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Applications of Ionizing Radiation in Mutation Breeding

Özge Çelik and Çimen Atak

Additional information is available at the end of the chapter

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Abstract

As a predicted result of increasing population worldwide, improvements in the breeding strategies in agriculture are valued as mandatory. The natural resources are limited, and due to the natural disasters like sudden and severe abiotic stress factors, excessive floods, etc., the production capacities are changed per year. In contrast, the yield potential should be significantly increased to cope with this problem. Despite rich genetic diversity, manipulation of the cultivars through alternative techniques such as mutation breeding becomes important. Radiation is proven as an effective method as a unique method to increase the genetic variability of the species. Gamma radiation is the most preferred physical mutagen by plant breeders. Several mutant varieties have been successfully introduced into commercial production by this method. Combinational use of *in vitro* tissue culture and mutation breeding methods makes a significant contribution to improve new crops. Large populations and the target mutations can be easily screened and identified by new methods. Marker assisted selection and advanced techniques such as microarray, next generation sequencing methods to detect a specific mutant in a large population will help to the plant breeders to use ionizing radiation efficiently in breeding programs.

Keywords: mutation breeding, *in vitro* mutagenesis, gamma rays, molecular markers, high-throughput technologies

1. Introduction

The worldwide population is expected to be nine billion at 2050. Conventional agricultural crops are inadequate to meet the current need to provide sustainable yield production. Therefore, crop improvement is getting an important need when we are not able to meet the demands of growing world population. For this reason, humans have begun to develop new

plant varieties for cultivation, and it is called as plant breeding. Numerous food, feed, and ornamental and industrial crops were improved via hybridization methods to meet the needs of human beings since many years. Over the last 15 years, development of new techniques became useful in breeding strategies to facilitate the improvement of new crop varieties.

Plant breeding methods and recent progress in biotechnology contribute greatly to friendly agriculture. The main point is to establish productive breeding strategies to improve crops.

Variation is the main point of the breeding that the plant breeders are focused. Genetic variation is a natural phenomenon. This variation is a natural result of genotypes, which have interactions with the environmental facts, get together. The recombination and independent assortment of the alleles are responsible to obtain new individuals from the population. Domestication of the crops is affected by several conditions such as ecological and agricultural. Selection of the adaptive genotypes is getting important in breeding of the cultivars. The main point is to achieve the production of higher-yielding crops [1], useful traits such as size of the fruits, and quality of the crops. The aim of the breeding is to combine various features of many plants in one plant. This method is general for breeding of the plants via sexual reproduction. During recombination of the alleles, offsprings carrying selectable variations for the several traits exist. Recombination is not responsible to produce new traits itself. Although genetic changes have provided the natural variation for species evolution, changes in species have not only been important for adaptation to natural environment. Mutations are the main reasons of genetic variabilities and cause new species eventually. Therefore, they have also been exploited by man in the agricultural processes of species domestication and crop improvement. As a new approach, manipulation of the cultivars through alternative techniques such as mutation breeding and Biotechnology are useful for especially some fully sterile plants [2].

Mutations have been shown as a way of procreating variations in a variety. They spontaneously occur in nature. Several mistakes can cause mutations during replication process. On the other hand, radiation is an efficient mutagen that the plants are exposed. The important point is the origin of the mutated cell. Somatic cell mutations are not easily traceable and cannot pass to the future generations; otherwise, embryonic cell mutations directly pass to the next springs. Spontaneous mutations occur without any human intervention and happen randomly with a low frequency. However, some mutagenic agents are known to induce mutations as an alternative to low incidences of spontaneous mutations to increase genetic variability by increasing the frequency of mutations. Using of mutagens propose the possibility of inducing desired characters that cannot be found in nature, in a variety or lost during the evolution [2].

A mutation is defined as any change within the genome of an organism, and it is not brought on by normal recombination and segregation [3]. The direct use of mutation is a very valuable supplementary approach to plant breeding. The main advantage of this technique is the shorter time required to breed a crop with improved character(s) than the hybridization process to obtain the same results.

Induced mutations consequently have a high potential for bringing about further genetic improvements. Induced mutations have played a significant role in meeting challenges related

to world food and nutritional security by way of mutant germplasm enhancement and their utilization for the development of new mutant varieties. A wide range of genetic variability has been induced by mutagenic treatments for use in plant breeding and crop improvement programs [1]. Physical mutagens are generally preferred by reason of being convenient, easily reproducibility, and user-/environment-friendly method. Ionizing radiation is used as a physical mutagen in breeding applications.

2. Types of ionizing radiation

Ionizing radiation (IR) is categorized by the nature of the particles or electromagnetic waves that create the ionizing effect. These have different ionization mechanisms and may be grouped as directly or indirectly ionizing. The physical properties of ionizing radiation types, namely gamma rays X-rays, UV light, alpha-particles, beta-particles, and neutrons, are different; therefore, their potential usage and bioapplicability to the breeding programs are different.

In the beginning of the twentieth century, ionizing radiation has been begun to induce the mutations. They can be particulate or electromagnetic (EM). Their specific feature is the localized release of large amounts of energy. These have different ionization mechanisms, and they can group as directly or indirectly ionizing. The physical and chemical reactions initiate the biological effects of ionizing radiations [4].

Mostly, X-rays had been used, and later gamma rays and neutrons have been preferred. Two forms of electromagnetic radiation, X-rays or gamma (γ) rays, are widely used in biological systems and most clinical applications. Cobalt-60 and cesium-137 (Cs-137) are the main sources of gamma rays used in biological studies. Cesium-137 is more preferred since its half-life is much longer than cobalt-60. Gamma rays are produced spontaneously, whereas X-rays are produced in an X-ray tube (accelerated electrons hit a tungsten target, and then they are decelerated. The Bremsstrahlung radiation is part of the kinetic energy, belongs to the electrons, and is converted to X-rays). Energy transfer is caused by the interaction, it cannot completely displace an electron, and it produces an excited molecule/atom; whenever the energy of a particle or photon exceeds the ionization grade of a molecule, ionization occurs. Ten electronvolt binding energy for the electrons is determined for biological materials, and higher energetic photons are considered as ionizing radiation, whereas the energies between 2 and 10 eV, which cause excitation, are called as nonionizing. Electrons, protons, α -particles, neutrons, and heavy charged ions are clinically used natural radiation types [4–6].

