Physical and Chemicals Mutagenesis in Plant Breeding

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Abstract

World agriculture faces the challenge of food security because of the rapid growth of human population, reduced arable land area, plant productivity and climate change. Since the pioneering work on mutation of Thomas Hunt Morgan in 1910 (Am Nat 44:449–496) and the first release of an improved cultivar in tobacco in the 1930s, plant mutation breeding has become an effective breeding method. It has produced direct mutant cultivars and provided materials for further breeding. It has contributed over 3330 cultivars in more than 220 plant species. Major advantages of plant mutation breeding are: (1) mutation induction in elite materials thus little or no additional breeding is necessary, (2) fastest way of developing new improved lines, (3) applicability to all plant species and (4) generation novel traits. Today, plant mutation breeding is a much-needed weapon to combat new challenges in agriculture such as direct and indirect effects of climate change. This chapter outlines and compares methods used for physical and chemical mutagenesis for crop plant improvement. The benefits of mutation induction needed to be assessed and placed in context with respect to other options (available resources, facilities, costs, pragmatism, etc.). In this chapter,

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the future of mutation breeding in the light of new and exciting advances in plant sciences and technologies are discussed.

Keywords

Mutagens · Physical and chemical · Mutation induction · Mutation detection · Mutagenesis · Plant mutation breeding · Crop improvement

3.1 Introduction

A mutation is a heritable change in the genetic material of an organism, which is independent of genetic segregation or genetic recombination. Mutation may be natural or induced. Deliberate mutation induction and selection has had a massive impact on agriculture in providing improved cultivars. Plant mutation breeding follows three strategies: induced mutagenesis, mutation detection and selection. Mutations can be classified into three main categories based on where they occur in: (1) genome, (2) chromosome and (3) gene mutation. Other types of mutations also occur such as non-nuclear mutations and gene copy number mutations, there are also mutation mimics, (reviewed by Lundqvist et al. 2012). Mutation is a natural process and has been a primary driver for speciation and evolution (Darwin 1868). Natural mutations have had a significant impact on the domestication and cultivation of wild species by man's selection of favoured traits in plants and animals, and thereby the evolution of mankind (Bronowski 1973). Mutation has been responsible for the abundant diversity of life on Earth.

Present agriculture is challenged by a massive world population, which is estimated to peak at nine billion people by 2050, and declining areas for farming. This bleak situation is further exacerbated by new environmental challenges: pests, diseases, drought, salinity, water-logging and abnormal temperatures (Rosenzweig et al. 2001; Roy et al. 2011; Islam et al. 2012). Thus, major crop plants have suffered a decrease in production and productivity with yield reduction of over 50% resulting in enormous economic losses worldwide and food shortages (Oerke et al. 1999; Rai et al. 2011).

Classical plant breeding, the improvement of crop plants through hybridization, transgressive segregation and heterosis to produce better genotypes has been reviewed recently by Makay et al. (2020). However, conventional approaches are slow and laborious, for annual crops it can take over 10 years to produce a new cultivar, for perennial crops this is much longer (Maluszynski et al. 1995). In addition, all plant breeding programmes are limited by the genetic variation available. Traditionally, this has led to germplasm collections which may be classed into elite, breeding, landrace and wild species groups. The further a breeder has to go to access a trait the longer and more complicated the breeding. Therefore, high genetic variability within the elite and contemporary breeding materials is preferred, and thus mutation induction in these groups is attractive.

Plant mutation breeding is a form of accelerated plant breeding (Forster et al. 2015). In addition to the benefits listed above, plant mutation breeding is considered a safe and accepted breeding method, which has been practiced since 1927 (Gager and Blakeslee 1927; Muller 1927). It has led to new traits that have provided yield and quality improvements and has revolutionized farming. Examples include: (1) semi-dwarf small grain cereals crops allowed mechanical harvesting (combine harvesters) and provided a yield advantage (Mlcochova et al. 2004; Rutger 2009), (2) rust-resistant wheat (Bado et al. 2015), (3) salt tolerance rice (Do 2009; Vinh et al. 2009), (4) high altitude farming of barley in Peru (Gómez-Pando et al. 2009), (5) seedless citrus fruit (Spiegel-Roy 1990; Vardi et al. 2008; Sutarto et al. 2009), (6) self-compatibility in apple, cherry and almond (Matthews and Lapins 1967; Monastra et al. 1998; Spiegel-Roy 1990; Tehrani and Brown 1992), (7) resistance to wilt disease and high yielding peppermint to sustain chewing gum production (Sigurbjörnsson and Micke 1974), (8) red grapefruit (FAO/IAEA 1991), (9) 742 improved cultivars in vegetatively propagated crops (ornamentals, food and fruit trees, Bado et al. 2017), (10) non-plant crops—high yielding and good quality fungi (Sathesh-Prabu and Lee 2011) and high yielding extracellular polysaccharides from the algae (Liu et al. 2015).

Currently, the stringent regulations imposed on genetically modified crops (that use recombinant DNA technology) do not apply to cultivars produced by mutation breeding. Mutation induction is particularly relevant to crops that do not have a sexual reproduction system, i.e. cannot be improved by normal breeding. Induced mutation has been an effective approach to broaden the genetic diversity to improve vegetatively propagated crops (VPCs) and to develop successful cultivars in food crops (banana, potato, sweet potato, cassava and wasabi), fruit trees (cherry, citrus, apple, almond, peach, apricot), ornamentals (chrysanthemum, rose, dahlia and many other flowers) and other crops (Bermuda grass, field mint, peppermint and tea) (van Harten and Broertjes 1989; Jankowicz-Cieslak et al. 2012; Bado et al. 2017; Chepkoech et al. 2019). An advantage of mutation breeding is that the starting (target) genotype is usually a top-performing or popular line or cultivar; any improvement is readily accepted, and the improved line may be developed into a new cultivar directly.

This chapter provides an overview of the current knowledge of physical and chemical mutagens and induced mutants, emphasizing their effectiveness in improved traits for agriculture, food production and meeting market demands. The principles of mutation breeding and genetics are not new though new methods in mutation induction and detection have been developed. There are several enabling biotechnologies, especially tissue culture that broaden the scope and make mutation breeding more effective and targeted. These are described and discussed with respect to need, available resources and the emergence of new and exciting plant biotechnologies.

3.2 History of Physical and Chemicals Mutagens in Plant Breeding

Plant mutation breeding has a long history in technology development and optimization, and the development and release of mutant cultivars (Table 3.1). Currently, over 3330 cultivars have been released worldwide with over 88% being derived from physical and chemical mutagens (https://mvd.iaea.org/).

3.2.1 Physical and Chemical Mutagens

Since discovering the mutagenic effects of X-rays (Muller 1927; Stadler 1928a, b), a large number of physical and chemical mutagens have been assessed in plant breeding. From the mutant cultivars (officially) released worldwide and registered in the IAEA database (>3330, https://mvd.iaea.org), the vast majority involved physical mutagenesis (>77%) followed by chemical mutagens (>11%) and their combinations (>1%) (Fig. 3.1). Other (>9%) mutant cultivars have been released through further breeding using these induced mutants (9.85%).

3.2.1.1 Physical Mutagens

Physical mutagens have had the greatest impact in plant breeding (Table 3.2). They can be classified into two groups: (1) Ionizing radiations and (2) Non-ionizing radiation. The ionizing radiations are composed of particulate or densely ionizing radiation (alpha ray, beta ray, heavy-ion beam and fast, slow and thermal neutrons), and electromagnetic or sparsely ionizing radiation (X-rays, gamma rays, cosmic rays, electron beams). This classification is mainly based on radiation capacity in producing ions and their ability (enough energy) to affect the structure of atoms of impacted materials, such as plant propagules (Mba et al. 2012).

With respect to mutation breeding, ionizing radiation causes biological injuries through two main interactions (physical and chemical) with DNA. Physical action is the result of the energy deposited which cause direct DNA damage. Chemical reactions are mainly due to highly reactive free radicals that are generated (OH^- and H^+) which cause indirect DNA damage (Rao et al. 1965; Reisz et al. 2014). Light of various wavelengths can also cause photochemical damage, typically purine or pyrimidine dimers, that result in point mutations in the DNA sequence (Kurowska et al. 2012).

In the presence of oxygen, radiation-induced free radicals form peroxy radicals that are highly damaging (Sparrow and Singleton 1953; Sparrow 1961; Esnault et al. 2010; Lagoda 2012). Thus, plant materials with high water content and/or some biochemical composition (e.g. oil) are more sensitivity to radiation. This may result in small to large mutations, from point mutations to deletion, single and double-strand breaks, and chromosomal rearrangement.

Physical mutagens have been applied to a wide range of plant materials from whole plants (in gamma fields) to single cells (in gamma cells). Soft materials such as in vivo (excluding seed), in vitro materials (e.g. embryogenic callus and embryos)

Table 3.1 Historical milestones in physical and chemical mutagenesis in plant breeding

Date	Milestone		References
	Physical mutagenesis	Chemical mutagenesis	
1838–1839	Description of the cell theory and suggestion of totipotency of cells		Scheiden (1838)
1868	Introduction of the 'histogen theory' to explain shoot apex behaviour in plants		Darwin (1868), van Harten (1998)
1895–1900	The discovery of various kinds of radiation		Forster and Shu (2012)
1901–1905	Suggestions and promotion of radiation in plant and animal mutation breeding		de Vries (1901, 1903, 1905)
1907	Proposition of the word 'chimera'		Cramer (1907)
1927	Attempts of induced mutations in seed propagated crops using gamma and X-rays		Gager and Blakeslee (1927), Muller (1927), von Sengbush (1927)
1928	Successful mutation induction and discovery of mutagenic effects of X-rays in barley and maize		Stadler (1928a, b)
1930s	Continued of deliberated mutation in plants The first official mutation breeding programmes: in Sweden, Germany, United States		Stadler (1929), Goodspeed (1929), Kharkwal (2012)
1931	Deliberate mutation induction on potato		Asseyeva (1931)
1932		Attempts to induce mutations using chemicals	Sakharov (1932), Klein (1932)
1934	Release of physically (X-rays) improved cultivar tabak 'Chlorina' cv. (Indonesia)		Rana (1965)
1941–1943		Description of the ability of chemicals to induce mutations, i.e. mustard gas	Auerbach and Robson (1942), Oehlkers (1943)
1942	Report of induced disease resistance in a crop plant; X-ray-induced powdery mildew resistance in barley		Freislebe and Lein (1942)
1944		First report on the deliberate use of chemical mutagens	Auerbach and Robson (1944)
1944–1946		Continuation of the demonstration of the mutagenic effects of chemicals	Auerbach and Robson (1946), Oehlkers (1946), Rapoport (1946)

