ANALYSIS OF THE SOMACLONAL VARIATION IN TWO IN VITRO REGENERATED AGAVE SPECIES†

[ANÁLISIS DE LA VARIACIÓN SOMACLONAL EN DOS ESPECIES DE AGAVE REGENERADAS IN VITRO]

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SUMMARY

Background: Plant tissue culture has been shown to be an efficient technique for the propagation of diverse Agave species using different in vitro regeneration processes. However, it has been demonstrated that genetic changes can occur in plants regenerated under these schemes, also called somaclonal variation. Objective: the objective of this study was to determine the genetic fidelity of plantlets regenerated from three different explants (mature zygotic embryonic axis, in vitro plantlet meristematic zone, and ex vitro plantlet meristematic zone) using two pathways of micropropagation (direct and indirect organogenesis) of A. salmiana and A. marmorata. Methodology: somaclonal variation of the obtained clones was evaluated using different DNA markers, such as anchored simple inter-sequence repeat (ASSR) and random amplified polymorphic DNA (RAPD). Results: the results show that only in those clones that undergo a callus phase and, consequently, indirect organogenesis, somaclonal variation was observed. In contrast, those clones obtained by direct organogenesis were genetically stable, it means not polymorphic bands were observed. Implications: it was achieved an efficient propagation protocol for A. salmiana and A. marmorata, maintaining genetic stability of regenerated plantlets as well as a possible alternative for genetic improvement by observing somaclonal variation via indirect organogenesis in both evaluated species. Conclusions: in this research, the micropropagation pathway (direct and indirect organogenesis) was the determining factor to maintain or not the genetic fidelity of the regenerated plants in both species of Agave used.

Key words: Agave salmiana, Agave marmorata, direct and indirect organogenesis, somaclonal variation, RAPD and ASSR.

RESUMEN

Antecedentes: El cultivo de tejidos vegetales ha demostrado ser una técnica eficiente de propagación en diversas especies de Agave mediante diferentes procesos de regeneración in vitro. Sin embargo, se ha comprobado que pueden ocurrir cambios genéticos en las plantas regeneradas bajo estos esquemas, también llamada variación somaclonal. Objetivo: el objetivo de este estudio fue determinar la fidelidad genética de plántulas regeneradas de tres explantes diferentes (eje embrionario cigótico maduro, zona meristemática de plántulas in vitro y zona meristemática de plántulas ex vitro) mediante dos vías de micropropagación (organogénesis directa e indirecta) en A. salmiana y A. marmorata. Metodología: se evaluó la variación somaclonal de los clones obtenidos usando diferentes marcadores de ADN, como los de inter-secuencia simple repetidas de tipo anclado (ASSR) y amplificación aleatoria de ADN polimórfico (RAPD). Resultados: los resultados muestran que solo en aquellos clones que pasaron por una fase de callo y consecuentemente organogénesis indirecta se observó variación somaclonal. Por el contrario, aquellos clones obtenidos por organogénesis directa fueron estables genéticamente, lo anterior significa que no se observaron bandas polimórficas. Implicaciones: se logró desarrollar un protocolo eficiente de propagación para A. salmiana y A. marmorata, manteniendo la estabilidad genética de las plantas regeneradas, además de considerar una posible alternativa de mejoramiento genético al observar variación somaclonal vía organogénesis indirecta en ambas especies evaluadas. Conclusión: en esta investigación la vía de micropropagación (organogénesis directa e indirecta) fue el factor determinante para mantener o no la fidelidad genética de las plantas regeneradas en ambas especies de Agave usadas.

Palabras clave: Agave salmiana, Agave marmorata, organogénesis directa e indirecta, variación somaclonal, RAPD y ASSR.

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INTRODUCTION

Among the most conspicuous plants in the Mexican landscape, especially in the arid and semi-arid areas of Mexico, are the agaves or magueys (García-Mendoza, 2007). These plants have important roles in wealth-generating economic activities, such as the industries of alcoholic beverage (tequila and mescal), fermented (pulque), natural fiber, construction material, paper, and therapeutic products, among others (Méndez-Gallegos et al., 2011). On the other hand, their slow growth and low rates of sexual and sexual reproduction make agaves difficult to reproduce massively. For this reason, in vitro propagation is a promising alternative for large-scale plant propagation (Domínguez-Rosales et al., 2008).