2.1. Effects of ionizing radiations

Ionizing radiation (IR) is known to effect on plants. Their effects are classified as direct and indirect. Stimulatory, intermediate, and detrimental effects on plant growth and development are based on dose of ionizing radiation applied to the plant tissues. The main point is to evaluate the impacts of ionizing radiation at genetic level. The severity of the impacts of radiation is in relation with the species, cultivars, plant age, physiology, and morphology of the plants besides their genetic organizations.

Ionizing radiation causes structural and functional changes in DNA molecule, which have roles in cellular and systemic levels. The nature of DNA modifications includes base alterations, base substitutions, base deletions, and chromosomal aberrations. These modifications are the reasons of macroscopic phenotyping variations [5, 6].

Interaction between atoms or molecules and ionizing radiations causes free radical production that damages the cells. Free radical is defined as an atom or group of atoms including an unpaired electron. Water in the cell accumulates energy initially and facilitates the production of reactive radicals, which oxidize and reduce. They have a role in direct and indirect actions of ionizing radiations. In direct action, a secondary electron reacts directly with the target to produce an effect, while in indirect action, free radicals produced via radiolysis of water interact with the target to comprise target radicals [6, 7].

There are substantial data indicating that the lethal effects of radioactive compounds accumulate in nucleus rather than other parts. Therefore, DNA is the main target as a result of ionizing radiation, and it targets DNA directly or indirectly and leads various alterations. Direct ionization of DNA, reactions with electrons or solvated electrons, reactions with OH or H_2O^+ , and reactions with other radicals can damage cellular DNA. There are some possibilities of DNA damages caused by ionizing radiation. IR and secondarily produced reactive oxygen species can cause changes in deoxyribose ring and structures of bases, DNA-DNA cross-links, and DNA-protein cross-links. Hydroxyl radicals react with bases. The reactive intermediates are produced as a result of this interaction [7, 8].

Hydroxyl radicals separate hydrogen atoms from the sugar-phosphate backbone of DNA to form 2-deoxyribose radical, which cause strong damages via attacking to oxygen or thiol groups [8]. Researchers have shown that purine and pyrimidine rings, single-strand breaks (SSBs), and base loss regions are damaged by DNA radiolysis products induced by free radicals. The amount of the yield of the individual products is important and reported to be different than produced during oxidative metabolism. Although free radicals attack on DNA and cause several DNA damages, they have not been thought to lead lethal and mutagenic results. Ionizing radiation-induced base damages are widely studied by *in vitro* studies. It is also reported by several studies that direct and indirect radiation effects may produce identical reactive intermediates. Oxygen is another key molecule that determines the biological effectiveness of the ionizing radiation. Oxygen can easily react with many free radicals. The amount of the radicals presents in deoxyribose or bases; harmful DNA damages occur [9–11].

If the damage site is deoxyribose, a strand brake directly forms. DNA base damages like ring saturation destabilize the N-glycosidic bonds, and abasic deoxyribose residues form. These regions can be converted into strand breaks. Double-strand breaks (DSBs) happen as a result of a localized attack by two or more OH radicals on DNA. Another potential reason can be defined as a hybrid attack that OH damages one of the strands, whereas the other strand exposes to a direct damage within 10 base pairs of the hydroxyl radical [12]. IR leads chromosomal aberrations during cell division. Chromosome malsegregation and defects in chromatid separation, bridge formation, chromosome exchange, chromosome breakage, and loss of chromosome fragments can be observed after IR treatment [13].

We can classify IR as an abiotic stress factor; therefore, the plants represent different levels of adaptive responses. DNA repair mechanisms and adaptive responses against radiation could protect the plant genome from excessive modifications. Natural ionizing radiation is supposed to play a significant role in the evolution of the plants. Homolog recombinations between the chromosomes would result in formation of new altered generations that show specific adaptive capabilities [13].

3. Mutation breeding

In nature, mutations acquired new survival traits to the crops against environmental stresses both biotic and abiotic. Many of these survival traits could be weakened or totally lost in time. Mutations are sudden changes at the genotype level and cause small and exquisite changes in phenotype, which cannot be detected by advanced molecular techniques. Identification of naturally mutated gene is inconvenient. When the breeders pinpoint the mutated gene, wild-type features have to be reestablished. This task is becoming increasingly infeasible due to long time, more human source, and increase in cost. That's why new breeding strategies were needed to be improved to fortify the crops. To achieve this mission, plant breeders should rebuild in crop plants several specific traits, which have role in survival of the plants under extreme conditions providing the other crop-specific traits such as quality, yield, etc. Phenotyping-based processes of conventional breeding strategies should have moved from base to a high level of genotype-based breeding methods [1, 14]. New technologies should be legal, economic, and ethical for the breeders and the consumers.

Under such circumstances, inducing mutations are potential applications to produce crops with desired traits and easily selected from the germplasm pool. As described above, radiation can cause several effects on genetic material due to the exposure dose. These effects can be classified in both positive and negative approaches. Beside the detrimental effects of radiation, plant breeders are focused on the effective usage of gamma radiation in breeding programs. Changes in agronomic characters can be transmitted to the next generations. Nuclear techniques are begun to be used in plant breeding mostly for inducing mutations. During the past 60 years, we observed a significant increase in the major crops. Ionizing radiations such as X-rays and gamma rays have been used for improvement of several crops such as wheat, rice, barley, cotton, tobacco, beans, etc. [15]. Plant breeders are also combined with this resource with different techniques to increase the efficiency and shorten the time. Induced mutagenesis and combined breeding strategies are effective to improve quantitative and qualitative traits in crops in a much shorter time than the conventional breeding procedure [6].

Gamma radiation is widely used to induce mutations in breeding studies than chemical mutagens. Ionizing radiation could cause several DNA damages randomly; therefore, several mutations (from point mutation to chromosome aberrations) could be induced. Over 3000 mutant varieties of major crops have been reported to be developed by ionizing radiation [2, 16].

Mutation rate/mutation frequency is defined as the ratio of mutation per locus and also termed as the number of mutant plant per M_2 generation [16]. It changes due to per dose and muta-

gen. The main point is to determine the best dose for inducing mutants rather than its type. From past to present, it is concluded that the doses between LD50 and LD30 (doses lead to 50% and 30% lethality) are generally useful in mutation breeding programs. The importance of convenient dose that depends on the radiation intensity and exposure time is gestured by the researchers [6].

