimeras are therefore produced, which are populated by cells of different origins (wild type and a range of mutants).

In seed propagated crops, the transmission of mutations to the next generation requires that mutant cells enter the germline and are transmitted to eggs and pollen grains. For vegetatively propagated plants the mutation must be transmitted to the vegetative propagule, such as buds.

Figure 8.4 illustrates the shoot apical meristems with the three distinct cell layers: the epidermal (L1) and the sub-epidermal (L2) layers form the outer layers called the tunica, while the inner L3 layer forms the corpus. The two inner layers, L2 and L3 contribute cells to form the body of the plant with the proportion of cells derived from each layer varying in different organ types. The chimera situation generates: i) genetically different cells (mutated or not) within a layer = mericlinal chimera; ii) genetically different cells involving several layers = sectorial chimera, and iii) genetically uniform mutated cells in a layer = periclinal chimera (Figure 8.4).

After irradiation the explant (M_1V_0) carries mutated and non-mutated cells, when the explant is sub-cultured (M_1V_1) a chimeric structure appears, the dissection of the variant and additional sub-cultures engenders: i) a non-mutated bud, or ii) a mutated bud at M_1V_1 (Figure 8.4a). On a transverse section of the irradiated bud several sectors would appear (Figure 8.4b). The challenge for the breeder working with *in vitro* mutation induction is to isolate the desired mutants. The process of separating and selecting only the mutated cell lines is referred to as: chimera disassociation or dissolution (Figure 8.5). In seed propagated crops the dissociation will occur naturally during the normal processes of sexual reproduction (see Chapter 5). In vegetatively propagated crops or in the case of *in vitro* mutation induction the disassociation of chimeras normally involves consecutive rounds of sub-culturing: M_1V_1, M_1V_2, M_1V_3, etc., (Geier, 2012).

8.4.HANDLING MUTATED PLANT POPULATIONS *IN VITRO*

The number of sub-cultures needed for isolating mutated sectors depends on the species, the plant regeneration methods used, and the types of mutagenized plant material.

Following the treatment with mutagenic agents, the newly formed shoot tips, for example, are transferred individually to fresh medium plates and incubated under

normal controlled culture environments. After about 4 weeks, the percentage of surviving plantlets is recorded and the ED (effective dose) level estimated. Individual shoots are dissected from M_1V_1 cultures and transferred onto shoot multiplication medium to generate the M_1V_2 generation. About one month after the transfer, a number of data are collected: mean shoot length, average number of shoots per explant and percentage of plants showing leaf abnormalities. All morphological changes (chlorophyll deficiency, morphological abnormalities, etc.) are carefully recorded this process might be repeated until, maybe the 5^{th} vegetative generations (M_1V_5) to identify any induced morphological variation which appears stable and transmissible to the next generation. The shoots dissected from M_1V_5 cultures may then be plated on a rooting medium for regenerating the whole plantlets. The time needed for roots initiation, development rate and the average number of roots/explant should be recorded. Well rooted mutated plants are then transferred and hardened in the greenhouse and maintained at normal temperatures for about a week to enhance the acclimatization process and carefully monitored until possible flowering and seed setting stages.

Initial morphometric analysis and evaluation for new phenotypes can be completed in the greenhouse at the whole plant level. Figure 8.6 illustrates the strategy for *in vitro* mutagenesis (from mutagenized organogenic calli or shoot meristems), handling of the mutated population and mutation recovery in a vegetatively propagated plant. The isolated putative mutants in the M_1V_2, and/or M_1V_3 generation can be evaluated for stability and multiplied to test their agronomic performance.

In addition to rounds of micro-propagation, Jain *et al.,* (2010) reported that embryogenic cultures (cell suspension or callus cultures) could be suitable for inducing mutations and provides mutated somatic seedlings in a short period, thus avoiding chimeras ,which otherwise require to multiply plants up to M_1V_4 generation for chimera dissociation.

Mass propagation of large numbers of shoot tips is used in order to induce direct shoot organogenesis and prevent callus formation. This system can be used for mutant plant multiplication in large numbers for further evaluation in which the target crop/genotype is recalcitrant to somatic embryogenesis. Successive rounds of isolation and division are performed to reduce genotypic heterogeneity, the number of individuals normally doubles or get even a larger increase (multi-apexing) at each generation. Tissue may be collected from M_1V_6 individuals, DNA extracted and screened (genotypically) for any induced mutations. The inheritance of isolated mutations is evaluated and confirmed in the M_1V_6 and subsequent generations.

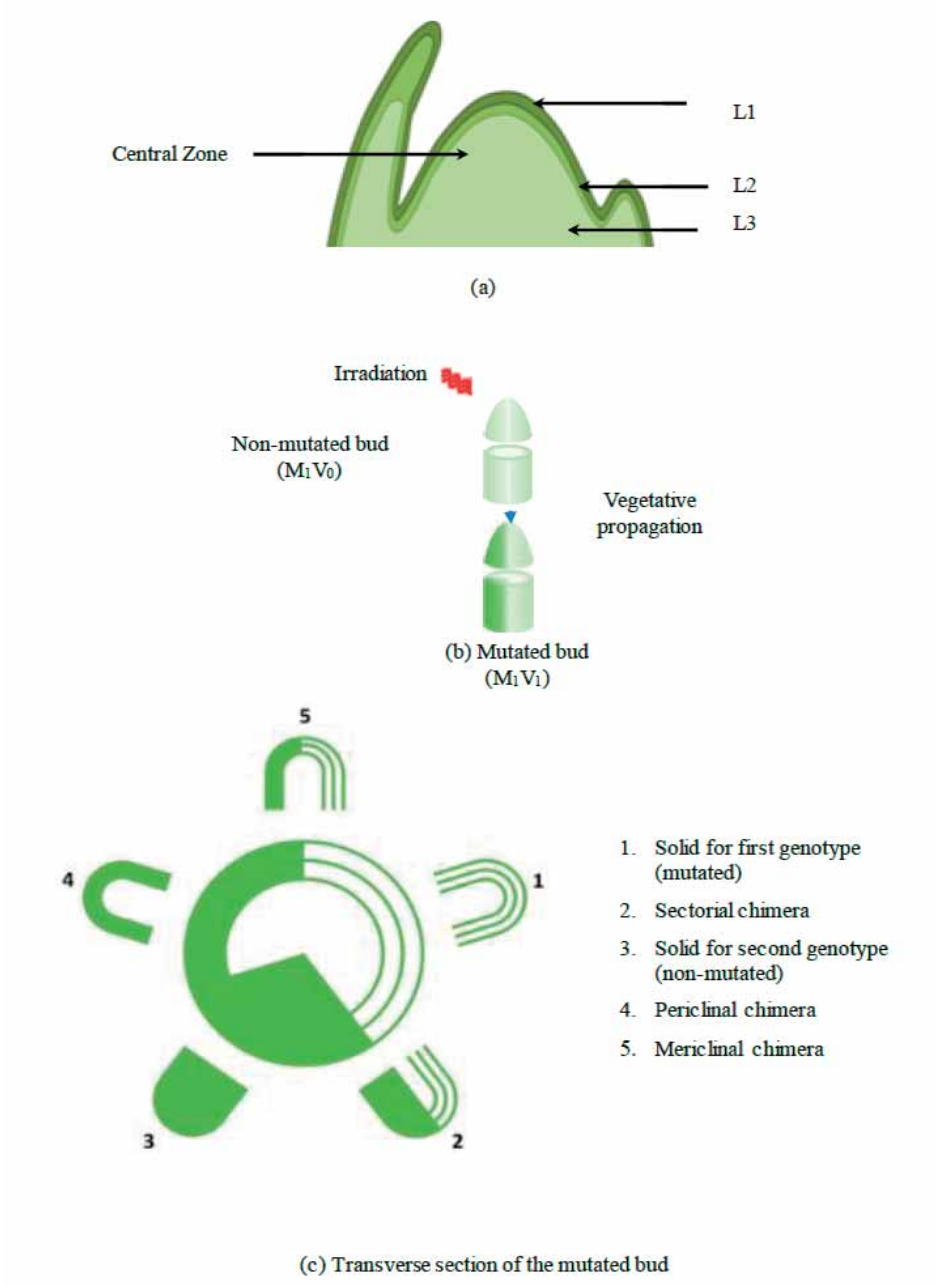


Figure 8.4. The development of mutant sectors in meristems: (a) Structure of the shoot meristem; (b) Effects of mutation on the mutated bud, and (c) Sectorial chimeras generated by mutagen treatment.

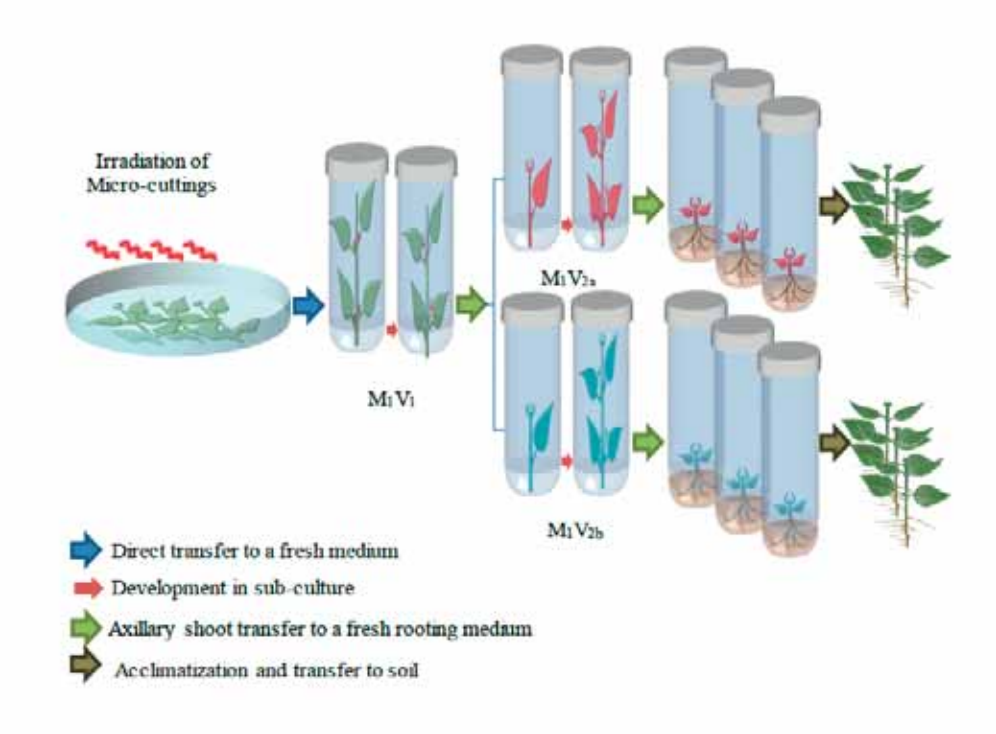


Figure 8.5. Disassociation of chimeras through successive sub-cultures.

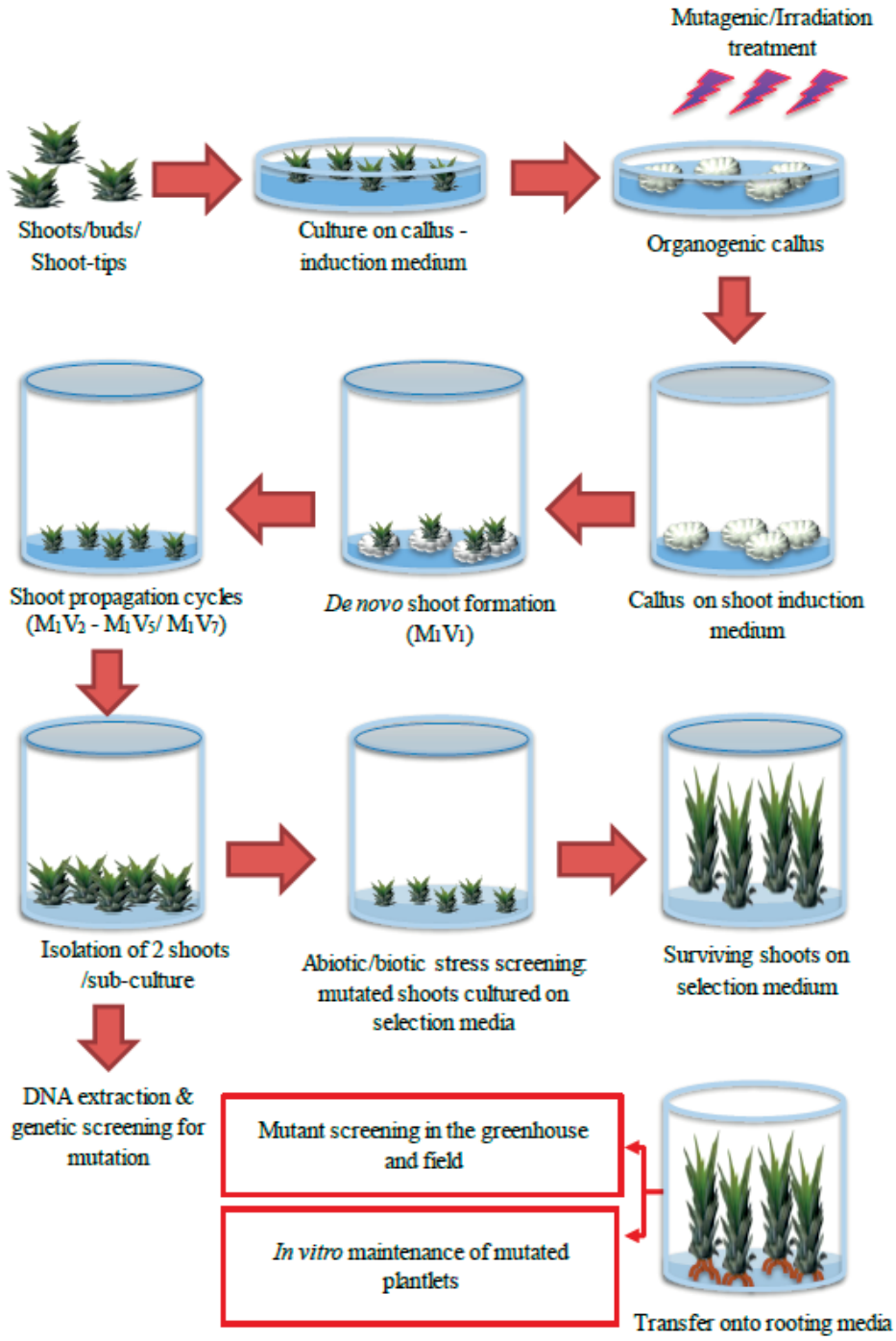


Figure 8.6. Theoretical schematic for the implementation in vitro mutagenesis programme (as modified from Duclercq et al., 2011).

8.5.*IN VITRO* MUTATION SCREENING METHODS

8.5.1. ***In vitro* screening techniques for abiotic stress**

In an extensive and well documented review by Rai *et al.*, (2011) the description and assessment of various methods applied for developing stress tolerant plants using *in vitro* techniques is provided. The responses to both biotic and the abiotic stresses have been investigated by applying the adequately selecting stress inducing agents, e.g. NaCl for salt tolerance (Figure 8.8), and polyethylene glycol (PEG) or mannitol for drought tolerance. Nikam *et al.*, (2015) reported on the selection of salt tolerant mutant clones of sugarcane (*Saccharum officinarum* L.) that were cultured on MS medium supplemented with 0, 50, 100, 150, 200 and 250mmol/l of sodium salt (NaCl) and displayed increased brix percentage and improved agro-morphological characters. Luan *et al.* (2007) also obtained salt tolerant cultivars of sweet potato (*Ipomoea batatas* L.) from EMS induced mutation by repeatedly (5 times every 20 days) screening for tolerant calli on MS medium supplemented with 200mM NaCl, prior to the regeneration of somatic embryos.

Vanhove *et al.*, (2012) devised a protocol using sorbitol as an agent to increase osmolality for drought experiments on various banana genotypes. The plantlets were cultured on liquid medium consisting of: (1) a standard control medium (containing 0.09M sucrose) and (2) a stress medium containing 0.09M sucrose and 0.21M sorbitol. The media were regularly refreshed every 2 weeks and the growth evaluated by calculating the difference between the fresh weight of the plantlets at the beginning and at the end of the experiment, i.e. after 48 days. Masoabi *et al.*, (2017) exposed 16mM EMS-treated sugarcane callus to different polyethylene glycol concentrations and reported the procedure as a suitable *in vitro* osmotic selection regime. The *in vitro* selected plants were further stressed by reducing the water supply in greenhouse pot trials to confirm drought tolerance.