_A. marmorata_, for manufacturing mescal (Nieto et al., 2016) and mostly for extracting pulque, _A. salmiana_ (Aguilar-Juárez et al., 2014), are representative species of maguey used to obtain beverages. However, in the in vitro propagation of plants, the most important concern is retaining the genetic integrity of the clones, with respect to the mother plant, since genetic instability can be a problem associated with the propagation industry (Pérez-Ponce, 1998). It is well known that in vitro culture techniques can induce genetic instability, that is, somaclonal variation (Larkin and Scowcroft, 1981). Among the strategies for evaluating somaclonal variations are phenotype identification and DNA analysis by using molecular markers. However, the latter is more effective since some changes induced by in vitro culture cannot be detected visually. When this occurs, somaclonal variation can be evaluated using DNA analysis (Palombi and Damiano, 2002). Among the molecular markers used are those based on PCR (polymerase chain reaction) and RAPD (Random Amplified Polymorphic DNA) that consist of amplifying DNA fragments with a primer of a length of ten pairs of bases with random sequences, which hybridize with the DNA (Williams et al., 1991), and SSR (Simple Sequence Repeats) that amplify genomic regions between two microsatellites with ASSR (Anchored Simple Sequence Repeat) type primers (Alcántara, 2007; Yamagishi et al., 2002). This technique is characterized as being rapid, due to its high rate of reproducibility, and efficiency in detecting polymorphism (Pradeep et al., 2002). Both markers have been widely used to evaluate variation generated by in vitro techniques (Agarwal et al., 2008; Hashmi et al., 1997; Palombi and Damiano, 2002; Rahman and Rajora, 2001; Victoria et al., 1994).

Although the genus Agave is considered to have a relatively stable karyotype because of its asexual propagation and it has been postulated that its speciation occurred as the result of determined mutations and DNA reordering (Cavallini et al., 1996), genetic variation has been detected using molecular techniques to distinguish among plants of different types and origins (Alfaro-Rojas et al., 2007; Rodríguez-Garay et al., 2008; Torres-Morán et al., 2010).

For this reason, genetic analysis is an important complement to propagation processes to elucidate changes that can occur in plants after their micropropagation by direct and indirect organogenesis techniques and correlate changes with the propagation method used. Thus we studied the genetic integrity of in vitro regenerated _A. salmiana_ and _A. marmorata_ plantlets using two types of molecular markers: RAPD and ASSR.

MATERIALS AND METHODS

Plant material

Leaf tissue from in vitro-regenerated plantlets of both species whose origin was three explants: mature zygotic embryonic axis from 240 seeds (E1), meristematic zone from 480 in vitro germinated plantlet of 45 days-old (E2), meristematic zone from 480 ex vitro plantlet of 6 months-old (E3), obtained by micropropagation techniques (direct (DO) and indirect (IO) organogenesis) of _A. salmiana_ (AS) and _A. marmorata_ (AM) (Table 1) were used. It should be noted that all explants used in this study were obtained from seeds. Wild _A. salmiana_ capsules were collected in the municipality of Toluca, State of Mexico (19°24'32.12" N and 99°41'26.80" W), and _A. marmorata_ seed was collected in Zimatlán, Oaxaca (16°52'8.18"N and 96°46'34.00"W). It is worth mentioning that the capsules of each species were collected from a single specimen.

The three explants: E1, E2 and E3 were established in MS medium supplemented with plant growth regulators (PGR), with concentrations of the cytokinin benzyl aminopurine (BA) and the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) as shown in Table 1. In the IO process two subcultures were performed, each with a duration of 30 days for organogenetic callus induction. The obtained calli were established in MS medium supplemented with BA cytokinin, and in the same way, two subcultures were done with the same duration (30 days) to initiate regeneration of shoots. The shoots obtained were established in MS rooting medium without PGR where they remained for 45 days, while the explants regenerated by OD followed the same process but without the callus induction phase. From all in vitro regenerated plantlets of each species, 15 clones from a single specimen for E1, E2, E3 of each of the techniques used (DO and IO)
were selected. It should be mentioned that the selected treatments were those that had the best shooting results (Arzate-Fernandez et al., 2020). In vitro plantlets were selected for their uniform size to form clones from which leaf tissue was taken to extract DNA.

