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ARTICLE

Selection and Analysis of Polymorphisms in Somaclonal Variants of *Agave americana* Resistant to *Fusarium oxysporum* via an Ethyl Methanesulphonate Treatment

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ABSTRACT

Agave americana L. callus were exposed to different concentrations of ethyl methanesulphonate (EMS) 0, 15, 30, 45 and 60 mM and to different times of exposure (2 and 4 h). The viability and capacity of shoot formation were shown to be affected when the callus were exposed to high concentrations (30–60 mM). Only the callus exposed to 15 mM EMS presented shoot formation; the exposure time of two hours produced the largest quantity of shoots regenerated per callus (21 shoots/callus). In order to generate somaclonal variants resistant to *Fusarium oxysporum*, a selection pressure was applied through of a culture filtrate (CF) of 100 ppm of the fungus. This was made in callus obtained in the treatment with 15 mM EMS during 2 h of exposure. The CF caused oxidation and necrosis in 71.25% of the callus; however, they were capable of generating shoots (3.5 shoots/callus). Molecular markers type RAPD, ISSR and DAMD were used to evaluate the genetic variation arising from the mutations caused by EMS on control plants and 16-month-old somaclonal variants. The polymorphic information content (PIC) for each one of the initiating groups was: 0.28 (DAMD), 0.09 (ISSR) and 0.14 (RAPD). DAMD revealed a greater percentage of polymorphism than RAPD and ISSR. Polymorphic bands were detected in the somaclonal variants. This indicated that the EMS caused genetic variation in the regenerated plants conferring resistance to them against *Fusarium oxysporum*.

KEYWORDS

Mutagenesis; polymorphisms; genetic variation; molecular markers

Abbreviations

CF	culture filtrate
DAMD	directed amplification of minisatellite DNA regions
EMS	ethyl methanesulphonate
ISSR	inter-simple sequence repeats
PIC	polymorphic information content
RAPD	randomly amplified polymorphic DNA

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1 Introduction

Agave americana L. belongs to the family *Agavaceae* and produces fructans as the main carbohydrate reserve [1]. The leaves are used to produce fiber [2]. It is also a plant with a high potential for producing biofuels [3]. In Chiapas (Mexico), it is used for the elaboration of an alcoholic beverage known as "comiteco" [4].

In Mexico, the alcoholic beverage industry derived from the agaves (tequila, comiteco, mezcal) has been constrained because of a recurring reduction in yield due to crop damage. One of the most serious phytosanitary problems that the *Agave* genus faces is the wilt and dry bud rot disease, which is mainly associated with the fungus *F. oxysporum*. The infection starts as a whitening of the basal leaves, followed by wilt, necrosis, and rot inside the bud, which eventually causes the plant death [5–7].

For the control of pests and diseases, the use of synthetic agrochemicals such as insecticides, bactericides and fungicides continue to be the most important for increasing crop yield. However, these products give rise to environmental problems and soil deterioration, generating resistance and increasing production costs [8]. The safest and most cost-efficient control method for the environment is the use of resistant cultivars [9].

Genetic improvement is an alternative to resolve the problems of diseases in crops, by generating plants resistant to plagues or by enhancing an important characteristic which can make the plant more productive. This can be carried out by means of plant biotechnology through plant tissue culture [10]. *In vitro* mutagenesis and somaclonal variation are the two most important techniques based on tissue culture for the generation of crops resistant to biotic and abiotic stresses. These methods facilitate the improvement of one or more traits of a genotype without altering the rest of them [11].

The mutations can be induced by physical (ionizing radiations, gamma rays, X rays, etc.) and chemical treatments (ethyl methanesulphonate (EMS), sodium azide, etc.). However, the chemical mutagenesis is preferred rather than the physical one. This is due to the fact that the first produces more specific and predictable mutations (point mutations) which are also easier to apply [12–14]. EMS is one of the most used mutagens because it provokes a high frequency of genetic mutations and a low frequency of chromosomal aberrations [15,16]. The use of EMS has caused mutagenesis in banana and sugarcane, and it has been useful for allowing plant resistance to *Fusarium* [17,18].

The induced mutations contributed to genetic variability, given that they produced changes in the DNA; these changes are known as polymorphisms of the DNA [19]. Polymorphisms can be at random or specific, depending on the type of primer used, the amplification conditions, method of separation and the detection of fragments [20]. A large number of molecular markers have been widely used to (1) detect and characterize somaclonal variation, (2) estimate genetic diversity and stability, and (3) identify and select mutant plants in different plant species [15,21–25].