8.5.2. ***In vitro* screening for biotic stress**

The advent of plant tissue culture techniques appeared early on in the mid-seventies as an excellent tool for developing and screening for biotic resistance on *in vitro* derived explants from (Rai *et al.*, 2011). The review described experiments on *in vitro* screening methods applied to organogenic and/or embryogenic calli, shoots, somatic embryos or cell suspensions by exposing them to toxins produced by the pathogen, to pathogen culture filtrate or to pathogen itself. Saxena *et al.*, (2008) reported *in vitro* screening of callus cultures of rose-scented geranium, *Pelargonium graveolens* cv. Hemanti against leaf blight disease caused by the fungal pathogen *Alternaria alternate*. Callus and regenerated plants were confirmed for resistance by exposure to culture filtrate of *A. alternate*. Semal (2013) described an easy and reliable method for screening resistance to fungus (Figure 8.8). Lebeda and Svabova, (2010) also

reported on mass screening methods for the selection of disease resistant dessert and plantain banana (Musa spp.); apple (*Malus domestica*); pineapple (*Ananas comusus* [L.] Merr.); and also, for pea (*Pisum sativum*); melon (*Cucumis* spp.); lettuce (*Lactuca sativa* L.); chickpea (*Cicer arietinum* L.); and various other tropical crops.

Jain *et al.,* (2010) in turn, presented results on irradiation with gamma-rays of axillary buds excised from *in vitro*-grown strawberry plants; where 5 percent of the plants survived the selection pressure of *Phytophthora cactorum* crude extract and these plants were also able to withstand drought for 5 – 6 days. Shoot tips and *in vitro* grown proliferating buds of banana cv. Rasthali (Silk, AAB) were treated with EMS, NaN_3 and DES and the mutated explants were screened *in vitro* for response to fusaric acid and fungus filtrate. Selected lines were confirmed for resistance to the disease in pot experiments and three resistant mutants were selected (Saraswathi *et al.*, 2016).

It is however, highly recommended to consider *in vitro* screening for both biotic and abiotic stress only as a pre-screen procedure, this is because quite often results may not continue in the field where the pressure can differ and other adverse factors may hamper the plant response.

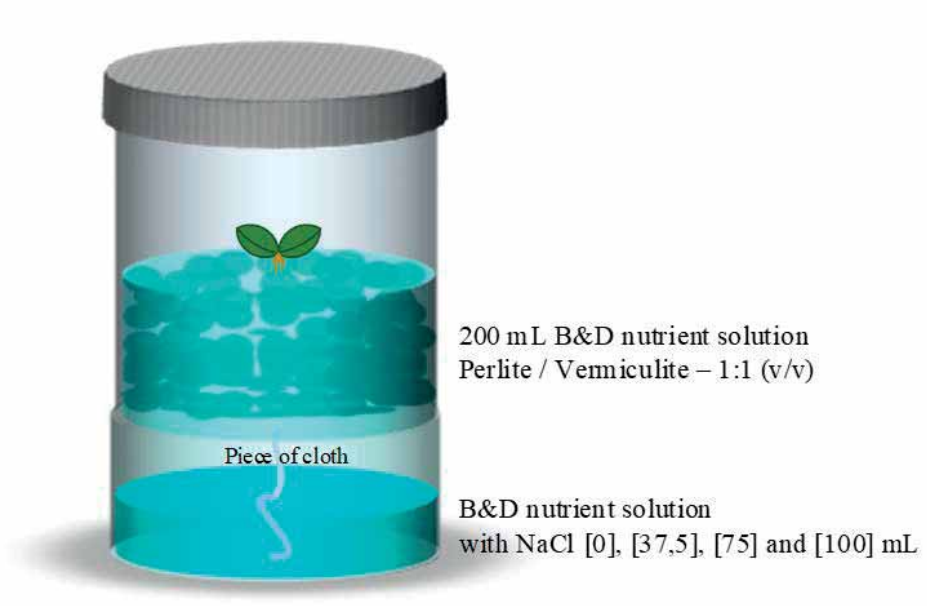
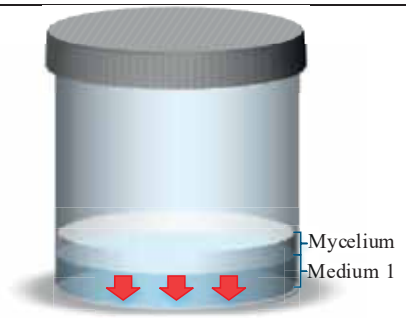


Figure 8.7. Screening for tolerance to abiotic stress (salt), as modified from Djilianov et al., (2003).

In the dual flasks, the lower level is filled with the nutrient solution to which salt is added in various concentrations. A piece of cloth connects to the upper level containing only the nutrient solution. The plantlets cultured onto this medium will then grow and response to the salt concentration may be visualized.

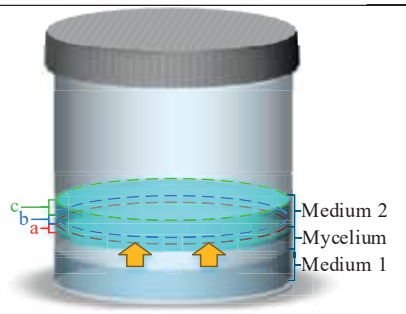
Phase 1

- Inoculation and mycelium development on the first layer medium 1.



Phase 2

- Addition of second layer of medium spiked with fungicide.
- Toxic material diffuses into the upper layer.
- The depth of the second layer may be adjusted to meet a given toxic effect (a, b, c).



Phase 3

- Callus culture on the second layer of medium 2.
- The Benlate (22 ppm) or Mycostatin (80 ppm) added into medium 2; inhibit the development of the mycelium.

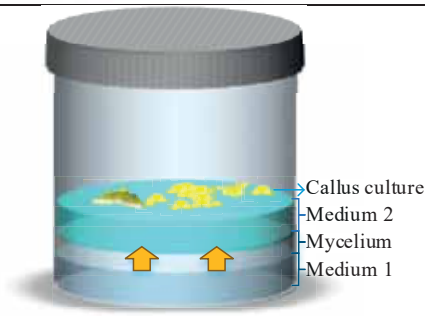


Figure 8.8. Screening for resistance to biotic stress (fungus) – Double Layer Technique (modified from Semal, 2013).

8.6.SOMACLONAL VARIATION

It has been abundantly documented that during the process of *in vitro* tissue culture some noticeable variations may appear, especially when long culture periods are involved; these *in vitro* induced variations are called somaclonal variations. Somaclonal variation is generally defined as genetic, epi-genetic and/or phenotypic

variations observed among the plants or plant progenies derived from *in vitro* cells, tissues and organs culture, probably as a result of gene spontaneous mutations or epi-genetic phenomenon such as DNA methylation (Delgado-Paredes *et al.*, 2017). Somaclonal variants are not always considered solid mutants; however, from the breeder's point of view these variants may show improved traits and are still valuable in crop improvement. Tripathy *et al.*, (2016) described somaclonal variation among regenerants in four genotypes of grass-pea (*Lathyrus sativus* L.) including a large seeded somaclone: NGOG 5 having high seed yield and low neurotoxin content, which was considered as a desirable candidate for future breeding programme of the species.

Thakur and Ishii, (2014) identified two narrow-leaf phenotypes among a population of hybrid poplar (*Populus sieboldii* × *P grandidentata*). These two variants appeared to be identical but arose from different parental lines. Using DNA markers, the authors demonstrated that these somaclonal variants, presented noticeable genetic and heritable variation for both qualitative and quantitative traits. Several authors have documented cytogenetic changes, single genes changes and also transposon mediated mutations in variants from tissue culture. In some crops somaclonal variation caused by methylation can be screened for, by simple DNA analysis. In fact, with the advances in molecular technologies, the genetic characterization of the mutations is becoming an attainable objective and could then lead to the validation of these variants as mutants.

Somaclonal variants have been isolated for a variety of valuable traits like disease resistance, insect resistance, stress (drought, salt, low temperature) resistance, improved yield anefficient nutrient uptake etc., (Tripathy *et al.*, 2016). Variations in flower colour and morphology has been frequently observed in horticultural plants such as cyclamen (*Cyclamen persicum*), carnation (*Dianthus caryophyllus*), chrysanthemum (*Chrysanthemum indicum*), gerbera (*Gerbera* spp.), and torenia (*Torenia fournieri*) have been commercially exploited through somaclonal variation (Singh, Kumar and Verma, 2017).

8.7.PROCEDURE FOR MUTATION INDUCTION IN *MUSA* SPP.

Banana and plantain (*Musa* spp.) are two important crops in which all cultivars are derived from spontaneous mutation without any breeding. Induced mutation techniques are particularly important for bananas as there is limited sexual reproduction and the technique can generate valuable genetic variation (Roux *et al.*, 2001).

The *Musa* spp. mutation induction system, based on *in vitro* techniques to recover mutant plants and micro-propagate desirable mutants, was pioneered by Novák and

collaborators in the in the Joint FAO/IAEA Plant Breeding and Genetics Laboratory. It is now applied worldwide in several *Musa* breeding programmes in dessert banana (*Musa acuminata* and *Musa balbisiana*) and plantain banana (*Musa paradisiacal* × *Musa* spp.*),* (Roux, 2004).

Step 1 – The process usually starts with *in vitro* the propagation of the plant material using: shoot tips, corms, and also embryonic cells suspensions. Shoot tips are found to be the most suitable and are the most commonly used hence, the protocol described below is primarily for mutation induction of shoot tips. The shoot apices can be excised either from suckers or male inflorescences. If embryogenic cell suspension is to be used, immature male flowers and/or shoot tip derived samples are generally used as initial explants. In these two methods, a few hundred initial explants are cultured over a period of 3 – 4 months followed by several sub-cultures to obtain good quality embryogenic callus and shoot developed. The choice between these methods will depend on the need and capacity of the laboratory.

Step 2 – The determination of an optimal mutagenic dose for physical and/or chemical mutagenesis. Success of any *in vitro* mutagenesis programme will depend on mutation induction at a workable frequency followed by selection for desirable mutants. An initial assessment of sensitivity of the plant part to specific mutagen treatments is normally contacted from which the optimal dose is determined in developing a mutant population.

Physical mutagenesis

- Shoot tips are treated with 10 doses: 10 to 100 Gy using a ^{60}Co gamma irradiation source at a dose rate of 44 Gy/min. For each *Musa* accession, at least 200 explants are typically treated for radio-sensitivity testing and 20 non-irradiated explants are used as controls. Immediately after irradiation, the explants are plated onto fresh semi-solid MS medium with 20 µM BAP. Radiation sensitivity and post-irradiation recovery are assessed by measuring survival and multiplication rates, shoot height and fresh weight 40 days after irradiation (Roux, 2004). The optimal dosage for mutation induction depends on parameter studied, although survival rate and fresh weight are preferred. The lethal dose at 50 percent (LD50) is calculated but relatively low doses are advised because they produce less chromosomal damage and less negative side effects than stronger treatments.
- After radio-sensitivity testing, the irradiation can be performed on about 2000 shoot tips, e.g. in batches of a few hundred depending on available resources, labour, space in field or in the greenhouse, etc. It should be noted that optimal dose depends on the variety/genotype but also on tissue culture conditions and handling that are laboratory specific.

Chemical mutagenesis

- Shoot tips propagated *in vitro* can also be submitted to chemical mutagen treatments. The optimal dosage for mutation induction is calculated as for physical mutagens. Sodium azide (NaN_3), diethyl sulphate (DES) and EMS can be applied as chemical mutagens, Bhagwat and Duncan, (1998) compared the effect of these three chemical mutagens, at various concentrations on shoot tips of *in vitro* grown cultures of banana considering two criteria as indicated in below formula: the number of apices that survive the treatment and the number of regenerated shoots, coupled with the factor of effectiveness (FE).

$$FE\ (\%) = \frac{\text{Total number of variations}}{\text{Total number of apices treated}} \times 100$$

Step 3 – The disassociation of chimeras and *in vitro* sub-cultures: M_1V_1 to M_1V_4

Several rounds of vegetative propagation should be carried out to disassociate chimeras, but the minimum number of cycles required depends on various conditions. The M_1V_4 stage or if needed, M_1V_6 stage, are commonly used to complete the process and these plantlets are transferred to a rooting medium.

Step 4 – Screening and selections may be conducted for biotic and/or abiotic stress in the laboratory or in the greenhouse.

Step 5 – Acclimatization and planting in field for screening/selection and assessment of agronomic characteristic.

Step 6 – Selected clones are propagated and evaluation is extended to field performance, this will include multiplication of the mutated clones (under field or laboratory conditions), confirmation of identified clones and evaluation for yield and yield components.

Step 7 – Multi-locational trials: selected clones should be tested in different locations before official testing and release (Table 8.2).

TABLE 8.2. THE CURRENT *IN VITRO* MUSA SPP. MUTATION-INDUCTION PROCESS USING SHOOT-TIP CULTURE AND FIELD SELECTION

Steps
Dissection of sucker from the field and shoot tip culture
↓
Radio-sensitivity test (minimum 200 shoot-tips/dose)
↓
Mutagenic treatment of minimum 2000 shoot tips with a LD_{50} dose
↓
Micro-propagation M_1V_1
↓
Micro-propagation M_1V_2
↓
Micro-propagation M_1V_3
↓
Rooting M_1V_4
↓
Acclimatization and transfer to soil
↓
Field selection
↓
Genetic confirmation and agronomic evaluation
↓
Micro-propagation of desired plants
↓
Multi-location trials

8.8.PRACTICAL EXAMPLES OF *IN VITRO* MUTAGENESIS

Case Study 1: *In vitro* mutation induction for the improvement of Melon (*Cucumis melo* cv. Yuva) initiated from the irradiation of plantlets and leaf or cotyledon fragments, at the Aegean Research Institute, and the Turkish Atomic Energy Authority, Sarayköy Nuclear Research and Training Centre, Turkey – Y. Kantoğlu and collaborators (Figure 8.9).

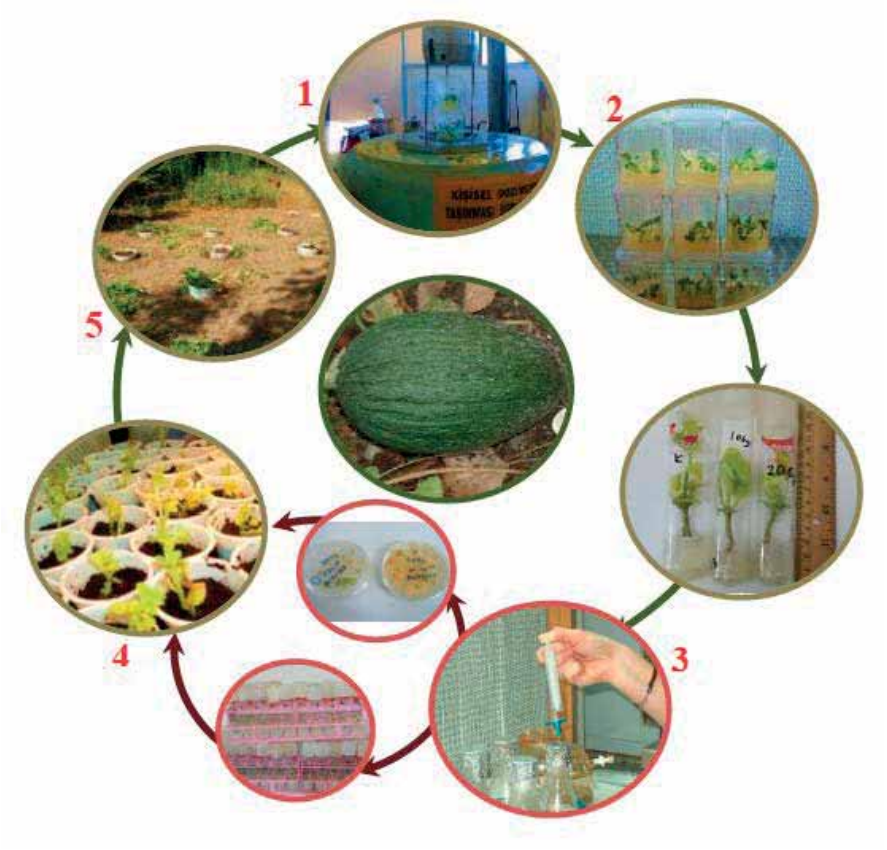


Figure 8.9. Scheme for mutation induction in melon.