**DNA extraction**

Genomic DNA was extracted for each clone using the cetyl trimethyl ammonium bromide (CTAB) method with slight modifications of the Zhou and Miwa (1999) procedure. The modifications consisted of macerating 150 mg fresh plant tissue in a porcelain mortar, previously frozen (-20 °C), and washing three times with Wash Solution (WS). The pellet was incubated with the active compound of CTAB and again washing twice with chloroform: isoamyl alcohol. RNA was eliminated with 1 µL RNase and later a wash with 96% ethanol to achieve its precipitation. Finally, the DNA was diluted in a buffer solution of Tris-EDTA (TE) to 70 µL and conserved at -20 °C in a General Electric® freezer until use.

**PCR amplification and DNA electrophoresis**

Ten primers of two types: five RAPD primers and five ASSR primers were assayed. The sequences of the RAPD-type primers were those used by Yamagishi (1995) and the ASSR primers by Yamagishi et al. (2002).

The PCR reaction was carried out in the total reaction of 10 µL, which contained 0.3 µL MgCl2, 0.2 µL four dNTPs, 0.5 µL of the primer (20 µM), 0.2 µL My TaqDNA polymerase (Bioline®), 1.0 µL My Reaction Buffer (Bioline®), 1.0 µL genomic DNA (10 ng) and 6.8 µL MilliQ water.

The RAPDs like primers (Yamagishi, 1995) and ASSR primers (Yamagishi et al., 2002) sequences are shown in Table 2.

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### Table 1. Origin of used explants and two morphogenetic processes applied and treatments assayed to regenerate in vitro plantlets of both agave species.

| Explant | Name of the material | Species | Explant used | Morphogenetic process (DO-IO) | Callus induction (2,4-D/BA mg L⁻¹) | Shoot regeneration (BA mg L⁻¹) |
|---------|----------------------|---------|-------------|-----------------------------|----------------------------------|-------------------------------|
| E1      | E1ASIO               | A. salmiana | Mature zygotic embryonic axis | IO                            | 1.0/15.0                          | 10.0                           |
|         | E1AMIO               | A. marmorata |                                          | 5.0/3.0                       | 5.0                              |
| E2      | E2ASDO               | A. salmiana | Meristematic zone from in vitro plantlets | DO                            | NA                              | 10.0                           |
|         | E2AMDO               | A. marmorata |                                          | NA                           | 5.0                              |
|         | E2ASIO               | A. salmiana |                                          | IO                            | 1.0/15.0                          | 10.0                           |
|         | E2AMIO               | A. marmorata |                                          | 5.0/3.0                       | 5.0                              |
| E3      | E3ASDO               | A. salmiana | Meristematic zone from ex vitro plantlets | DO                            | NA                              | 10.0                           |
|         | E3AMDO               | A. marmorata |                                          | NA                           | 5.0                              |
|         | E3ASIO               | A. salmiana |                                          | IO                            | 1.0/15.0                          | 10.0                           |
|         | E3AMIO               | A. marmorata |                                          | 5.0/3.0                       | 5.0                              |

NA= not applicable

### Table 2. Sequence of the used primers for PCR amplification of DNA from two agave species.

| Primers     | Primer sequence |
|-------------|-----------------|
| RAPD Y24    | AACC CGGC TTC   |
| RAPD Y29    | TCCG GGC CGT    |
| RAPD Y37    | TACCG CCG GC    |
| RAPD Y38    | TAAC CGC GC     |
| RAPD Y41    | GCCT CTC GGG    |
| 3'-ASSR02   | 5'-(CT) ATC-3'   |
| 3'-ASSR15   | 5'-(CT) ATG-3'   |
| 3'-ASSR20   | 5'-(CT) GCA-3'   |
| 3'-ASSR29   | 5'-(CT) GTA-3'   |
| 3'-ASSR35   | 5'-(CT) TGA-3'   |

For the RAPD primers, the thermal cycles were 94°C for 5 min, 54°C for 1 min, 72°C for 2 min for the first cycle, followed by 41 cycles of the thermal profile: 94°C for 1 min, 54°C for 1 min, 72°C, all for 2 min, and a final cycle of 72°C for 10 min, 54°C for 1 min and 72°C for 5 min. The amplification cycles for both ASSR primers were those described by Yamagishi et al. (2002) beginning at a temperature of 94°C for 9 min, followed by 45 cycles of the thermal profile: 94°C, 46°C, 72°C, all for 1 min, and a final cycle of 72°C for 10 min.