Among the different molecular markers based on DNA, the randomly amplified polymorphic DNA (RAPD) and the inter-simple sequence repeats (ISSR) have been the most used to detect genetic variation and polymorphism. This is because they are simple to use, rapid, reliable and reproducible [26–28]. The methods of single primer amplification reaction (SPAR) based on PCR include a combination of RAPD, ISSR and DAMD markers (the direct amplification of mini-satellite regions of DNA). In recent years, they have become an important tool for the analysis of genetic diversity in plants, and collectively provide a complete description of the nature and scope of the diversity, its genetic relationship and germplasm management [29–32].

To date, no reports are available regarding the generation of *A. americana* L. plants resistant to *F. oxysporum* through *in vitro* mutagenesis. The aim of this study was to induce *in vitro* mutagenesis in

callus of *A. americana* L. with the use of EMS to generate somaclonal variants resistant to *F. oxysporum* carrying out the analysis with molecular markers for the detection of polymorphisms.

2 Materials and Methods

2.1 Callus Induction

Basal explants containing several lateral meristems from 8-month old plantlets of *A. americana* L. obtained *in vitro* were placed on MS medium [33]. Vitamins, sucrose (30 gL^{-1}), myo-inositol (0.228 mM), sodium phosphate (0.362 mM), and phytagel (2.5 gL^{-1}), were added to that medium, supplemented with 0.11 µM 2,4-Dichlorophenoxyacetic acid (2,4-D) and 44 µM 6-Benzylaminopurine (BAP). Explants were incubated for two months at 25°C ± 2 under continuous light [34].

2.2 Treatment with Ethyl Methanesulphonate

Following the method of Koch et al. [35], 0.2 g of callus were weighed and subsequently exposed to different concentrations of EMS (0, 15, 30, 45, 60 mM), and to different times of exposure (2 and 4 h). The callus was then rinsed three times with half strength MS liquid media without regulators. The treated callus was transferred to glass flask containing MS medium without growth regulators. All treatments were carried out in triplicate. After a period of 4 weeks, the number of regenerated shoots per treatment was evaluated.

2.3 F. oxysporum Culture and Filtrate Preparation

The strain *F. oxysporum* was isolated from infected *Agave americana* L. with an accession number in the GenBank (MT791313) [5]. It was cultured in potato dextrose agar (PDA) over a period of 5 days, after which a mycelial square was cut from the edge of the colony, planted in 250 ml of Czapek Dox broth, and shaken at 145 rpm for 5 days. The supernatant was filtered through a paper filter at constant weight. In order to obtain the dry mass of the culture, the mycelium was dried at 80°C for 24 h. The concentration of each lot of the culture filtrate (CF) was expressed as the fungal dry mass/volume of broth used in the liquid culture of *F. oxysporum*. The culture filtrate was centrifuged at 4,000 rpm for 45 min, after which it was passed through a Whatman No. 1 paper. The filtration was carried out sequentially using a millipore filter of 0.45 and another of 0.22 µm. Finally, the culture filtrate was centrifuged at 15,000 rpm for 30 min at 20°C, and the supernatant was transferred to a sterile Falcon tube for subsequent use. Different concentrations of CF were used to determine the best concentration (data not showed).

2.4 Effect of the F. oxysporum Filtrate on the Shoot Regeneration of A. americana L.

All the callus exposed to EMS, at four weeks of culture, which were maintained in MS medium free of growth regulators, were transferred to an MS medium supplemented with vitamins, Sucrose, (30 gL^{-1}), ammonium nitrate (1.65 gL^{-1}), potassium nitrate (1.9 gL^{-1}), Phytagel (2.5 gL^{-1}), and 2,4-D (2.05 μML^{-1}). Also, 100 ppm of the culture filtrate of *F. oxysporum* were added over a period of 8 weeks, following the methodology proposed by Mahlanza et al. [18] with some modifications. The flasks were incubated at 25°C ± 2 under continuous light. For the regeneration of shoots, the callus was transferred to an MS medium without growth regulators for 4 weeks of culture. Thereafter, the number of regenerated shoots for each treatment was calculated.