A. *In vitro* culture and irradiation

1. Irradiation of 7 days-old *in vitro* plantlets using a vertical ^{60}Co gamma ray irradiator.
2. Radio-sensitivity tests performed on 30 days old (M_1V_1) plantlets and optimal dose determined as: 22 Gy.

B. Screening for resistance to wilt disease

3. Filtrates of *Fusarium oxysporum melonis* race1, 2 were prepared for screening for the tolerance to the Fusarium wilt disease. After the treatment with the filtrate, 6-month-old rooted shoots and 3 weeks old embryogenesis derived surviving shoots and/or plantlets plants were sub-cultured several times and transferred to hardening conditions in the greenhouse.

C. Acclimatization and transfer to the field

4. 36 to 42-month-old M_1V_6 plantlets transferred to the greenhouse are one more time subjected to inoculation with *Fusarium oxysporum* fungus filtrate in pots.

5. A few plants, which maintained tolerance/resistance and set fruits; were then tested for agronomic and commercial value in various fields.

Case Study 2: Sugarcane improvement through *in vitro* mutagenesis – Bhabha Atomic Research Centre, India (Suprasanna *et al.*, 2008; Suprasanna, 2010) (Figure 8.10).

Embryogenic callus cultures of a widely grown sugarcane (*Saccharum officinarum* L.) var. Co 86032 were established from spindle leaf discs on callus induction medium. Gamma radiation was applied to cultures at 10, 20, 30, 40 or 50 Gy (~ 100 embryos per dose) and the LD50 dose was determined as 20 Gy. Plantlets were regenerated from the callus and rooted on specific rooting medium, and then transferred for hardening in the greenhouse. About 5,000 putative mutant plants were planted in the field and at maturity data were recorded on agronomic traits including number of millable canes, stool weight, number of internodes, cane weight, cane diameter and Brix (a measure of total soluble solids). A total of 900 variants that performed better in comparison to check varieties were selected and re-evaluated in the field during the 2007 – 2008 to 2011 – 2012 growing seasons. Eleven selected clones were isolated that were found to be superior for morphological, quality and yield contributing characters (Table 8.3).

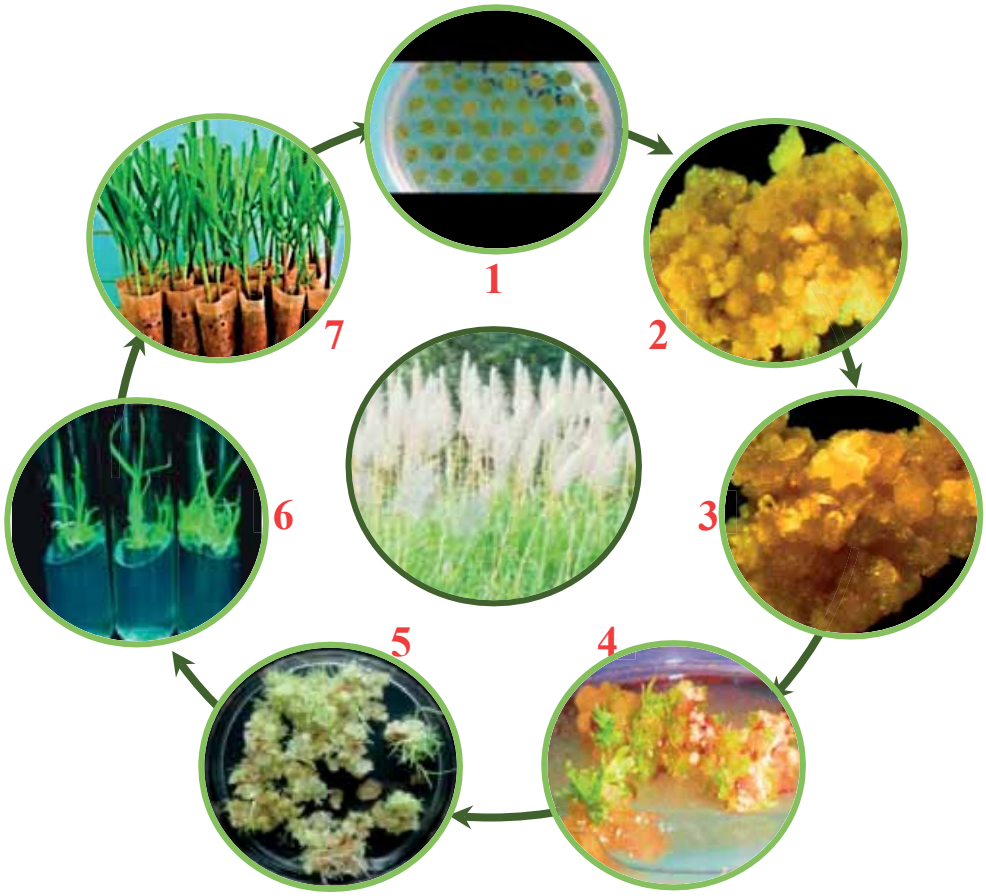


Figure 8.10. Radiation mutagenesis in sugarcane (Saccharum officinarum L.) var. Co 86032. ***1.*** *Leaf discs of the var. CoC671, 86,032 were plated onto specific embryogenic medium;* ***2.*** *Callus was irradiated at 10, 20, 30, 40, or 50 Gy and the LD50 dose determined as 20 Gy;* ***3.*** *Callus cultures were sub-cultured on new medium containing added NaCl (salt) at various concentrations for three subsequent subcultures;* ***4.*** *Tolerance to salt was measured by various parameters including cell membrane damages, electrolyte leakage and free proline production;* ***5.*** *After 30 days somatic embryos which survived the salinity stress were transferred to embryo induction medium;* ***6.*** *Fully developed plantlets were scored and then transferred to acclimatization conditions then to hardening;* ***7.*** *Selected vigorous plants were later transferred to the field for trials and assessment of agronomic and commercial value.*

TABLE 8.3. MUTAGENESIS PROGRAMME IN SUGARCANE

Steps involved	Generation	Time duration
Establishment of *in vitro* embryogenic callus cultures from young leaf roll explants collected from field grown mother plants	V_0	2-3 months
Establishment of high frequency *in vitro* plant regeneration and mass multiplication	V_0	2-4 months
Establishment of radio-sensitivity tests with 100 cultures (per dose) and optimization of LD50 dose	V_0	2-4 months
Mutagenic treatment of 500 – 1000 cultures with LD50 dose and selection	M_1V_0	6-8 months
First sub-culture of selected callus lines	M_1V_1 to M_1 V_2	2 months
Third sub-culture of selected callus lines	M_1V_3 to M_1 V_4	2 months
Plant regeneration from selected irradiated cultures	M_1V_4	2-4 months
Hardening of plantlets and evaluation in the nursery on the basis of quality and phenotype	M_1V_4	4-6 months
Evaluation under row trials on the basis of quality and phenotype	M_1V_4	12 months
Evaluation of selected clones in replicated trial based on agronomic and biochemical characters	M_1V_4	12 months
Vegetative propagation and maintenance of selected clones –based on agronomic characters and selection for stable phenotypic character	M_1V_4	12 months
Vegetative propagation and maintenance of selected clones based on agronomic characters and selection for stable phenotypic characters	M_1V_4	12 months
Multi-location trials, selection of clones and evaluation in replicated trials based on agronomic characters and stable new phenotypic character	M_1V_4	12 months

B) HAPLOIDS AND DOUBLED HAPLOIDS IN MUTATION BREEDING

8.9.INTRODUCTION

A general principle in biology is that most organisms contain genetic contributions from a male (sperm) and a female (egg) parent. The male and female gametes (sperm- and egg cells, respectively) are the products of meiosis and contain half, i.e. n or one set of chromosomes rather than the complete 2 sets or 2n chromosomes of the parent somatic cells. The sporophyte of higher plants has paired chromosomes in their genomes, abnormal plants with only a single (gametic) set of chromosomes are called haploids and these can occur naturally at very low frequencies or be experimentally induced in large numbers (see Section 8.2.2.).

Dunwell (2010) has provided as excellent recent review on haploids (Hs) and doubled haploids (DHs). The first haploid described was a dwarf mutant form of cotton with half the normal chromosome number (n), discovered in the early 1920s (Dunwell, 2010). In another pioneering study spontaneous haploids of *Datura stramonium* were described by Blakeslee *et al.* (1922), that had 12 (n) chromosomes instead of the normal 24 (2n) complement. It took four more decades until the first experiments were under-taken in haploid induction in plants (Guha and Maheshwari, 1964) using anthers of *Datura innoxia*. Haploidy/doubled haploidy in plant genetics and breeding was first discussed seriously in a specially organized symposium in Guelph, Canada in 1974 after the production of the first cultivar derived from a rapeseed (*Brassica napus*), cv. Maris Haplona in the UK in the early 1970s (review by Forster *et al.*, 2007).

The main characteristic and advantage of haploids (Hs) is their potential for conversion into doubled haploids (DHs) as these are genetically pure, homozygous and fertile; haploids are of little use (other than their conversion to DHs or as vegetatively propagated ornamentals) as they are often weak and infertile. Doubled haploidy is the fastest route to homozygosity, a condition prized by plant breeders as DHs can be the end products – cultivars – in the breeding of many crops such as rice, wheat, barley, and rapeseed or provide parental lines in the production of F_1 hybrid genotypes (cultivars) such as maize and various vegetables, e.g. eggplant, pepper, melon and tomato. Doubled haploids can also be maintained indefinitely by selfing or clonal propagation.

In mutation breeding, it is known that most mutations in plants, spontaneous or induced are genetically recessive and, accordingly, are only expressed in the homozygous condition (see Chapters 1 and 2). This is one reason why mutant traits are rarely observed in M_1 populations as the individuals are usually heterozygous. The development of homozygosity is therefore important in observing and assessing

mutant phenotypes. Thus, the targeting of haploids for mutation induction and their conversion into doubled haploids is of immense interest in plant mutation breeding.

8.10. METHODS FOR HAPLOID/DOUBLED HAPLOID PRODUCTION

The main methods in H/DH production in crop plants are described below.

8.10.1. **Haploids via androgenesis**

As stated earlier, the first experimentally produced haploid plants were obtained through anther culture; these cultures were, *in vitro* induced to produce embryos from male gametic cells (androgenesis) from *Datura innoxia* (Guha and Maheshwari, 1964). Androgenesis is to date, the easiest and most common procedure for the generation of haploid in large numbers and in a wide range of plants. Careful histological studies showed that the haploid embryos produced arise from microspores at the mid to late uni-nucleate stage in the development of pollen grains (Szarejko, 2012). Specialized cultural conditions of these microspores with specific hormones and nutrients divert the natural development from pollen grains into haploid embryos (Figures 8.11 and 8.12). Great care is required as anther walls include diploid (parental) tissue which may be confused with DH production. To circumvent this, scientists initiated *in vitro* culture of isolated microspores obtained through a gentle homogenization of anthers using a mortar and pestle or a blender for cell separation (Szarejko, 2012).

This technology (Figures 8.13 and 8.14) requires skilled operators to produce high quality, of healthy isolated microspores. Szarejko and her team with extensive experience in the use of haploids in mutation breeding emphasized the need to confirm the haploidy stage of the plants using either direct cytological or flow cytometry techniques for chromosome counting and/or ploidy-level determination (Szarejko *et al.*, 1995; Szarejko, 2003; Szarejko, 2012). Ploidy confirmation may also be verified using simpler, indirect methods based on guard cell size and plastids dimensions, which are correlated with ploidy-level (Yuan *et al.*, 2009). DNA markers can be used to confirm homozygosity in potential DHs, one useful method is enzyme mismatch cleavage of amplified DNA described by Till *et al.*, (2004) and applied later for DH determination in tef (*Eragrostis tef*), (Till *et al.*, 2017). These methods provide evidence that informative markers (unlinked and polymorphic in parental lines) are homozygous and that the line is a doubled haploid with the following probability.

Number of informative homozygous markers	Probability of being a DH
1	50.000%
2	75.000%
3	87.500%
4	93.750%
5	96.875%
6	98.437%
7	99.218%
8	99.608%
9	99.803%
10	99.900%

Plants responsive to androgenesis include: rice (*Oryza sativa*), common wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), maize (*Zea mays*), barley (*Hordeum vulgare*), triticale (x triticosecale), rye (*Secale cereale*), timothy (*Phleum* spp.), ryegrass (*Lolium multiflorum*), rapeseed (*Brassica nap*us), broccoli (*Brassica oleracea*), tobacco (*Nicotiana tobacum*), potato (*Solanum tuberosum*), linseed (*Linus usitatissimum*), asparagus (*Asparagus officina*lis), and apple (*Malus domestica*), aspen (*Populus* spp.), oak (*Quercus* spp.) and citrus (*Citrus* spp.).

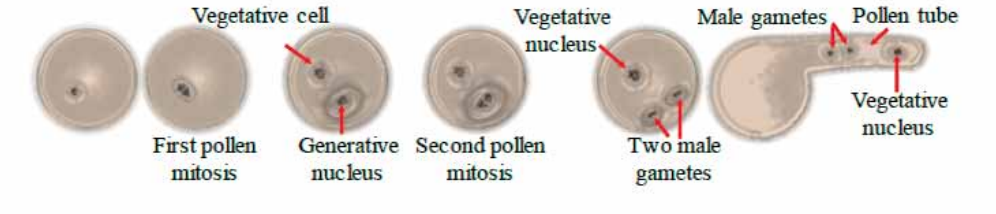


Figure 8.11. In vivo gametophytic development of a microspore.

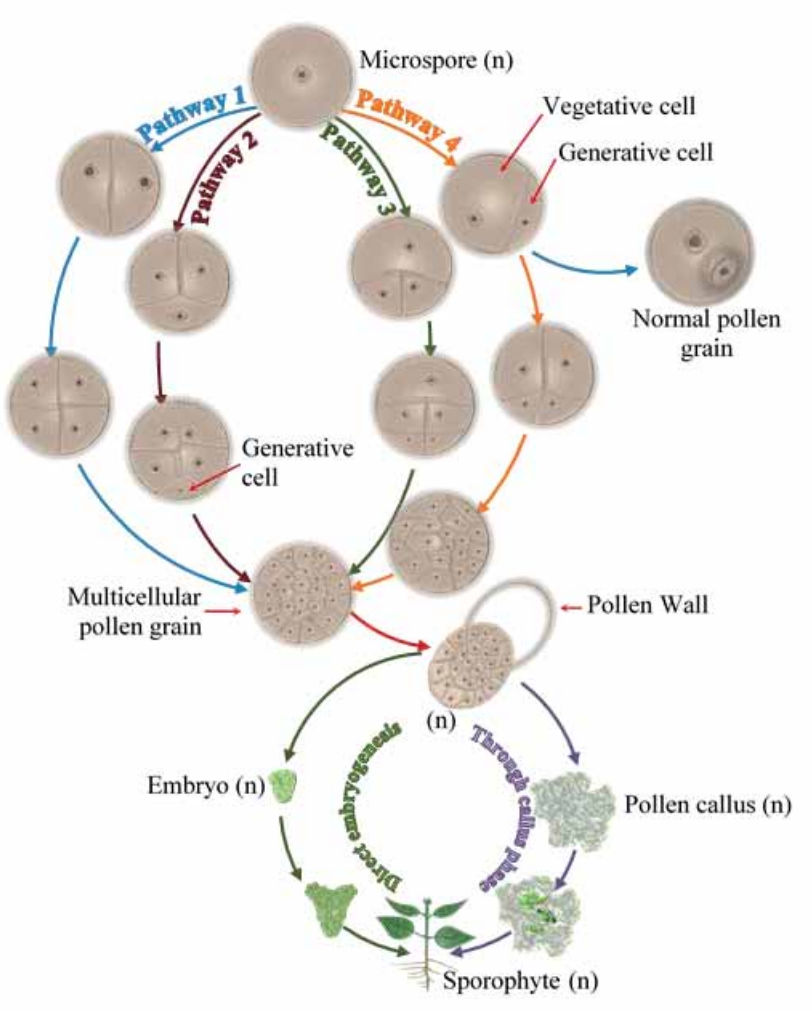


Figure 8.12. Alternative Pathways for haploid development from a microspore (modified from Bhojwani and Razdan, 1983).

