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# The Use of Gamma Irradiation in Plant Mutation Breeding

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## Abstract

In plant breeding programs, one of the oldest methods is mutation breeding. Currently, mutation breeding has become popular among the breeders and scientists again with its use in plant biotechnology and due to some restrictions on the other techniques such as hybridization, cross breeding, and transgenic plants. Physical mutagens (X-rays, UV light, neutrons-alpha-beta particles, fast and thermal neutrons, especially gamma rays) are used more widely than chemical (ethyl methanesulfonate [EMS]) ones to artificially induce mutations (mutagenesis). However, among the physical mutagens, gamma-rays are widely used. During the irradiation of the seeds with ionizing radiation to generate mutants with desirable traits, reactive oxygen species (ROS) or free radicals can generate in cells. Although, these radicals/species generally can be very dangerous for the cell compartments, they can take an important role as a signal molecule activation of genes of antioxidant enzymes and proline, which are defense systems against these radicals in plant cells. In this chapter, usability of gamma-irradiation to provide the permanent gene expression of antioxidant enzymes and proline through the production of reactive oxygen species (ROS) is discussed.

**Keywords:** gamma rays, plant mutation breeding, reactive oxygen species, gene expression, antioxidant enzymes

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## 1. Introduction

As one of the earliest agricultural activities, plant breeding has begun in very ancient times (early on Neolithic revolution, 10,000 BC) parallel to human culture [1, 2]. For the plant breeding, genetic variations are the pre-request. Mutations (natural process that creates new variants [alleles] of genes) are the main source of all genetic variation in plants as well as in any other organisms [3, 4]. According to the historical records (an ancient book "Lulan"), the first spontaneous mutant plants (cereal crops) are found in China 2317 years ago [5, 6]. After that, a few

researchers reported spontaneous variation in plants between 1590 and 1968 [6]. However, the first publications of induced mutations (through X-rays) for the plant breeding was published 89 years ago by Muller and Stadler [7, 8]. As a result of these studies, mutation breeding as a new approach was added to other plant breeding methods. Thereafter, a large amount of genetic variability has been induced by various mutagens (physical and chemical) and contributed to modern plant breeding. *Nicotiana tabacum* was the first commercial mutant cultivar (called: chlorine type), which produced by inducing mutations [6]. Induced mutations were used to improve tolerant plant varieties over the past 50 years in all over the world [8]. Currently, 3246 registered mutagenic plant varieties are there in FAO/IAEA mutant data base [9].