DNA amplification was performed in a thermocycler Labnet International Inc. (MultiGene optiMax®). Separation of the DNA fragments was achieved in electrophoresis chambers using 1% type II agarose gel (Sigma®) to which 3 µL ethidium bromide (0.5 µg/ml) (Sigma®) was added. The run conditions for each sample were 80V and 120 mA for 80 min. The amplified fragments were visualized in a transilluminator BioDoc-It Imaging System (UVP®). Size of the amplification products was estimated with a low-range (2,500 pb) and a high-range (15,000 pb) ladder marker (Fermentas®).
Statistical analysis

Data were analyzed with a binary data matrix (BDM) that signals the presence (1) or absence (0) of bands for each individual considering all the loci identified in the sample. The results obtained in the BDM were estimated using genetic distance values, according to Nei (1972). To visualize similarities among individuals more appropriately, a dendrogram was constructed using a similarity matrix with the unweighted pair grouping method with arithmetic means (UPGMA) in POPGENE software (version 1.32; Molecular Biology and Biotechnology Center, University of Alberta and Center for International Forestry Research, AB, Edmonton, Canada) (Yeh and Boyle, 1999). To determine the reproducibility and consistency of the results obtained with both types of primers (RAPD and ASSR), the DNA was extracted from the same samples and amplified in triplicate with the same primers. Only those primers that amplified clear banding patterns were used.

RESULTS AND DISCUSSION

In this study it was demonstrated that the DNA from A. salmiana (AS) and A. marmorata (AM) was amplified selectively by PCR when different combinations of primers were used, indicating that the method of DNA extraction was satisfactory. However, only two RAPD type primers (Y24 and Y41) and two ASSR type primers (ASSR20 and ASSR29) managed to amplify clear banding patterns, which were enough for the analysis of the somaclonal variation in both species of agave.

Size of the fragments amplified by both primers RAPDs (Y41 and Y24) in the two agave species ranged between 400 and 1500 pb, coinciding with those reported (< 2000 pb) when this technique was used in similar studies of other plant species (Yah-Chulim et al., 2012). For example, in a study verifying DNA extract quality, Zambrano et al. (2002) amplified DNA extract from Saccharum spp., Musa sp. and Minihost escalenta, with product size ranging from 200 to 1750 bp. While the size of fragments observed by the ASSR primers (ASSR20 and ASSR29) was 400-1750 pb.

Amplification of bands with each of the primers revealed differences between the groups of clones studied. A total of 196 bands were amplified (Tables 3), coinciding with those results from Hedrick and Miller (1992) who obtained reproducible bands using RAPD and SSR markers.

The percentage of polymorphism observed was different with each primer, depending on the type of explant used. The highest percentage of polymorphism was 100% in E1ASIO and E1AMIO generated by the primers Y41 and ASSR29, respectively. Of all the explants, the largest range of polymorphism (66.66-100%) was observed in plants regenerated from the mature zygotic embryonic axis (E1), followed by those

Table 3. Level of polymorphism observed with both types of markers (RAPD and ASSR) in A. salmiana and A. marmorata derived from three different explants and two distinct processes of regeneration.

| Name of material | Number of bands | Polymorphic bands | Polymorphism (%) | Number of bands | Polymorphic bands | Polymorphism (%) |
|------------------|-----------------|-------------------|-----------------|-----------------|-------------------|-----------------|
| E1ASIO           | 7               | 5                 | 71.42           | 3               | 2                 | 66.66           |
| E1AMIO           | 5               | 4                 | 80              | 5               | 5                 | 100             |
| E2ASDO           | 2               | 0                 | 0               | 5               | 0                 | 0               |
| E2AMDO           | 3               | 0                 | 0               | 5               | 0                 | 0               |
| E2ASIO           | 2               | 0                 | 0               | 15              | 12                | 80              |
| E2AMIO           | 8               | 5                 | 62.5            | 12              | 10                | 83.33           |
| E3ASDO           | 2               | 0                 | 0               | 5               | 0                 | 0               |
| E3AMDO           | 2               | 0                 | 0               | 3               | 0                 | 0               |
| E3ASIO           | 2               | 0                 | 0               | 7               | 6                 | 85.71           |
| E3AMIO           | 2               | 0                 | 0               | 3               | 0                 | 0               |
| Total            | 35              | 14                | 40              | 63              | 35                | 55.55           |