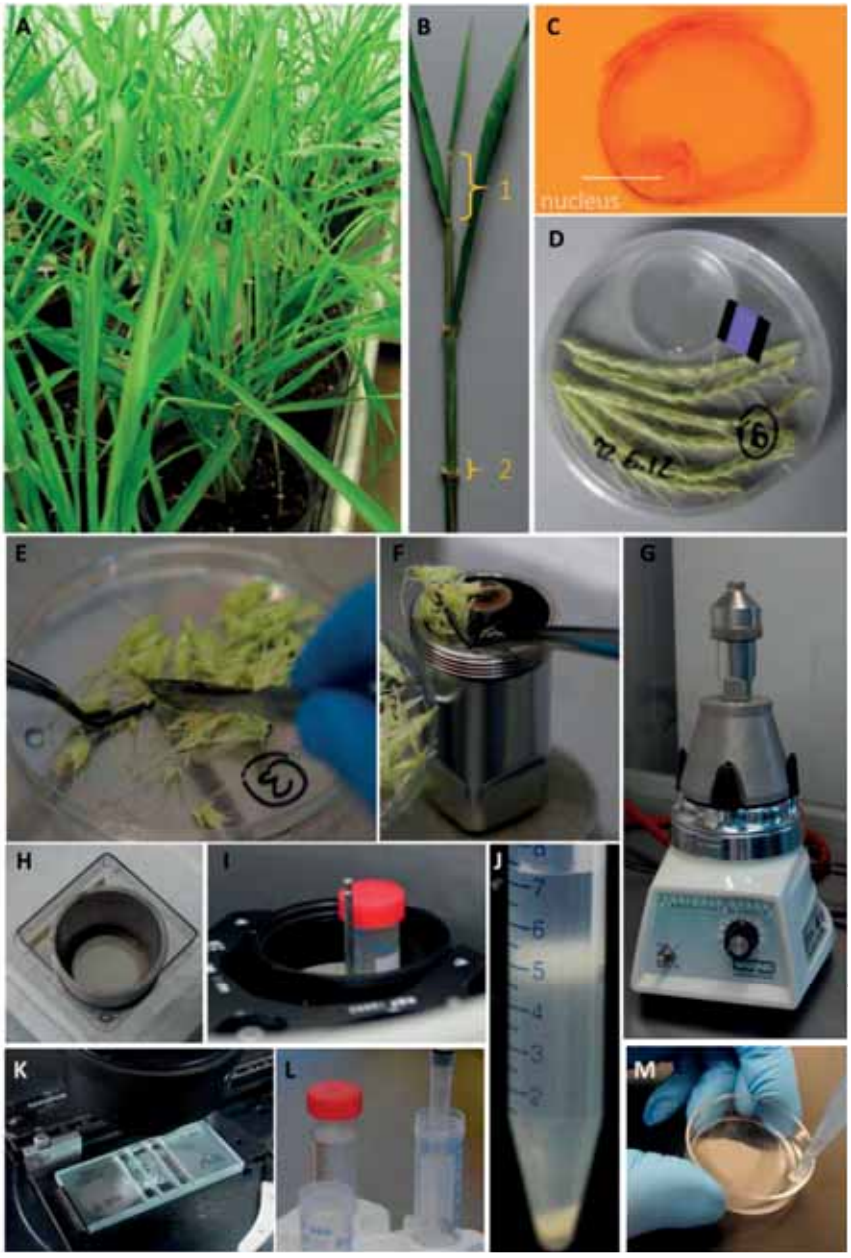


Figure 8.13. Isolation of microspores from barley spikes. (A) Barley donor plants growth in a chamber at 16/12ºC and 16/8h photoperiod. (B) Morphological features associated with microspore developmental stage. (C) Microspore at mid-late developmental stage. (D) Pretreatment of spikes for 2 weeks at 4 °C. (E) Cut spikes on 1cm fragments before blending. (F) Placement of spikes to the blender chamber. (G) Blending spikes for 15 secs at low speed in 0,4 M mannitol. (H) Filter through 100 µm nylon mesh. (I) Centrifugation for 10 min at 110 × g (J). Viable microspores located in the interphase after centrifugation in density gradient. (K) Determination of the number of microspores using counting chamber. (L) Microspores suspension in an appropriate amount of induction medium. (M) Supplementation of microspores with induction medium. Courtesy of M. Gajecka.

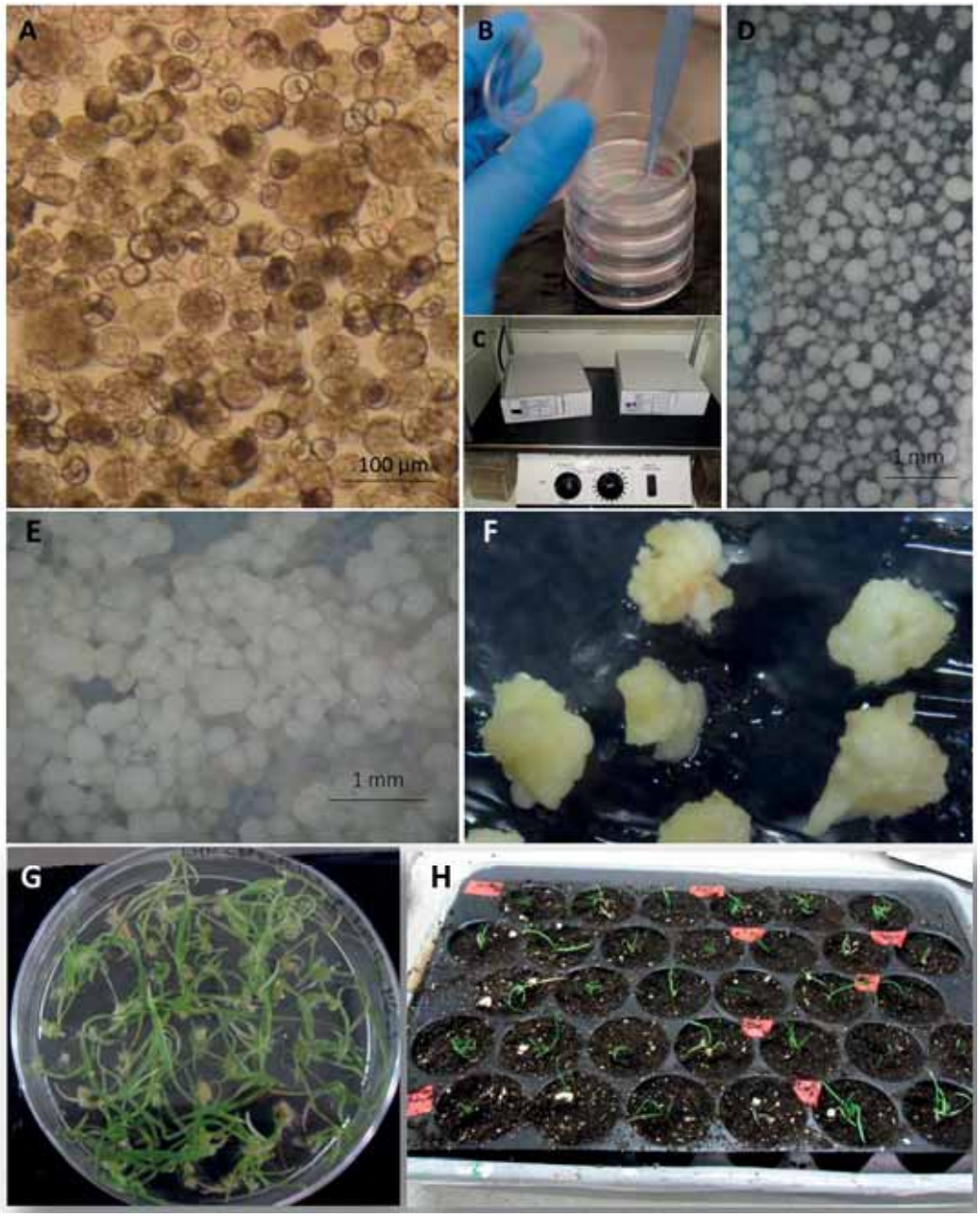


Figure 8.14. Microspore in vitro culture. (A) Microspores after 7 days of culture in induction medium. (B) Supplementation with medium after 7 days. (C) Continuation of culture at 65 rpm and 25°C in the dark for 2 weeks. (D) Microspore culture in induction medium after 21 days. (E) Microspores-derived embryos after 14 days of culture on differentiation medium. (F) Androgenic embryos cultured on regeneration medium at 25°C in the dark for 5 days and continued in the light. (G) Growth of androgenic plantlets in in vitro culture. (H) Growth of plantlets in the soil. Courtesy of M. Gajecka.

8.10.2. **Haploids via gynogenesis**

Haploid plants may also be induced from maternal gametic cells, e.g. the haploid cells produced after meiosis of the megaspore which comprise the ovule. However, these cells, typically the egg, are produced in very small numbers compared to those produced for pollen production. Thus, the frequency of haploids *via* gynogenesis is often far lower that of androgenesis, but considering that some species are recalcitrant to androgenesis, gynogenesis offers a good alternative (Dunwell, 2010; Chen *et al.*, 2011; Germana, 2012).

Plants responsive to gynogenesis include: onion (*Allium cepa*), sugar beet (*Beta vulgaris*), pepper (*Capsicum annuum*), maize (*Zea mays*), sweet potato (*Ipomea batatas*), tulip (*Tulipa generiana*), barley (*Hordem* vulgare) and cucumber (*Cucumis sativus*).

8.10.3. **Haploids via wide crosses followed by chromosome elimination**

In many species, egg cells may be stimulated to develop into haploid embryos after pollination with pollen of another-, but related species as it is the case, for example, when barley (*Hordeum vulgare*) is pollinated by the related species, *H. bulbosum*. The fertilization occurs, but after the chromosomes *from H. bulbosum* get eliminated during the early cell divisions of the embryo development (Kasha and Kao, 1970). Similarly, wheat crosses with maize (*Zea mays*) lead to fertilization of an egg cell by maize sperm followed by elimination of maize chromosomes in subsequent mitotic divisions at early stages of embryo development.

These haploid embryos need to be rescued and cultured *in vitro* as the endosperm does not developed and the embryo frequently aborts *in vivo* (although some of them may survive, see *Spontaneous haploids, 8.2.2.4.* below).

Plants responsive to chromosome elimination after pollination with alien pollen include: wheat (*Triticum* spp.), barley (*Hordeum vulgare*), pearl millet (*Pennisetum spp*), sunflower (*Helianthus annuus*), strawberry (*Fragaria spp*), and maize (*Zea mays*). This method is usually deployed in situations where other H/DH methods fail, however in wheat the pollination with maize is commonly used in breeding as the main method of DH production. For reviews see, among others: Devaux and Pickering(2005); Forster *et al.* (2007); Dunwell (2010); (Dunwell *et al.*, 2010) and Szarejko (2012).

8.10.4. **Spontaneous haploids**

Reproduction in higher plants is normally characterized by double fertilization: resulting in 1) a zygote with 2n chromosomes (union of sperm- and egg cells) and

2) the endosperm with 3n chromosomes (union of sperm and two central nuclei of the ovule), both occur in the ovule, which then develops into a seed. However, in some species seeds may contain more than one embryo, polyembryony, in quite a few instances the additional twin embryo is a haploid. Polyembryony was first described in 1719 by Leeuwenhoek in orange seeds containing twin embryos. The phenomenon was later observed in numerous species, genera and families of both gymnosperms and angiosperms (Koltunow, Hidaka and Robinson, 1996). This is a natural phenomenon, which occurs in many species a rate of about 1 in 100,000 seeds, for example in oil pam (Forster *et al.*, 2007). This frequency was considered too low for practical purposes, but in recent years high-throughput detection methods, for example the use of flow cytometry have allowed the efficient screening and detection of haploids in oil palm (Nasution *et al.*, 2013), and such methods may be applied to other species.

Species amenable to haploid production via spontaneous production in seeds include, among others: oil palm (*Elaeis guineensis*), pepper (*Capsicum annuum*), coffee (*Coffea arabica*), and cotton (*Gossypium hirsutum*).

8.10.5. **Haploids via aberrant pollination**

In the early 1900s, Hertwig (1911) observed that frog sperm exposed to radium retained their ability to fertilize eggs but lost their genetic function. This was called then the "Hertwig Effect". This observation demonstrated that eggs may be stimulated to develop spontaneously without being fertilized after what is called: aberrant pollination. Later, the technology was adapted to tobacco (*Nicotina tabaccum*) by Pandey and Phung, 1982 as cited by (Sato *et al.*, 2000; Germana, 2012) who worked on carnation (*Dianthus caryophyllus*) and fruit crops, respectively. The examples of successful application of radiation-stimulated parthenogenesis include citrus (*Citrus clementina*) and rose (*Rosa sp.*). Gynogenetic haploid plants in muskmelon (*Cucumis melo*) were also induced by pollination with pollen irradiated by ^{60}Co gamma-rays and *in vitro* culture of fertilized ovules or immature embryos by (Sauton and Dumas de Vaulx, 1987). This methodology was later improved by Yetisir and Sari (2003), who found that ɣ-ray doses higher than 30 Krads (300 Gy) were necessary to avoid normal fertilization and obtain only haploid embryos (all haploids showed expected maternal phenotypes).

Further studies in maize (*Zea mays*) showed the development of DHs expressing various types of mutation, namely chromosomes aberrations, and some of these mutants were viable and fertile (Viccini and de Carvalho, 2002). The same effect was obtained in a few other crops, including: apple (*Malus domesticus*), and barley (*Hordeum vulgaris*).

8.10.6. **Haploid inducer genes**

Ravi and Chan (2010) indicated that haploid plants in the model species, *Arabidopsis thaliana* can be regenerated by crossing *cenh3* null mutants expressing altered CENH3 proteins with wild type. This was an important breakthrough for *Arabidopsis* research as *Arabidopsis* is recalcitrant to other H/DH systems described above. Chromosomes from the mutant (*cenh3*) line are eliminated as they are not able to attach to the metaphase spindle apparatus.

This method is yet to be applied to crop plants, though homologous of the *cenh3* mutation are being searched for in crop plants.

8.11. MAIN APPROACHES IN HAPLOID AND DOUBLED HAPLOID MUTAGENESIS

In recent years, haploid (H) and doubled haploid (DH) production of plants gained increased popularity and is now applied to a wide range of crops, mostly for breeding cereals, grasses, oil and other industrial crops, tubers and vegetables, trees, and ornamentals. It is important to call plants experimentally doubled from haploids: doubled haploids (DHs) as they are completely homozygous and true breeding and thus, distinct from normal diploids, which have the same ploidy but may contain heterozygous loci. The doubling of the chromosome sets can occur spontaneously during mitosis, or be induced, e.g. by a treatment with colchicine (Yuan *et al.*, 2009). Maluszynski, *et al.,* (2003) provided many protocols for H/DH production in crop plants and extensively described their use in plant breeding.

There are many examples of successes in crop breeding using H/DH methods and these include an important number in mutation breeding (Maluszynski, *et al.,* 2003; Jain and Spencer, 2006; Szarejko and Forster, 2007; Dunwell, 2010; Szarejko, 2012; and Mba *et al.*, 2012).

Vos *et al.,* (2009) described induced mutations from spontaneous doubled haploid embryos in oranges (*Citrus* spp.). The mutants displayed excellent fruit characteristics, good yield, and also resistance to some diseases. An alternative approach for inducing mutation from haploid cells is to irradiate or treat with chemical mutagen agents, anthers or microspores cultures *in vitro* (Szarejko, 2003 and 2012).