The final target is to select the desired mutants in the second and third generations (M_2 and M_3). It is effective to select the mutants treated by the mutagens with a high mutation frequency from the M_1 population. M_1 population consists of heterozygous plants. That means during the treatment one allele is affected by the mutation, and it is impossible to discriminate the recessive mutation in this generation. Therefore, the breeders should sift out the next generations to identify the homozygotes for both dominant and recessive alleles [6]. M_2 population is the first generation that the selection begins. Physical, mechanical, phenotypic, and other methods are used for the selection of the mutants. When the plant breeder finds a mutant line, the next step is the multiplication of the seeds for further field and other studies. The main theme is to develop a mutant, which has a potential to be commercial variety surpassing the mother cultivar or a new genetic stock having improved properties.

According to the 2015 data of Food and Agriculture Organization/International Atomic Energy Agency (FAO/IAEA), over 232 different crops including wheat, rice, sunflower, soybean, tomato, and tobacco were subjected to mutation breeding programs and over 3000 mutant varieties with improved properties in over 70 countries [6]. The mutant plant production distribution worldwide is given in **Figure 1**.

Sixty-one percent of these varieties was improved by using gamma radiation. **Figure 2** represents the maximum plant species improved via mutation breeding.

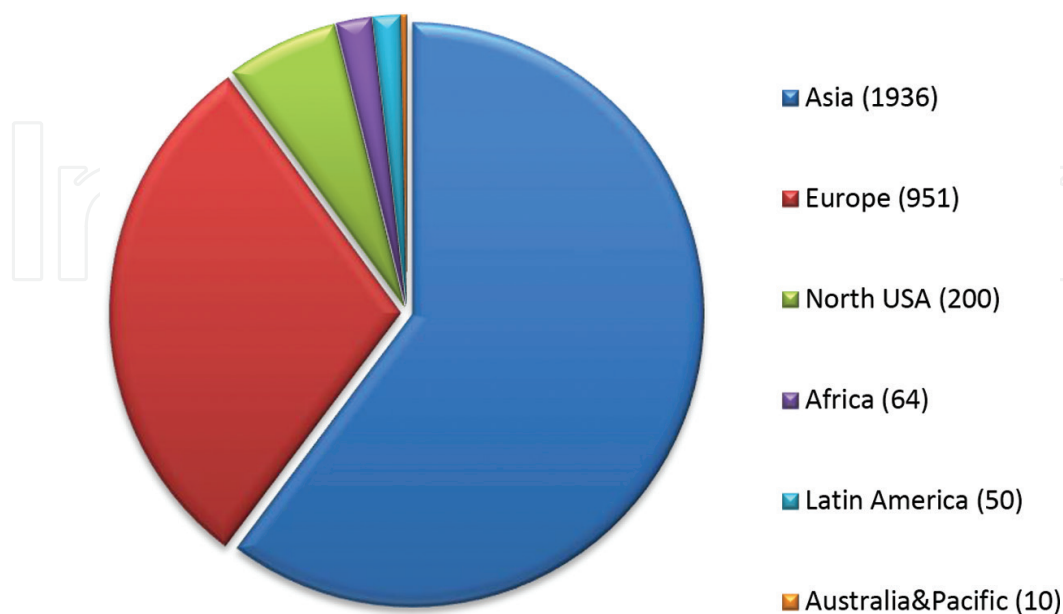


Figure 1. The number and the rate of the mutant cultivar production rate worldwide [17].

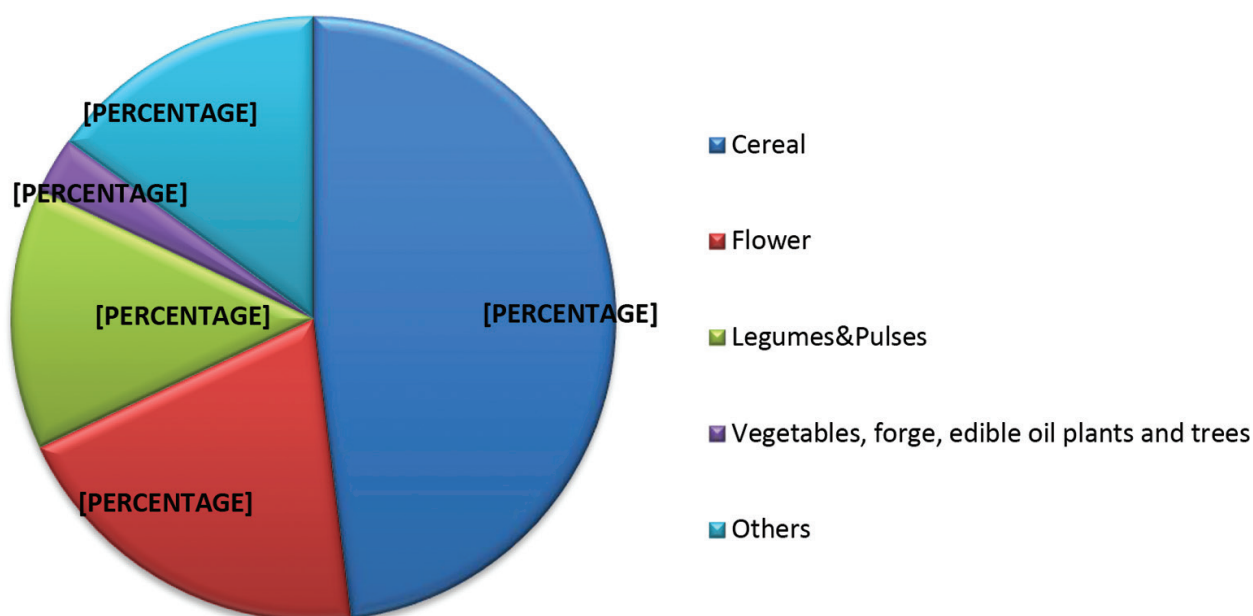


Figure 2. The maximum plant species improved via mutation breeding [17].

Mutation breeding studies are widely preferred to improve cultivars tolerant or resistant to various abiotic stresses and biotic stresses such as bacteria, viruses, and pathogens and to improve the quality and the agricultural traits of the crops such as oil, protein, and yield [6]. The most improved features in some plant species by gamma radiation were given in **Table 1**.

Instead of waiting for natural mutations to generate a desired trait, creating a mutation with different tools may promote to the breeding studies. The simplicity and low cost of mutation treatments and gamma radiation became an effective tool to improve new agronomic traits in various crops. It may be evaluated as an alternative to genetically modified plants. The released mutation breeding-derived varieties showed the potential usage of mutation breeding as a flexible and available accession to any crop supplied for desired purposes, and discriminating techniques are successfully combined.