(continued)

Table 3.1 (continued)

Date	Milestone		References
	Physical mutagenesis	Chemical mutagenesis	
1949	Start of the gamma irradiation in plant mutation breeding Released of mutant in vegetatively propagated crop by X-rays: Tulip cv. 'Faraday' in Netherlands		Sparrow and Singleton (1953), https://mvd.iaea.org/
1954	Officially improved food crop plant by X-rays, pea mutant 'Stral-art' cv.		https://mvd.iaea.org/
1961	Officially released introgressed mutant cultivar Antirrhinum cv. 'Juliva'		https://mvd.iaea.org/
1963	Release of mutant from gamma ray: mutant variety 'Mori-hou-fu 3A' cv. of apple		https://mvd.iaea.org/
1964	Release of mutant cultivar in sweet cherry cv. 'Compact Lambert' in Canada		Sigurbjörnsson and Micke (1974)
1964	Set up of the FAO/IAEA Joint Division with a mandate to support the production of induced mutations for food security issues in developing countries		Forster and Shu (2012)
1966	UN Geneva Conference on 'Peaceful Application of Atomic Energy'		Donini and Sonnino (1998)
1966		Official release of the chemically (diethyl sulphate, dES) improved cultivar of barley, cv. Luther in the United States	Nilan and Muir (1967)
1968	Release of the mutant cultivar (X-ray) potato cv. 'Mariline 2' in Belgium		van Harten (1989)
1969–1970	The first FAO/IAEA international training course on crop mutation breeding Pullman Symposium on plant mutation breeding • Publication of first classified list of mutant cultivars • Publication of the first Manual on Mutation Breeding		IAEA (1970), Kharkwal (2012)
1972	Start of 'Mutation Breeding Newsletter' published by FAO/IAEA		Forster and Shu (2012)

(continued)

Table 3.1 (continued)

Date	Milestone		References
	Physical mutagenesis	Chemical mutagenesis	
1975	Initiation of coordination research programme organized by FAO and IAEA on 'Improvement of vegetatively propagated plants through induced mutations'		FAO/IAEA (1975)
1977	Publication of the second edition of Manual on Mutation Breeding		IAEA (1977)
1978	Start of space mutation breeding in China		Xianfang et al. (2004)
1980–1981	• Introduction of tissue culture (biotechnology) for in vitro mutation induction • First mutation breeding report on VPCs • FAO/IAEA first symposium on 'Use of induced mutations as a tool in plant research' in Vienna, Austria		Broertjes (1982), Kharkwal (2012)
1989–1990	Use of RIKEN RI-Beam Factory (ion beam) for radiation biology research and plant breeding		Abe et al. (2012)
1993	FAO/IAEA Mutant Variety Database (https://mvd.iaea.org/)		Forster and Shu (2012)
1998	Release of the rice mutant cv. 'Hangyu' 1 from cosmic irradiation		https://mvd.iaea.org/
2000–2005	TILLING (Targeting Induced Local Lesion in Genome) to induce and study mutation-phenotype association		McCallum et al. (2000), Colbert et al. (2001)
2002	Release of ion beam commercial mutant cv. 'Temari Bright Pink'		Abe et al. (2012)
2005–2010	Biotechnologies for targeted mutation discovery and wide spectrum establishment: • Direct genomic selection (DGS) method • Exome capture sequencing for mutation screening and functional genomic analysis • High-throughput sequencing and mutation discovery methods based on massive parallel sequencing • Using next-generation sequencing (NGS) for rapid detection of rare mutation in targeted gene		Basiardes et al. (2005), Okou et al. (2007), Mamanova et al. (2010), Gilchrist and Haughn (2010), Tsai et al. (2011)
2008	International Symposium on Induced Mutation in Plants in Vienna, Austria. Induced Plant Mutation in the Genomics Era.		Shu (2009)
2018	• IAEA/FAO International Symposium on Plant Mutation Breeding and Biotechnology • Publication of the third edition of the Manual on Mutation Breeding • New breeding techniques (e.g. genome editing) are not exempted from the current EU GMO legislation • Physical or chemical treatments are explicitly exempted from the EU GMO legislation		FAO/IAEA (2018), Court-of-Justice-of-the-European-Union (2018), Holme et al. (2019)
2020	Emmanuelle Charpentier and Jennifer A. Doudna won the 2020 Nobel Prize in Chemistry for the development of a method for genome editing (CRISPR/Cas)		Wu et al. (2020)

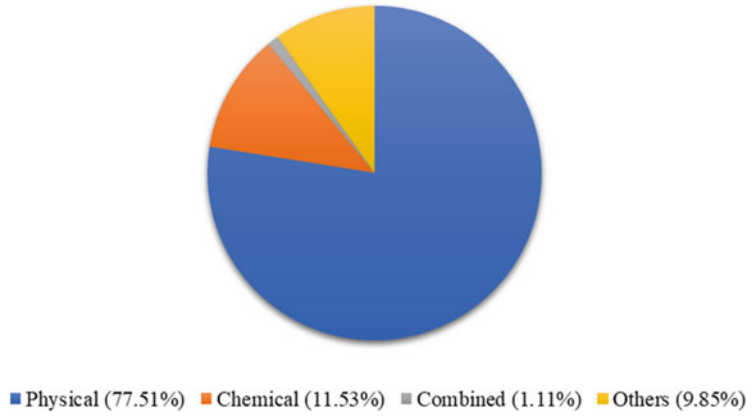

Fig. 3.1 Distribution of improved mutant crop cultivars developed by using different mutagenic agents. (Data from IAEA mutants' database: https://mvd.iaea.org/, accessed on 13 July 2020)

require relatively lower dosage of physical mutagens compared to seeds because of their high water content. Other variables include: storage time, applied mutagen dose, activity level of radioactive source and temperature influence physical mutagenesis (Mba et al. 2010). The material treated and the treatment itself are critical factors determining success.

The efficiency and effectiveness of physical mutagens have been described and are well known (Snyder et al. 1961; Lagoda 2012). A key feature is linear energy transfer (LET) of various mutagens, such as fast neutrons, ion beams, X-rays, gamma rays and UV light (in order of high to low LET) (Magori et al. 2010; Tanaka et al. 2010). The effects from the same dosage of different types of mutagens can be evaluated as relative biological effects (RBEs), which is a more pragmatic indicator for plant breeding (e.g. post-treatment survival and growth).

Gamma rays have been the most widely used physical mutagens in plant mutagenesis (Beyaz and Yildiz 2017; Li et al. 2019). Gamma rays are emitted from radioactive isotopes, mostly commonly Cobalt, ^{60}Co (with a half-life of 5.3 years) and Caesium, ^{137}Cs (with a half-life of 30 years). Because of their shorter wavelength (3×10^{-14} m), and uniform penetrating power, they are capable of reaching and changing DNA in many biological samples. Gamma rays gained popularity (after 1970s) during the promotion of peaceful uses of radioactive elements (following US President Dwight D. Eisenhower 'Atoms for Peace' speech on December 8, 1953), with an emphasis on world hunger concerns. Recently, gamma sources have become a security risk, and strict international regulations are imposed on the shipment and installation of new gamma sources and the refurbishment of old gamma irradiators. However, interest in induced mutation has not waned and other methods have been developed.

There has been a resurgence in the use of X-rays. X-ray irradiation does not involve radioactive isotope sources. X-rays represent the second most commonly used physical mutagen applied in plant mutation breeding, with over 21% of

Table 3.2 Comparison of various physical mutagens with respect to their respective advantages and disadvantages in plant breeding and genetics

Mutagen	Advantages	Disadvantages	Comments
Gamma rays	• Low LET radiation • Optimal procedures published and available • Regional, national and international services available • Highly reproducibility • Deep penetration in multi-cellular systems • Choice of high or low DNA damage • Cause single nucleotide substitution, inversion and deletion	• Requires a radioactive source • Many restrictions for new source and refurbished sources • Problem of high dose rate of new (highly reactive) sources • Requires specialized physical structure/laboratories and technicians	Most successful, has resulted in 66.91% of officially released mutant cultivars
X-rays	• Non-radioactive • Low LET radiation • Easy accessibility • Penetrates tissues from a few millimetres to a few centimetres	• Requires optimal settings for uniform irradiation • Optimized procedures are generally not available	Second most popular physical mutagen with 21.89% of officially released mutant cultivars
Neutron rays (fast, slow, thermal)	• High LET radiation • Penetrates tissues from a few millimetres to a few centimetres • Saturate genome for genetic studies • Cause point mutation, A/T to G/C transition, insertion, inversion, translocations tandem duplication and deletion • Gene knock-outs	• Requires nuclear reactor or accelerator • Relative high cost • Can create large deletions (>1 Mb) • Can delete multiple genes at a time • Difficulty of absorbed dosage estimation due to surface contamination	Third most popular physical mutagen with over 5.23% of officially released mutant cultivars
UV light	• Very low LET radiation • Available • Activation of transposable elements (indirect gene mutation) • Effective in pollen and fungal mycelium • Relative low cost	• Low penetration • Limited to single cell layer or sensitive material	Very limited effectiveness and use in plant mutation breeding
Ion beam	• High LET radiation • Large biological effects • High mutation rates • High survival rates of M_1/M_1V_1 • Wide and new	• Deposit high energy/damage • Not tested on a large number of species • Laborious sample preparation	• High installation cost of the accelerator • Highly effectiveness in ornamentals • Good for genetics studies

(continued)

Table 3.2 (continued)

Mutagen	Advantages	Disadvantages	Comments
	phenotypic variation • Targeted trait specificity	• Low accessibility of ion beam irradiators/facilities • Anatomy of seed and meristematic tissue can be limiting	
Cosmic rays	• High-energy ion radiation • High LET radiation • Tested on various plant species	• Requires space flight • Massive investment costs and intensive technological support • Very limited accessibility • Mechanism of space-induced mutation not fully understood • Limited to plant materials in dormant state (not actively growing)	• Access to space programme • Possibility to induce novel variation • Possibility to obtain rare mutants • Effective ground simulation of space environment factors may increase this approach

released mutant cultivars worldwide (https://mvd.iaea.org). It should be noted that X-rays were the first physical mutagen proven to induce mutation in crop plants in the early plant mutation era with mutational effects on barley described in 1928 (Stadler 1928a).

Today, ion beam is emerging as an effective physical mutagen (after its biological effects were discovered in the mid-1980s, Abe et al. 2012). Ion beam treatments have resulted in the release of several improved cultivars in rice, wheat, potato, tomato, soybean, tree crops (ginkgo, Chinese chestnut, sea buckthorn), and many ornamental plants (e.g. chrysanthemum, carnation, dahlia, lily, petunia, rose and cyclamen) (Tanaka 2009; Abe et al. 2012; Feng and Yu 2012; Yamaguchi 2018).