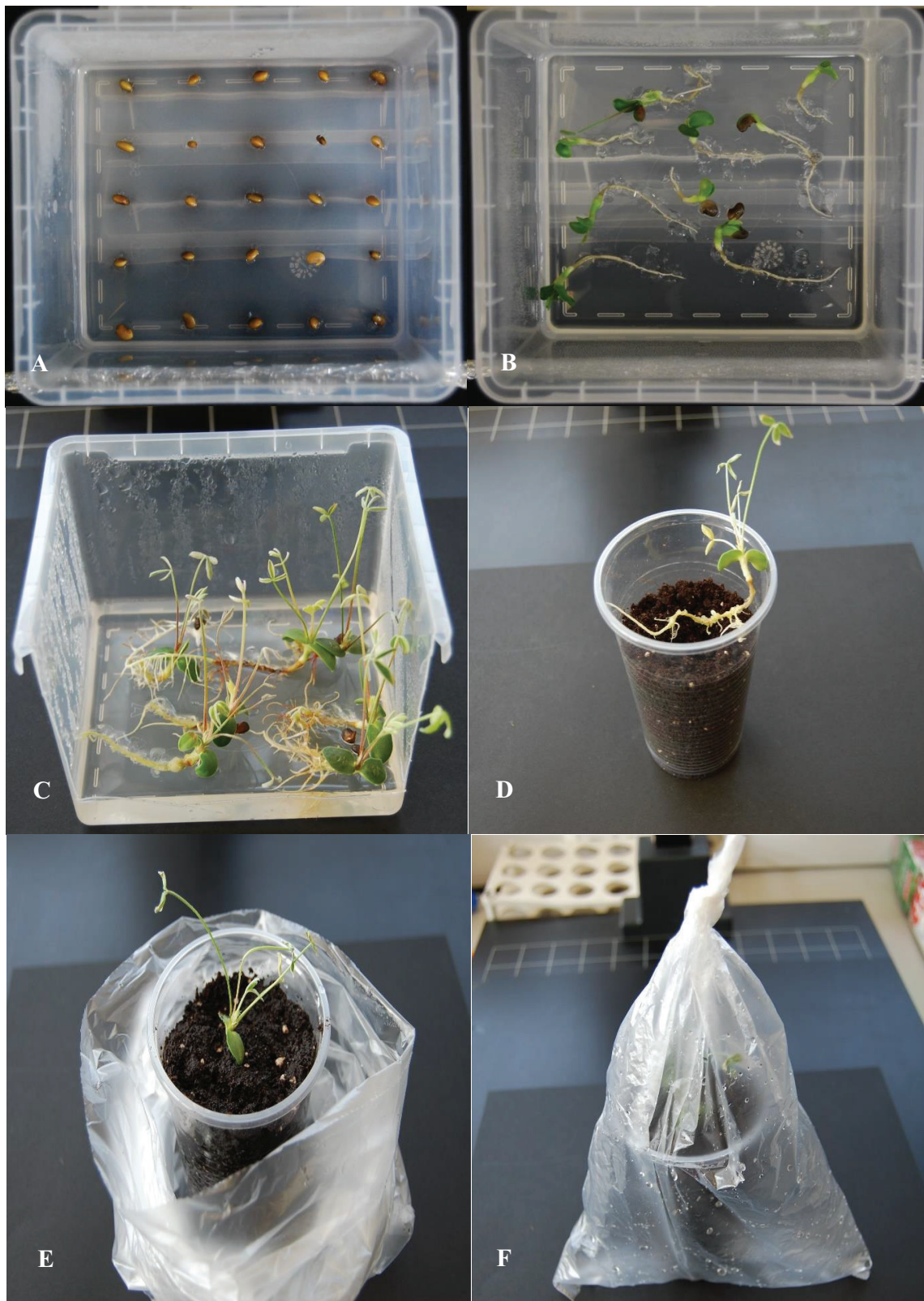
Increasing crop yields is a major demand for assuring food security. Mutagenesis is an important tool to improve crops and has not got any regulatory restrictions as genetically modified organisms (GMOs) [10]. Plant breeding is based on the genes. Initially, breeders select new phenotypes with valuable characters without knowing the genetic constitution. The emergence of molecular genetics is parallel to understand the details of inheritance of desirable/undesirable traits and genetically controlled, modern biotechnological breeding has paved a wide road. By using DNA recombinant technologies, the gene encoding a trait precisely manipulated to create novel phenotypes. The cloned gene in respect of the source or recipient of the genes can be transferred by breeding technology known as transgenic technology. In transgenic technology, the key step is the integration of desired foreign genes into the host plant genome. For plant transformation, there are primary tree methods such as the Agrobacterium-mediated, particle bombardment, and protoplast transformation. The Agrobacterium-mediated gene transfer method is one of the most practical and suitable method [2]. The first transgenic plant (tobacco [*N. tabacum*], which contain antibiotic resistance gene) was obtained in 1980 by Marc De Block through Agrobacterium-mediated method [11]. Breeders can transfer encoding genes of new characters into plants genome through transgenic approaches. Its precision and the betterment of a trait without changing the genetic makeup of genome in elite genotypes are the main advantages [2]. Although transgenic technology has significant achievement in improving crops and has substantial commercial value, this technology has some technical obstacles. For example, in terms of highly recalcitrant to genetic transformation and regeneration, there are many economically important plant species or elite varieties of species. In addition to the technical obstacles, there are some debates about unpredictable risks of transgenic technology on environment and food safety, even though many of these debates are baseless. However, more advanced technologies have been developed to solve these ideas [2]. On the other hand, plant breeders use mutagenesis in plant breeding programs without restrictions such as the legislative constraints, licensing costs, and societal opposition of transgenic technology [10]. Although still limited to the content of the endogenous genome, mutagenesis and high-resolution screening will supply a very good complement to recombinant DNA technologies and genetically modified organisms (GMOs) in further improved new plant forms that are better adapted to change conditions of environment and the increasing global population [1].