8.11.1. **Doubled haploid production from mutant plants**

Doubled haploidy has relevance in plant mutation breeding as a means of fixing mutations in a homozygous state. The DH lines can be produced from plants at any generation from M_1 to M_n, but for accelerated breeding the sooner the better. Theoretically, all the methodologies cited earlier (Section 8.2.2.) for haploid plant

production can be used for inducing haploids/doubled haploids; however, in practice most of the published examples of H/DH mutant genotypes have been obtained through androgenesis, notably in rice, wheat, barley, vegetables and medicinal plants (Szarejko, 2012). M_1 plants are therefore of interest, but there are two major constraints: 1) preferably only plants carrying interesting mutants should be selected and this would require genotypic screening (see Section 8.3.), and 2) M_1 plants are often weak and therefore not good donors for haploid/doubled haploid methods. Despite this, DHs can be produced, see Section 8.2.5.1., for a protocol. Stable mutants have been produced from fertilized egg cells of a japonica rice (*Oryza sativa*) variety treated with ethyl methane sulphonate (EMS) and N-methyl-N-nitrosourea (MNU). The doubled haploids (DHs) derived from anther culture of these M_1 plants were stable and could be used as new breeding materials (Lee, Cheong & Kim, 2003).

However, M_2 plants (and subsequent generations) provide a more practicable option. In addition to fixing the mutant gene the H/DH plants produced will be the result of one round of meiosis from M_1 plants, two rounds for M_2, three form M_3 etc., and therefore DHs produced carrying the mutation of interest can also be screened for variation in background mutation load, i.e. selection of mutant lines with the minimal background disturbance (see Section 8.3.).

The production of haploids from the M_1 generation still offers great promise, as any mutation existing in a homozygous genotype is likely to be confirmed and visualized phenotypically as soon as the doubling of the chromosomes number is achieved as there would be no segregation. This also means that in only one generation after irradiation stable mutants can be obtained, thus short cutting considerably the breeding cycles needed for obtaining true breeding material. Methods for mutant DH production from M_1 populations vary depending on the species, but the basic protocols are the same and here we take barley protocols as an example.

8.11.2. **Mutagenesis of haploid cells**

Irradiation of haploid cells (anthers and/or pollen grains) has been used for: 1) enhancing the understanding of pollen grain germination and its possible impact of flowering and maturity in crops; and 2) for the induction of mutation of haploid plant materials. In general, these studies showed that when working with haploid cells in mutagenesis attention must be paid to:

- the methods for the H/DH production;
- the choice of genotype (should be responsive to H/DH method);
- the choice of the mutagenic treatment (physical, chemical, biological);
- the assessment of effective mutagenic doses;

- the screening process to be used to detect mutants (genotypic and/or phenotypic); and
- the selection of the optimal developmental stage to be used for mutation induction.

8.12. HAPLOID CULTURE AND MUTATION BREEDING

The advantages of the application of haploid methods in plant breeding have been reviewed extensively (Maluszynski *et al.,* 2003); Szarejko and Forster, 2007; Dunwell, 2010). For plant breeding purpose, the development of haploids is exploited to produce:

- pre-breeding plant material;
- rare varieties or when variety improvement is not attainable from conventional crosses, in developing stable pure (homozygous) lines, and
- accelerated breeding cycles.

Coupling H/DH technology with mutation induction provides a rapid means of producing mutant lines in a homozygous condition, thus speeding up the development of new varieties with mutant traits.

Care is required for physical irradiation and chemical mutagenic treatments as reproductive cells and tissues are fragile. Although all the known physical and chemical mutagens have been applied to haploid cells, the most used agent is a gentle treatment by UV light (see Chapter 1). This mutagen agent has many advantages as it is readily available in most plant tissue culture laboratories, it is relatively cheap, and the radiation doses are generally in the lower energy range. When a treatment is too severe (e.g. gamma- or X-rays) it may cause sterility and viability problems and may result in a low density of mutations, which in turn will require more screening to identify the specific mutation. The low penetration of UV light irradiation requires a large amount of pollen grains and the strict use of a one cell layer to ensure efficient induction of mutation.

The irradiated material then needs to go through regeneration steps to produce H/DH plants that would then be subjected to mutant gene/trait screening and selection processes (Maluszynski, *et al.,* 2003); Forster *et al.*, 2007). It is therefore advisable to mutagenize several batches over a range of mutagen dose rates and concentrations, then choose the batch that shows the best trade-off between, stability, fertility and mutation damage/induction.

8.13. PROTOCOLS IN MUTAGENESIS USING HAPLOID/DOUBLED HAPLOID SYSTEMS

Haploids have been targeted by plant mutation breeders since the first haploid/doubled haploid technologies were developed as they provide many advantages. Chief among these is the immediate production of homozygous mutant alleles on doubling haploid cells/tissues treated with a mutagen. Mutation induction using microspores was first deployed using responsive model species such as barley and brassicas see Maluszynski, *et al.,* (2003); Forster *et al.*, (2007); and Szarejko, (2012) for reviews. Further studies in maize showed the development of DHs expressing various types of mutations, namely chromosomes aberrations, and some of these mutants were viable and fertile.

8.13.1. **Barley: mutant haploid/doubled haploid production from M_1 populations**

In this approach, the M_1 mutant population is produced by standard methods (see Chapters 1, 2 and 4), but DHs are produced from M_1 plants. The example given here involves mutation induction via seed mutagenesis (Figure 8.15).

- Mutagenic treatment

  Seeds (M_0) of an elite line are selected for mutagenesis and treated as described in Chapters 1, 2 and 4 to produce M_1 seeds. Care is needed to select the optimum dose treatment (usually in the range LD30 – LD50) to obtain a mutant population in which the frequency of the desired mutant(s) is detectable.

- Propagation of M_1 plants

  The M_1 plants suffer from physiological disorders as well as chimeras and they need to be grown up in optimal conditions, normally a growth chamber or a greenhouse with controlled temperature and lighting. The M_1 also carries mutations in a heterozygous condition. It is essential that healthy plants are produced as these are the best donors for haploid production.

- Doubled haploid production

  Doubled haploid methods are applied directly on plants from the M_1 population. The choice of method (androgenesis, gynogenesis, aberrant pollination etc.) depends on the species and genotype, a wide range of species specific protocols is provided in the manual: Doubled Haploid Production in Crop Plants (Maluszynski *et al.*, 2003). Ideally only plants carrying

mutations of interest should be selected as donors for DH production, but in the M_1 this is restricted to genotypic selection (see Section 8.3.).

- Selection of DH mutants

  The DHs produced may be selected during the culturing process both genotypically and phenotypically. Screening may also be done genotypically and phenotypically on hardened plants produced from *in vitro* culture, normally in a greenhouse. However, it should be noted that phenotypic testing in culture and immediately on plants derived from culture is unreliable as the plants can suffer from physiological disorders and somaclonal variation, therefore phenotypic testing is best done on later generations.

8.13.2. **Barley: *in vitro* haploid/doubled haploid mutant production**

Microspore culture is the best system, though anther culture can also be used and can be applied to any species where these methods work. Mutation induction is targeted at the single cell microspore stage shortly after isolation (uni-nucleate stage, see Figure 8.16). The factors to consider are listed below.

- Mutagenic treatment

  The mutagenic treatment must be applied at the uni-nucleate stage of microspore development. Since the microspore stage is normally stage material is sampled mutagenic treatment must be applied immediately or soon after sampling. If the microspore is allowed to develop and produce more nuclei they approach becomes use-less as more than one nucleus may carry mutations and chimeras will develop. It should be noted that the mutation treatment (physical or chemical) will drastically reduce the viability of microspores and subsequent embryogenesis. This is one of the reasons why UV light is a favoured (gentle) mutagen for microspores. If gamma is used then a gamma source with a low activity should be used. Radiosensitivity of microspores is normally determined by measuring lethality, "killing curves". The dose selected should allow for sufficient embryogenesis in producing a DH M_1 population that can be screened *in vitro* and eventually in the field.

  Physical and chemical mutagens can be used though chemical mutagens are more difficult to handle in terms of treatment and washing to remove chemicals from cultures, discard of waste toxic materials and human health and safety (see Chapter 2). EMS, ENU, MNU and sodium azide have been used in barley and *Brassica* microspore mutagenesis (summarized by Szarejko, 2003).

Although microspores are the preferred materials for haploid/doubled haploid production other reproductive tissues and cells can be targeted such as immature inflorescences and isolated ovules.

- Selection in culture

  Some traits may be selected for in culture, e.g. drought and salinity (see Section 8.1.), these are usually classed as pre-screens as the acid test for phenotypic traits is screening in the field.

- Regenerate doubled haploids (DH M_1 mutants)

  In some species, notably barley, there is a high rate of spontaneous doubling *in vitro* during embryogenesis. This can attain more than 60 percent and thus, precludes the need for artificial doubling with chemical agents such as colchicine and oryzalin, which may be applied *in vitro*, or later on seedling plant stage.

- Primary evaluation of plants in field conditions

  A great advantage of DH mutants is that DHs can be multiplied and tested repeatedly, e.g. in replicated, multi-locational and multi-season trials. However, sufficient materials must first be generated and this is usually done by growing the first generation from tissue culture in optimal greenhouse conditions to maximize seed production.

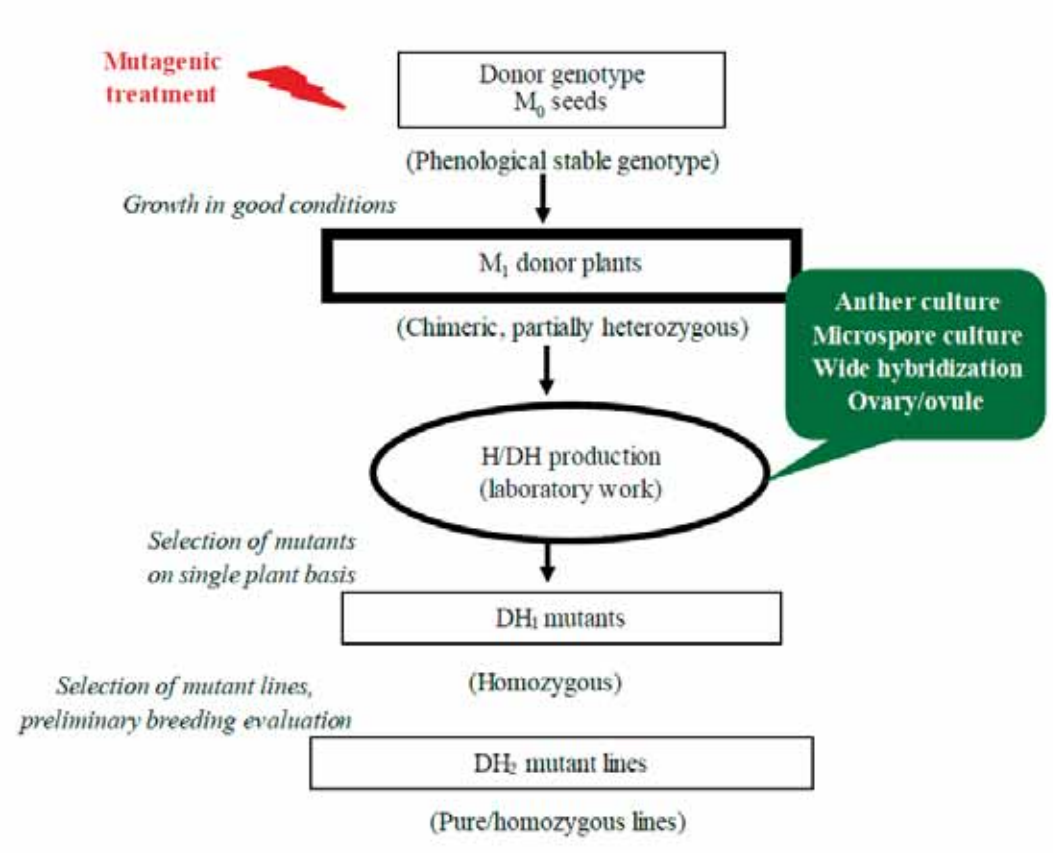


Figure 8.15. Development of mutant lines using seed mutagenesis and DH systems, modified from (Szarejko, 2003).

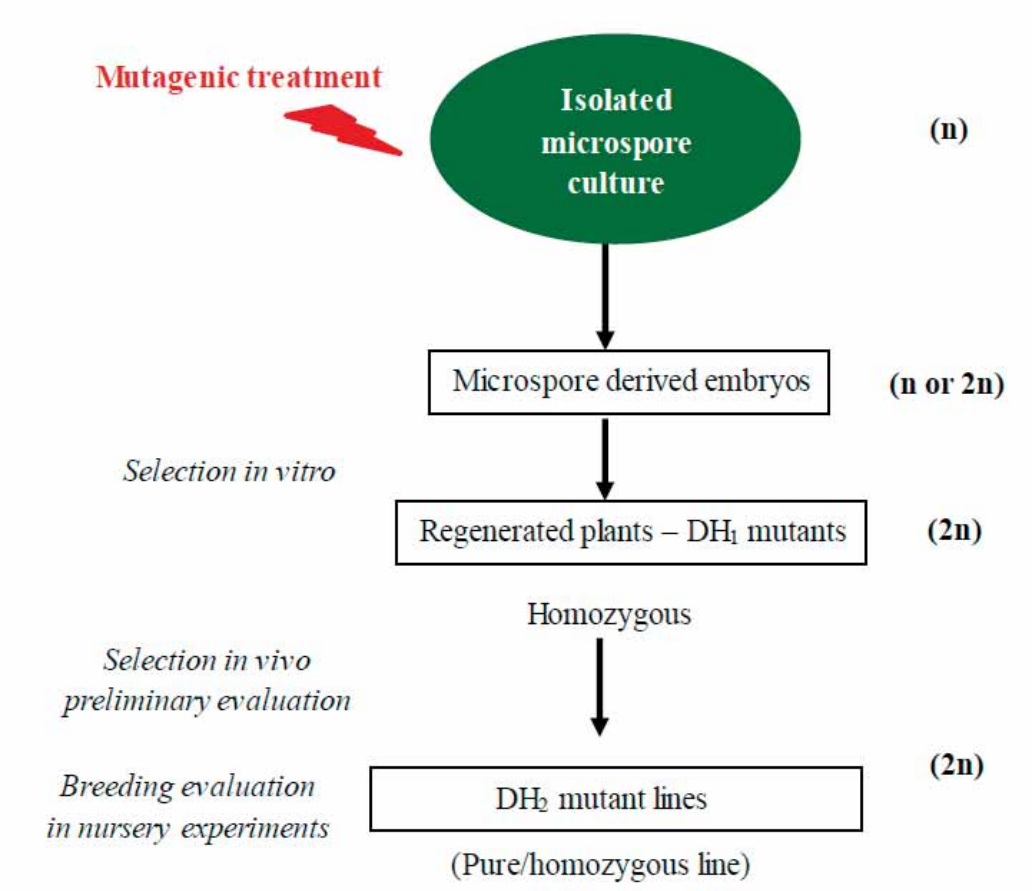


Figure 8.16. In vitro mutagenesis using isolated microspore culture from Szarejko (2003).

8.13.3. **Doubled haploids in pedigree mutation breeding**

Each breeding method is based on a wide genetic background. In case of mutation breeding, there are different methods to increase the genetic variation/segregation via different mutagenic agents. Two breeding methods are proposed. The first is to use DH techniques in M_1/M_2 generations. In some exemption, when heterozygosity still exists in M_{3-4} generations, alternative method is to release DHs from later generations (M_3/M_4), when the breeder has important positive data about the selected but segregated mutant lines (Pauk *et al.* 2004). The success of the techniques depends on the used protocol (see Maluszynski, *et al.,* 2003) and the routine and skills in the particular laboratory. It should be noted that, such methods are continually updated and verified against new publications to improve the laboratory protocol step by step standard procedures.

In the mutation pedigree breeding methods two breeding alternatives are integrated. The first breeding alternative is the early-generation haploid induction, and the other one is the late-mutant generation induction. These two breeding alternatives have different advantages and disadvantages as stated below.

- Releasing mutant DH lines from early generation (M_1 or M_2) of mutated population

  Spontaneous and colchicine treated mutant DH lines after one-year evaluation and seed propagation are passed to the replicated and multi-location performance tests. By this alternative 3 – 5 selection steps can be saved in the breeding process.

- Releasing mutant DH lines from late generation (M_3 or M_4) of mutated population

  The second alternative shows, the selection in early generations (M_1, and M_2) and starts the H/DH lines induction from only later mutant (M_3 or M_4) generations. In this alternative the DHs are induced from selected population genetically more valuable population than in the previous alternative.

- Evaluation of using DH lines in mutation breeding

  Benefit of the 1st breeding alternative is the perfect homogeneity, significantly shorter breeding time. Disadvantages are high number of unusable DH mutants relatively, because of the lack of selection for agronomic characters in selection process.