Ultrasound, UV light, accelerated ageing and combined ^{32}P and gamma rays were used for plant mutagenesis with limited effectiveness recorded (http://mvd.iaea.org).

3.2.1.2 Chemical Mutagens

Several early researchers attempted to induce mutations in animals and plants by chemical mutagens (Morgan 1911; Sakharov 1932; Klein 1932). Frequently used chemical mutagens in plants include, alkylating agents, e.g. mustard gas, ethyl methane sulphonate (EMS), ethyleneimine (EI), *n*-ethyl-nitrosourea (NEU), *n*-methyl-nitrosourea (NMU), 1,4-bis-diazoacetyl butane (DAB), hydroxylamine (HA), diethyl sulphate (DES), dimethyl sulphate (DMS), methyl methane sulphonate (MMS), maleic hydrazide (MH), hydrogen fluoride (HF) and sodium

azide (SA) (Shkvarnikov and Morgun 1975; Leitão 2012). These cause point mutations, and their effects are often silent or missense mutations (50%), with only about 5% nonsense mutations (Viana et al. 2019). Alkylating agents have one, two, or more reactive groups known as mono, di, or polyfunctional that react with DNA (Davies 1966). When the chemical agents interact with genetic materials, they generate alkylated O^6guanine, N^3adenine and N^3cytosine. During the repair of the alkylated base, O^6guanine tends to convert G:C to A:T base-pairs, N^3adenine gives rise to A:T to T:A transversions and N^3cytosine often results in C:G to T:A transitions and C:G to G:C transversions (Minocha and Arnason 1962; Leitão 2012). These single nucleotide changes generate truncations and missense mutations and can broaden allelic variation. Nonsense mutations may cause premature stop codons and splice site deletions (Tadele et al. 2009). The chemical mutagens are specific in their action, whereas radiation activity is random (Kempner 2011).

Ethyl methane sulphonate (EMS) is one of the most popular alkylating agents in plant mutation breeding and genetics. EMS produced over 25% of the chemically improved mutant cultivars due to its effectiveness and ease of handling, especially its detoxification by hydrolysis for disposal (Pathirana 2011). Other effective chemical mutagens include: *n*-ethyl-nitrosourea (NEU), nitroso methyl urea (NMU), colchicine, ethyleneimine (EI) and dimethyl sulphate (DMS) with over 14%, 10%, 9%, 7% and 4% of total released mutant cultivars, respectively. Over 70% of mutant cultivars produced through chemical mutagenesis are from these six chemical agents (http://mvd.iaea.org). Other non-alkylating mutagens include (1) nitrous acid and nitric oxide, (2) base analogues and related compounds, (3) some antibiotics, e.g. streptozotocin, mitomycin C or azaserine and (4) intercalating agents and topoisomerase. Their mutagenic effects and their applications in plant mutation induction have been reviewed by Leitão (2012).

From the non-alkylating mutagens, sodium azide (SA) (a common bactericide, pesticide and industrial nitrogen gas generator) is one of the most commonly used mutagens in plant breeding (Kleinhofs et al. 1974; Gruszka et al. 2012). Common chemical mutagens with their mode of action are listed in Table 3.3. However, it is essential to note that most of those chemical agents are hazardous to health and are carcinogens. An exhaustive list of the chemical mutagens used in mutation breeding with their effectiveness can be found in IAEA mutant database (http://mvd.iaea.org).

The following parameters need to be considered to establish optimal procedures for the chemical mutagenesis:

- Dose (concentration × time)
- pH
- Physical and chemical properties of the agents
- Interaction with the culture media
- Post-treatment condition

Table 3.3 Classes of chemicals with some examples of chemical mutagens and their mode of action

Chemical class	Chemical mutagens	Mode of action
Base analogues and related compounds	5-Bromouracil, 5-bromo-deoxyuridine, 2-aminopurine, maleic hydrazide, 8-ethoxy caffeine	Deletion, addition, frameshift, Chromosome breakage transitions (purine to purine or pyrimidine to pyrimidine) and tautomerization
Acridines	Acridine orange, acriflavine, ethyleneimine, proflavine	Transitions AT to GC, GC to AT
Mustards	Sulphur mustard, nitrogen mustard	Alkylate various sites in DNA
Nitrosamines	Dimethyl nitrosamine, nitrosoguanidine, nitroso methyl urea	Alkylate various sites in DNA
Alkylating agents	1-Methyl-1-nitrosourea, 1-ethyl-1-nitrosourea, methyl methane sulphonate, ethyl methane sulphonate, dimethyl sulphate, diethyl sulphate; 1-methyl-2-nitro-1-nitrosoguanidine, 1-ethyl-2-nitro-1-nitrosoguanidine, *N*,*N*-dimethyl nitrous amide, *N*,*N*-diethyl nitrous amide and others	Alkylate various sites in DNA
Epoxides	Ethylene oxide, diepoxybutane	Alkylate various sites in DNA
Azide	Sodium azide	Generates azidoalanine causing G/C to A/T transitions. Gene mutation
Miscellaneous	Nitrous acid, maleic hydrazide, hydrazine, hydroxylamine, antibiotics, free radicals	Transitions AT to GC, GC to AT, Chromosomal aberrations
Intercalating agents and topoisomerase	Ethidium bromide and proflavine daunorubicin, 4′,6-diamidino-2-phenylindole (DAPI)	Block transcription and DNA replication
Colchicine	–	Chromosome doubling affects spindle microtubule formation and promotes chromosome doubling

Some other influencing factors need to be considered for effective treatments:

- Pre-soaking
- Treatment duration
- The temperature during treatment
- Hydrogen ion concentration
- Dry back
- Post washing
- Storage of treated materials

Table 3.4 Advantages and disadvantages of physical and chemical mutagens

Mutagens	Advantages	Disadvantages
Physical	• Tested, proven, robust, ubiquitous application, non-hazardous, environmentally friendly, transferrable, cost-effective, non-regulated, produce rare mutant events and low mutational load (with specific doses) • Reproducible • High and uniform penetration in multi-cellular plant materials (except UV)	• Mutagenesis is random, thus selection methods for desired mutations are required • Mutation spectra are not well known, except for fast neutrons • High degree of sterility in treated plants • Dose rate needs to be determined for each genotype • Chromosomal and gene mutations can occur simultaneously
Chemical	• Tested, proven, robust • Point mutation predominant • Relatively less chromosomal damage • High mutation rates and densities are useful for gene-phenotype association studies • Different mutation spectra • Identification of multiple mutant alleles	• Mutagenesis is random and widespread • Chemicals are very hazardous, non-environmentally friendly and regulated • Penetration difficulties in multi-cellular plant parts • Difficulties in reproducibility • Sensitivity of some target tissues and organs • Difficult to assess effective dose and reproducibility • The dose rate needs to be determined for each genotype • Stability of the mutagen

3.2.1.3 Advantages and Disadvantages of Physical and Chemical Mutagens

The choice of physical or chemical mutagen depends on the objective and all have advantages and disadvantages (Table 3.4). Ionizing radiations have benefits that they can be used for every plant material, and protocols have been developed that provide a degree of accuracy and reproducibility. Irradiation services are available at local, regional, national and international levels, with many supported by the International Atomic Energy Agency or national atomic energy institutes.

In addition to the random action and the ability to produce small mutagenic effects in an otherwise unaltered genetic background, there are some limitations and challenges. Limiting factors for gamma ray emitters (typically ^{60}Co and ^{137}Cs) is that new and refurbished sources are highly radioactive, so shields need to be installed to allow low dose treatments recommended for plant materials with a high-water content. More importantly, there are now restrictive international regulations on the transport of radioactive isotopes, and this complicates or prevents the setting up of new gamma sources or the refurbishment of old ones.

A concern for fast neutron treatments is they cause a low level of temporary radioactivity in the irradiated materials that may pose a safety problem in post-irradiation handling and absorbed dose assessment. X-rays being non-radioactive are

more user-friendly and accessible as they are used widely in other uses worldwide (e.g. blood irradiator, sterilization and radiotherapy in hospitals). However, there is a problem with uniformity using large samples. Thus, X-ray irradiators need to be equipped with sample rotating devices to provide even irradiation exposure.

Ion beam mutagenesis suffers from laborious sample preparation, limiting treated sample size, e.g. a plastic box with 5 × 7.5 × 1.25 cm, or plastic Petri dishes of 3, 6 and 9 cm in automatic ion beam irradiation system (Abe et al. 2012) and results in severe damage to treated material DNA. Despite the effectiveness of ion beams in generating beneficial and a new spectrum of mutations (e.g. Khazaei et al. 2018), it has a major disadvantage in that there are a limited facilities compared to gamma and X-rays.

Chemical mutagens are known to be effective in gene mutation and permit the identification of new alleles, which is of high interest to plant geneticists. Their major drawback for plant breeding is they produce a heavy mutational load. The chemicals used are extremely hazardous and require suitable infrastructure/laboratories for safety use. Furthermore, these chemicals have an impact on the survival of the treated plant materials and sensitive materials such as in vitro cells (e.g. microspores and protoplasts), tissues consisting of single cell layers (somatic and gametic cells) and immature embryos.

3.3 Determination of Optimum Dosage for Mutation Induction

Cells of higher plants respond to physical and chemical mutagens with various biological changes influenced by the developmental stage, environment and biological activity of the plant materials to be targeted. These factors significantly affect the efficiency and effectiveness of the mutation treatment and are defined as:

Efficiency—the ratio of desirable mutagenic changes to undesired effects
Effectiveness—the number of mutations produced per unit of dose

Damaging effects are proportional to the dose (physical) or concentration (chemical) received. However, mutagens can directly or indirectly damage cellular components and can cause perturbations in metabolic and physiological functions as well as genetic changes. Therefore, before developing mutagenic populations or mutation studies, it is recommended to conduct radio-sensitivity (physical) or toxicity (chemical) tests to determine suitable treatments. The optimum irradiation dose or chemical treatment should balance the required mutation frequency and plant injury. The ultimate goal is to induce enough interesting new variations in a viable population and in a population size that is manageable with breeding or genetic resources. In general, the lower the density of mutations the more mutant lines that need to be screened. To determine optimal treatments, it is advisable to evaluate the effects of a range of treatments. The type of plant propagule to be treated has a strong effect; some examples follow. In ionizing irradiation for seeds higher doses are used (e.g. 50 to >600 Gy for dry small grain cereal seeds). In contrast, low dose ranges are

recommended (10–100 Gy, or less) for tissues with high-water content, e.g. stems, buds, embryos, anthers, microspores and floral tissues. For fast neutrons, typical doses are between 5 and 50 Gy for sunflower seed (Gvozdenic et al. 2009). When using chemical mutagens, such as EMS, for seed mutagenesis, the concentration ranges from 0.05 to 0.1 M (0.25% to >2%) for a duration of 0.5–24 h with a temperature of 25–35 °C whereas for in vitro plant materials a concentration up to 1% is normally applied (Kodym and Afza 2003; Mba et al. 2010). In sodium azide (SA) toxicity, a range of 1–50 mM for duration 2–24 h, e.g.: 1–4 mM at pH 3 for 3–6 h was effective for dry barley seed, but for in vitro plant materials the concentration ranged from 0.1 to 1.0 mM for 10 min for leaf callus of sugarcane and 6 h for barley anthers, and 0.1–10 mM for 1 h for immature maize embryos (Gruszka et al. 2012). Maghuly et al. (2016) reported that an EMS range of 0.4–1.6% for 1.5 h was optimal for jatropha in vivo cuttings.