2.5 Isolation of DNA and Reactions of Amplification by PCR

DNA was obtained from leaf tissues of 4 regenerated plants of *A. americana* L.. These plants were 16-month-old, and were obtained with 15 mM of EMS + 100 ppm CF. Control plants from the *in vitro* culture were not treated with the mutagen. The extraction of DNA was performed in accordance with the methodology of Keb-Llanes et al. [36].

RAPD markers (random amplified polymorphic DNA): four primers were used; the hybridization temperature was established in accordance with each primer: 50°C for (GAC) 5 and (GTG) 5; 42°C for (TCC) 5 and (GACAC) 3. The amplification by PCR was performed in a reaction mixture of 25 µL containing 1 × PCR buffer (10 × 200 mM Tris-HCl, 500 mM KCl, pH 8.4; Invitrogen), 0.25 mM of each dNTP (Invitrogen), 2 mM $MgCl_2$, 0.8 µM primers, 10 ng of DNA and 1 U of Taq polymerase (Invitrogen). The amplification of DNA was carried out using a GeneAmp 9700 (PerkinElmer) thermocycler. The thermo cycles were at 95°C for 5 min, and 40 cycles of 40 s at 95°C, 60 s at the temperature of appropriate alignment, 60 s at 72°C and 5 min at 72°C.

DAMD (directed amplification of minisatellite DNA regions): DAMD six primers were used: HVR, 33.6, YNZ22, M13, HVA and HBV. The PCR reactions were carried out in volumes of 25 µl which contained 1 × PCR buffer (Invitrogen) (Tris-HCl 10 × 200 mM, KCl 500 mM, pH 8.4), 0.2 mM of each dNTP (Invitrogen), $MgCl_2$ 2 mM, primers 0.2 µM, 60 ng of DNA and 1 U of DNA Taq polymerase (Invitrogen). DNA amplification was carried out using a GeneAmp 9700 (Perkin–Elmer) thermocycler. The conditions of the reaction cycles of amplification were 95°C for 5 min; followed by 40 cycles of 1 min at 95°C, 2 min at 55°C, 2 min at 72°C, and 7 min at 72°C.

ISSR (inter-simple sequence repeats): The ISSR was performed with six markers: (GACA)4, (AG)8TA, (GA)6GG, (TG)8GT, (TG)8GT and (AG)8TC. PCR reactions were carried out in volumes of 25 µl which contained 1 × PCR buffer (Invitrogen) (Tris-HCl 10 × 200 mM, KCl 500 mM, pH 8.4), 0.2 mM of each dNTP (Invitrogen), $MgCl_2$ 2.5 mM, primers 0.7 µM, 50 ng of DNA and 1.25 U of Taq polymerase DNA (Invitrogen). The amplifications reaction cycle conditions were at 94°C for 5 min, followed by 33 cycles of 1 min at 94°C, 75 s at 48°C, 4 min at 72°C, and 7 min at 72°C. The amplification of the DNA was performed using a GeneAmp 9700 (Perkin-Elmer) thermocycler.

2.6 Electrophoresis

All the amplified fragments of DNA were separated by electrophoresis in agarose gel at 1.5% (p/v) using TBE buffer at a constant voltage of 5 V/cm. The gels were dyed with ethidium bromide and the bands were visualized under a UV transilluminator. The images were taken with a Gel DocTM EZ (BioRad) image generator. Two replications were made per gel.

2.7 Data Analysis

The EMS effect on the formation of the number of shoots in callus and the effect of the culture filtrate of *F. oxysporum* on the percentage of oxidation/necrosis in callus were determined by analysis of variance (ANOVA) with a significance level of 95%. Mean comparisons were conducted by the least significant difference test, using the STATGRAPHICS® Centurion XVI.II software.

For molecular analysis, the data were qualified as discrete variables using "1" to indicate the presence and "0" to indicate the absence of a fragment. The binary data obtained from the qualifications of the profiles RAPD, DAMD and ISSR with different primers, individually and cumulatively, were subjected to the construction of a similarity matrix. With this purpose, the similarity coefficients for the method of unweighted peer groups using the method of mathematic averages (UPGMA) were used. The UPGMA method was used to generate the dendrogram based on the coefficient of similarity in the module of groups in hierarchical clusters and nested sequential agglomerate (SAHN) of the software version for PC NTSYS, version 2.0. The data were also used to calculate the content values of polymorphic information content (PIC) in accordance with the equation following Anderson et al. [37]:

where: Pi was the frequency of the ith allele, and K was the total number of different alleles at the locus.

