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REVIEW

In vitro Mutagenesis for the Improvement of Agave Genus

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ABSTRACT

Biotechnological techniques provide a viable alternative to help improve and increase the production of plant species of agricultural and economic importance, which have been affected over the years by climate change, increasing their susceptibility to pests and/or diseases, generating losses in production as well as a decrease in their regenerative and genetic diversity. The application of biotechnological techniques such as *in vitro* mutagenesis offers a viable option for the generation of crops that are resistant to the different factors caused by abiotic and biotic stress. *In vitro* mutagenesis has been used in an efficient way to generate genetic changes in different plant species. However, these methods have not been studied thoroughly in crops of agro-industrial interest, such as agave, which represents an economic resource of national importance and are considered as endemic species of Mexico. Therefore, this literary review aimed to focus on the studies that have been used for the genetic improvement of this species via mutagenesis techniques in plants in the agave genus. Therefore, the objective was to set a precedent for future genetic studies that aim to obtain more productive regenerants for various industries, such as food and pharmaceutical. It is also of great interest to compile information from basic research that helps understand and elucidate a model of possible defense mechanisms that are activated in the Agave genus.

KEYWORDS

Biotechnology; genetic variations; stress; mutations; resistance

Nomenclature

BA	6-benzyl adenine
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
IAA	Indoleacetic acid
PAL	Phenylalanine ammonia lyase
PEG	Polyethylene glycol
PRs	Pathogenesis-related proteins
ROS	Reactive oxygen species
TIB	Temporary immersion Bioreactor

1 Introduction

Genetic improvement in plants began with the domestication of wild ancestors of maize (*Zea mays* L.) [1,2]. In agriculture, breeding by mutations plays a fundamental role in the development of new cultivars with an improvement in yield, quality, resistance and/or tolerance to biotic and abiotic stress through novel tools [3], whereby mutation is the main source of genetic variations in plants [4]. Numerous works have been reported in literature where mutagenesis has been implemented in plants. However, regarding the Agave genus, there are few reports using *in vitro* mutagenesis for genetic improvement, probably because it is a crop that presents problems in its traditional propagation and low genetic variability [5,6], as well as affectations caused by abiotic and biotic stresses. Genetic improvement programs in agave are relevant, as it is an important crop because of applications as a raw material in the traditional production of alcoholic beverages, food, and fibers [7,8]. Thus, the culture of plant tissues facilitates the use of *in vitro* mutagenesis [9] when carrying out genetic improvement in the study of plants of agricultural and economic interest.

2 Genetic Improvement in Plants

Genetic improvement is a tool that allows to increase genetic diversity in different plant species [10], which can cause new biological functions [11,12]. Biotechnological techniques, such as the implementation of Plant Tissue Culture (PTC), have facilitated the use of *in vitro* mutagenesis, the combination of these two techniques has shown the efficacy to induce variability in a short period of time. Research with cultures has been reported where mutagenesis has been used to induce useful features, such as quality improvement and resistance to diseases, this technique is used at various levels of tissue differentiation (cell cultures, callus, somatic embryos and seeds) [13].

Induced mutagenesis produces macromolecular changes, which lead to physiological and morphological alterations of plant genotypes [14,15]. The most important factors to be considered for *in vitro* induced mutagenesis are the selection of the mutagen, dose and exposure time, type of plant, as well as the determination of LD50. The latter has great relevance, as high concentrations and long periods of exposure can cause significant effects on plant material mortality [16,17].

Genetic hereditary variations created by mutagens are very efficient as the frequency of spontaneous mutations is minor [18,19]. It is well documented that induction of mutagenesis using *in vitro* culture techniques favors the development of improved cultivars in less time [19,20].

3 *In vitro* Cultures as a Tool for Agave Propagation

The culture of plant tissues is a tool of great importance that is fundamental for the propagation and improvement of plants of interest [21]. It is used as a feasible alternative for the *in vitro* production of disease and pest-free plants [22,23], rapid multiplication, genomic transformation, and production of various specialized metabolites of interest [24,25]. It is regarded as an important technique for large-scale micropropagation of plants [26], which are generally difficult to propagate using traditional methods [27,28]. It is also considered important for the conservation of important, threatened, or endangered species [29], promotes a fast and reliable system for obtaining genetically uniform and disease-free clone plants [30,31], without seasonal variations but rich in bioactive compounds [32].

Regarding the Agave genus, studies have been reported in recent years about the use of *in vitro* culture techniques, which are shown in Table 1.

On the other hand, it is well known that the use of temporary immersion systems increases the production and improves the quality of micropropagated explants. Some publications with the Agave genus are listed in Table 2.