The acidity of SA and the alkalinity of EMS solutions give rise to different post-treatment response in germination and seedling development. SA treatments also affect cellular metabolism and mitosis (Gruszka et al. 2012). In general, vegetative materials with active growth (high mitotic rate) and reproductive cells (meiotic cells) are more susceptible to mutagen treatment than dormant or quiescent materials such as seed (Lagoda 2012).

Plant materials for testing should be: clean, genetically and morphologically uniform, disease and pest free, homogenous with high viability/vigour and health. Radio-sensitivity or toxicity tests (as for mutation treatments) are influenced by biological activity (e.g. mitotic rate), genotype, and by environmental factors such as oxygen, water content, post-irradiation storage, and temperature (Mba et al. 2010). The suitable dose and choice of mutagen also depends on the aim, chiefly whether this is for breeding or genetic studies. The effects of mutation treatments are also subject to the variability in plant genera, between and within species, and the genotype.

Response to treatment is assessed in the first (treated) generation, i.e. M_1 of seed propagated plants and M_1V_1 of vegetatively propagated plants. Typically, measurements are taken on biomass (germination and growth rate, multiplication rate, seedling height, hypocotyl, and root length, reviewed Bado et al. 2015). One measure of sensitivity is the lethal dose (LD) for 50% of treated materials (LD_{50}) or 50% growth reduction (GR_{50} or RD_{50}) though alternative LD_{30} or GR_{30} may also be useful. For seed, early stages in germination and seedling growth are often measured, such as germination/growth rate, seedling height (for monocots, Figs. 3.2 and 3.3a), or hypocotyl length (for dicots, Figs. 3.2 and 3.3b). This allows treatment effects in the M_1 samples to be distinguished from delayed germination and seedling biomass (Kodym et al. 2012). These data are then expressed as a percentage over untreated samples (control) to estimate the sensitivity parameters LD_{50} or GR_{50}, or their alternatives at 30%, for mutation induction. The plotted data is typically a curve (Fig. 3.2) with stimulated growth at low doses, however for practical purposes a straight line is often fitted (Mba et al. 2010) or else a probit analysis which is a function of the inverse cumulative distribution function or quantile function associated with the standard normal distribution (Surakshitha et al. 2017). Examples

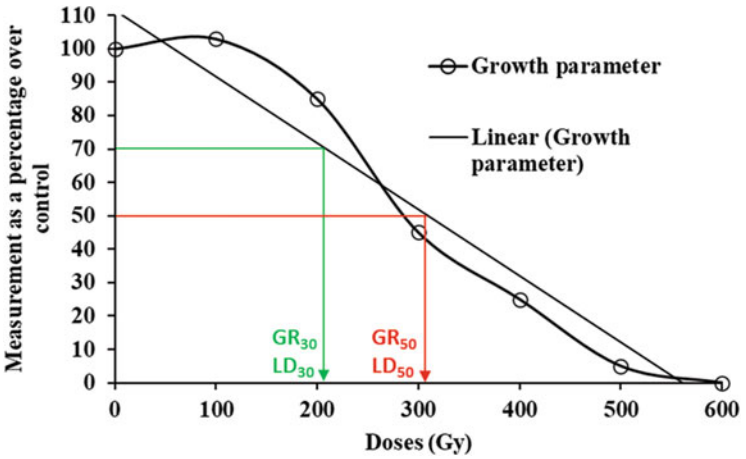

Fig. 3.2 Theoretical curve showing effects of irradiation on plant growth parameters (e.g. reduction of germination rate, seedling height, hypocotyl length, fresh weigh), note low doses can stimulate plant growth whereas optimal dosage for mutation induction causes reduced growth. Example of the GR_{30} or LD_{30} (green) and GR_{50} or LD_{50} (red)

of radio-sensitivity tests are given in Fig. 3.3, showing a theorical radiosensitivity test of monocot versus dicot seed to physical or chemical mutagens.

3.4 Mutagenesis

To improve crop plants, breeders and researchers have until recently relied on three main options: crossing, mutation and transformation. The choice is mainly guided by the following considerations: (1) is the target trait under genetic control, (2) can desired alleles be manipulated easily by crossing, (3) does linkage drag results in unwanted traits and (4) does crossing result in the break-up of refined linkage groups required for successful cultivars. If the choice is plant mutation breeding, then issues focus on the ability to produce a desired mutation with little or no effects on the background genome of the elite line selected. Other considerations include: space to grow on mutant generations (this is particularly problematic for tree crops that require large areas), time, resources and facilities for screening large mutant populations (either phenotypically or genotypically). The target genotype is normally a top performing cultivar or elite breeding line (that is deficient for a particular trait). Target genotypes are often popular with respect to market demands and adapted to local environments or favoured non-field growing conditions (e.g. hydroponics and indoor farming).

In principle, any live plant material can be used for physical and chemical mutagenesis; however, the plant material must have the ability to grow. Thus, most materials include a meristem (apical and side meristems or embryo), or tissue

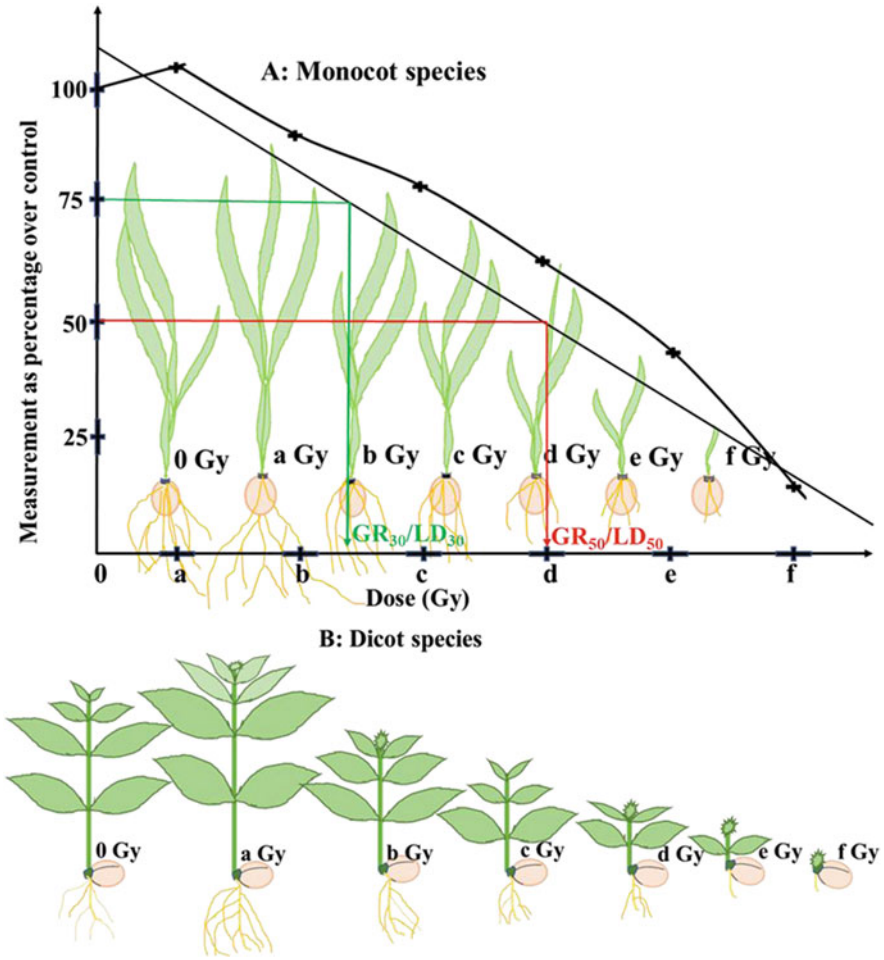

Fig. 3.3 Radio-sensitivity or toxicity test of a seed plant crops: (**a**) Monocot species with measurements of seedlings, (e.g. rice, wheat, maize, sorghum) and in vitro (e.g. banana, ginger and cocoyam); and (**b**) dicot species assessed by hypocotyl or epicotyl length, inter node (e.g. peanut, Bambara groundnut, pea, sunflower) and in vitro (e.g. potato, sweet potato, yam and cassava). Example of a, b, c, d, e and f for monocot species (0, 100, 200, 200, 300, 400, 500 and 600 Gy) and dicot species (0, 50, 100, 150, 200, 300, 400 Gy) and in vitro materials (0, 5, 10, 15, 20, 40 and 60 Gy)

culture methods in which cells, tissues or organs can be cultured. Totipotency of plant cells can be exploited to regenerate plants after mutagenesis via a range of techniques (Jankowicz-Cieslak et al. 2012; Chen et al. 2013; Bado et al. 2016; López et al. 2017; Suprasanna et al. 2012). A summary of live plant materials in plant mutation breeding is given in Fig. 3.4.