As mentioned above, mutation breeding studies are provided to numerous researches in terms of developing applications for plant biotechnology, plant tissue culture, and mutation treatments to improve new cultivars. Therefore, research and developmental studies are widely associated to combined techniques including in vitro culture and molecular techniques through mutation breeding. In vitro mutagenesis applications are becoming important at this point.

3.1. In vitro mutagenesis applications

Induced mutagenesis is a widely used method to identify and isolate the plant genes in combination with molecular accessions. These kinds of studies supply a clear prehension into the relation of genes and functions of the genes that have role in growth and development under several conditions [12].

Crops	Improved traits
Apple	Early maturing, red fruit skin color, variegated leaf, dwarf, compact tree, resistance to powdery mildew and apple scab
Apricot	Earliness
Banana	Earliness, bunch size, reduced height, tolerance to <i>Fusarium oxysporum</i> f. sp. cubense, large fruit size, putative mutant resistant to black sigatoka disease
Barley	Phytate (antinutrient)
Canola	Oil quality improvement
Chickpea	Resistant to Ascochyta blight and Fusarium wilt
Citrus	Seedless, red color fruit, <i>Xanthomonas citri</i> disease resistant, resistant to tristeza virus
Cotton	Resistant to bacterial blight, cotton leaf curl virus, high fiber
Indian jujube	Fruit morphology, earliness
Loquat	Fruit size
Maize	Resistant to pathogen <i>Striga</i> , acidity, and drought tolerance; improvement of protein quality
Mung bean	Resistant to yellow mosaic virus
Pineapple	Spineless, drought tolerance
Tomato	Resistant to bacterial wilt (<i>Ralstonia solanacearum</i>)
Rapeseed	Resistant to powdery mild
Rice	Resistant to blast, yellow mottle virus, bacterial leaf blight and bacterial leaf stripe, semidwarf/dwarf cultivar, lodging resistance, acid sulfate soil tolerance; tolerant to cold and high altitudes, salinity tolerance, early maturity, high-resistant starch in rice for diabetes patients, giant embryos of eight more plant oil, low amylose, low protein)
Soybean	Resistant to Myrothecium leaf spot and yellow mosaic virus, oil quality improvement, oilseed meals that are low in phytic acid desirability, poultry and swine feed
Strawberry	Thick and small leaf, light leaf color, white flesh and long fruit, <i>Phytophthora cactorum</i> resistance Salinity tolerance
Sunflower	Oil quality improvement, semidwarf/dwarf cultivars
Wheat	Resistant to stripe rust

Table 1. Applications of induced mutagenesis for improved features in plant breeding [6, 17].

In vitro culture methods appear to have opportunities to display the useful variants. The recent improvements on in vitro technology acquired an importance to enlarge the aim of mutation breeding applications. The use in conjunction of in vitro tissue culture and mutation breeding methods makes a significant contribution to improve new crops and new varieties. Jain [18] reported the importance of this technique for ornamental plants beside the crops.

It is known that genetic variabilities may occur during in vitro culture conditions without any application of mutagens, spontaneously. The frequency of the variants still indeterminable,

and there are many parameters to depend on. Application of the mutagens can increase the rate of genetic variability via inducing the frequency of the mutations.

The progress in recombinant DNA technologies and genes can be easily cloned from a genome into a genome of an organism. Genes can be purified in vitro in small amounts, and therefore the potential of inducing mutations has significantly broadened. In a controlled experimental environment, it is available to change the sequence of the nucleotides of DNA. In vitro mutagenesis studies systemically and efficiently focus on the potential ways of inducing mutagenesis. Some applications of mutagenesis depend on using isolated DNA molecule. In contrast to conventional mutagenesis, in vitro mutation breeding can be thought as a practicable and achievable technique to improve new genetic variabilities. Only few traits can be modified, and the remaining is not altered by the treatment.

In vitro mutagenesis have some properties such as increased mutation rate, uniform mutagen treatment, needs of less space and time for large populations, and opportunity to keep the plant material disease-free. On the other hand, one of the main restrictions of mutation breeding application is the formation of chimeras as a result of the treatment. At this point, mutant selection process is becoming important.

In vitro culture methods are more useful in mutation studies. Totipotency is a natural feature of the plant cells. By using one plant cell, it is possible to produce a whole plant, induce regeneration of the tissues and micropropagation of the plants in large volumes, and give opportunity to use different parts of the plants (stem, leaves, cuttings, apical and axillary buds, and tubers) to induce mutagenesis easily. Another advantage of in vitro culture is to screen the populations after mutagenesis to select the variant/mutants before giving a whole plant. Different plant tissues can be propagated to produce different tissues by using several combinations of plant growth regulators. Callus is an important cell organization. Cell suspension culture technique is started by using callus tissue to separate the single totipotent cells. Every plant cell can be differentiated into somatic embryos which is a useful tool for mutagenesis [4, 9].

The target of the studies is to isolate the non-chimeric mutants from the irradiated explants to obtain desired mutants via repetitive selection processes. Meanwhile, duration of the culture and the selective traits that mutated are the main factors effect these processes [4]. M_2 generation of the culture is the earliest step that the predominantly recessive mutants could be determined. Mutagen treatment can be applied at different stages of the cultures.

3.1.1. Selection of species and explant types for in vitro culture

Correct choice of the plant species due to economic, commercial production capacity and agricultural importance is the first step of an in vitro mutagenesis study. The selection of the plant material is related to the success of the in vitro culture. Seed, callus, node, shoot, and root tip cultures are the most commonly preferred plant material for in vitro mutagenesis applications. The genotype of a plant has a role in in vitro culture studies. The studies showed that different explants of same plant had different responses to the same radiation dose [19, 20]. Therefore, it is necessary to design an in vitro mutagenesis experiment in a proper combination of dose and explant type.

3.1.2. Determination of proper gamma radiation dose

The most important subject of in vitro mutagenesis is to select the suitable radiation dose to obtain the maximum viability. In the beginning, assessment of the LD₅₀ value is needed to optimize the exact mutation dose. The sensitivity of the plants changes due to the species, cultivars, and current physiological environment. A preliminary dose experiment should be performed to define the appropriate dose. Reduced growth and seedling damages may be seen as traces of the genetically damaged plants after irradiation [21]. IAEA/FAO reported the average doses for crop species and summarized in **Table 2**.