## 2. Application of gamma rays to induce mutation and *in vitro* selection

As mutations may occur spontaneously, it can induce artificially. Artificially induced mutations can be created by physical mutagens, such as X-rays, gamma rays, and neutrons, and chemical

mutagens, such as ethyl methanesulfonate (EMS), in plant mutation breeding [12]. Although, various mutagens (physical and chemical) are used for the induction of mutation, physical mutagens (radiation: gamma rays and X-rays) was used more frequently as compared to chemical mutagens. However, among the physical mutagens, gamma rays (1604 improved mutants) are used more widely than X-rays (561 improved mutants). Gamma rays are ionizing radiation and used in inducing mutations in seeds and other planting materials such as cuttings, pollen, or tissue-cultured calli [4, 13]. In the early twentieth century, plant biologists discovered method that the frequency of genetic variations could increase in treated seeds with chemical compounds or radioactive rays. Mutation induction has become a powerful tool for developing new and novel plant germplasm [14]. Radiation-induced mutation is one the most widely used method to improve direct mutant varieties compared to acclimatization, selection, hybridization, which are laborious, time consuming, and also with limited genetic variation [15]. This discovery later referred as plant mutation breeding. As an *in vitro* culture techniques, *in vitro* selection can be use to obtain plant genotype tolerance to adverse environment conditions such as drought, high salinity [16]. Numerous mutant or genetic material is possible due to *in vitro* selection [17]. *In vitro* selection is the greatest method to obtain the desired traits of plant, because it has the capacity to manipulate the variation to the expected result. Both abiotic and biotic tolerant plants could be obtained from the media which contain the selection agent [16]. *In vitro* selection has been practiced for desirable traits and success has been achieved in several crop plants [18–20]. Easy application of *in vitro* selection method is one of the most important requirements for the achievement of *in vitro* selection technique and to obtain the tolerant plants. Exposed biological materials by mutagens can be reliable and easy screening in a comparatively small space, which can save time, money, and space under *in vitro* conditions when comparing the greenhouse and field [21].

Beyaz [22] reported easy and reliable *in vitro* selection protocol and optimization for the creation of irradiation-based mutagenesis in sainfoin (*Onobrychis viciifolia* Scop.). Sainfoin (*O. viciifolia*) is a significant forage legume species, which grown widespread than the other *Onobrychis* species, agriculturally [23]. The seeds of sainfoin (*O. viciifolia* Scop.) were irradiated to 0, 400, 500, and 600 Gy using an experimental  $^{60}\text{Co}$  source (dose rate of  $0.483 \text{ kGy h}^{-1}$ ) at the Sarayköy Nuclear Research and Training Center (SANAEM) of the Turkish Atomic Energy Authority (Ankara, Turkey). Irradiated and unirradiated (control) seeds of sainfoin were sown into Murashige and Skoog (MS) medium supplemented with 150 mM NaCl (**Figure 1A**). Seeds were allowed to germinate and develop at  $20 \pm 1^\circ\text{C}$  under cool white fluorescent light ( $27 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) with a (16 h light/8 h dark) illumination period for 10 days. After that, selected plantlets were transferred to MS medium without NaCl for continuous development for 2 weeks (**Figure 1B**). For acclimatization, following steps were applied. Advanced plantlets (**Figure 1C**) were transferred to plastic glasses (**Figure 1D**). After that, plastic glasses placed in freezer bags (**Figure 1E and F**) to provide suitable moisture and adaptation to external condition for 1 week grown in the plant growth chamber. Plantlets in freezer bags were transferred to plant growth chamber (**Figure 2A**) and the mouths of the freezer bags slowly open during 1 week period, and at the end of 1 week, all plantlets were removed from freezer bags (**Figure 2B**). Every 2 days for 2 weeks, acclimatized plantlets watered with 30 ml tap water containing 150 mM NaCl for *in vivo* selection. Most of the plantlets died (**Figure 2C**) during treatments of NaCl, survived plantlets were selected (**Figure 2D**). Survived plantlets (**Figure 2E**) were transferred to plastic glasses which contain soil without NaCl for more