  Benefits of the 2nd breeding alterative are perfect homogeneity, more usable DH lines (positive mutants) in breeding, compared with the previous alternative. Disadvantage of the 2nd alternative is that, there is no significant time saving (only some years) in the breeding process.

8.14. SCREENING FOR HAPLOID/DOUBLED HAPLOID MUTANTS

8.14.1. **Phenotyping**

As in any other crop breeding programme, the production of haploid then doubled-haploid mutant plants needs a thorough screening for the selection of the desirable mutant trait. Haploids of higher plants can be distinguished from their diploid equivalent in many ways. Most obviously from the perspective of phenotype, as they are usually smaller in appearance, partly because of their smaller cell size; bearing in mind that in general terms, cell volumes in plants are positively correlated to ploidy level. The most widely used of these phenotypic methods is the measurement of

stomatal guard cell length and chloroplast content in these cells, nevertheless none of these phenotypic predictors of haploidy is absolutely reliable. Methods providing direct measurements of genome size provide are a far more reliable diagnosis of haploid status. These include direct measurement of the chromosome number, using conventional chromosome counting techniques (cytology) and measurement of the DNA content using micro densitometry, or flow cytometry (Dunwell, 2010; Szarejko, 2012).

It should be noted that the frequency of mutated DH lines, be it in natural conditions or experimentally induced is generally very low or low: ~ 0.00001 in natural occurrences and 0.8 to15.8 percent in haploid experimental production. Increasing the number of haploids is the key priority when searching for a viable and ultimate haploid. Several methodologies have been assayed for increasing this rates; Szarejko *et al.*, (1995) reported rates at up to 25 percent resulting from the use of sodium azide and NMU (N-methyl-N-nitrosourea). Nasution *et al.*, (2013) with a rigorous and high throughput approach using flow cytometry screening obtained up to 1/1000 seedlings among natural off-types oil palm individuals.

Extensive review books and articles are available among which provide instrumental protocols and tips for developing and identifying haploid and doubled haploid plants for breeding purposes (Maluszynski *et al.,* 2003; Forster *et al.*, 2007; and Szarejko, 2012).

8.14.2. **Genotyping**

For scientists, as well as for breeders it is imperative to validate, at the earliest stage possible the genetics of the lines in the pipeline for selection and/or for genetic research. Thus, the plant populations on which to perform such validation where formally defined including: F_2, recombinant inbred lines, near-isogenic lines, etc. Haploid and doubled-haploid populations have been recognized as the best plant material for such purpose as they have simpler genome (n) or stable homozygous genome (2n). In mutation breeding this becomes even more important as any recessive mutation in the haploid stage readily appears in the doubled-haploid stage. So, numerous tentative for applying genotyping techniques have been made. However, they were not always successful nor were they worthwhile to apply in terms of costs and labor.

The first approaches for validating haploid plants were: cytology, flow cytometry, and from the onset of the tools in molecular biology and genetic markers some acceleration was observed. Genetic maps for various crops where then developed using different types of markers, including to haploid and doubled-ãhaploid plants (Kuchel *et al.*, 2007). DHs are invaluable in genetic mapping and trait mapping as they ae “immortal” and can be used repeatedly, especially for new marker and new

trait screening. Numerous DH mapping populations exist in barley, rapeseed, and wheat but are rare in triticale oat, rye, and others (Tuvesson *et al.*, 2007; Seymour, Taylor and Tucker, 2012). Genome sequencing and high throughput genotyping methodologies contributed to an additional push and allowed the development of markers for specific genes and/or QTL.

Close *et al.*, (2009) in an in-depth critical review of the development of genetic linkage maps in barley, provided a high-density consensus map based only on complete and error-free datasets supported by a readily available SNP genotyping resource. Till *et al.* (2017) likewise developed a low cost protocol for validation of doubled -haploid plants by enzymatic mismatch cleavage in barley.

Genomic selection (GS) is an emerging as a new tool in plant breeding. Thus, GS uses comprehensive marker information to calculate breeding values for complex crops traits (Cros *et al.*, 2015; Heffner *et al.*, 2010). Genotypic selection is particularly relevant for DH mutation programmes as DNA can be extracted from cultured materials (callus, embryos, and plantlets) and early selection made for advancing material of interest. Also, newly regenerated plants grown on in greenhouse conditions often suffer from physiological disorders and therefore cannot be screened reliably for phenotypic traits, but DNA analysis and selection for mutation can be performed.

C) DNA MARKER AND GENOTYPING APPLICATIONS FOR MUTATION BREEDING

8.15. INTRODUCTION

The value of genetic markers as indirect selection indicators in plant breeding has been known for over 90 years. However, it was not until the mid-1980s that abundant molecular markers became available for reliable selection of agronomically important traits in breeding programmes. Since then, indirect selection using DNA markers has significantly increased the efficiency and speed of plant breeding. The turn of the 21st century witnessed another major leap forward with the advent of automated technologies, next-generation DNA sequencing and enabling statistical and bioinformatics tools. With regards to plant mutation breeding, the imminent impact of these new concepts and approaches will be in: 1) marker-assisted backcrossing for introgression or pyramiding of mutant alleles; 2) increased speed and precision to detect mutations in genes underlying important traits enabling genotypic selection; and, 3) improved design of mutation breeding programmes. For example, reverse genetics techniques are providing new insights into the landscape of induced mutations in plants, enabling a more rational choice and dosage of the mutagenic agent. Identification of causative mutations at agronomically important loci can now be performed with unprecedented speed and precision. This knowledge can then be translated into functional markers, which show complete linkage with trait locus alleles. In one application, genotyping assays can be used for marker-assisted selection of mutant traits, in a similar way as done in cross breeding. In addition, direct molecular screening for induced mutations in known target genes can enable selection of candidate mutants at the initial stages of a mutation breeding programme. This can significantly enhance the efficiency of mutant selection and expand the scope of mutation breeding to crops or trees that have a long juvenile stage and which have hitherto lagged, behind the annual crops. This chapter introduces selected concepts and applications in the use of molecular tools and techniques for experimental and applied plant mutagenesis. Two protocols are provided as examples: 1) a procedure for identifying small sequence variations in large mutant populations using high-throughput sequencing of PCR amplicons that can be applied to diploid crops; 2) genotyping assays for diagnostics and marker-assisted selection of shell thickness in fruits of oil palm (*Elaeis guineensis* Jacq.).

8.16. ADVANTAGES AND USE OF MOLECULAR TECHNIQUES FOR PLANT MUTATION BREEDING

As set out at the beginning of this manual, mutations are heritable changes in the DNA. DNA-based molecular markers are polymorphic DNA fragments/sequences

that have been used widely for genotyping and diversity analysis (Staub, Serquen and Gupta, 1996).

DNA-based markers offer several advantages compared to morphological markers:

1) Increased reliability: phenotypic assays are affected by environmental conditions, heritability of the trait and other factors; thus, genotyping scores based on DNA markers tend to be more reliable compared to measurements of phenotypes.

2) Increased efficiency: DNA markers can be scored at seedling stages thus considerable time and space can be saved, especially for traits expressed at later stages of development (e.g. flower, fruit or seed characteristics).

3) Reduced cost: for example, PCR-based assays can be cost-effective compared to phenotyping, especially in a high-throughput setting.

4) Uniqueness: DNA markers are unique for each gene/allele and have the power to enable identification of more than one mutation for the same trait. Thus, markers or genotyping assays can be used to screen simultaneously for multiple genes or alleles which are useful for example in gene pyramiding.

A review of the types and characteristics of DNA markers and their applications in mutation is provided by Wu *et al.* (2012).

The rapid development of DNA-marker techniques and genomics over the past 2 – 3 decades is changing the way traditional and mutation-based plant breeding is being practiced. For example, acquisition of nucleotide variations via sequencing allows the development of simple PCR-based markers for genotyping assays such as allele-specific amplification, high-resolution melt analysis (HRM), cleaved amplified polymorphic sequences (CAPS), and other assays.

Molecular methods for crop improvement are becoming increasingly automated and reliable, and costs have come down. Significantly for mutation breeding, high throughput methods are available which enable thousands of samples to be analysed in a matter of weeks. While many methods have been described, the trend is that direct DNA sequencing is becoming the standard platform from which new approaches are being developed. The use of sequencing therefore provides new and precise tools, and concomitantly new strategies, for both mutation detection and marker-assisted selection.

Figure 8.16 illustrates the different stages of a plant mutation breeding programme from the initial step of mutation induction to mutant selection and the subsequent

utilization of mutant alleles for introgression or pyramiding into elite germplasm. DNA marker techniques and high-throughput sequencing methods can be applied at different stages to facilitate some of the investigations or accelerate specific steps in the mutation breeding programme. Note that in case of functional markers, the molecular marker shows complete linkage with the mutant allele (Andersen and Lübberstedt, 2003).

With respect to plant mutation breeding, two important applications of DNA markers and high-throughput DNA sequencing are: 1) detection of mutations in target genes of known sequence that control important traits to enable genotypic screening at the early stages of a mutation breeding programme; and, 2) the use of markers for introgressing or pyramiding important mutant traits through marker-assisted backcrossing. For marker development, appropriate experimental populations should be developed from mutants and wild-type parents to enable identification of the causative mutation(s) and to confirm linkage with the trait (phenotype).

Additional general applications of DNA markers for mutation breeding include, for example, the identification of off-types or contaminants that may have been introduced during the mutation breeding programme (see Chapter 5) and the use of markers to reduce the non-desirable mutations and maintain the elite genetic background.

These applications are described briefly in the following sections followed by two examples of protocols. In addition, genotyping using DNA markers can be considered the most reliable method for the identification of mutant lines and varieties. Therefore, molecular tools are also useful for identification of the genotype/cultivar, and when appropriate, for seed purity analysis prior to mutagenesis.

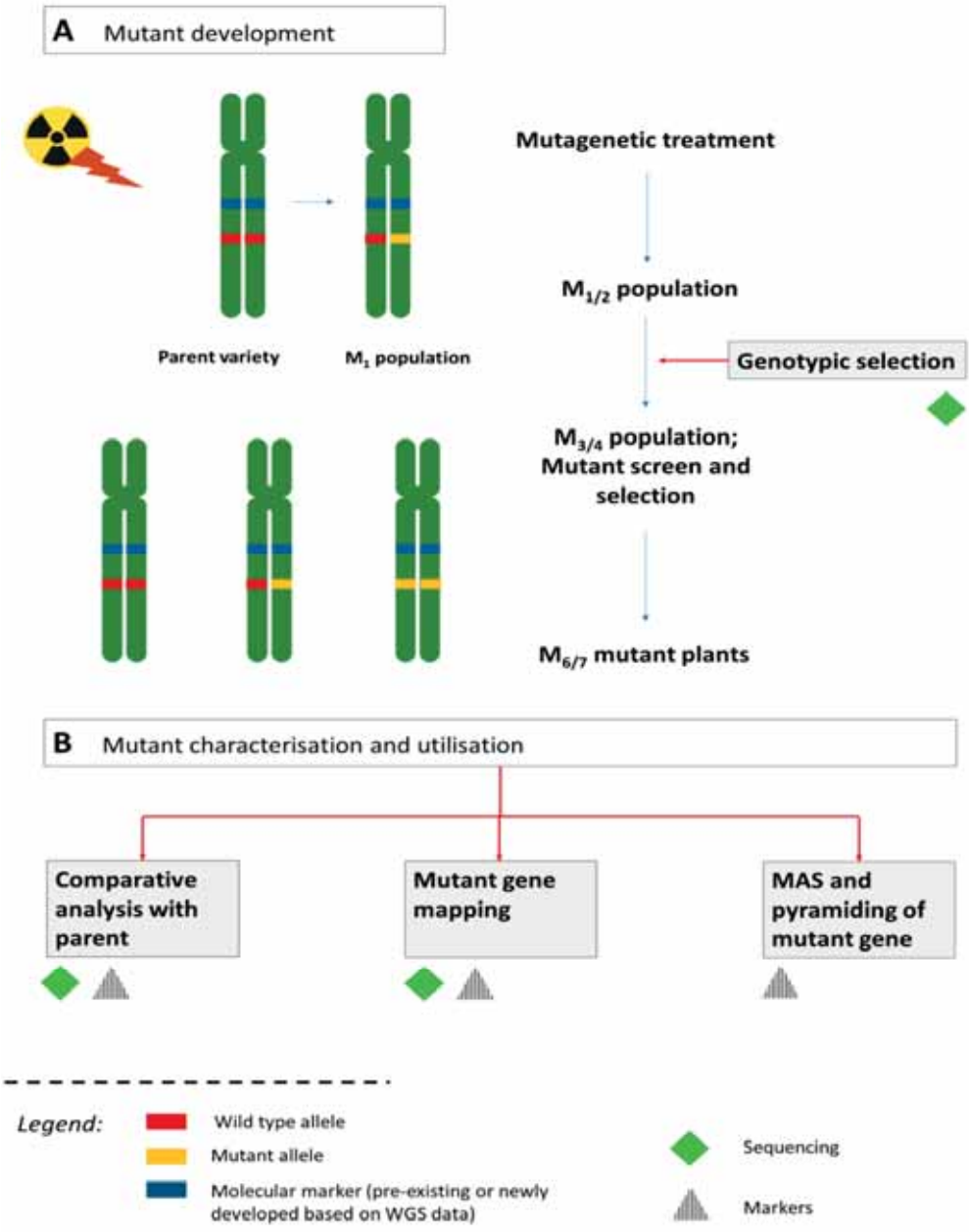


Figure 8.16. Molecular marker and high-throughput sequencing applications in plant mutation research or breeding. Adapted from Wu, Shu and Li (2012).

8.17. MARKER-ASSISTED BACKCROSSING

Marker-Assisted Selection (MAS) is a process whereby a marker is used for indirect selection of a genetic determinant(s) of a trait of interest. The method involves selection of plants carrying genomic regions that are involved in the expression of the targeted trait(s) through the application of molecular markers. The availability of molecular markers along with dense genetic maps enabled MAS for major genes and quantitative trait loci. Thus, with the application of MAS, individual plants can be selected based on their genotype during the selection process. MAS is widely used to increase the effectiveness of backcross breeding to accelerate the introgression or pyramiding of traits of agronomic interest from a donor parent to an elite, recurrent parent (Das, Patra and Baek, 2017). Marker applications are particularly significant for the selection of mutant traits as these are predominantly recessive and can only be recognised phenotypically in the homozygous state. The process of selecting for new traits from a donor parent using markers that are tightly linked to the gene of interest is referred to as 'foreground selection' while 'background selection' refers to using markers to select against other DNA from the donor parent, i.e. to maintain the elite genetic background of the recurrent parent.

An important mutant trait in oil palm is shell thickness in fruit. The wild-type fruit has a thick protective shell around the kernel (Dura fruit type, genotype *Sh*/*Sh*, Figure 8.17a); mutant fruit is shell-less (Pisifera, genotype *sh*/*sh*, Figure 8.17b). Commercial oil palm is the heterozygote between these two and has thin-shelled fruit (Tenera, *Sh*/*sh*, Figure 8.17c) and is produced from Dura × Pisifera crosses. Tenera fruits yield 30% more oil than Dura fruits. The mutant Pisifera suffers from female sterility and is used as a male (pollen) parent in commercial Tenera seed production. The shell thickness gene (*Sh*) is the most economically important gene in oil palm production and has been the subject of intense genetic studies (Singh *et al.*, 2013a). The *Sh* gene has been sequenced and DNA diagnostic markers have been developed for wild-type and mutant alleles (Singh *et al.*, 2015a).

Figure 8.17. Shell thickness phenotypes of oil palm fruits: a) Dura wild-type fruit with a thick shell protecting the kernel; b) Pisifera mutant-type fruit with no shell around the kernel; and c) Tenera thin-shelled commercial fruit. Courtesy of B.P. Forster.

Genetic markers are important in screening for fruit type in oil palm as phenotyping can only be done on mature palms and this takes 4 – 5 years from sowing seed. Genotyping saves time and space, and can be done on seedlings, and/or selections, made in the nursery, i.e. prior to field planting. Screening for fruit type is important for various reasons: 1) to check the purity of Teneras in commercial plantations; 2) to check for purity of Teneras in commercial seed production (Kelanaputra *et al*., 2018 in press); 3) to select for fruit types in progenies from breeding programmes and select specific types for field trialling (Setiawati *et al*., 2018 in press); and 4) to screen for new induced mutations in the shell thickness gene of oil palm (Nur *et al*., 2018 in press).