3 Results and Discussion

In the genetic improvement by means of induced mutations, it is important to optimize the dosage of the mutagenic agent and the exposure time. In general, the dosage is optimized with the use of various concentrations of the mutagen or the duration of the treatment, or a combination of both [25]. In this study, different concentrations of EMS and different times of exposure were employed (Tab. 1); it was possible to observe how the viability and capacity of shoot formation was affected in the callus of *A. americana* L. which were treated with ethyl methanesulphonate at different concentrations and exposure times. Only the callus treated with 15 mM of EMS presented shoot formation, and no response was observed at higher concentrations (30–60 mM). Moreover, the number of shoots either diminished or did not vary as the exposure time of the callus to EMS increased (Tab. 1).

Table 1: Effect of EMS on the number of shoots in callus of *A. americana* L. treated with different concentrations of ethyl methanesulphonate (EMS) at different times (2 and 4 h)

Treatment	EMS (mM)	Time (h)	Number of shoots
1	15	2	21 ± 3.0^{b}
2	15	4	9 ± 1.52^{c}
3	30	2	0 ± 0^{d}
4	30	4	0 ± 0^{d}
5	45	2	0 ± 0^{d}
6	45	4	0 ± 0^{d}
7	60	2	0 ± 0^{d}
8	60	4	0 ± 0^{d}
Control	0	0	54 ± 2.64^{a}
DMS (0.05)			2.44

Note: *Values with the same letter are not significantly different between treatments. DMS: minimum significant difference ($p < 0.05$).

Other studies also indicate that the survival of explants treated with chemical mutagens and their capacity to regenerate in plants is reduced with an increase in the concentration and time of exposure to the mutagen [38]. In soybean (*Glycine max*) cell suspension cultures it was possible to observe a reduction in the survival of cultures when the EMS dose was increased [15]. The same behavior was presented in *Vitis vinífera*, as well as a reduction in the capacity to regenerate shoots when the dose of the mutagenic agent increased [39]. The use of callus as plant material for mutation induction is very effective. This is because the callus is a population of partial-unorganized mass of cells that have not undergone differentiation and divide continuously. The callus was also very sensitive to the mutagen because the cells are actively dividing so that the chance of a mutation is very large [40].

The callus immersed in the EMS solution showed different growth responses depending on the time and concentration used. Callus exposed to 15 mM of EMS for 2 h presented an average of 21 shoots per explant; a longer exposure time to the EMS (4 h) decreased the number of shoots obtained by the explant (Tab. 1). The reduction in shoot formation is probably caused by the use of alkylating agents which provoke the generation of toxic products and subsequently block the replication, suppressing cellular division as well as cell differentiation [41].

For obtaining somaclonal variants resistant to *F. oxysporum*, it was used 15 mM de EMS + culture filtrate (CF) of *F. oxysporum* in callus. The culture filtrate provoked oxidation and necrosis in the control callus (Fig. 1B). However, the percentage of oxidation and necrosis was lower in the callus treated with 15 mM of EMS than in the control (Fig. 1C). Callus oxidation and necrosis in the presence of the culture filtrate of *F. oxysporum* is due to the content of toxins and enzymes implicated in the pathogenesis, which induce oxidative stress during the *in vitro* selection pressure. In this regard, establishment of an appropriate phytotoxin concentration that negatively affects cells, tissues, organs and whole plants increases the probability of obtaining tolerant lines [42]. *Fusarium* spp. culture filtrates and purified toxins have been used widely in callus [43,44], root growth [45,46] and leaf necrosis [43] tests to select tolerant genotypes. Callus control in contact with the culture filtrate of *F. oxysporum* suffered necrosis (Fig. 1B); thus, shoot generation was not observed. However, in callus treated with 15 mM of EMS, despite the oxidation, the cells had the capacity to regenerate shoots which were tolerant to *F. oxysporum* (Fig. 1C). This was because they were able to survive this selection pressure (Tab. 2). The somaclonal variants obtained after the mutagenesis process and the selection pressure with the pathogen filtrate presented a normal morphology to the control plants.