According to the findings of the preliminary studies done with gamma radiation, it has been reported that there is no linearity between the radiation dose and the variance. The experimental gamma radiation treatments were summarized in **Table 3**.

Seed, callus, shoot tips, node cultures, and bulblets were frequently used for irradiation of different species. ¹³⁷Cs and ⁶⁰Co gamma sources were used to induce mutagenesis at different doses depending on the radiosensitivity of the explants. Atak et al. [24] used 100–500 Gy radiation doses produced by ¹³⁷Cs gamma source for soybean seeds, while Singh and Datta [29] used ⁶⁰Co gamma source at different doses ranging between 10 and 100 Gy for *Triticum aestivum* seeds. Ulukapı et al. [21] also used ⁶⁰Co gamma source at 80–240 Gy radiation doses to induce genetic variability for *Solanum melongena* L. Çelik and Atak [23] used 100, 200, 300, and 400 Gy gamma rays by ¹³⁷Cs to determine the effective radiation dose for breeding studies of two Turkish tobacco varieties. They irradiated the tobacco seeds and selected the salt-tolerant mutants in M₃ progeny.

Seetohul et al. [35] used 0–60 Gy gamma doses of ⁶⁰Co gamma source to induce mutations for shoot tip explants of Taro plant. Jain [26] irradiated shoot tip explants of *Musa* spp. by Cesium-137 at 10–50 Gy doses, while Baraka and El-Sammak [33] used 0.25–1 Gy for *Gypsophila paniculata* L. shoot tip explants by ⁶⁰Co gamma source. Atak et al. [25] used shoot tip explants of *Rhododendron* varieties to induce mutants at 5–50 Gy of gamma rays of ¹³⁷Cs source.

In tissue culture treatments, different synthetic chemicals show similar effects as plant growth regulators which have abilities to induce growth of the tissues as desired. In mutation-based

Species	Useful mutation breeding dose (gray)
<i>Oryza sativa japonica</i>	120–250
<i>Oryza sativa indica</i>	150–250
<i>Triticum aestivum</i>	40–70
<i>Hordeum vulgare</i>	30–60
<i>Glycine max</i>	100–200
<i>Phaseolus vulgaris</i>	80–150
<i>Nicotiana tabacum</i>	200–350
<i>Medicago sativa</i>	400–600

Table 2. Gamma radiation radiosensitivity of some crop species [22].

Species	Plant material	Gamma ray source	Gamma radiation dose (Gy)	Reference
<i>Nicotiana tabacum</i> L.	Seeds	¹³⁷ Cs	100–400	[23]
<i>Glycine max</i> L. Merr.	Seeds	¹³⁷ Cs	100–500	[24]
<i>Rhododendron</i> spp.	Shoot tip	¹³⁷ Cs	5–50	[25]
<i>Musa</i> spp.	Shoot tip	¹³⁷ Cs	10–50	[26]
<i>Solanum tuberosum</i> L. “Marfona”	Node	¹³⁷ Cs	5–50	[27]
<i>Saccharum officinarum</i> L.	Leaf primordia	¹³⁷ Cs	10–50	[28]
<i>Triticum aestivum</i>	Seed	⁶⁰ Co	10–100	[29]
<i>Paphiopedilum delenatii</i>	PLBs, shoot buds, in vitro plantlets	⁶⁰ Co	10–80	[30]
	Embryogenic callus	⁶⁰ Co	50–400	[31]
<i>Saccharum officinarum</i> L.	Embryonic callus culture	⁶⁰ Co	10–80	[32]
<i>Solanum melongena</i> L.	Seed	⁶⁰ Co	80–240	[21]
<i>Gypsophila paniculata</i> L.	Shoot tips and lateral buds	⁶⁰ Co	0.25–1	[33]
<i>Etilingera elatior</i>	Axenic culture	⁶⁰ Co	10–140	[34]
<i>Colocasia esculenta</i> L. Schott	Shoot tips	¹³⁵ Co	0–60	[35]
<i>Chrysanthemum grandiflora</i>	Shoots	⁶⁰ Co	5–30	[36]
<i>Chrysanthemum morifolium</i>	Ray florets	⁶⁰ Co	0.5–1	[37]
<i>Lilium longiflorum</i> Thunb. Cv. White fox	Bulblets	⁶⁰ Co	0.5–2.5	[38]
<i>Rosa hybrida</i> L.	Single-node cuttings	⁶⁰ Co	5–80	[39]

Table 3. In vitro mutagenesis protocols for some crop species.

selection of the plants with desired characters using in vitro cultivation methods for vegetative plants, clonally reproduction of the plant parts is needed in order to detect the mutant generations via using easy stability tests [27]. The schematic diagram representing the usage of gamma radiation for in vitro mutagenesis applications is given in **Figure 3**.

3.1.3. In vitro selection of the mutants

The selection of the desired mutants is an essential and important part in a mutation breeding program. In vitro mutagenesis applications give opportunity to the breeders to select the mutants in a controlled environment. The plant breeders can work with a large population of plant material. Different culture techniques such as suspension cultures and protoplast cultures can be widely preferred to have a genetic uniformity in the selection studies.