The major decisions for adopting tissue culture-based approaches for a mutation breeding are given in a flow diagram (Fig. 3.5). These include access to local

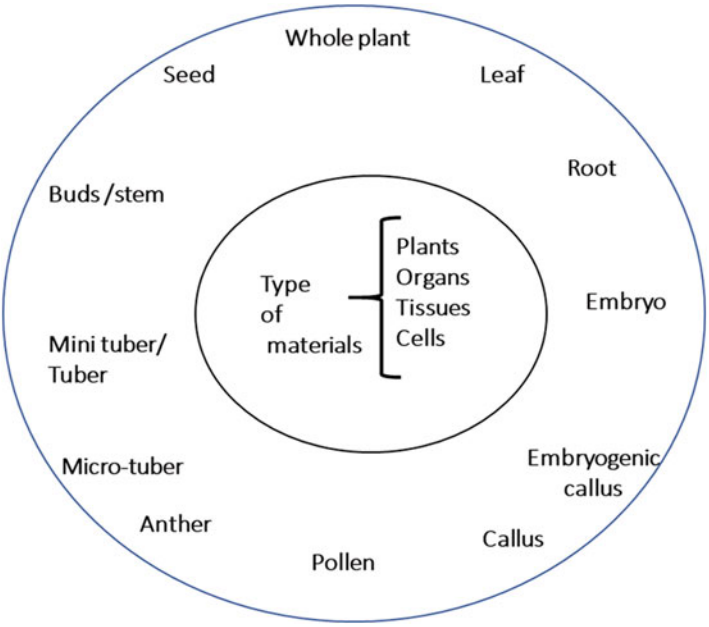

Fig. 3.4 Plant materials used in mutation breeding: single cells (somatic and gametic cells, protoplast, leaf disc, etc.) and multi-cellular systems (whole plant, seed, bud, stem cutting, graft, tuber, mini- and micro-tubers, etc.)

mutation induction facilities and restrictions in out-sourcing, chemical mutagens may have an advantage because of their wide diversity and availability. Single-cell propagules favour multi-cellular tissue culture systems because in vitro screening can be done easily and on large numbers of callus, embryogenic callus and plantlets. Gametic cells, being haploid, have an advantage in that on chromosome doubling, e.g. in doubled haploid production mutation events are made homozygous instantly and thus the mutant phenotype can be seen relatively quickly (in other materials mutations occur in one allele and since most induced mutations are recessive these materials need to be selfed to produced individuals that carry homozygous mutant genes which show the changed phenotype). However, mutation breeding using single-cell propagules is often laborious with low regeneration into plantlets and requires skills, resources, and facilities; often the target genotype is one which is responsive to tissue culture but not one that is in demand by breeders and end-users. This explains the limited adoption and success of single-cell systems in plant mutation breeding, which have generated less than 9% of improved cultivars in vegetatively propagated crops (Bado et al. 2017). However, tissue culture offers great potential especially for vegetatively propagated plants.

In developing a mutation breeding programme, one must be aware of the likelihood of success and the numbers of individuals and progenies that need to be screened. The most common mutant traits induced are late flowering, pale green

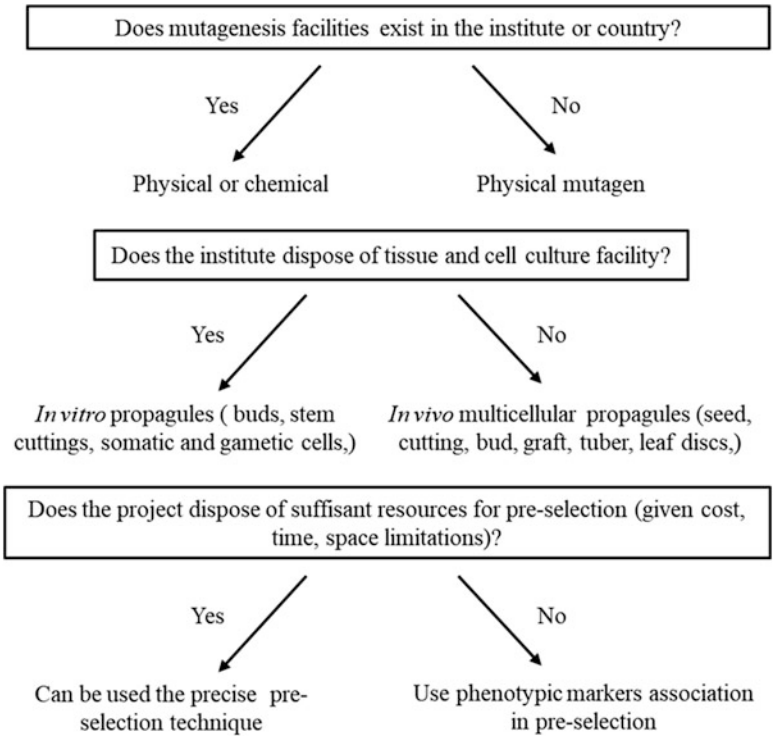

Fig. 3.5 Decision diagram for the development of mutation breeding and mutation genetics programmes

leaf colour, short stature and sterility, collectively these can make up over 50% of all mutants generated (Forster et al. 2012). To induce lodging-resistant mutants in oat, barley, bread and durum wheat with physical and chemical mutagens about 1200 seeds are required, whereas in the production of pest and disease resistance about 10,000–20,000 seeds are needed for mutation treatment (Donini and Sonnino 1998). Since induced mutation is a random process, large mutant populations need to be developed for screening. The success of mutation breeding depends on multiple factors, either external (i.e. resources, space, time, and facilities) and internal (i.e. choice of plant materials and mutagens, cost and space in laboratories, nurseries and field). The mode of mutagen application involves internal factors (breeder's choice) and external factors (availability of the mutation induction facility).

3.4.1 Mutagenesis of Seed

Seeds are the most commonly used targets for mutation treatment. This is because they are a common material used by breeders, they are in a dormant/sessile state and can be easily transported, treated, returned, stored and grown on. As stated above a

radiosensitivity or toxicity test is recommended as a first step (Sect. 3.3). The seed for treatment should be healthy and uniform (both genetically and physiologically). For physical mutagenesis water content must be controlled, this is normally done in a desiccator. General pre-treatment procedures (parchment removal, storage desiccation, pre-soaking, etc.) can be found in Mba et al. (2010 and references therein).

After treatment, the parental M_0 seeds is referred to as M_1 seeds, i.e. the first mutant generation. In addition to carrying mutations, these seeds are also physiologically weak due to treatment and require to be grown in optimal conditions. Physiological imbalance can promote out-crossing and isolation (bagging) of floral parts may be required to prevent this (Ukai and Nakagawa 2012). In addition to physiological aberrations, M_1 plants are often chimeric and since most mutations are recessive and in a heterozygous state, thus M_1 plants cannot be used for phenotypic screening. It has become a dogma in plant mutation breeding that the earliest selection for mutant traits cannot be carried out until the M_2 at the earliest (Fig. 3.6, Black arrow scheme). However, with the advent of DNA analysis, mutations in specific genes can now be screened for and individual M_1 plants carry mutations in target genes can be selected and grown on. This is a major change in philosophy, as selection can be done a generation earlier, thus (1) eliminating the vast number of M_1 plants of no interest and (2) saving time (to produce subsequent generations), costs and subsequent laboratory, nursery and field space (Fig. 3.6, red arrow scheme). These factors make mutation breeding in perennial crops, e.g. trees more attractive, as an example mutation breeding in oil palm is now a reality (Nur et al. 2018). A limitation in genotypic selection is that DNA needs to be extracted and analysed, but there are an increasing number of service providers and such analyses can be out-sourced.

In mutagenesis of haploids (e.g. gametic cells), any interesting and exciting observations in M_1 materials can be fixed at an early stage through doubled haploid technologies (Fig. 3.6, green scheme, Zhao et al. 1994). M_1 plants (either the whole population or selected individual mutant genotypes) are grown up till maturity. Depending on the breeding system of the species (in-breeder or out-breeder), the M_1 plants may be selfed or crossed to produce the M_2. Although genotypic selection can be practiced in the M_1, it is the M_2 generation that provides the earliest display of the mutant phenotype, which is the ultimate target for breeders and geneticists. Space is therefore required to grown up the M_2 plants, and since many traits of interest are related to yield (or impact on yield indirectly) this requires growing to maturity and harvest. M_2 individuals may be grown on to produce M_3 families (and above) containing specific mutant traits (a common practice in gene-phenotype studies, e.g. Targeting Induced Local Lesions in Genomes (TILLING) or used directly in plant breeding).

3.4.2 Mutagenesis of Vegetative Propagules

The number of mutant cultivars in vegetatively propagated crops lags way behind seed propagated crops by 793 (23.80%) to 2539 (76.20%). However, the picture is

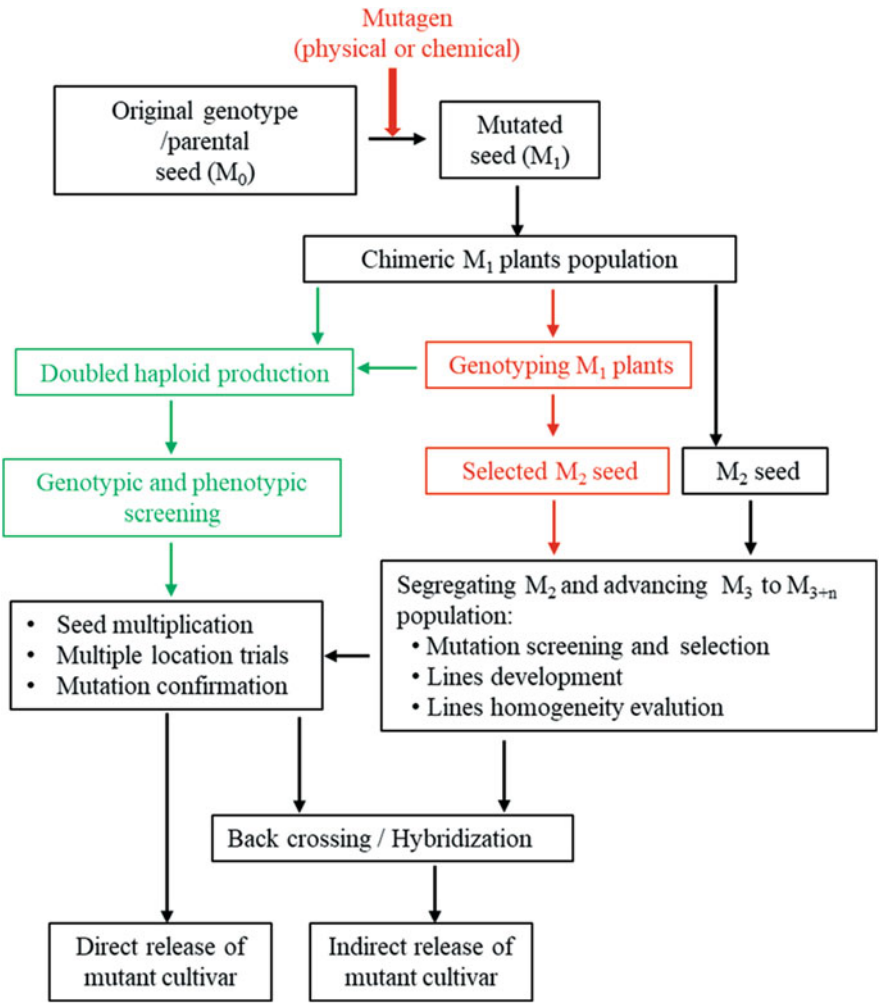

Fig. 3.6 Scheme of mutation breeding using seeds as plant propagules for mutation induction, showing: the commonly adopted strategy (previous dogma in black arrow), accelerated scheme mode through doubled haploidy (green arrow) and M_1 genotypic screening and selection

changing as advancements in cell/tissue culture techniques enable new approaches. Vegetative propagules, for mutation induction can be classified into two categories: (1) single-cell propagules (somatic and gametic calli, embryogenic calli, cell suspensions, microspores and protoplasts) and (2) multi-cellular propagules (stem cuttings, buds, mini-tubers, micro-tubers, tubers, whole plants, scions, grafts, bulbs, etc.) (Fig. 3.7). Vegetatively propagated plants usually have no or a very limited breeding system. Therefore, a major objective in producing mutations (the vast majority of which are recessive) is to knock-out the dominant allele at heterozygous loci of interest, thus producing a double recessive locus. This can be done with