8.18. GENOTYPIC SELECTION

Phenotypic selection of mutants is the cornerstone of a mutation breeding programme, typically requiring extensive resources and time to select and progress mutant populations up to the stage of advanced mutant lines or varietal release. Direct (phenotypic) selection remains the main process by which desirable mutants are selected and advanced. Indeed, it is a sensible way to proceed as ultimately it is the traits that are important in developing superior varieties.

However, molecular screening for induced mutations in known target genes allows genotypic selection of candidate mutants in a mutation breeding programme. Indeed, Next Generation Sequencing (NGS) platforms have facilitated high-throughput discovery of induced mutations in plant genomes (Tsai *et al.*, 2011; Du *et al.*, 2014; Yuan *et al.*, 2014; Li *et al.*, 2016; Gupta *et al.*, 2017; Datta *et al.*, 2018). Importantly, genotypic selection requires knowledge of the DNA sequence of the allele(s) that are causative for the desired trait. The efficiency of traditional gene mapping and cloning

for marker development can be greatly improved using whole genome sequencing to evaluate segregating populations (Abe *et al.*, 2012; Schneeberger *et al.*, 2009). Alternatively, reverse genetic methods can be employed to create and identify mutations in specific genes to assay gene function. Mutation detection in targeted genes of large populations using complex DNA pools has been adopted for this purpose. This method essentially combines chemical mutagenesis with high throughput sequencing of DNA fragments amplified through PCR, or enriched via probe capture assays (Tsai *et al.*, 2011; Henry *et al.*, 2014; Krasileva *et al.*, 2017). In this way, large mutant populations comprising thousands of individual plants can be effectively screened for natural or induced sequence variations in a short time (see Section 8.20.1).

Chemical mutagens have been most commonly used in reverse genetic studies because they induce primarily single point mutations, and current technologies can be easily adapted for their discovery (Jankowicz-Cieslak and Till, 2015b). Treatment of plants with physical mutagens can result in more diverse spectra of lesions including SNPs, small and large insertions and deletions and genomic rearrangements (Yuan *et al.*, 2014; Henry *et al.*, 2015; Li *et al.*, 2016a, 2016b; Datta *et al.*, 2018). As the cost of sequencing drops, whole genome sequencing approaches can be applied to recover all types of induced lesions. It is likely that varying dosages will affect the types of mutations that accumulate and with stable inheritance. Therefore, dosage optimizations may be carried out to enrich the allele types desired for a specific reverse genetics project.

As stated earlier, the prerequisite for genotypic selection is a full understanding of the molecular and genetic architecture of the trait of interest. Candidate genes are currently available for many traits of interest such as, for example, traits involved in crop domestication. Still, more efforts are needed to link allelic diversity to phenotypes in germplasm used in crop improvement programmes.

Importantly, new genotyping methods can be applied to select mutants at the early stages of the mutation breeding programme, thus saving years of this process. As described in Chapter 5, the M_1 population consists of chimeric tissues. In case of autogamous seed crops, this chimerism is largely resolved at the M_2 stage after selfing of the M_1 plants. Therefore, genotypic selection is preferable, and may be performed at the M_2 stage where all mutations identified should be meiotically heritable.

It is also possible to select at the M_1 stage. This approach of cataloguing potentially interesting mutant alleles in the M_1 is particularly relevant for perennial crops or trees that have a long juvenile stage (in some cases 5 – 10 years). In addition, for large perennial crops such as trees, space can be saved in growing out only the selected (10 – 30) individual M_1 plants and not the complete M_1 population (normally about a

thousand individuals) as selection can be made from leaf samples taken from nursery plants before transplanting to the field (Nur *et al*., 2018 in press). However, the risk is that some mutations selected may not be heritable and thus may not be passed on to the next generation, but at least some will be. Also, possibly some interesting mutations may be missed when performing the selection at the M_1 step.

Three major factors have contributed to genotyping as the choice method for mutation detection: 1) the availability of sequence data of plants and crops species; 2) reverse genetic studies that relate genes to functions; and, 3) a drastic reduction in cost of genotyping assays. Together these methods provide sequences of target genes and information on sequence variations produced through mutations (phenotypes of the various mutant alleles). Selected individuals with sequence variation in the target genes will be sorted out for the evaluation of expression of the traits. It is, however, expected that not all the sequence variants will be expressed in the targeted trait.

Before deciding to set up a genotypic screen, practical concerns and comparative cost-benefit analysis with direct (phenotypic) selection need to be evaluated. If a cost-effective and efficient phenotypic screen is available that can test thousands of plants in a mutant population, it is advisable to carry out phenotypic selection. This is especially the case if there is no knowledge of the genes involved in the trait of interest. In addition, genotypically selected mutants need to be tested in the field eventually to confirm the capture of the desired phenotype and to assess field performance. As knowledge about the genes underlying traits of interest grows, more gains in streamlining and efficiency will be achieved through application of genotypic selection.

8.19. ADDITIONAL APPLICATIONS OF DNA MARKERS FOR MUTATION BREEDING

8.19.1. **Excluding off-types using DNA markers**

During the process of mutant population development, there is ample opportunity to introduce off-types or contaminants from external sources such as inadvertent outcrossing (see Chapter 5). Such contaminants may also result from contamination of the seed stock that was used for mutation induction. Often a comparative analysis between a mutant and its parent needs to be conducted. Such comparative analysis may serve different purposes. For example, to identify causative mutations underlying important traits, it may be necessary to conduct comparative genomics between the mutant and its parent variety (Abe *et al.*, 2012; Schneeberger *et al.*, 2009). The scientific rigour of such comparative studies relies entirely on the true origin of the mutant and the parent. In this regard, standard DNA markers such as SSRs can be used to ensure the mutant being studied is truly a direct mutant of the parental variety. Indeed, Fu, Li and Shu, (2008) demonstrated the occurrence of outcross-derived

contaminants which were frequently selected as 'mutants' in several rice populations. They further showed that such false mutants can be easily identified using SSR markers. For example, in case of SSRs if a mutant shows a high degree of polymorphism (e.g. >5%) compared to its parent, it may not be a true mutant and is more likely a contaminant (Fu *et al.*, 2007).

8.19.2. **Background selection for clean-up of mutational load**

Another area where molecular markers can have a major impact in plant mutation breeding is in background selection, i.e. the selection and preservation of elite genetic backgrounds of the parents with a minimal mutational load. In addition to marker-assisted selection for a desired mutant gene (foreground), the genetic background can be monitored and selected efficiently, thus reducing the rounds of selfing, backcrossing or top-crossing. Molecular markers can also be used in genome scans to select those individuals that contain both the introgressed allele and the greatest proportion of the recurrent parent genome, thus combining foreground and background selection in a single scan.

8.20. EXAMPLE PROTOCOLS

8.20.1. **Identification of SNPs and indels in large mutant populations using high-throughput amplicon sequencing applicable to diploid plant species**

The below protocol was adapted at the Joint FAO/IAEA Plant Breeding and Genetics Laboratory, Seibersdorf, Austria using the Illumina MiSeq sequencing platform. The protocol has been used in the context of TILLING experiments (Gupta *et al.*, 2017) to analyse M_2 populations from mutated tomato and barley, and also to evaluate natural nucleotide variation in cassava (Duitama *et al.*, 2017). Mutant populations were developed using chemical mutagenesis to enrich for single nucleotide mutations. Screening assays are typically carried out on several hundred mutant lines simultaneously. Guidelines and protocols for chemical mutagenesis are outlined in Chapter 2 of this manual.

Note: Before proceeding with Next Generation Sequencing (NGS) approaches for mutation identification in specific target genes, please review the newest kits available on the market and carefully read the technical notes from the respective kits used at different stages of this protocol. Additional protocols for the recovery of chemically induced mutations by NGS can be found in (Burkart-Waco *et al.*, 2017).

This protocol consists of four major steps outlined in Figure 8.18. The first step, DNA extraction and polymerase chain reaction (PCR) amplification is the longest and critical for the subsequent steps and for effective mutation detection. Step 2: Library

Preparation and Sequencing, is being conducted using commercial kits. This step can take between 1 to 2 days plus the run time of the DNA sequencer. In Step 3: Variant Calling, various platforms can be used for mutation detection in pooled samples (Gupta *et al.*, 2017). In the final Step 4, candidate mutations are validated using Sanger sequencing (Sanger, 1981) and phenotyping methods.

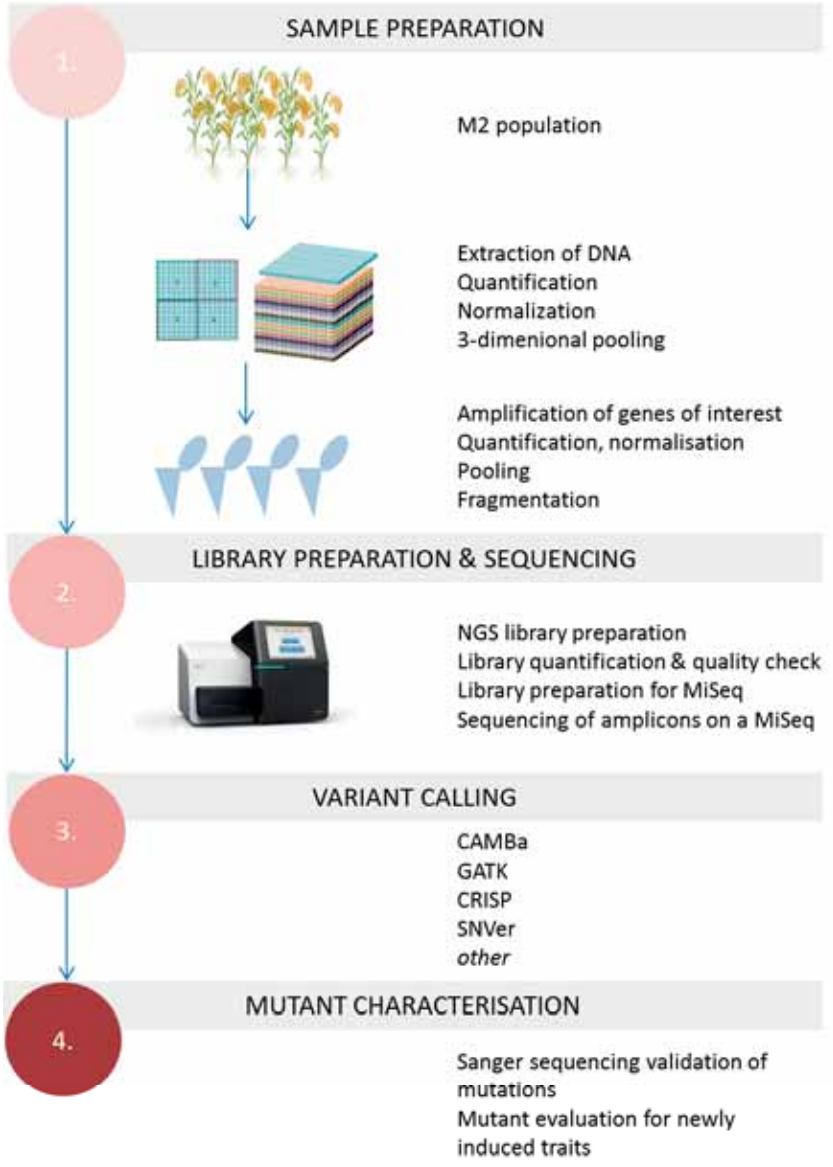


Figure 8.18. Flowchart outlining the different steps for high-throughput detection of SNPs and small Indels in large mutant populations of diploid plants.

Step 1 – DNA extraction and PCR amplification

Step 1.1 – DNA extraction, quantification, normalization and 3-dimensional pooling

Proceed with collection of tissue, e.g. leaf tissue from individual M_2 plants. Care should be taken to avoid cross-contamination and mislabelling. Several DNA extraction methods are available. Test these with your selected crop. Genomic DNA yield and stability at different storage temperatures should be established. We recommend testing with approximately 10 primer pairs in PCR reactions to ensure that the chosen DNA extraction method does not produce any contaminants that may inhibit PCR. Usually the M_2 population consists of a minimum of few hundreds up to thousands of plants. Therefore, a kit such as Qiagen 96 where 96 samples can be processed in parallel is recommended.

The DNA quality and quantity is very important to obtain high quality sequence data. Accurate determination of the concentration of template DNA is essential to ensure that all samples in a pool of genomic DNA are represented equally so that mutations from every pool can be identified in the assay. To verify DNA quality and quantity we suggest using agarose gel electrophoresis as it allows evaluation of DNA degradation as well as determination of concentration. Free software can be used to determine DNA concentration from gels, and tools have been developed to facilitate the normalization of all DNA samples to a single concentration prior to pooling (Huynh *et al.*, 2017) . If choosing another method of DNA concentration determination, such as spectroscopic approaches, we suggest testing several methods side-by-side to ensure that the chosen method is both accurate and precise for the measurement of intact genomic DNA. It is advisable to normalize DNAs to a concentration higher than that used in PCR. This allows flexibility in experimental design. Array DNA of the M_2 population into 8 × 8 grid format plates (Till *et al.*, 2006). For DNA pooling and multiplexing follow the protocol described by Tsai *et al.* (2011). This pooling strategy utilizes 12 plates of 8 × 8 grid format. This equates to 768 individuals being pooled for one MiSeq run with 44 sequencing libraries. There are a range of different pooling strategies that can be applied. For example, higher-fold pooling has been described that can increase throughput and reduce costs (Duitama *et al.*, 2017; Gupta *et al.*, 2017; Pan *et al.*, 2015) . Symmetrically pooled samples may also be advantageous. However, we suggest starting with the 12-plate approach to establish optimal parameters for your plant material prior to attempting higher-fold pooling experiments.

Step 1.2 – PCR amplification of genes of interest, quantification, normalisation, pooling and fragmentation

Design primers to amplify specific fragments. Note that direct sequencing of smaller PCR fragments (500 – 600bp) is possible when using the MiSeq (Gupta *et al.*, 2017;

Pan *et al.*, 2015). However, throughput is increased by designing primers to amplify 1500 bp or more of the targeted genes followed by fragmentation of the PCR products (Slota, Maluszynski and Szarejko, 2017). Carry out PCR using pooled DNA (in this case, 44 pools) for every target separately. Quantify PCR amplicons using for example agarose gel electrophoresis. Throughput can be increased using multi-channel pipettors and 96-well format gels. Normalise all PCR products to the same concentration. It is essential that the concentration of all amplified genes in all pools is the same to ensure accurate mutation calling. Carefully pool amplicons of all gene targets resulting in 44 pools: all amplicons produced from the same genomic DNA pool are pooled.

Fragment every individual pool through sonication using e.g. the Covaris ultrasonicator M220 (Covaris, Inc. USA). The following settings are recommended: run time 30 sec; peak power 50; duty factor 40, cycles/burst 200. The fragmented PCR products can be visualised via standard gel electrophoresis, or by using automated equipment such as the Advanced Analytical Fragment Analyzer™ (ThermoFisher Scientific). The pools containing the fragmented PCR amplicons are then used for the library preparation.

Step 2 – Library preparation and sequencing

Step 2.1 – NGS library preparation, quantification and preparation for MiSeq run

Proceed with library preparation. In this protocol indexed DNA library for NGS is prepared using the TruSeq Nano DNA HT Library Preparation Kit (Illumina, Inc.) with 200 ng of input PCR product.

Check the quality and quantity of prepared library. Library sizing can be performed using gel electrophoresis or automated equipment such as the Fragment Analyzer with High Sensitivity NGS Fragment Analysis Kit (1 – 6000bp) as illustrated in Figure 8.19.