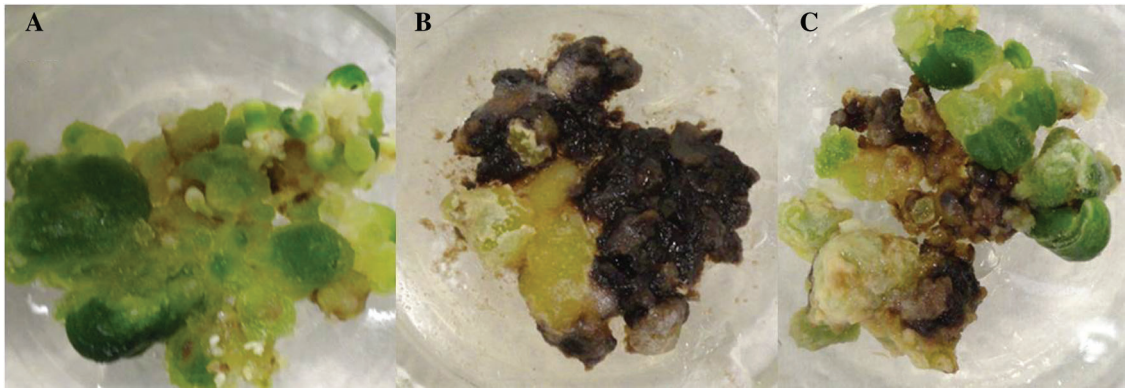

Figure 1: Effect of the culture filtrate of *F. oxysporum* on callus of *A. americana* L. after four weeks from treatment initiation. (A) Callus control, (B) Callus control + 100 ppm culture filtrate of *F. oxysporum*, (C) Callus treated with 15 mM of ethyl methanesulphonate + 100 ppm culture filtrate of *F. oxysporum*.

Table 2: Effect of the culture filtrate of *F. oxysporum* on the percentage of oxidation/necrosis of callus and on the number of shoots in *A. americana* L. after eight weeks from treatment initiation

Treatment	Oxidation/Necrosis %	Number of shoots
Control	0 ± 0^{c}	54.25 ± 1.47^{a}
Control + 100 ppm CF	100 ± 0^{a}	0 ± 0^{c}
15 mM of EMS + 100 ppm CF	71.25 ± 8.53^{b}	3.5 ± 1.11^{b}
DMS (0.05)	7.9	2.3

Note: CF: Culture filtrate, Values with the same letter are not significantly different between treatments. DMS: minimum significant difference ($p < 0.05$).

The regeneration ability of cells to form shoots depends on the mechanism of tolerance or/and resistance of each cell to cope with biotic or abiotic stress on the selection media [44]. The mutation induced by the EMS and the *in vitro* selection pressure through culture filtrates of fungi (CF) have been reported to generate both genetic variation and somaclonal variation, and acquisition of lines resistant to *Fusarium* [17,18,47]. In addition, *in vitro* use of culture filtrates have demonstrated to be adequate agents of selection in studies of disease resistance [48].

The analysis of genetic variability in four somaclonal variants of *A. americana* L. obtained with the treatment of 15 mM of EMS and CF of *F. oxysporum* (Fig. 2), and in control plants which were not treated with the mutagen was carried out with the use of DAMD, ISSR and RAPD markers.