| Primer          | 3´-ASSR20 | Polymorphism (%) | 3´-ASSR29 | Polymorphism (%) |
|-----------------|-----------|------------------|-----------|------------------|
| E1ASIO          | 13        | 12               | 92.30     | 12               | 100              |
| E1AMIO          | 7         | 3                | 42.85     | 2                | 50               |
| E2ASDO          | 3         | 0                | 0         | 4                | 0                |
| E2AMDO          | 3         | 0                | 0         | 5                | 0                |
| E2ASIO          | 8         | 5                | 62.5      | 6                | 0                |
| E2AMIO          | 2         | 0                | 0         | 5                | 4                | 80              |
| E3ASDO          | 4         | 0                | 0         | 3                | 0                | 0               |
| E3AMDO          | 2         | 0                | 0         | 2                | 0                | 0               |
| E3ASIO          | 4         | 0                | 0         | 6                | 5                | 80              |
| E3AMIO          | 5         | 3                | 60        | 2                | 0                | 0               |
| Total           | 51        | 23               | 45.09     | 47               | 22               | 46.80           |
regenerated from the meristematic zone (MZ) of *in vitro* plantlets (E2) (62.5-83.3%), while those obtained from MZ *ex vitro* plantlets (E3) had lower polymorphism (60-85.71%) with the four primers tested. The range observed for both types of primers in the three assayed explants was similar to those reported by Palombi and Damiano (2002) in regenerated kiwi (*Actinidia delicosa* A. Chev.) plants, obtaining 55.05-85.07% polymorphism using RAPD and SSR type primers.

In contrast, polymorphism was not observed in plantlets of both agave species regenerated by direct organogenesis (DO) with any of the primers used. Therefore, we suggest that somaclonal variation (SV) was not observed. However, in those plantlets that underwent a phase of callogenesis and consequently indirect organogenesis polymorphism was observed with at least one of the primers used, indicating the presence of genetic variation in the plantlets regenerated depending of propagation method (Table 3). This agrees with Venkatachalam *et al.* (2007), who found in *Musa* spp. uniform results with RAPD and SSR primers and did not detect somaclonal variation in plants regenerated by direct organogenesis.

It is well known that genetic variation can be induced by different genetic and epigenetic mechanisms, which are likely reflected in the amplified band pattern using different systems of markers (Sahijram *et al.*, 2003), such as those shown in Fig. 1A and Fig. 2A. In our study, the common factor in the explants that passed through IO was 2,4-D, which has been associated with genetic abnormalities, mutations (Ladyżyński *et al.*, 2002; Mohanty *et al.*, 2008; Kunakh *et al.*, 2005), and DNA methylation that produces changes in the phenotype (Chakrabarty *et al.*, 2003; Regalado *et al.*, 2015). This has been reported in species such as *Cinchona officinalis* L. (Armijos-González, 2016), *Aloe Vera* (Rathore *et al.*, 2011), *Annanas comosus* (Soneji *et al.*, 2002), and *Bletilla striada* (Wang and Tian, 2014). However, our results differ from González *et al.* (2003), who did not detect genetic variability when they used 2,4-D to induce somatic embryogenesis in henequen (*A. fourcroydes* Lem.).

The dendrograms of genetic distance resulting from UPGMA grouping analysis reveal three main groups in both species (Fig. 1B, C and 2B, C). However, there is formation of multiple subgroups, the individuals of which had similar band patterns, mainly those regenerated via direct organogenesis by MZ *in vitro* and *ex vitro* (E2ASDO, E3ASDO, E2AMDO and E3AMDO). It should be pointed out that, in some samples obtained via indirect organogenesis, no genetic differences were found using either type of marker. This confirms what several authors have reported: when using molecular markers, at least two methodologies should be combined to corroborate the results and avoid false positives (Chen *et al.*, 1998; Ooms *et al.*, 1987).

The largest genetic distance (GD) was 1.00 in both species: in AS generated with the primer Y41 (Fig. 1B) and in AM with the primer ASS29 (Fig. 2C). In contrast, there were no groups of clones obtained with DO; their Nei (1972) GD was 0.0 and the regenerants exhibited genetic fidelity.

Torres-Morán *et al.* (2010) observed genetic variability in *A. tequilana* plants obtained in the field by asexual propagation (rhizome suckers), as well as in plants regenerated by *in vitro* culture methods (somatic embryogenesis and axillary buds) using ISTR-type markers. Our results agree with them, since genetic variation in plantlets regenerated by *in vitro* culture, as well as with those reported in other species of the genus, such as *A. fourcroydes* (González *et al.*, 2003; Infante *et al.*, 2006), *A. cocui* (Osorio and Infante, 2006), *A. americana*, *A. angustifolia*, *A. deserti* and *A. sisalana* by evaluating with AFLPs and ISTR (Infante *et al.*, 2006) using MZ as initial explant. Unlike other research, the comparison with a control plant was not possible given the origin of the explants (seed), it is important to remember that the genetically analyzed clones came from tissues (main explant) which went through an identical *in vitro* regeneration process as the shoots obtained.