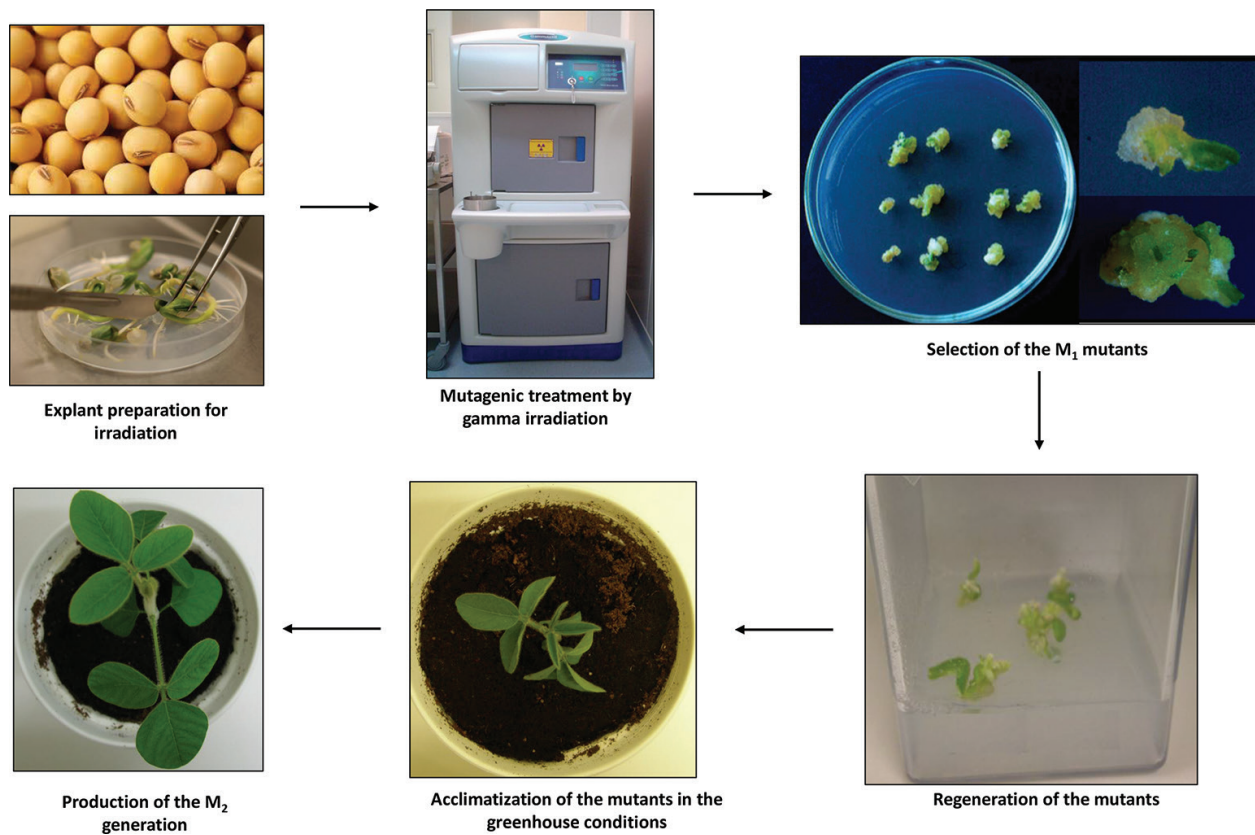


Figure 3. The representative schematic diagram of an in vitro mutagenesis application.

In vitro selection studies have some advantages. These can be classified as follows:

1. Easiness of the application
2. Reduced time of the selection
3. Availability to use some selective agents in culture conditions

In vitro selection studies can be performed in two types: single step and multistep [4]. In single-step selection procedure, the inhibitor agent is added to the culture environment and the subcultures used for the selection studies. In multistep method, the dose of the selective agent below lethal dose is added to the culture, and the concentration of the inhibitor is gradually increased in subcultures. The selected mutant by this method has been defined as more stable than selected via other methods [4].

Food and ornamental plants are widely assessed for nutritional quality, early ripening, better flower, and biotic/abiotic stress tolerance capacities [4]. For abiotic stress treatments, it is more convenient to control the culture conditions than in the field environment [20, 40]. Salt, drought, cold, and heavy metal tolerance have been successfully performed in many plants. Callus, suspension cultures, or protoplast cultures were used for in vitro selection analysis by adding the selective agents reducing the growth such as mannitol and polyethylene glycol for drought tolerance; NaCl for salt tolerance; boron, aluminum, and nickel for metal tolerance; or changing the temperature of the cultures to select cold/high-temperature-tolerant plants [4, 41]. Both selection strategies, single step and multistep, can be used. The main point is to

inhibit the false-positive selection responses due to epigenetic alterations in long-term culture conditions. When the plants are subject to long-term stress treatments with gradual increase of the selective agent, non-tolerant cells can experience stable epigenetic alterations, which can be inherited by mitosis. In order to avoid this period, preference of single-step selection procedure is suggested to be efficient during mutation breeding programs [41].

In selection studies, the main criterion is to define the exact selective agent. This means that the molecular mechanism of the desired trait should be clearly understood by the plant breeders. Morphological and physiological changes should be used in combination to discriminate the mutants. All the parameters such as leaf injury, slower growth, average number of shoots per explant, survival percentage of the plants, fresh weight of the explants, leaf photosynthetic capacity, antioxidant defense system, and accumulation of osmolytes should be investigated in detail especially for stress tolerance studies [40, 41].

3.2. Mutational genomic analysis

Mutational genomics is becoming a valuable tool to differentiate the mutants improved via mutation breeding programs. It is also an important tool to understand the molecular basis of the plant stress response based on the data gathered from mutants of model plants and an easy way to determine the genetic similarities and characterize the variations between the mutants at the DNA level.

The mutants were identified based on morphological characters, traditionally. The new developments in DNA technologies give opportunity to the plant breeders to make it quick and definite.

Molecular markers are widely used to differentiate the genetic differences between the mutant and the mother plants through characterizing the variations at DNA level. High-throughput genomic platforms such as random amplified DNA polymorphism (RAPD), cDNA-amplified fragment length polymorphism (AFLP), single-strand conformational polymorphism (SSCP), microarray, differential display, targeting induced local lesions in genome (TILLING) and high-resolution melt (HRM) analysis allow rapid and in-depth global analysis of mutational variations [4].

Among these methods RAPD, inter simple sequence repeat (ISSR), and AFLP have been frequently used in genomic classification of the mutants [15, 42]. RAPD is an inexpensive and a rapid method to use in many fields of biotechnology. There is no need for genome information. It has been widely used to determine the genetic diversity in mutation breeding programs of many plants. RAPD is an efficient method to detect DNA alteration via using random primers. It has been started to use in earlier studies of genetic variabilities obtained by radiation treatments in *Chrysanthemum* [36, 37], soybean [24], sugarcane, sunflower, groundnut [43], tobacco [23], potato [27], *Rhododendron* [25]. ISSR method is another molecular marker method widely used in plant biotechnology applications. It is also easy to apply more informative than RAPD, reliable, and inexpensive [44, 45]. ISSR primers are designed by using microsatellite sequences to amplify the genomic regions flanked by microsatellite repeats. By using one primer, it is possible to amplify multiple fragments as a result of ISSR analysis [46, 47]. The information obtained from ISSR analysis is more reliable than RAPD to provide supplementary data of the genetic variations of the mutants from the nonoverlapping genome regions [48].

Xi et al. [38] reported an in vitro mutagenesis protocol for *Lilium longiflorum* Thunb. cv. White fox. They used 0, 0.5, 1.0, 1.5, 2.0, and 2.5 Gy gamma rays to observe the effects of radiation on adventitious bud formation from bulblet-scale thin cell layers. 1.0 Gy was determined as the most effective dose due to survival rate of the bulblet-scale thin layers. They also evaluated the morphological mutants using ISSR DNA fingerprinting method.