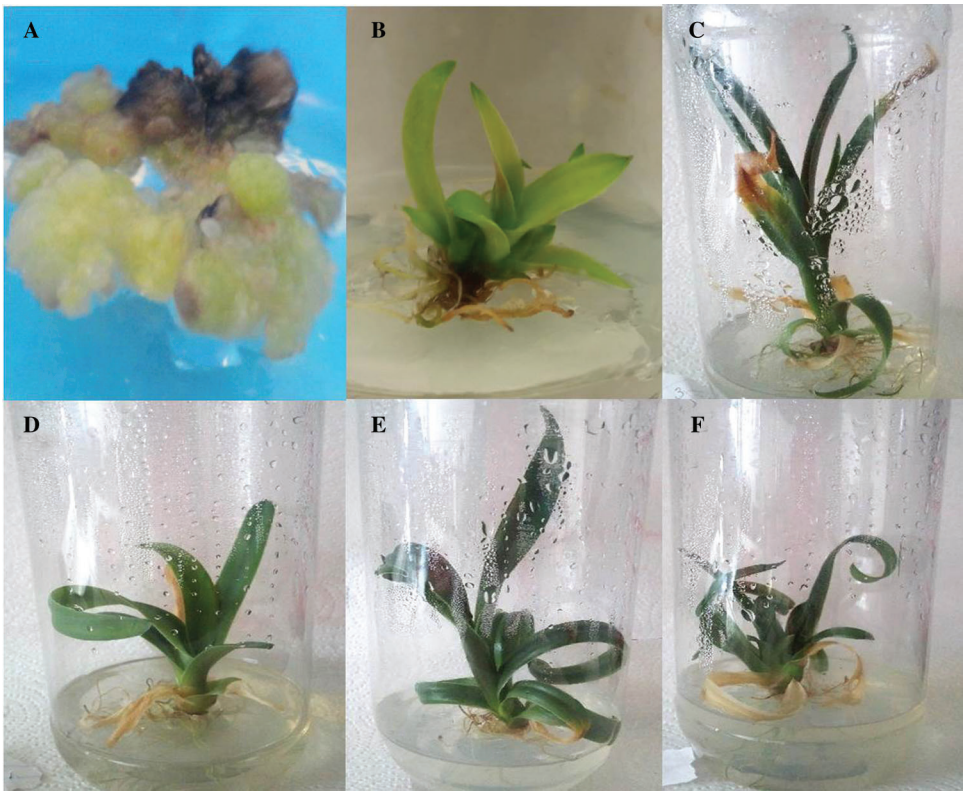

Figure 2: Somaclonal variants resistant to *F. oxysporum* on *A. americana* L. (A) Callus treated with 15 mM of ethyl methanesulphonate + 100 ppm culture filtrate of *F. oxysporum*, (B) shoots regeneration at eight weeks, (C) Clone 1, (D) Clone 3, (E) Clone 6, (F) Clone 7. All clones are 16-month-old

Fig. 3 shows the amplification profiles generated by the molecular markers used. Of the four primers used with RAPD, only the primer $(TCC)^5$ revealed a polymorphic fragment of ~2500 pb (Fig. 3A) on *A. americana* L. plants treated with 15 mM of EMS. Similarly, the primer (AG)8TA (of the six primers used for ISSR), generated a polymorphic fragment in ~2800 pb (Fig. 3B). This fragment is absent in the control plants and present in the plants treated with 15 mM of EMS. However, with the DAMD markers, polymorphic fragments were revealed with two of the five primers used (Fig. 3C), with the primer 33.6 a polymorphic fragment was generated at ~4800 pb. This fragment was absent in the CL1 and present in the control plant, and it was also present in the somaclonal variants CL3, CL6, and CL7. With the HVR primer, a polymorphic fragment was generated at ~2800 pb, present in three of the somaclonal variants (CL3, CL6, CL7) and absent in the control plant and in CL1. The polymorphic fragments observed with

the different molecular markers used can indicate the presence of genetic differences between the control plants and those obtained with 15 mM of *A. americana* EMS. These differences were caused by the treatment with the chemical mutagen. In other words, the polymorphisms obtained were caused by the mutations produced at the sites of union of the primer, resulting in an increase or decrease in the total number of union sites of the primer and, in consequence, in the number of fragments amplified [15,26]. The direct-acting alkylating agents, such as the EMS, mainly cause point mutations which are the result of an elimination, addition or substitution of only one pair of bases [49].