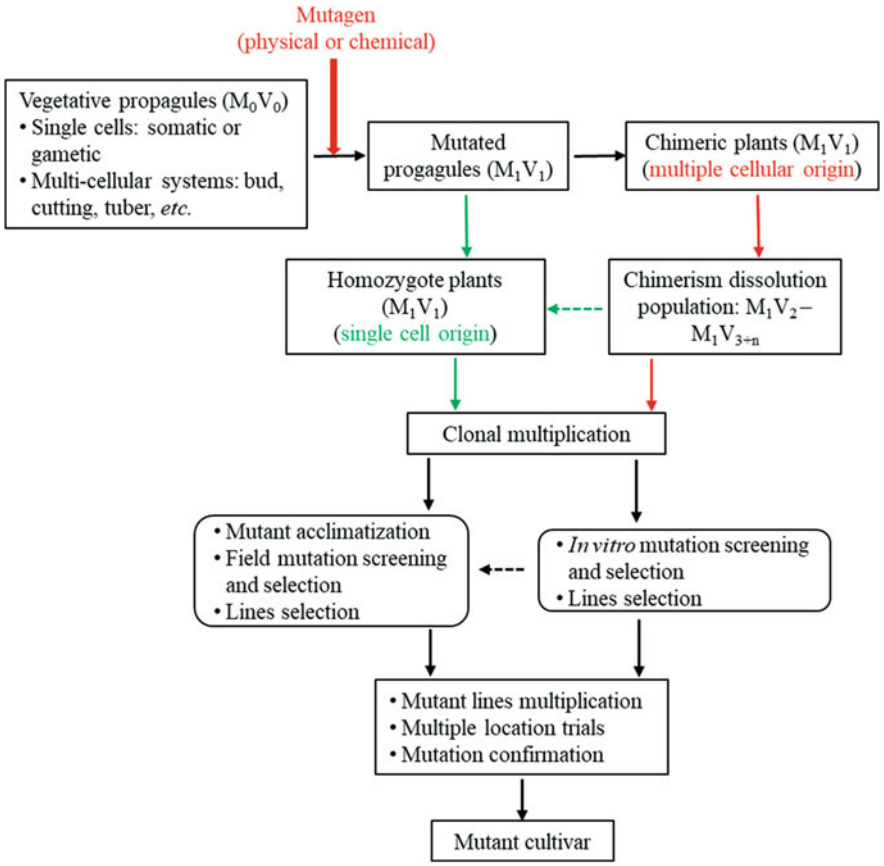

Fig. 3.7 Scheme of mutation breeding using vegetative materials for mutation induction showing the commonly adopted strategies and an accelerated mode through double haploidy

gamma and X-ray irradiation, and chemical mutagenic treatments using LD_{50} or LD_{30} treatments. Alternatively, high dose treatments and ion beam irradiation can be used to delete parts of the genome. As with seed treatments, mutagenesis of multi-cellular explants produces chimeric plants (M_1V_1). Outer layer cells/tissues may constitute a barrier to the target meristematic cells in both physical and chemical mutagenesis. A comparison of recommended/commonly used treatments is given by Bado et al. (2017) for several vegetatively propagated crop species.

The starting materials for treatment are termed M_0V_0, which become M_1V_1 after mutagenesis (Fig. 3.7). Treated multi-cellular explants (M_1V_1) normally have to undergo multiple sub-cultures to produce homohistont explants (solid mutants in which chimeras have been dissolved) which are uniform for the mutation and phenotype. At least three sub-cultures or propagation cycles are recommended in treated buds or stem explants to stabilize the mutated sectors before mutant selection (Broertjes and van Harten 1988; Roux et al. 2003; Sutarto et al. 2009; Jankowicz-

Cieslak et al. 2012). The more heterozygous are genotypes the more phenotypic mutations will be (Spiegel-Roy 1990). An alternative method for mutation event isolation is the detachment of the variegated sectors of mutated explants (possible in the M_1V_1 generation, but more normally practiced in higher generations, e.g. M_1V_{3+n}) to develop homohistonts through regeneration or somatic embryogenesis (Fig. 3.7, dash arrow), this has been a successful technique mutation breeding in ornamental plants (Dwivedi et al. 2000; Datta et al. 2005; Datta 2020).

Single cells have the advantage of generating solid mutant plants directly. The regenerated plants (M_1V_1) may be screened early and desired mutants selected (Suprasanna et al. 2012). The advanced and selected lines can then be micropropagated to produce enough plants for acclimatization and multi-locational field trialling. Stabilized mutants with suitable yield may then be released as direct mutant cultivars.

Other propagules of vegetative propagated plants can include seed (in facultative species) as well as somatic or gametic cell cultures of seed propagated plants (Saleem et al. 2005; Serrat et al. 2014). For facultative species cross breeding is possible to introgress, the mutation into other cultivars.

3.5 Combined Mutagenic Treatments

Combining two or more mutagenic treatments date back to the 1950s (Oster 1958; Moutschen 1960; Konzak et al. 1961; Merz et al. 1961; Moutschen and Moutschen-Dahme 1961; Soriano 1963; Favret 1963; Valeva 1964; Wallace 1965; Aastveit 1968; Doll and Sandfaer 1969). Two improved ornamental cultivars of streptocarpus, 'Cobalt Nymph' and 'Purple Nymph' were produced in The Netherlands in 1969 by combining physical (X-ray) and chemical (colchicine) treatments (http://mvd.iaea.org). Combined mutagenic treatments have not been as attractive in plant mutation breeding as single-acting mutagens. To date, only 37 improved cultivars have been documented out of a total of over 3330 (>1%) registered mutant cultivars (http://mvd.iaea.org, Fig. 3.2). Reports of combined mutagenic treatments in plant mutation breeding have declined with only three improved cultivars released after 2000. However, there are many recent reports on the effects of combined treatments by researchers and breeders (Mehandjiev et al. 2001; Bhat et al. 2007; Goyal and Khan 2010; Khan and Tyagi 2010; Makeen and Babu 2010; Srinivas and Veerabadhiran 2010; Channaoui et al. 2019). The highest number of combined mutagens resulting in a successful mutant cultivar involved five chemical mutagens (NEU, NMU, DES, DMS, and EI) resulting in the high yielding cultivar 'Prikarpatskie 4' of field bean in Russian Federation in 1986 (http://mvd.iaea.org). Konzak et al. (1975) were among the first to report a synergistic effect of SA on mutation yields following MNH treatment. Synergistic increases in the frequency of chimeras, chlorophyll-deficient mutations, and morphological mutations were observed in both SA post-irradiation treatments and pre-EMS treatments with gamma irradiation of barley seed by Cheng and Gao (1988). Wani (2009) also recorded synergism by combining gamma rays and EMS treatments in chickpea.

The efficiency and effectiveness of the combined mutagens, as with single treatments is influenced by the applied doses/treatments (Olejniczak and Patyna 1981; Srivastav and Raina 1981; Chauhan and Patra 1993; Olejniczak 1994), the mode of application (pre, post, or simultaneous), and the plant materials (seed and vegetative propagules). For instance, in seed mutagenesis with ionizing radiation, water content is an influencing factor affecting efficiency and effectiveness. Jafri et al. (2013) recorded a better response inducing chromosomal aberrations with combined gamma ray and EMS treatments of coriander. The combined mutagens (fast neutrons + NEU) for mutation induction in pea were the most efficient and produced the highest number and the widest of spectrum mutations. Other examples include barley (Aastveit 1968; Mohan 1972), black gram (Lal et al. 2009) and rapeseed (Channaoui et al. 2019).

However, non-synergistic effects have been observed in several other studies of seed treatments (e.g. Olejniczak 1994; Girija and Dhanavel 2009). In vegetative propagules, combined mutagenic treatments were applied to detached leaves of *Streptocarpus* sp. and rooted cuttings of *Bougainvillea* sp. As previously mentioned, the combination of X-rays and colchicine produced two mutant cultivars 'Cobalt Nymph' and 'Purple Nymph' (http://mvd.iaea.org). In bougainvillea, the combined treatment of gamma rays and colchicine resulted in two mutant cultivars, 'Silver Top' and Lady Hudson, released in 1978 and 1979, respectively (http://mvd.iaea.org). With these four exceptions there are no other published reports (to our knowledge) of this kind for other vegetatively propagated plant or vegetative propagules.

3.6 Mutations Screening

Mutagenic populations for desired traits and alleles can be selected for by classic phenotypic and/or more modern genotypic screening. The previous dogma was that mutation selection could only be performed at the M_2. Screening in the M_1 has been considered heresy. This is because M_1 plants contain chimeras, physiological disorders and usually do not express the mutant phenotype (as the vast majority, >90%, are genetically recessive). However, with the meteoric rise in DNA analytical methods and an increasing focus on DNA sequencing in plant breeding and genetics, high-throughput and affordable genotypic screening methods are now available (e.g. High Resolution DNA Melt and Diversity Arrays Technologies) that can be applied in mutation detection. Genotypic screening of mutants can be applied to M_1 generations as it is independent of plant health, physiology, phenotype and environmental effects. This presents a massive step forward for plant mutation breeding as selection of (usually rare) plants carrying mutations of interest can be selected earlier, and the vast majority of plants of no interest rejected, thus saving not only time, but future investment in development. This opportunity can be applied to all plant species, but is of particular relevance to perennial crops, e.g. trees that have long generation times, and which require a lot of time and space in nurseries and in the field. A limitation is that the target gene for mutation is known and sequence data are available, but the genomes of most major crop species and important genes are

being revealed and other crops will follow suit. It is true that some selected M_1 plants will carry chimeras, but these can be dissolved in producing the next generation (by sexual or vegetative propagation), the most important issue for the breeder is to capture and exploit as early as possible the mutant alleles of interest.