Sianipar et al. [49] used RAPD method to detect the genetic variability between the mutant plantlets improved from gamma-irradiated rodent tuber calli. They obtained 69 fragments from 11 mutant plantlets by using 10 RAPD primers.

Barakat and El-Sammak [33] irradiated shoot tips and lateral buds of *G. paniculata* with four different gamma radiation doses between 0.25 and 1 Gy. They detected the genetic polymorphisms among the mutants by RAPD analysis. They obtained 105 different amplification products from 10 random primers. RAPD is evaluated as an efficient molecular marker technique to detect the variations. Atak et al. [25] used RAPD method to show the genetic similarities of the *Rhododendron* mutants improved via gamma irradiation. They used 0–50 Gy gamma radiation doses to improve the shoot and root regeneration rates of *Rhododendron* plants. RAPD detected higher genetic variability among the *Rhododendron* mutants. Yayıcı and Alikamanoğlu [27] observed 89.66% polymorphism rate with six primers among the mutant potato plants, which were improved as salt tolerant via gamma radiation treatment. Kaul et al. [36] used in vitro mutagenesis in *Chrysanthemum* cv. Snow Ball by irradiation of the in vitro shoots, and genetic polymorphisms among the mutants and the control plants were assessed by RAPD. They reported that 10 Gy gamma irradiation was found as the most effective dose to induce genetic variation in morphological traits, and they observed 100% polymorphism among the mutants. Gamma radiation-induced salt-tolerant oriental tobacco mutants were improved by Çelik and Atak [23]. Salt tolerance of the mutants was controlled by the callus induction in the presence of high salt concentration. The genetic similarities of the mutants were determined by RAPD analysis. The relationships between the salt-tolerant mutants and controlled tobacco varieties were shown in Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram. Some representative RAPD profiles of the mutants developed by in vitro mutagenesis were given in **Figure 4**.

Sen and Alikamanoğlu [44] used ISSR method to differentiate the drought-tolerant sugar beet mutant improved via irradiation of shoot tip explants by gamma radiation. They obtained 91 polymorphic bands of 106 PCR fragments with 19 inter simple sequence repeat (ISSR) primers.

Perera et al. [40] applied in vitro mutagenesis treatment to an important energy crop giant miscanthus (*Mischanthus × giganteus*) to induce variation in cultivar Freedom. ISSR markers were used to determine the variations in the mutant plants. The putative mutants were selected due to the results of molecular marker analysis to use for further bioenergy researches. Wu et al. [50] used ISSR analysis to show the genetic similarities between the mutants. For this reason, they used 60 ISSR primers, and 60 polymorphic bands of 392 were evaluated to have information on the molecular level of mutation breeding. Atak et al. (unpublished data) [51] used ISSR marker method (with 61 ISSR primers) to define the genetic variation among the 8 salt-tolerant mutant soybeans obtained from in vitro mutagenesis treatment by using ¹³⁷Cs gamma source. The representative results of ISSR amplification of 8 salt-tolerant mutant soybeans were given in **Figure 5**.

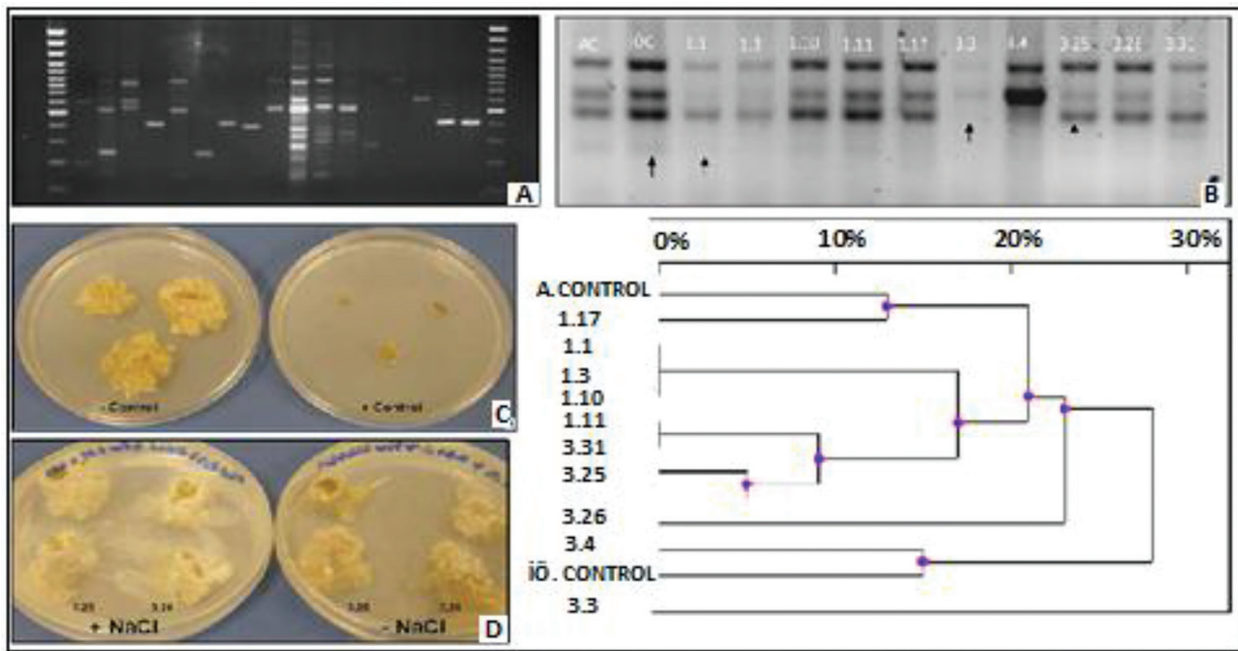


Figure 4. Evaluation of salt-tolerant tobacco mutants improved via in vitro mutagenesis application. A and B represent the RAPD profiles of the mutants. C shows the callus growth of control plants under in vitro salt stress. D shows the callus growth profiles of the mutants under salt stress [23].