In our study, probably the population studied in each experiment was small, however, variability in the response of the evaluated materials from indirect organogenesis was evident. It is known that the development pattern of an explant during morphogenesis *in vitro* is a key element related to SV since, when a highly differentiated tissue passes through a stage of dedifferentiation with a high rate of cell division, more SV can occur than when regeneration develops directly from axillary buds or embryos (Cardone *et al.*, 2004; Sahijram *et al.*, 2003). This can explain the results obtained in our study since, although efficient results were obtained in sprouting with the indirect organogenesis system in both species (Arzate-Fernandez *et al.*, 2020), genetic analysis with both types of molecular markers found instability in all the clones that passed through a phase of indirect tissue organogenesis (Armijos-González, 2016; Oliveira *et al.*, 1995). Moreover, it can also be explained by the heterogeneity of the callus cells and the possible accumulation of genomic alterations (Kuznetsova *et al.*, 2006) during long-term culture (Bublyk *et al.*, 2012). It might also add that the non-meristematic parts and the intermediate callus stages have a high risk of genetic instability among the regenerated plants (Martinez-Palacios *et al.*, 2003), while the culture of meristematic zones that do not undergo a state of
Figure 1. PCR amplification products using RAPD-type primers, Y24 (a) and Y41 (b), and ASSR-type primers, ASSR20 (c) and ASSR29 (d), in *A. salmiana* clones obtained from three different explants (E1=mature zygotic embryonic axis, E2=MZ of *in vitro* plantlets, E3=MZ of *ex vitro* plantlets), and two regeneration process (IO=indirect organogenesis, DO=direct organogenesis), M1 and M2= low and high range ladder-type molecular markers, respectively (A). Dendrograms showing clusters of *A. salmiana* clones of three assayed explants obtained from analysis with RAPD-type primers (Y24 and Y41) (B), and with ASSR-type primers (ASSR20 and ASSR29) (C), based on Nei (1972) genetic distance and using the UPGMA method.
Figure 2. PCR amplification products using RAPD-type primers, Y24 (a) and Y41 (b), and ASSR-type primers, ASSR20 (c) and ASSR29 (d), in *A. marmorata* clones obtained from three different explants (E1=mature zygotic embryonic axis, E2=MZ of *in vitro* plantlets, E3=MZ of *ex vitro* plantlets), and two regeneration process (IO=indirect organogenesis, DO=direct organogenesis) M1 and M2= low and high range ladder-type molecular markers, respectively (A). Dendrograms showing clusters of *A. marmorata* clones of three assayed explants obtained from analysis with RAPD-type primers (Y24 and Y41) (B), and with ASSR-type primers (ASSR20 and ASSR29) (C), based on Nei (1972) genetic distance and using the UPGMA method.
dedifferentiation may or not produce variation, compared with those explants that do (Bayliss, 1977; D’Amato, 1985; Karp and Bright, 1985).

It should be pointed out that the regenerated plantlets used for the SV analysis in this study were obtained from multiple subcultures. Rodríguez et al. (2014) mentioned that the larger the number of subcultures, the larger is the risk of genetic variation. This may be due to an increase in the duration of exposure to stress-causing factors, such as plant growth regulators (PGR). Several studies have reported that mutations accumulate sequentially with culture time; regenerated plants cultured for three months can contain a small number of mutations, and after several subcultures, mutations can occur (Armijos-González, 2016; Kaepller et al., 2000; Peng et al., 2015). This point can be another possible factor that may have affected our results since, in the case of IO regenerated plantlets, the treatment of callus induction lasted 60 days (two subcultures), the treatments of shoot regeneration 60 days more and plantlets passed 45 days in a rooting medium. In contrast, DO-regenerated plantlets were not exposed as long to PGR.

Several studies on genetic variation using RAPDs have reported genetic stability in in vitro regenerated material, for example, plantlets regenerated from pseudobulbs of Bletia purpurea Lam. (Yah-Chulim et al., 2012) in which no genetic alterations were found using AIA and BA as PGR for the proliferation of DO shoots. Armijos-González (2016), using SSR, did not observed SV in direct shoots using combinations of BA/NAA in Cinchona officinalis L., and Kajla et al. (2015), using RAPD and ISSR markers, did not detect polymorphism in DO regenerated Musa sp. cv. Robusta. These reports agree with our results; according to band patterns observed with RAPD and ASSR markers (Fig. 1A and 2A), SV was not found in shoots regenerated by direct organogenesis of either species assayed, confirming findings of Peschke and Phillips (1992), who reported that direct organogenesis using MZ is associated with high genetic stability.