Table 1: Application of *in vitro* culture in the Agave genus

Agave species	Type of explant	Plant grows regulators /Factor	Response	Reference
A. americana Lineo.	Meristems	2,4-Diclorofenoxyacetic acid (2,4-D) y 6-Benzyladenine (BA)	Callus induction by using 2,26 µM of 2,4-D y 11,0 µM BA Greater number of shoots per explant with 0,11 µM of 2,4-D and 44 µM BA after 36 weeks	Reyes-Zambrano et al. [33]
A. angustifolia Haw.	Seedlings	Temperature	Greater carbon substrate-converting efficiency in a temperature range between 25°C–35°C	Millán-Soto et al. [34]
A. americana L.	Meristems	2,4-Diclorofenoxyacetic acid (2,4-D) y 6-Benzyladenine (BA)	Maximum percentage of shoots using 0,11 mM of 2,4-D and 73,3 mM BA	Lecona-Guzmán et al. [35]
A. marmorata Roezl.	Seedlings	6-Benzyladenine (BA) and Indole-3-Acetic Acid (IAA)	Greater length of shoots, greater root number and length by adding 10 mg L^{-1} of IAA	Aguilar Jiménez [36]
A. sisalana Perr.	Seedlings	Sodium chloride (NaCl) and polyethylene glycol (PEG 6000)	Hyperhydricity was reverted by using medium enriched with 0.2% NaCl and 0.1% PEG	Nikam et al. [37]
A. angustifolia Haw.	Callus	Vitamins (myo-inositol, pyridoxine and thiamin)	Greater number of somatic embryos per explant by using 2.5–3.0 mg L^{-1} of thiamin in medium	Reyes-Díaz et al. [38]

Table 2: Use of temporary immersion systems for agave micropropagation

Agave species	Temporary immersion system	Immersion frequency and time	Reference
A. tequilana Weber.	BioMINT®	1 min immersion every 12 h	Monja-Mio et al. [39]
A. guiengola Gentry.	Temporary immersion Bioreactor (TIB)	1 min immersion every 6 h	Chávez-Ortiz et al. [40]
A. marmorata Roelz.	Temporary immersion Bioreactor (TIB)	2 min immersion every 8 h	Martínez-Martínez et al. [41]
A. angustifolia Haw.	Recipient for Automated Temporary Immersion (RITA®)	1 min inmersion every 6 h	Monja-Mio et al. [42]

4 Genetic Transformation

Genetic transformation is one of the technologies used to reveal or modulate gene function [43]. Transformation by *Agrobacterium tumefanciens* is the most popular method for transferring genes of

desirable characteristics to plants [44,45], this technique has proven to be a strategy with great potential for increasing specialized metabolites in different plants [46,47]. Suárez-González et al. [48] described the genetic manipulation of *Agave salmiana* via two transformation systems. By using mediation by *A. tumefanciens* and bioballistics, 31 transgenic plants with small roots using *Agrobacterium* were obtained, suggesting that the combination of callus of *A. salmiana* with *A. tumefanciens* is suitable for the transgenesis, which helps to tolerate diseases in agave plants and facilitate to overexpress endogenous genes, such as those related in the biosynthesis of fructans. The use of molecular markers has been shown to be useful for evaluating the genetic fidelity of plants [49], caused by transformation by *Agrobacterium* and by genetic variation experiments, such as somaclonal variation [50].

5 Somaclonal Variation

Somaclonal variations were described by Larkin et al. [51]. These variations refer to induced abnormalities that become genetic and heritable in crops [52,53], being a phenomenon observed in cells and tissues *in vitro* [54]. They are essential and of great importance for genetic improvement and for obtaining new cultivars, generating morphological and physiological changes, and for creating resistance or tolerance to biotic and abiotic stress [55]. Kashtwari et al. [9] described that the efficiency of mutation induction *in vitro* allows large populations to be managed.

6 *In vitro* Mutagenesis

The application of *in vitro* mutagenesis has been used more frequently for improving the quality and increasing to resistance in plants [56]. This has occurred by inducing heritable changes in the genetic constitution of cells through the alteration of their deoxyribonucleic acid (DNA) [57].

The use of *in vitro* mutagenesis is a strategy that can accelerate breeding programs, as the resulting genetic variations can lead to the development of new varieties [10]. This method has been used mainly with the aim of obtaining better-adapted varieties through the alteration of traits, such as the period of maturity and flowering, seed size, resistance to diseases, yield components, as well as tolerance to abiotic and biotic stress [58,59].