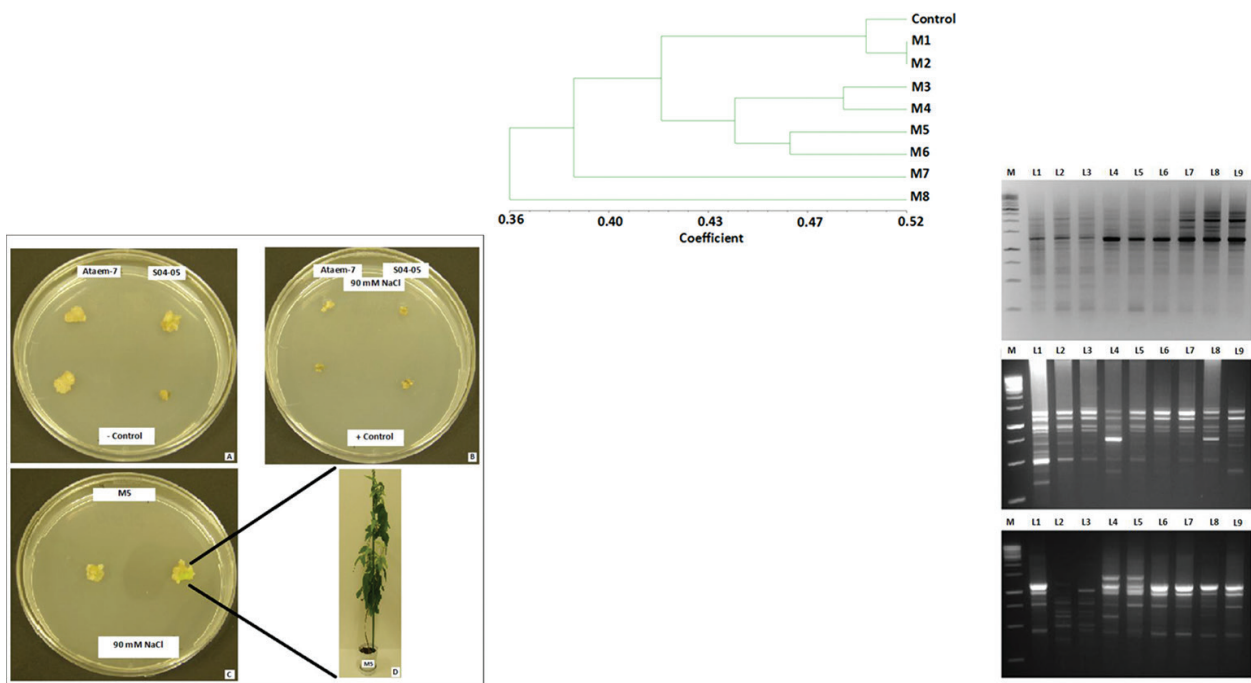


Figure 5. The representative results of evaluation of 8 salt-tolerant mutant soybean plants improved by in vitro mutagenesis treatment. A. The callus growth of Ataem-7 and S04-05 soybean cultivars. B. The callus growth of Ataem-7 and S04-05 soybean cultivars under 90 mM NaCl. C. Callus growth of M5 under 90 mM NaCl. D. The whole plant M5.

Single-strand conformational polymorphism (SSCP) is another strength method to identify the variations between the mutant and mother plants in amplified DNA samples. It is widely

used to determine the genetic mutations in several organisms. It is also an effective method to find a potential genetic marker which is in relation with a desired trait to use in selection studies of agricultural populations [52]. Irradiation of the plant tissues can cause mutation between the allelic gene copies [single-nucleotide polymorphism (SNP)]. SSCP is an efficient method to detect these polymorphisms. It is possible to detect relations between SSCP polymorphisms and quantitative traits [53].

These methods can only be able to detect the genetic variations of the mutants in accordance with the mother plants. There are a number of methods to screen the causal mutation at a desirable phenotype. Molecular markers that are in relation with the mutation are known to be able to segregate in the next progenies. The main point is to make the functional analysis of the mutant genes that have role in acquiring the new desired characters. To identify a mutant, the number of the genes controlling that specific phenotypic character is deterministic [54].

In a mutation breeding program, identification of differentially expressed genes, the biological processes they have role in, or the metabolic pathways of interest should be carried out through modern genomics and system biology. To achieve this, there are specific tools to discriminate with the use of next-generation molecular techniques. In microarray systems, it is available to detect the gene expressional differences between the mutants and control plants. Thousands of spots on a microarray chip containing a few million copies of identical DNA molecules buried on each spot are related to each gene of a plant genome. If it is a targeted mutation, it is possible to show the expressional differences between them by microarray technique. In general, spontaneous mutations cannot be detected at microarray systems. Sequencing methods are more efficient in the meanwhile. Mutant plants can now easily sequence by next-generation sequencing (NGS) techniques to define the mutations [55]. To apply these methods, there is no need for a reference genome. These analyses can be classified as forward genetic screening methods that give opportunity to improve the knowledge about the genes that control specific biological roles in mutant plants. In contrast to forward genetic, reverse genetic is more popular to detect the function of a gene. In mutation breeding programs, the plant breeders are focused to identify the individuals from a population that have an allelic variation of a gene. As mentioned previously, these individuals are improved by mutagenic treatments. TILLING method is available to determine the mutants with specific phenotypes. In tomato, approximately 3000 mutant lines that were improved by chemical mutagens on fruit ripening trait were identified by this method. This method is used for barley to screen the homeodomain-leucine zipper protein mutants. Recent progresses in NGS technologies and TILLING which is in relation with these technologies make it possible to screen the potential genes [54, 56].

4. Discussion and the conclusion

The increasing importance of plant breeding studies in correlation with biotechnology and molecular genetics is attempted to meet the requirements of increasing population for food and crop plants. Therefore, mutation breeding treatments have become more frequent and alternative to classical breeding and genetically modified plants. The main aim is to com-

bine several features of many plants in one super plant. In vitro mutagenesis has become an efficient tool for this purpose. Plant breeders are focused to crop improvement techniques to improve genetic variations of useful traits by using next-generation molecular methods.

Using these advanced genomic techniques, new molecular mechanisms and new genes can be potentially identified by the plant breeders as a result of in vitro mutagenesis treatments. To gain more data, additional needs of various comparative and descriptive experiments can be upgraded to acquire more specific points to build the relations between the regulatory mechanisms. Therefore, the recent progress in mutation breeding studies in relation with new technologies is quite important to contribute new advancement to plant breeding programs.

Author details

Özge Çelik* and Çimen Atak

*Address all correspondence to: ocelik@iku.edu.tr

Department of Molecular Biology and Genetics, Faculty of Science and Letters, TC İstanbul Kültür University, Istanbul, Turkey

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