Differences in the stability of tissue cultures produced from different explants are often due to pre-existing variability. The most widely recognized case of this fact is polynathy (when diploid and polyploid cells coexist in the same tissue). This condition can be found in more than 90% of plant species (D’Amato, 1985). Van den Bulk et al. (1990), using tomato cv. Moneymaker seeds, showed that the hypocotyl is polysomatic, while other explants, such as leaf and cotyledon had few or no diploid cells. This may be another reason for the difference in percentages of polymorphism observed between the regeneration processes (DO and IO) and the type of explant used (E1, E2 or E3) since, of the three explants assayed, plantlets regenerated using as the explant the mature zygotic embryonic axis from seed showed the highest genetic variation. This may also contribute, in general, to the variation found in the three explants of both species since all of them came from seed. On the other hand, the three explants maintained their capacity of morphogenetic response as well as their genetic stability in shoot regeneration via direct organogenesis, and therefore, this propagation method is not a determining factor in SV.

It is worth mentioning the importance of following up the regenerated plant material since the somaclonal variation is generally spontaneous, and changes may or not be inheritable (Anu et al., 2004; Bray and Jain, 1998; Kaepller et al., 2000; Larkin and Scowcroft, 1981; Navarro and Perea, 1996; Pierik, 1997; Sahijram et al., 2003). Duarte-Aké et al. (2016) studied the epigenetic and physiological differences in regenerated A. angustifolia plants. The epigenetic analysis revealed an increase in DNA methylation during the first two subcultures. However, after a time, the levels of methylation began to decrease.

With the results obtained, it can be suggested that the indirect regeneration process of A. salmiana and A. marmorata is not recommendable when the objective is to conserve the original genetic characteristics of the species. However, it could be interesting for the generation of variants with agronomic or ornamental value since genetic variability provides opportunities to study topics related to plant quality (Domínguez-Rosales et al., 2008), and it may be possible to obtain desirable agronomic characteristics (carbohydrate content, maturation period, resistance to disease, and others) (Valenzuela-Sánchez, 2006).

CONCLUSIONS

The study of genetic integrity of regenerated plantlets using three distinct explants and two ways of propagation (direct and indirect organogenesis) of A. salmiana and A. marmorata, using two types of molecular markers (RAPD and ASSR), showed homogeneous amplification profiles in those plantlets obtained through direct organogenesis. In contrast, the plantlets from indirect organogenesis had genetic differences in their banding patterns, suggested as, somaclonal variation evidence.

It was possible to establish an efficient propagation protocol for A. salmiana and A. marmorata, maintaining genetic stability via direct organogenesis, as well as a possible alternative for genetic improvement through somaclonal variation that occurs in both species when organogenesis is indirect.

This is the first study on genetic stability in regenerated shoots of A. salmiana and A. marmorata cultured in vitro through direct and indirect organogenesis.
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Data availability. Data is available with Dr. Amaury Martín Arzate Fernandez (amaury1963@yahoo.com.mx), upon reasonable request.

Author contribution statement (CRediT).  I Martínez-Velasco - Data curation, Formal Analysis, Investigation, Visualization, Writing – original draft., AM Arzate-Fernández – Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing

REFERENCES

Arzate-Fernández, A.M., Martínez-Velasco, I., Álvarez-Aragón, C., Martínez-Martínez, S.Y., Norman-Mondragón, T.H., 2020. Morphogenetic response of two Agave species regenerated in vitro. Tropical and Subtropical Agroecosystems, 23(2), pp. #47. Available at: https://www.revista.ccba.uady.mx/ojs/index.php/TSA/article/view/3201.

Agarwal, M., Shrivastava, N. and Padh, H., 2008. Advances in molecular marker techniques and their applications in plant sciences. Plant Cell Reports, 27(4), pp. 617–631. https://doi.org/10.1007/s00299-008-0507-z

Aguilar Juárez, B., Enríquez Del Valle, R., Rodríguez-Ortiz, G., Granados Sánchez, D. and Cerero, B.M., 2014. El estado actual de Agave salmiana y A. mappisaga del valle de México. Revista Mexicana de Agroecosistemas, 2(1), pp. 2007–9559. Available at: https://www.voaxaca.tecnm.mx/revista/docs/RMAE%20vol%201_2_2014/RMAE-2014-11%20Agave.pdf

Alfaro Rojas, G., Legaria Solano, J.P., Rodríguez Pérez, J.E., 2007. Genetic diversity in populations of pulquero agaves (Agave spp.) in northeastern Mexico state. fitotecnia Mexicana, 30(1), pp. 1–12.