To improve the frequency of mutations *in vivo* and *in vitro* random mutagenesis, as well as for expediting selections of desired traits, traditional mutation and *in vitro* mutagenesis employ various physical and chemical mutagens, which generate phenotypic and genotypic variations [60]. An adequate dose of mutagens, as well as exposure time, are important factors affecting *in vitro* mutagenesis, depending on the phase of tissue differentiation, species, and genotype [61].

6.1 Type of Mutagens Applied in vitro

In vitro mutations can be generated by physical means (gamma rays, ultraviolet light) or chemical (ethyl methanesulfonate, sodium azide, 5-azacitidine and salicylic acid) among others [62].

Physical mutagens consist in exposing parts of the plant to radiation, which causes the breakdown of the DNA chain, thus generating the loss of one or more nucleotides [63], causing modifications in DNA, cell membranes, lipids, enzymes, and other cellular constituents [64].

Chemical mutagens can be broadly classified into alkylating agents, intercalating agents, deaminating agents and base analogues. Alkylation causes the link-up of a hydrocarbon group with low molecular weight to the bases, which changes their pairing properties since true DNA replication depends on precise base mating. Base alkylation causes incorrect pairing and will therefore induce mutations [63].

The development of mutant plants has been described from *in vitro* mutagenesis techniques that have used both chemical and physical agents, whereby plants have been generated with desirable characteristics, some of these features include resistance to diseases, early maturity, and tolerance to stress biotic [65,66]. Among the most used mutagenic agents in monocotyledonous plants are those described in Table 3.

Table 3: Mutagenic agents used in monocotyledonous plants *in vitro*

Species	Mutagenic agent	Tissue	Response	Reference
A. tequilana Weber.	Gamma Rays ^{60}Co	Vitroplants	Changes in injury diameter, as a response to defense mechanisms against *Cercospora agavicola*	Ángeles-Espino et al. [67]
Saccharum spp.	Ethyl methanesulfonate	Callus	Increase drought tolerance	Masoabi et al. [62]
Aloe vera L.	Colchicine	Vitroplants with roots	Increase in the polyploid cells	Motero Paredes et al. [68]
A. americana L.	Ethyl methanesulfonate	Callus	Dwarfism, different foliar shapes, fructans increase and in PAL activity	Reyes-Zambrano et al. [69]
Saccharum (cv. C-1051-73).	Sodium azide	Explants	Differences in the shoots multiplication rate, fresh weight of raceme, aldehyde levels, phenols, carotenoids, and chlorophyll	Gómez et al. [70]
A. tequilana Weber.	Gamma Rays ^{60}Co	Vitroplants	Development of somatic embryos	Espino et al. [71]
Zingiber officinale Rosc.	Gamma Rays ^{60}Co	Shoots	Resistance to yellow disease	Sharma et al. [72]
Saccharum spp. hybrids.	Ethyl methanesulfonate	Callus	Agronomic improvements (early maturity, high saccharose content) and resistance to carbon disease	Dalvi et al. [66]

7 Biotic and Abiotic Stresses in Plants

Stress in a biological system induces a response with various negative morphological, physiological, and biochemical changes, which put the vital form of the organism at risk [73]. Plants grow in poor climatic conditions that prevent them from acquiring their full genetic capacity to grow and develop, which imposes a severe impact on their economic performance [74]. Plants are continuously exposed to a large amount of stress [75], which can be classified in abiotic stress and biotic stress. It is called abiotic stress, if it is caused by non-living factors such as drought, exposure to heavy metals, cold, heat and salinity. But if caused by pathogenic or parasitic organisms, such as fungi, viruses, bacteria, or insects it is called biotic stress [76]. These organisms can reduce the photosynthetic activity and crop yield [77] (Fig. 1).