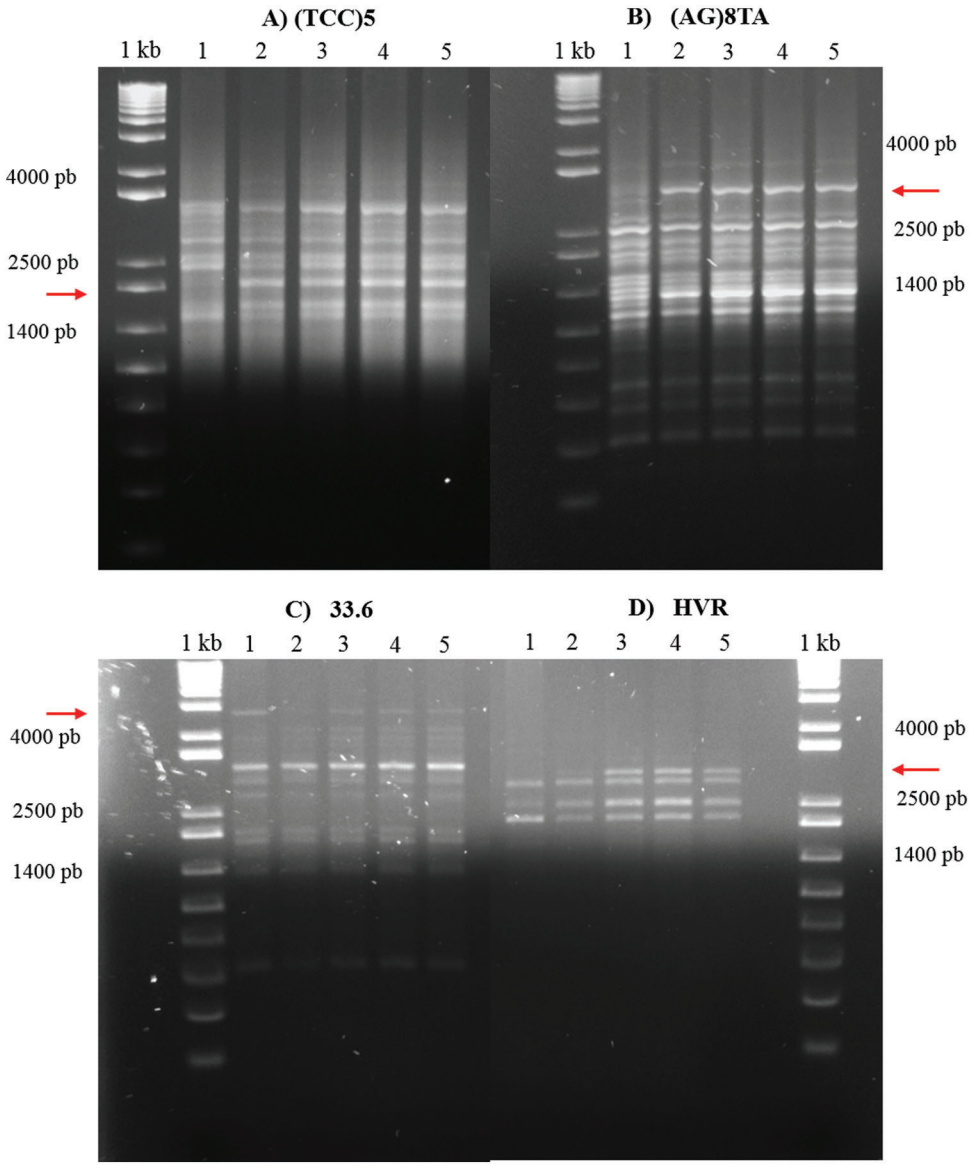

Figure 3: Amplification profiles obtained from different somaclonal variants of *A. americana* L. in agarose gel. (A) RAPD, (B) ISSR, (C–D) DAMD, Line 1: CONTROL, Line 2: CLONE 1, Line 3: CLONE 3, Line 4: CLONE 6, line 5: CLONE 7. *Red arrows:* indicate absence/presence of bands. (A) $(TCC)^5$, (B) (AG)8TA (C) 33.6 (D) HVR

The three types of molecular markers employed in the present work revealed that the polymorphic information content (PIC) for each one of the initiating groups was: 0.28 (DAMD), 0.09 (ISSR) and 0.14 (RAPD). This may be due to the fact that the amplicons generated by RAPD are obtained in regions widely distributed in the genome, whereas the amplicons generated by ISSR and DAMD are obtained from portions of the genome rich in microsatellite [29].

The three types of markers used were capable of detecting polymorphisms in the somaclonal variants obtained by mutagenesis with EMS. DAMD revealed a higher percentage of polymorphism than RAPD and ISSR. This suggests that DAMD generated greater information for the determination of the genetic variation in plants regenerated from *A. americana* L. treated with ethyl methanesulphonate. The UPGMA dendrogram (Fig. 4) demonstrated that the level of similarity between the control plants and those treated with EMS was high (0.92–0.99), and it was distributed in two main groups: in Group 1 the control plants, and in Group II the plants treated with EMS (Fig. 4). In Group I the control plants were found with a similarity coefficient of 0.92 with respect to the rest of the somaclonal variants. Group II was subdivided into two subgroups: (IIa), where the somaclonal variant CL1 was found and (IIb) where the remaining somaclonal variants analyzed were found (CL3, CL6 and CL7). These later somaclonal variants presented the greatest similarity between them (coefficient = 0.986). This indicated the existence of genetic variation between the somaclonal variants. Such variation was originated from the mutations caused by the EMS.