**Figure 1.** Irradiated and unirradiated seeds of sainfoin in MS-basal selection media supplemented with 150 mM NaCl (A). After 10 days of seed germination, selected plantlets and advanced plantlets in MS-basal medium without NaCl (B, C). An acclimatization process of plantlets to external conditions (D–F).



**Figure 2.** General view of plantlets in freezer bags (A). Plantlets which removed freezer bags in plant growth chamber (B). Dead plants as a result of salt application (C). Survived plantlets (D). Survived plantlets after treatments of NaCl (E). Washing root of survived plants with tap water to get rid of NaCl and transferred to new soil without NaCl (F and G).

development (**Figure 2E and G**). Superior plants (putative mutants) in terms of salt tolerance were moved to a growth chamber (**Figure 3A and B**) for a while, and after that, they were transplanted in field (**Figure 3C–F**).



**Figure 3.** General view of survived plants of sainfoin in growth chamber (A and B). Transferred putative mutants against to salt stress in the field (C and D). Putative mutant sainfoin plants in the field after 1.5 months (E) and 2 months (F) (Location: The University of Ankara, Faculty of Agriculture, Department of Field Crops, Turkey, photos were taken on 21 March 2013).

### 3. Ionize radiation, reactive oxygen species (ROS) and defense systems of ROS

Ionizing radiation causes biological injury in exposed biological materials. The first target of ionizing radiation is water molecule, which is ubiquitous in any organisms. The cell is composed of ~80% water [24]. As a result of excitation and ionization reactions, water molecule ( $H_2O$ ) and  $H\cdot$  and  $OH\cdot$  radicals are generated [25]. Gamma rays cause to produce free radicals (free radicals like  $O_2^{\cdot-}$  and  $OH\cdot$  and nonradicals like  $H_2O_2$  and  $^1O_2$ ) as known reactive oxygen species (ROS) through direct interactions of radiation with target macromolecules or via products of water radiolysis [13, 24, 26]. The formation of reactive oxygen species (ROS) occurs in the general metabolism of the plant cell.

However, such as other environmental stress, radiation lead to increase the formation of ROS in plant cell due to damage of cellular homeostasis and cause progressive oxidative damage and finally cell death [27].

Reactive oxygen species (ROS) control many different processes in plants [28]. Plants has two antioxidant machinery, one of them is antioxidative enzymes, including ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR). The other one is nonenzymatic antioxidants like ascorbic acid (AA), reduced glutathione (GSH),  $\alpha$ -tocopherol, carotenoids, flavonoids, and the osmolyte proline [26]. ROS depends on ionizing radiation level that causes damage or modification of components in plants, ultimately affecting morphology, physiology, anatomy, and biochemistry of plants [13, 29]. Currently, scientific evidence shows that ROS play an important signaling role in plants and regulate biological activities such as growth, development, and especially response to biotic and abiotic stress factors [26, 27]. ROS can induce injury of cell compartment, but on the other hand, they induce new gene expression in cells [30]. However, Esnault et al. [31] hypothesized that ROS (mainly  $H_2O_2$ ) can play a secondary role are in signaling process of cell. And after a first stress, plants can be more tolerant to a new stress synthesis due to secondary metabolites. Moreover, using gamma rays can create a permanent gene expression of antioxidative enzymes for scavenging "oxidative stress" start from the first generation of plants. And this provides to improve superior plants varieties against biotic and abiotic stress factors.