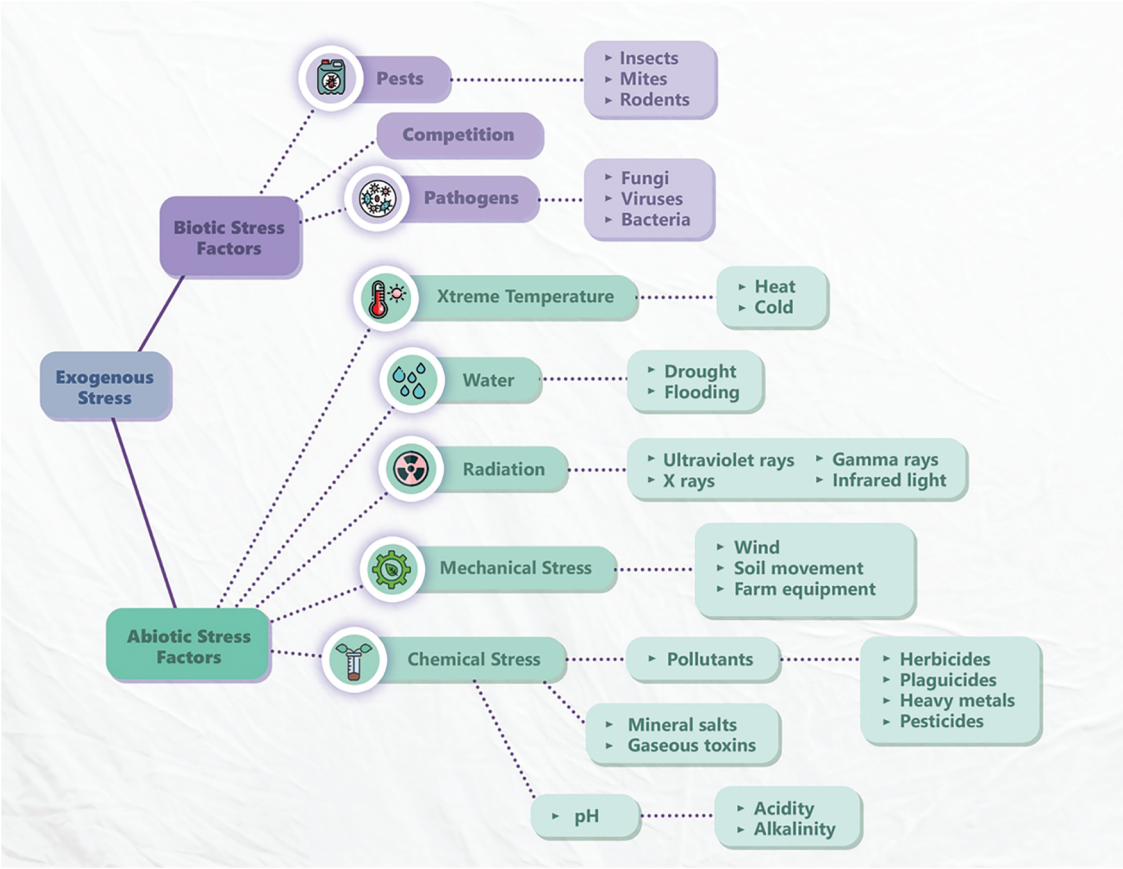

Figure 1: Environmental factors that create stress in plants (biotic and abiotic) modified from Schulze et al. [78]

7.1 Abiotic Stress

Abiotic stress, such as high salt content, extreme temperatures, and drought, can be unfavorable to plants because they cause oxidative stress, cause cell damage, and produce reactive oxygen species (ROS) [79,80], causes modifications in different physiological processes (stomata opening, photosynthesis, cell expansion, positive regulation of antioxidants and accumulation of organic solutes such as amino acids, polyamines and carbohydrates) [81]. In turn, these can cause a reduction in the yield of some crops depending on when this stress occurs [82,83].

Abiotic stress is also known to interfere with the transportation of extracellular and intracellular molecules, which are essential for optimal plant maintenance, development, and growth [84]. The plasma membrane is a barrier that separates and protects the plant's internal cellular content from external environments and is important for receiving signals that are often mediated by ionic charge and solute fluctuations. These ion and solute concentrations received by the plasma membrane help during modulated absorption of inorganic nutrients and cause changes in secondary messenger concentrations that drive osmotic gradients essential for cell expansion [85,86]. Abiotic stress directly affects the components of the photosynthetic apparatus, causing a reduction in the photosynthetic efficiency of the plant, changes in the amount of CO_2 and stomatal conductance, and the reduction of CO_2 leads to a greater activity of the RUBISCO enzyme in the photorespiration process, as a result, it is seen an accumulation of salt and a decrease in stomatal conductance [81].

7.2 Biotic Stress

Biotic stress is induced by nematodes, fungi, bacteria viruses, invasion of parasitic plants and pest attacks [87], which cause severe yield losses or crop failures during infestation [83,88].

To overcome biotic stress, plants use multiple classes of proteins: catalytic enzymes involved in cell wall modifications, ROS, pathogenesis-related proteins and phytohormones, as well as post-translational factors, receptors, and receptor-like kinases [87,89–91]. The pathogen recognition by the plant generates disturbances in the potential of the cell membrane, which causes an increase in Ca^{2+} at the intracellular level, which acts as a second messenger to activate calcium-sensitive proteins and signal transduction pathways [92]. It has been described that plants generally produce responses mediated by Salicylic Acid to counteract the damage caused by biotrophic pathogens, while to combat necrotrophic agents presence, responses are mediated by Jasmonic Acid and Ethylene [93].