Mutants derived from gametic cells and somatic cells can also be screened at the first generation, and these do not produce chimeras. Gametic cells and doubled haploidy have been of interest as mutation produced in haploid gametic cells are fixed immediately as homozygotes in the doubled haploid and thus the mutant phenotype can be observed (Fig. 3.6, green scheme, Castillo et al. 2001). In addition, cell culture allows the production of large population sizes in a short time for mass in vitro mutation induction and screening. Early examples of doubled haploid mutation screening in vitro include salt tolerance in wheat (Zhao et al. 1994, 1995). However, it is essential to note that many agronomically useful traits (e.g. resistance to biotic and abiotic stresses, and notably yield) are not expressed in single cells or callus or young plants (Maluszynski et al. 1995; van Harten 1998).

Regarding phenotypic screening of traits, some may require simple procedures such as visual selection, others need more elaborated investigations (see Sects. 3.4.1 and 3.4.2). It is important to point out that although mutation detection can now be performed in the M_1 via genotypic selection, the ultimate test is the observation of the desired phenotype and no detrimental effects. Thus, plant performance of M_2 individuals and M_3 families remain key assessment stages in plant mutation breeding.

Phenotypic traits may be qualitative or quantitative traits (Donini and Sonnino 1998; Makay et al. 2020). For qualitative (single gene) traits (e.g. morphological and physiological traits, response to daylength, vernalisation), the mutation can be detected on a single plant based on visual inspection or combined with appropriate (e.g. physiological) screening procedures. These screens are often destructive and can be carried out only on generations above the M_2 for seed crops or M_1V_3 for vegetatively propagated crops. For phenotypic quantitative trait screening (e.g. yield, quality, biochemical content, mineral content, seed or fruit size, weight, and density), mature plants are generally required though biochemical, biometric, physical, or mechanical methods may be applied as indicators. Here the traditional practice is to screen a mutant family usually the M_3 of seed propagated plants and the M_1V_3 of vegetatively propagated plants, but if the target gene is known the M_1 plants may be screened directly (see above).

Most mutant cultivars have been developed from forward-genetic (phenotypic screening) (Pathirana 2011). However, DNA technologies now allow the evaluation of mutant individuals and populations genotypically. One of the first examples has been the identification of a novel allele ($waxy_{E1100}$) in the *granule-bound starch synthase I* gene (waxy) from seed EMS mutagenesis. The novel allele was introgressed into elite breeding lines lacking granule-bound starch synthase I protein activity to produce genotypes with high-amylopectin starch (Muth et al. 2008).

Mutation screening based on heritable phenotypes remains useful and also essential for performance testing of potential cultivars, and this does not require genetic information. It is a useful and traditional method (Oladosu et al. 2016) and

this has been the traditional selection method of breeders for decades. However, the specific mutation(s) mediating the phenotype may remain undiscovered. Therefore, screening a mutagenized population by phenotype should be followed by characterization of the molecular event underlying the modified character if possible (Sikora et al. 2011, see below for methods).

Several methods have been developed to identify a mutation in a gene of interest, but all have their limitations. Techniques rely on mobility change during gel electrophoresis of DNA, such as single-strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE), these provide a rapid screening method but they do not show the location nor the type of variation in the DNA fragment (Gilchrist et al. 2006). Methods that are based on polymerase chain reaction (PCR) require the knowledge of the target DNA sequence and only work for small fragments of DNA. Array hybridization techniques are efficient but require a large amount of genetic material and have low sensitivity (discovering approximately 50% of the single nucleotide polymorphism (SNPs)). Conventional sequencing (Sanger) is the most accurate approach for candidate genes from multiple genotypes; however, efforts and cost for detecting multiple loci in large numbers of individuals are relatively high (Gilchrist et al. 2006).

A number of rapid and sensitive reverse genetic screening methods, like denaturing high-pressure liquid chromatography (dHPLC), a post PCR method such as high-resolution melting (HRM), and single strand-specific endonucleases like CEL I, have been used for mutation detection (Shu et al. 2012a, b). However, changes in DNA base modification after the initial screening for the HRM and CEL I methods are required and normally confirmed by re-sequencing (Szurman-Zubrzycka et al. 2017).

TILLING (Targeting Induced Local Lesions in Genomes) as a reverse genetic technique holds much potential for examining natural and induced variation and bridging the phenotype-genotype gap (Gilchrist and Haughn 2005; Till et al. 2003; Comai et al. 2004). Many of the species for which complete genome sequence data are available, including the tree crops such as papaya, cocoa, peach, apple, avocado and oil palm *are* amenable to classical mutagenesis and genetic analysis. For these organisms, identifying natural genetic variants can provide much information about gene function and can also be useful for association mapping and linkage disequilibrium analysis. TILLING techniques are able to detect small deletions, insertions, and microsatellite (SSR) polymorphisms in addition to single-bp changes in the DNA sequence. New methods were introduced by Albert et al. (2007), who enriched genomic loci for NGS using Roche NimbleGen oligonucleotide arrays, and Hodges et al. (2007), who applied the arrays to capture the full human exome. Since then, methods requiring less hands-on work and smaller amounts of input DNA have been developed (Sulonen et al. 2011). A solution-based oligonucleotide hybridization and capture method based on Agilent's biotinylated RNA baits was described by Gnirke et al. (2009) and developed further (Hoischen et al. 2010; Harbour et al. 2010). These methods were used in soybean (Haun et al. 2011; Bolon et al. 2011) to find the genomic variation among individuals of reference cultivar Williams 82 and of a Fast Neutron mutant population, respectively. To characterize large genomes like wheat

(Winfield et al. 2012) and pine (Neves et al. 2013; Henry et al. 2014; Hussain et al. 2018) exome capture sequencing (ECS) helped to identify a large number of novel SNPs. In general, most of the functional variation resides in the coding regions of the genome. Thus, it is logical, to begin with, exons for the discovery of the causative genetic variants of important traits (Cosart et al. 2011; Bamshad et al. 2011).

Significant efforts towards large-scale characterization, screening, identification, and discovery of SNPs-mutants and short InDels were made with success in crop plants using TILLING combined with Next Generation Sequencing (NGS) (Gupta et al. 2017; Tsai et al. 2011). Fortunately, recent advances in genomic technology and computational methods make it possible to identify the genetic basis of variation using genome-wide approaches more cost-effectively (Hillier et al. 2008; Ng et al. 2009), such as WG (whole-genome) sequencing (Hillier et al. 2008), RNA-seq (Hansey et al. 2012), methylated DNA (Brunner et al. 2009), genotyping-by-sequencing (GBS) (Huang et al. 2009), and restriction-site associated DNA (RAD) sequencing (seq) (Baird et al. 2008). Compared to WG sequencing, the approaches mentioned above are more cost-efficient to provide genetic information through: (a) minimizing representation of uninformative genomic regions and (b) enabling efficient sample pooling strategies (Beissinger et al. 2013; Schlötterer et al. 2014). While RAD-seq involves sequencing DNA fragments with high coverage, GBS's focus is to interrogate DNA sequences with low target coverage and minimize reads in repetitive sequences (Beissinger et al. 2013). Both RAD-seq and GBS identify genetic markers that are usually not functionally relevant (Maghuly et al. 2018). Since RNA-seq involves the sequencing of mRNA, it targets only expressed transcripts. ECS focuses on sequencing and analyses of the genomic regions most likely to be functionally relevant (Schlötterer et al. 2014). However, identifying specific genome modification demands further reference genome, which is commonly not given (Grohmann et al. 2019).

In recent years, due to rapid advancement in NGS and bioinformatic tool kits, several novel mutation mapping strategies such as a forward genetic systems for pinpointing causal mutations have been developed including, mutational mapping (MutMap), MutMap-Gap, and mutant chromosome sequencing (MutChromSeq) (Periyannan 2018; Kumawat et al. 2019). They provide enormous information about the function of most of the unknown genes. While the MutMap method is suitable for identifying a causative mutation in the plants with small genomes with available high-quality reference genome sequences, the MutMap-gap method is helpful in the absence of a reference genome sequence, where gaps or rearrangement at the targeted sequence will be filled by de novo assembly. In plants with complex genomes or high ploidy levels, DArTseq (Diversity Arrays Technology) and MutChromSeq provide better alternatives to detect mutations (Baloch et al. 2017; Periyannan 2018). These techniques reduce genome complexity and facilitate rapid gene isolation by a combination of chromosome purification with mutagenesis. Although MutChromSeq is a powerful method for large complex genomes, it requires preliminary knowledge of targeted genes' map position on the chromosome and a reference genome.

All the methods mentioned above rely on short-reads, which are cost-effective, provide accurate data, and are supported by various bioinformatic tools; however, this platform is not suitable for long insertion sequence and structural variation detection. In this case, long reads of third- and fourth-generation sequencing can offer more accurate genome assemblies, detect structural variations, and preserve base modification by eliminating amplification bias in the case of native DNA and RNA. But the error rate of long-read sequencing platforms is high. Therefore, a combination of short- and long-read sequencing can overcome the weakness of both techniques (Grohmann et al. 2019; Zhao et al. 2019; Amarasinghe et al. 2020).

As stated above, it is recommended that mutants detected via phenotypic screening should be investigated to discover their underlying genetic modification. This is not simply for academic understanding but is of practical significance. Genetic markers can be developed from the identified mutational event, and these can be used in selection in subsequent cycles of plant breeding (marker-assisted selection). Although field trialling remains an essential stage in the development of new cultivars, the number of lines that are advanced to this stage can be focused on those carrying elite (proven) genetic backgrounds (defensive breeding) and which carry new mutant alleles (targeted breeding) to meet new market demands, and for this there is an increasing emphasis on breeding by genotype.

3.7 Impacts of Plant Mutation Breeding in Crop Plant Improvement

A major objective of mutation induction has been to improve well-adapted plant cultivars/elite lines with a deficiency in one or two traits, either directly or through the production of pre-breeding materials. Mutation has resulted in new and now established ideotypes, e.g. semi-dwarfs for mechanical harvesting, and such mutations are now present in many crops. The original mutant cultivar is termed a foundation cultivar (Mlcochova et al. 2004; Rutger 2009). Significant mutant traits include yield, plant morphotype (growth habit, semi-dwarf), flowering time, abiotic and biotic resistance, physiological traits, nutritional value, or processing and sexual reproduction (autogamy or allogamy) mutants (Konzak et al. 1984; Bado et al. 2015; Oladosu et al. 2016).