**Here in the main question:** Can we use ionize radiations (especially, gamma rays) to generate mutants with desirable characteristics via supply permanent gene expression of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) and also osmoprotectants such as proline and transmissible to the progeny in plants?

Although there are lots of works related to changing transcriptional regulation of various types of genes (especially genes of antioxidative enzymes ) due to gamma irradiation, there are limited reports on permanent and transmissible increased transcript levels of genes, which induced by gamma rays, of antioxidative enzymes in plants.



Beyaz et al. [32] reported that permanent production of antioxidant enzymes and proline in M1 plants of sainfoin (*Onobrychis viciifolia* Scop.) under *in vitro* conditions. In the study conducted by Beyaz et al. [32], the aim was to investigate effects of gamma radiation on physiological responses of the M1 sainfoin plants. Seeds of sainfoin ecotype 'Koçaş' were exposed to 0, 400, 500, and 600 Gy from a  $^{60}\text{Co}$  source at a dose rate of  $0.483 \text{ kGy h}^{-1}$ . Irradiated and unirradiated seeds were sown into culture vessels containing MS-basal medium to be cultured for 30 days under *in vitro* conditions. At the end of this period, seedlings, which germinated from the radiated and unirradiated seeds, were transferred into pots in a growth chamber for 30 days more. Chlorophyll contents, activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as contents of malondialdehyde (MDA) and proline were examined in unirradiated and irradiated 60-day-old seedlings. Overall, the activities of the antioxidant enzymes (SOD, CAT, and GR) and contents of chlorophyll and proline in the leaves tended to increase after irradiation in a dose-dependent manner. By contrast, the activity of APX decreased. The lipid peroxidation characterized by the MDA content remained unchanged, except after irradiation to 500 Gy. The highest CAT activity and the highest proline content were observed after irradiation to the highest dose of 600 Gy. The highest SOD and GR activities were observed after irradiation to the lowest tested dose of 400 Gy. This is the first study that provided basic information on the impact of gamma radiation on physiological responses of sainfoin and its radiosensitivity. These findings will be useful in development of a mutation breeding program of sainfoin.

Also Zaka et al. [33] query the same questions in their investigation and reported that the low chronic ionizing radiation provide genetically transmissible gene expression of antioxidant enzymes such as (SOD, GR, CAT, POD, and GPDH) to the progeny of *Spila capillata* (Poaceae), which grown two contaminated areas ( $5.4$  and  $25 \mu\text{Sv h}^{-1}$ ) on the Semipalatinsk nuclear test site in Kazakhstan. They considered evolutionary point of view and answered their observation with the natural populations that can change their genetic structure under environmental constraints and facilitates adaptation. The selective pressure of low radioactive contamination levels (leading to gamma-irradiation dose rates as low as  $4.5$  and  $25 \mu\text{Sv h}^{-1}$ ) may have played an important role during 50 years. However, Çelik et al. [25] reported that a high level of ascorbate peroxidase activity (APX) in the leaves generated from irradiated soybean seeds, compared to the unirradiated group. The APX activity increased in the unirradiated group over the experimental period and was increased by 3.6-fold at 14 days and 3.8-fold at 21 days after irradiation ( $P \leq 0.05$ ). Kim et al. [34] found that gene expression of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) can be induced depending on increasing gamma-irradiation dosage level in Triticeae species. Plant cells change their protein metabolism under main stress conditions. Resistant or stress proteins are induced in response to gamma-irradiation stress, and this defense mechanism change patterns of gene expression, especially stress-inducible gene expression, which produces qualitative and quantitative changes in total soluble protein [34, 35]. The different gene expression pattern of antioxidant enzymes can be observed in the irradiated plant species [36].