Biotic agents are classified into biotrophs, necrotrophs and hemibiotrophs, according to their type of association with the host plant. Biotrophic pathogens evade surveillance and/or suppress the defense response, whereas necrotrophic pathogens take advantage of natural wounds and openings to penetrate plant tissues, grow intercellularly and produce enzymes and/or toxins that cause tissue death and nutrient outflow. Finally, another group of pathogens behaves like biotrophs during the early stages of infection by bypassing the plant's defense response. During the late stages of the disease, they display characteristics of necrotrophs, and the death of plant tissue occurs. These pathogens are called hemibiotrophs and include various fungi and bacteria [94,95].

8 Application of *in vitro* Mutagenesis in the Agave Genus

Agaves are a source of different products in the food and drug industry, production of biofuel, among other uses [96]. Its greatest application is the production of alcoholic beverages such as mezcal, bacanora, tequila, raicilla, pulque and comiteco [6,7,33,35].

There has been an increase in interest in the antioxidant, antimicrobial, antidiabetic, anti-inflammatory, antitumor properties of agave, among other factors.

However, there are few reports on the genetic improvement of species of the Agave genus, despite being a crop of agronomic and economic importance, and which has little natural regeneration and low genetic diversity. An example is the studies carried out by Trejo et al. [97], report genetic variations in seventeen agave varieties, classified into three clusters, describing that the high number of Agave races without differences in their morphological diversity or genetic reserves, can be explained by the plasticity of morphological traits and the events of gene flow.

On the other hand, regarding the application of *in vitro* mutagenesis in Agave, Díaz-Martínez et al. [98], described that regeneration process from different explants of *Agave tequilana* Weber var. Azul, it could reveal details about gene expression as a mechanism to cause the silencing of elements and avoid possible mutational effects.

Tejavathi et al. [99] reported genetic variability in *Agave vera-cruz* Miller plants, which were obtained through direct and indirect organogenesis and grown with arbuscular mycorrhizal fungi (AMF). Plants were also grown with tissues derived from exposure to gamma radiation at 15 Gy for 20 min in 2-min intervals and from exposure to ethyl methanesulfonate in intervals going from 0.1% to 1% for 1 to 5 h. It was observed that a distinct phenotypic variant deriving from indirect organogenesis presented more polymorphism, followed by samples treated with mutagens. Plants obtained of direct organogenesis and subsequently treated with AMF generated a separate subgroup that indicated an alteration. It was thus proposed that the use of *in vitro* culture methods, along mutagenesis and AMF symbiosis, are a tool in increasing the genetic variability of propagated plants.

On the other hand, Reyes-Zambrano et al. [69] reported that the use of ethyl methanesulfonate induced variations in the morphometric and morphological parameters of the seedlings obtained from *Agave americana* L., where 60% of the seedlings presented dwarfism and different foliar forms, with a lack of spines. Also, an increase in the fructan content of 30% with respect to the seedlings in the control treatment could be observed, along with an increase in PAL activity.

Studies carried out by Espino et al. [71], described the obtention of somatic embryos and mutant seedlings from tissue irradiated with gamma rays ^{60}Co. It was mentioned that the radiation generated mutations that altered competition in the cells, thus decreasing embryogenic callus and the development of somatic embryos. For their part, Reyes-Zambrano et al. [100], described that chitinase activity increased significantly in *Agave americana* L. plants, infected with *Fusarium oxysporum* at 15 days after inoculation (DAI) regarding uninfected plants, while β-1, 3 glucanase activity showed no statistically significant difference with control plants the results suggest that, in response to *F. oxysporum* infection, *A. americana* L. activates chitinase-like PRs proteins.

Recently, Reyes-Zambrano et al. [101] reported obtaining somaclonal variants in *Agave americana* L., that were resistant to *F. oxysporum*. These resulted from the induction of mutations with EMS at a concentration of 200 mM during an exposure time of 2 h.

9 Conclusions

Currently, *in vitro* mutagenesis represents an effective biotechnological tool to be used in genetic improvement programs, this technique becomes more relevant when it is applied to species of agronomic importance such as those of the Agave genus, which are characterized by having little genetic variation and slow natural regenerative capacity.

In vitro mutagenesis in combination with plant tissue culture and large-scale micropropagation systems such as temporary immersion systems can be used to obtain plants with outstanding characteristics such as resistance to biotic factors and/or tolerance to abiotic factors, fiber production, increase in the content of fructans and other specialized secondary metabolites such as inulin, antioxidant compounds among others.

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