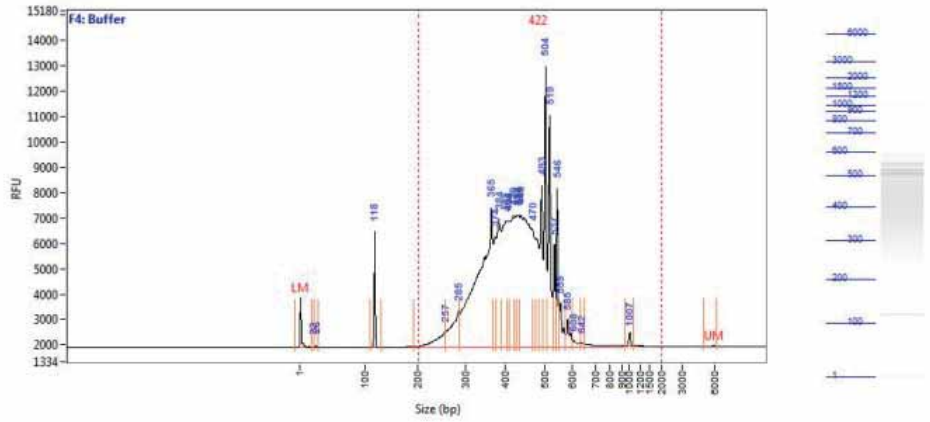


Figure 8.19. Qualitative and quantitative visualisation of NGS libraries with the use of Fragment Analyser and the High Sensitivity NGS Fragment Analysis Kit.

The concentration of each prepared library should be determined and samples normalized to a common concentration prior to pooling. While DNA concentration can be determined using qPCR or with the Fragment Analyzer, we typically use Qubit® dsDNA HS Assay Kits (ThermoFisher Scientific). The concentration of each library is converted from ng/µl into nM, based on the size of the DNA amplicons.

Dilute each library to a concentration higher than that recommended by the kit protocol. For example, with the current sequencing chemistry a final concentration of 4 nM is required. In this case libraries are diluted to 6nM. This allows for variations in the pipetting process without producing a library below the set concentration. Next, pool the indexed and normalised libraries (same volume and same concentration) in a single Eppendorf tube. This will result in all 44 libraries being pooled together. Re-quantify using the Qubit® (ThermoFisher Scientific) and adjust the pooled library to the required concentration. Follow the library denaturation and dilution guidelines for the kit and sequencing chemistry used.

Step 2.2 – Sequencing of amplicons on a MiSeq

Proceed with sequencing of denatured and diluted libraries. Follow guidelines for preparing the sequencing sample sheet. If using the Illumina MiSeq, a 2 × 300 PE chemistry can be employed. Note that shorter read lengths are needed for higher-throughput Illumina platforms. When running the MiSeq, reads can be de-multiplexed automatically with FASTQ files produced for downstream analysis. Note that when outsourcing sequencing some facilities will not provide FASTQ files but rather unaligned BAM files. FASTQ files can be generated (e.g., using bamtofastq), taking

care of appropriate parameters such as paired end reads. Note also that some tools such as GATK (The Broad Institute) require read-group information.

Step 3 – Variant calling

TILLING by Sequencing data produced by the Illumina machines can be processed further with the use of numerous bioinformatic platforms. Prior to the analysis, evaluate the run statistics on the MiSeq. Important parameters to evaluate the quality of your run are cluster density and Phred base quality scores. Optimal values for both parameters may vary depending on machine and sequencing chemistry used.

Note that at the time of writing, the MiSeq software can produce variant call files (VCF) using GATK. However, it does not take into account the complex pooling scheme incorporated into the project run. You will have to make new files with software where the pooling strategy can be addressed.

Transfer fastq.gz files from the MiSeq sequencer (for a full description of file types, see manufacturer's website and https://www.ncbi.nlm.nih.gov/sra/docs/submitformats/). Prepare a reference file (.fa). You have the option of using a whole genome or simply amplicons. The easiest method is to make one file with a list of all amplicons as this works with the multiple pipelines and is required for CAMBa (http://web.cs.ucdavis.edu/~filkov/CAMBa/). Process fastq.gz and reference (.fa) files following the below presented workflow (Figure 8.20). Note that most software is run on the command line and detailed instructions are available for each tool. If you have not yet established a computational platform for data analysis, we suggest the Linux operating system owing to its ease of use and the fact that most tools are compiled for this operating system.

Useful links: Many tools are available from the GitHub development platform (e.g., https://github.com/lh3/bwa, https://github.com/broadinstitute/picard).

CAMBa and related tools can be found at http://comailab.genomecenter.ucdavis.edu/index.php/TILLING_by_Sequencing and under "The TILLING Pipeline" click Pipeline download.

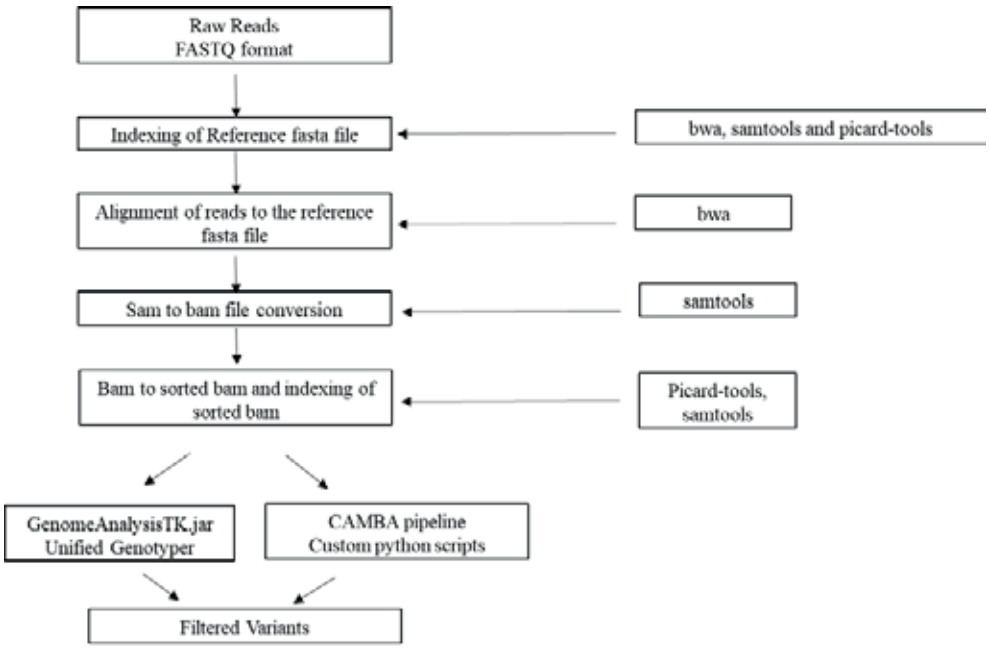


Figure 8.20. Bioinformatic analysis utilising CAMBa and GATK pipelines. Figure adapted from Gupta et al., 2017.

Step 4 – Molecular and phenotypic validation

Step 4.1 – Validation of mutations using Sanger sequencing

Candidate sequence variations are amplified using standard PCR techniques and sequenced using standard Sanger DNA sequencing to verify the mutations (Sanger, 1981) .

Step 4.2 – Phenotypic evaluation of mutant plants

Individual plants selected with sequence variations in the target gene will need to be phenotyped for the targeted trait as compared with the wild parent. Previous studies suggest that approximately 10% of recovered chemically induced SNP mutations identified in a gene-specific PCR amplicon may affect gene-function. Thus, the number of putative mutants that show altered phenotypes in the laboratory and screen-house and finally confirmed in the field is expected to be small.

8.20.2. **Genotyping for shell thickness selection in oil palm**

Single nucleotide polymorphisms in the shell thickness (*Sh)* gene are responsible for the difference between Dura (thick shell) and Pisifera (shell-less) fruit types (Figures 8.17; 8.21) and these can be detected using High Resolution DNA Melt (HRM) curves. Cleaved amplified polymorphism (CAP) analysis can be used to verify the genotypes. The protocol below is that used by the Plant Genomics Laboratory at Verdant Bioscience, Indonesia.

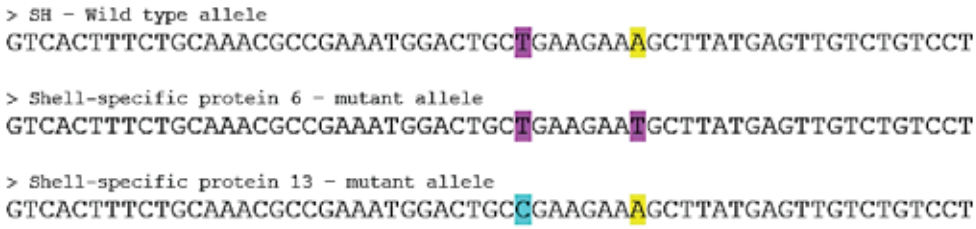


Figure 8.21. Single nucleotide polymorphism between Dura and Pisifera fruit types in the Sh gene.

Step 1 – Look for SNP variation in the shell thickness gene sequence in the oil palm genome

The shell thickness gene sequence of oil palm can be found in the oil palm genome GenBank (https://www.ncbi.nlm.nih.gov/) by typing in the keywords "elaeis guineensis shell thickness" in the search box. This will show the complete oil palm shell-specific gene information that has been deposited in GenBank. By performing a multiple sequence alignment for all coding sequences of shell thickness the position of single nucleotide polymorphisms (SNPs) can be found. SNPs which are responsible for shell thickness phenotypes are identified and DNA primers are designed targeting the SNPs (Figure 8.21).

Step 2 – Design primers for shell thickness alleles

A wide selection of web-based primer design tools can be applied to generate allele-specific PCR primers. For example, Primer 3 [bioinfo.ut.ee/primer3-0.4.0/] is one such tool. Primer dimers and hairpin loops of designed primers can be checked using web-based tools such as OligoAnalyzer web tool from IDT SciTools (Owczarzy *et al.*, 2008) and OligoCalc (http://biotools.nubic.northwestern.edu).

Step 3 – Oil palm leaf sampling

Suitable material for the extraction of genomic DNA is young leaf tissue with a sample size approximately 3cm^2. Samples are typically taken from seedlings in the nursery and placed in zip-sealed plastic bags and deep frozen in a -80°C freezer if not used immediately.

Step 4 – DNA Extraction

The leaf samples are punched using a leaf puncher tool and the leaf discs (2 – 3 to 5mm diameter discs) are placed in tubes. Samples are homogenized in a tissue disruptor. DNA extraction is carried out with a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. The quantity and quality of DNA can be checked by spectrophotometry (e.g. NanoDrop). Purified genomic DNA is used as a DNA template for genotyping.

Step 5 – Shell thickness detection using the HRM technique, validated by CAP analysis

High Resolution Melt PCR can be used for shell thickness genotyping (Dura, Pisifera and Tenera). Multiplex PCR can be performed which uses 3 pairs of allele-specific primers. From HRM melt curves, three genotypes of shell thickness can be distinguished (Figure 8.22). HRM requires real time PCR machines, such as the Rotor-Gene Q and can be used for large sample sizes in high-throughput screening.

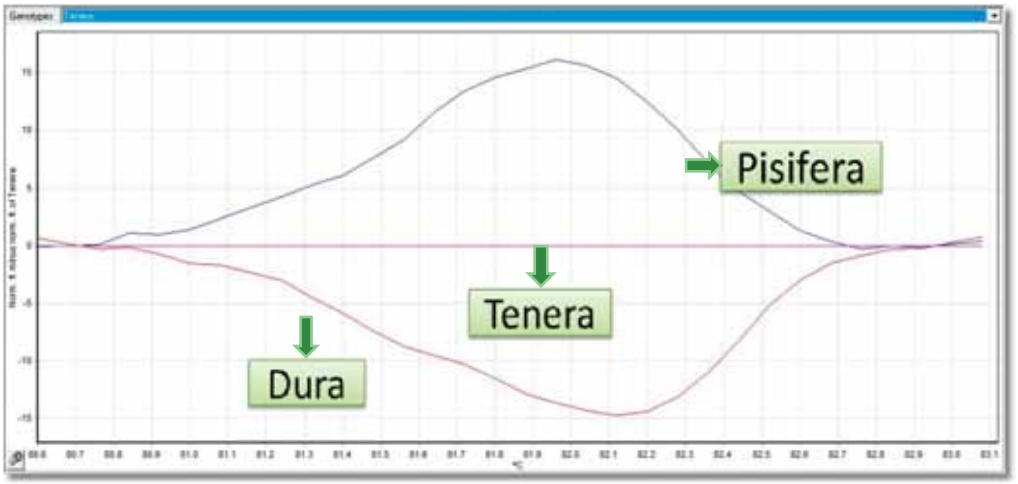


Figure 8.22. HRM curves of Dura, Pisifera and Tenera are easily distinguished (here using Tenera as a base line).

If a SNP occurs within the recognition site of a restriction enzyme, it is much easier to use cleaved amplified polymorphic sequences (CAPS). Shell thickness gene sequences have a specific restriction site for *Hind*III, this restriction site allows CAPS to distinguish shell alleles. Figure 8.23 shows a clear-cut differentiation of Dura, Pisifera and Tenera genotypes using CAPS markers. CAPS analysis involves a simple PCR protocol using conventional PCR equipment and banding patterns are visualized in simple gel-based systems. However, CAPS analysis is slow compared to HRM and not suited to high-throughput.

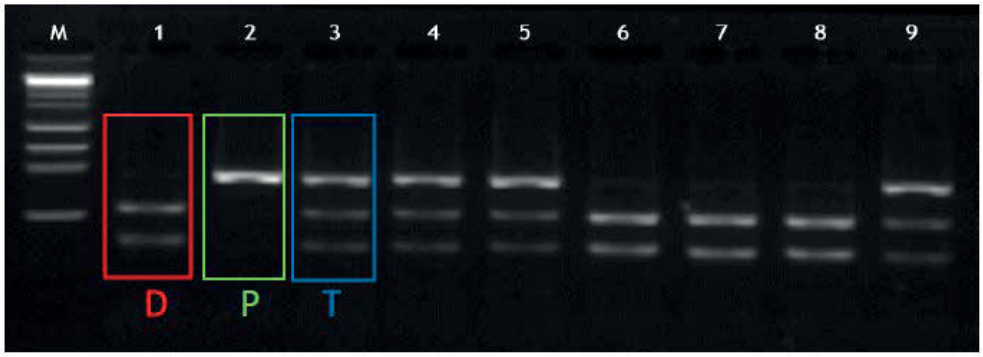


Figure 8.23. Banding patterns of CAPs in verifying Dura, Pisifera and Tenera fruit types.

In addition to the in-house protocols, such as that described above, *Sh* determinations can be done using outside services such as Orion Biosains (www.orionbiosains.com). Orion Biosains also provides services to screen for another important mutation in oil palm, virescent fruit (*Vir*), which changes the fruit colour during ripening from black-red to green-yellow (Singh *et al.*, 2015b).

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MANUAL ON MUTATION BREEDING
THIRD EDITION

Edited by Madeleine Spencer-Lopes, Brian P. Forster and Ljupcho Jankuloski

Co-published by the International Atomic Energy Agency (IAEA) and the Food and Agriculture Organization (FAO) of the United Nations.

The previous (2nd) edition of the *Manual on Mutation Breeding* was published in 1977. After nearly 40 years it is time to up-date this manual as there have been several major developments in the intervening years. There has been an upsurge in the use of induced mutations – for both classical crop improvement and for functional genomic studies – and at the same time, there is an ever increasing and urgent need to generate novel variation in improving crop production: more nutritious, hardy, input use-efficient and productive crop varieties, to underpin 'green' sustainable food production for the 21st century, especially in safe-guarding food security which is challenged by climate change, hunger and world population growth.

This 3rd edition of the *Manual on Mutation Breeding* that you now peruse, describes advances in plant mutation breeding, including basic irradiation techniques as well as chemical mutagenesis, in both seed-propagated and vegetatively propagated crops. The manual provides comprehensive overviews and guidelines for new high-throughput screening methods – both phenotypic and genotypic – that are currently available to enable the detection of rare and valuable mutant traits and reviews techniques for increasing the efficiency of crop mutation breeding. Most of all the manual provides practical, hands on, methods in plant mutation breeding techniques, with clear illustrated step-by-step protocols.

The Manual on Mutation Breeding is founded on the experience and knowledge of the staff of the Plant Breeding and Genetics Section and Laboratory of the Joint FAO/IAEA Division – past and present – and has been written in collaboration with external scientists who are internationally renowned experts in specific fields. The combined contributions, backed by the richness of the Mutant Variety Database of the Joint FAO/IAEA Division, represents a valuable resource for all those interested in the resurgence of plant mutation breeding.

ISBN 978-92-5-130526-3

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I9285EN/1/05.18