According to Mohammed et al. [37], a substantial variation of the protein patterns of cowpea seeds occur by gamma rays induced, and this variation has occurred because of the new expression of some polypeptide, the silence of others, and overexpression of a third class polypeptides. Aly and El-Beltagi [38] showed that increase in GST, CAT, SOD, and POD

activities in *Vicia faba* L. seeds could be attributed to ionizing irradiation stress. Cho et al. [39] investigated the expression patterns of diverse genes at various time points after gamma irradiation of young tobacco plants and found three different gene expression pattern (increased, decreased, and no response) of antioxidative enzymes (CAT, SOD, and GST). However, Al-Rumaih and Al-Rumaih [40] reported that gamma irradiation affected antioxidant enzyme activities in the three investigated species of wheat. Moreover, the increased activity of antioxidant enzymes (SOD, CAT, POX, APOX) in response to gamma-irradiation treatment in many plant species (*Nicotiana*, *Triticum aestivum*, sugar cane, *Phaseolus vulgaris*, radish, groundnut, and pepper) were reported [41–46].

As a result of metabolic pathways of aerobic cells, free radicals and ROS are occurred and influenced most of the biological activity. On the other hand, products of oxidation reactions play an important role some biological process such as aging, some degenerative diseases, differentiation, and development, including serving as subcellular messengers in gene regulatory and signal transduction pathways. Several studies reported the hypothesis. The hypothesis is that shifts oxidant/antioxidant equilibrium in cell may also affect developmental pathways in a different of tissues from phylogenetically diverse organisms [47].

Over expression of antioxidant enzyme genes likely arise due to an efficient regulatory mechanism to provide cells with resistance [33, 40]. Sen et al. [48] reported that the activities of several antioxidant enzymes were evaluated in both vegetative and flowering stages, and mutant lines (wheat irradiated by 200 Gy) showing the highest biochemical performance. These studies clearly indicated that gene expression of antioxidant enzymes can be made permanently in genome of progeny of plants by gamma-rays irradiation.

#### 4. Conclusion

It is estimated that the population of the world will be 9.1 billion, which is compared to 34% today, in 2050. Populations of developing countries will be the biggest in this increase. Urbanization will continue at an increasing rate and the majority of the world's population (~70%) will live in urban (compared to 49% today) [49]. However, food production is not increasing parallel to the human population and a large part of the population of today's world is not already well fed. Therefore, humanity has two critical problems such as controlling population growth and increasing food production. It is important for food safety that people are economically and easily accessible to food. For the food security, physical availability and affordability of food are the most important criteria. Induced mutations have a vital role in increasing world food security, because of induced mutations provide new food crop varieties, contributed to the significant increase in crop production and supplied directly accessible of food for the locations people [8]. Mutagenesis has become widespread again in plant breeding during the last decades. Plant mutagenesis, which creates new variation in crop plants, coupled with *in vitro* selection and plant biotechnology methods allows breeders to select for characters that were tough obtain in breeding for only a few decades ago [1]. This is a viable option that increases productivities via "smart" plant varieties that can produce more yield. However, the genetic

similarities among crop varieties or unvarying parental materials, which are weaker than the same stress, are limited to uncover new alleles of genes in cropping systems. Therefore, creating new combination of genes causes to break yield plateaux and enhance tolerance. Induced mutation uncovers novel alleles that are used to breed superior crop varieties [50]. The uncovering of new genetic variation in inbred elite cultivars supplies a unique possibility to characterize novel traits, while the lines protect their excellence in the agriculture. Depending on the increasing technology such as whole-genome sequencing and high-resolution analytical techniques, we accumulate more genetic information from a wide range of crop plants and also gain both money and time compared to traditional breeding techniques. However, generated markers in this process offer to the stack of the useful characters, paving the way for developing complex multigenic characters such as abiotic stress resistance [1].

As a results of biotic and abiotic stress factors, the production of reactive oxygen species (ROS) lead to increase in plant cells and cause oxidative stress. Scavenging mechanisms such as antioxidant enzymes keep plants from adverse effect of oxidative stress [30]. If we succeed supplying permanent gene expression of antioxidant enzymes and osmoprotectants such as proline and glycine-betaine by gamma radiation in the plant cell, we can provide tolerances of plants to almost adverse environmental stress factors. For the future, it seems that mutagenic crops continue their important role in plant breeding.

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