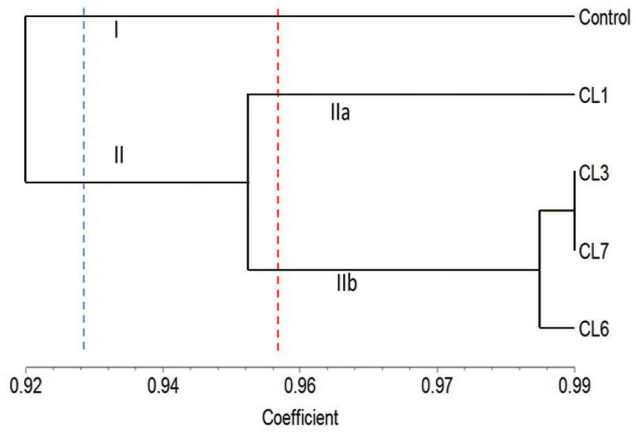

Figure 4: UPGMA dendrogram originated from the amplification profiles generated by the three types of molecular markers

There are very few reports in the genus *Agave* where the application of physical or chemical mutagens are used to generate resistance or tolerance to various types of stress (biotic and abiotic). We found that there was a decrease in the number of regenerated shoots in calluses treated with EMS. In embryogenic calluses of *Agave tequilana* Weber var. Azul, obtained by means of a physical mutagen such as gamma irradiation, an irradiation of 20 Gy caused an embryogenic callus induction percentage of 44.44 after 28 days from induction; callus that were not irradiated presented a percentage of embryogenic callus induction of 88.89. This decrease in the percentage of induction of embryogenic callus indicates that the acquired mutations cause changes in the capacity of cell division. Due to this, the development of somatic embryos is also affected as well as the conversion to plantlets at low radiation doses (10 and 20 Gy) [50].

Pathogenicity tests of *Cercospora agavicola* in detached leaves of vitro plantlets of *A. tequilana* irradiated with gamma rays Co^{60} have shown that this physical mutagenic agent causes significant

changes in the diameter of the lesion as a response of the defense plant mechanisms. This indicates additive genetic behavior (horizontal resistance) [51].

In the genus *Agave*, the induction of mutants by physical (gamma rays) and chemicals (EMS) mutagenic agents has recently been used. EMS causes phenotypic modifications in the morphological and morphometric parameters, as well as an increase in the content of fructans and fructose; it also increases the activity of the enzyme phenylalanine-ammonium lyase (PAL) which is related to a higher concentration of anthocyanins in *A. americana* L. plants [52]. The application of low doses of gamma rays Co^{60} on *in vitro* plants of *A. tequilana* induces genetic variability and stimulates the synthesis of fructooligosaccharides [53].

Recently Reyes-Zambrano et al. [5] published a study on plants obtained from the *in vitro* culture of *A. americana* L which were six-month-old and had acclimatized 3 months in a greenhouse environment. These plants showed the activity of PRs proteins (chitinase and β-1,3 glucanase), which act as a defense mechanism against infection of a *F. oxysporum* strain. These authors report that the specific chitinase activity showed a significant increase (63.43 μmol min^{-1} μg protein) in the evaluated plants after 15 days from infection with the *F. oxysporum* strain (accession number in GenBank MT791313) with respect to the uninfected plants (27.24 μmol min^{-1} μg protein). Such increase in activity may be due to the fact that some PRs proteins are expressed constitutively at basal levels, but their expression increase in response to a pathogen attack with the subsequent activation of the systematic acquired response (SAR).

Mutagenesis through chemical and physical processes can be used in the genus *Agave*. Both processes generate changes in DNA and can be used as a tool to generate genetic variation and phenotypic and morphological changes as well as to improve biochemical and physiological processes [5,53].

4 Conclusions

In this mutagenesis study on callus of *A. americana*, ethyl methanesulphonate and a selection pressure by means of a culture filtrate of *F. oxysporum* were used to generate somaclonal variants resistant to this pathogen. The obtained results demonstrated that the EMS causes point mutations in the callus cells of *A. americana*, which confer resistance to the pathogen. EMS was capable of generating genetic variation in these calluses. This was observed in the somaclonal variants on regenerated plants through the detection of polymorphisms using molecular markers (e.g., RAPD, ISSR and DAMD). DAMD was the marker which provided most information for the determination of the genetic variation in somaclonal variants of *A. americana* treated with EMS. These results can be considered as the basis for future genetic improvement programs on *A. americana* and the *Agaveceae* family. This will be through the development of cultivars resistant to *F. oxysporum*, the implementation of techniques for the massive micropropagation of plants, and the generation of models for studying the genes of resistance involved in the pathogenesis of *F. oxysporum*.

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