Food security has received particular attention from the joint FAO/IAEA division of the United Nations which has provided services, training and capacity building in plant mutation breeding and genetics for member states. There have been successes in a range of crops: cereals (>47% of induced mutant cultivars), ornamentals and flowers (>21%), legumes and pulses (>13%), oil crops (>3%) and others including medicinal plants, fodder crops, (>13%). Achievements can also be classed by mutagen treatments. Single physical treatments account for 77.51% of mutant cultivars, single chemical treatments for 11.53%, combined mutagens (1.11%) and somaclonal variations (spontaneous mutation, about 1%) (Fig. 3.8).

Mutation induction by physical and chemical mutagens has resulted in the widening of genetic variability in over 220 plant species (Bado et al. 2015,

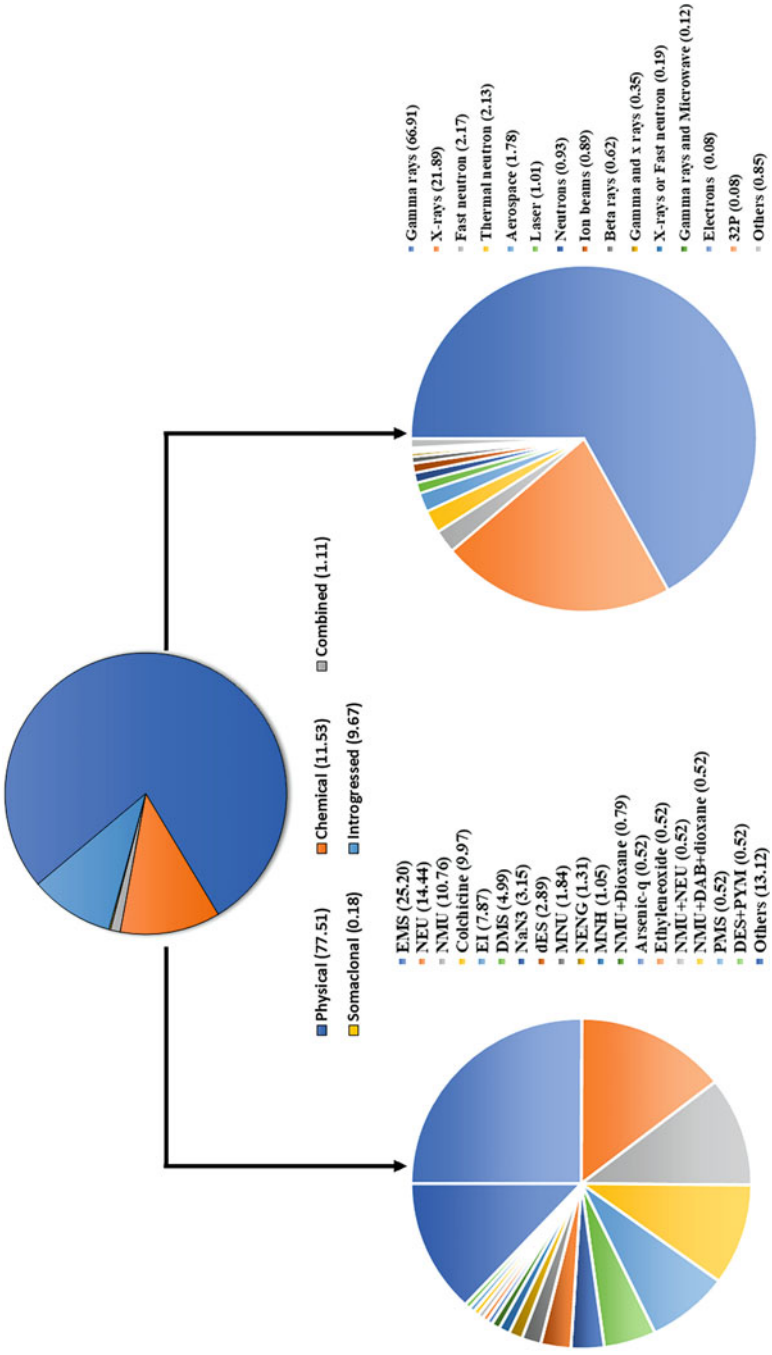

Fig. 3.8 Distribution of mutant cultivars as a percentage of the total number if mutant cultivars (From IAEA data base, accessed July 2020)

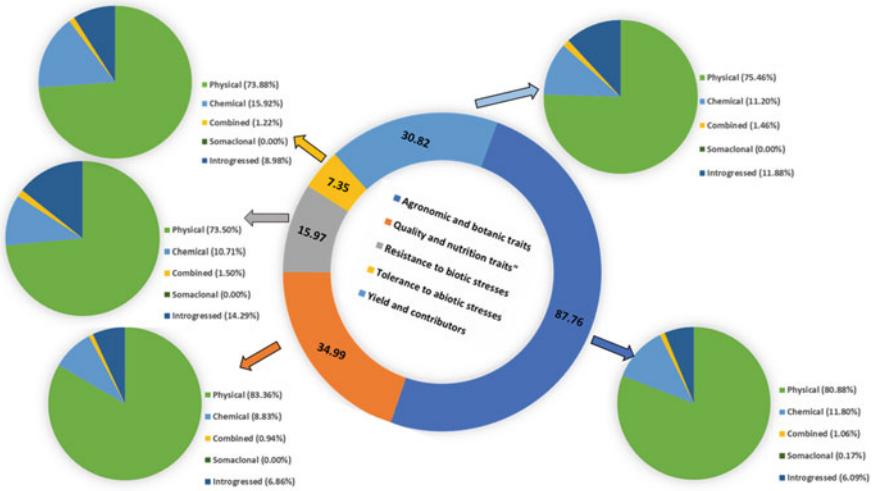

Fig. 3.9 Impact of physical and chemical mutagens (single and combination treatments) in inducing important crop traits (From IAEA data base, accessed July 2020)

Fig. 3.9). Agronomic and botanic traits modified include yield, quality, morphotype, life cycle, seed shape and size, seedling vigour, high biomass, flower size, shape and colour, fruit morphology and anatomy, shattering, early flowering and maturity, late maturity, winter hardiness, resistance/tolerance to abiotic stressors, wide adaptability, improved taste, elimination of toxins, etc. Physical mutagens have been the most effect in producing new traits, followed by chemical mutagens, combined treatments and finally somaclonal variation (Fig. 3.9). Improved mutant cultivars have a presence worldwide with over 60%, 28%, 7%, 2% and about 1% in Asia, Europe, America, Africa and Australia and Pacific continents, respectively. These new cultivars have contributed to wealth at all levels, from producer, to farmer, to the end user and nationally (see reviews from Kume et al. 2002; Ahloowalia et al. 2004; Bado et al. 2015, 2017; Oladosu et al. 2016).

3.8 Future Outlook of Plant Mutation Breeding

Plant production worldwide has led to narrow bottleneck in genetic variation among cultivars and breeding lines. This low genetic diversity represents a severe limitation to crop improvement and has led to extensive gene capture expeditions to centres of natural genetic variation among landraces and wild species. Various consortia (national and international) have been set up in germplasm collection of various crops, but the further a breeder has to go genetically to find a trait/allele of interest the more breeding effort is required. Moreover, countries housing wild germplasm are becoming increasingly protective about their natural resources and strict treaties have been signed in this respect (Moore et al. 2007).

Mutation induction is an attractive alternative to broadening genetic variability in elite lines as it permits the improvement of plant genotypes quickly (Roychowdhury and Tah 2013). This chapter as focused on the induction of mutations for plant breeding using physical and chemical mutagens. These approaches are relatively fast, cheap and simple and have served plant breeding well and will continue to do so for some considerable time. New methods in mutation detection, via genotyping, are set to have an impact not only on more rapid and specific mutation detection, but also application to wider groups of plants, notably perennial crops which have lagged behind annual crops especially in genotypic mutation selection. There are also exciting developments in mutation induction via gene editing, e.g. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas) (Deltcheva et al. 2011; Jinek et al. 2012) for which the inventors Emmanuelle Charpentier and Jennifer A. Doudna won the 2020 Nobel Prize for Chemistry (Wu et al. 2020). Advantages of plant mutation breeding include: (1) use of any type of plant material (Fig. 3.5) (2) can induce novel alleles in a gene and does not require knowledge of the gene controlling the trait, which is prerequisite when considering other methods; and (3) plant mutation breeding is an accelerated form of breeding (e.g. especially relevant to perennial crops). However, typical of innovation, new methods are normally initially expensive and require specialized facilities.

3.9 Conclusion

Traditional plant mutation breeding has had significant impacts worldwide. The effectiveness of physical and chemical mutagenesis has increased through enabling technologies, particularly tissue culture for vegetatively propagated plants and mutation detection and selection via high-throughput phenotyping and genotyping. Thus, more plant species can benefit from these approaches. Considerations for plant mutation breeding are:

1. Mutation induction: Greater use of locally accessible mutagen facilities. The resurgence of X-ray irradiation for mutation induction as an alternative to gamma irradiation. Less toxic and more environmentally friendly chemical mutagens will improve the diversity in the choice of mutagens by breeders and researchers.
2. Plant propagule: The increasing availability of improved methods in cell, tissue and organ culture of crop species which are not genotype dependent.
3. Selection: High-throughput phenotyping and genotyping and the development of marker-assisted selection. In vitro selection, especially for vegetatively propagated crops.

Mutation breeding is and remains a relevant and practical breeding option. Today, a major challenge for breeders is to respond rapidly to effects of climate change, which is bringing new diseases and pests, and changing environmental conditions. In general, induced mutation and its exploitation in plant breeding aims to achieve

resilient crops with improved quantitative and qualitative traits within a short time. Due to its relative simplicity, flexibility, practicability, low cost, long safety record and universal acceptance, chemical and physical mutagens continue to provide solutions for plant breeding objectives.

Although physical and chemical mutagenesis have been used in many crop species, they still face challenges, preliminary in terms of (1) precision and efficiency, (2) high-throughput of whole-genome functional screening of modified genes or DNA sequences, (3) high-throughput phenotypic screening of a large population (accessible facilities), and (4) effective tissue culture systems for perennial crop species. Various new approaches are still needed to overcome the challenges of today and tomorrow to enhance mutation induction and mutation detection in generating desired effects. Physical and chemical mutagenesis remains an effective low-cost weapon in the armoury of plant breeders, and methods continue to improve.

Dedication The authors would like to dedicate this chapter to Udda Lundqvist who died recently. Udda Lundqvist was a pioneer in plant mutation genetics, especially in barley, which became a model crop for mutation studies. She inspired many and will be greatly missed.

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