Anu, A., Babu, K.N. and Peter, K.V., 2004. Variations Among Somaclones and its Seedling Progeny in Capsicum Annuum, Plant Cell, Tissue and Organ Culture, 76(3), pp. 261–267. https://doi.org/10.1023/B:TIQU.0000009246.24216.ea

Alcántara, M.R., 2007. Capítulo 18; Breve revisión de los marcadores moleculares. Parte III: Las herramientas moleculares. In: C. A. para la I. y D. de La eds. Biotecnología y Mejoramiento Vegetal II, Argentina, pp. 541–566.

Armijos-González, R., 2016. Conservación de plantas regeneradas in vitro y análisis de la variación somaclonal de Cinchona officinalis, Linneo. Universidad politécnica de Madrid. https://doi.org/10.20868/UPM.thesis.39541.

Bayliss, M.W., 1977. The effects of 2,4-D on growth and mitosis in suspension cultures of Daucus carota. Plant Science, 8, pp. 99–103. https://doi.org/10.1016/0304-4211(77)90018-9

Bray, D.S. and Jain, S.M., 1998. Somaclonal Variation: Mechanism and Applications in Crop Improvement. In: Jain, S.M., Brar, D.S., Brar, S.A. (eds), Somaclonal Variation and Induced Mutations in Crop Improvement, Current Plant Science and Biotechnology in Agriculture, 32, pp. 15–37. https://doi.org/10.1007/978-94-015-9125-6_2

Bublyk, O., Andreev, I.V.S. and Kunakh, V., 2012. Genetic variability in regenerated plants of Ungernia victoris. Biologia Plantarum, 56, pp. 395–400. https://doi.org/10.1007/s10535-012-0106-2

Cardone, S., Olmos, S. and Echenique, V., 2004. Variación Somaclonal. In: Echenique LV, Rubinstein C, Mroginiski (eds), Biotecnología y mejoramiento vegetal, Argentina: Consejo Argentino para la Información y el Desarrollo de la Biotecnología, pp. 81–96.

Cavallini, A., Natali, L., Cionini, G. and Castorena-Sanchez, L. 1996. Cytophotometric and biochemical analyses of DNA in pentaploid and diploid Agave species. Genome, 39, pp. 266–271. https://doi.org/10.1139/g96-036

Chakrabarty, D., Yu, K. and Paek, K., 2003. Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (Eleutroccoccus senticosus). Plant Science, 165, pp. 61–68. https://doi.org/10.1016/S0168-9452(03)00127-4

Chen, W., Chen, T., Fu, Y.M., Hsieh, R. and Chen, W., 1998. Studies on somaclonal variation in...
Phalaenopsis. Plant Cell Reports, 18, pp. 7–13. https://doi.org/10.1007/s002990050523

D'amato, F. and Bayliss, M.W., 1985. Cytogenetics of plant cell and tissue cultures and their regenerates. Critical Reviews in Plant Sciences, 3, pp. 73–112. https://doi.org/10.1080/07352688509382204

Domínguez-Rosales, M.S., González-Jiménez, M.D.L.L., Gómez-Rosales, C., Valles-Quíones, C., Delgadillo-Días de León, S., Mireles-Ordz, S.J. and Pérez-Molphe, B.E., 2008. El cultivo in vitro como herramienta para el aprovechamiento, mejoramiento y conservación de especies del género Agave. Investigación y Ciencia, 41, pp. 53–62. Available at: https://www.redalyc.org/articulo.oa?id=67404109

Duarte-Aké, F., Castillo-Castro, E., Pool, F.B., Espadas, F., Santamarí, J.M., Robert, M.L. and De-la-Pen, C., 2016. Physiological differences and changes in global DNA methylation levels in Agave angustifolia Haw. albino variant somaclones during the micropropagation process. Plant Cell Reports, 35, pp. 2489–2502. https://doi.org/10.1007/s00299-016-2049-0

García-Mendoza, A.J., 2007. Los agaves de México. Ciencias, 087, pp. 14–23. Available at: http://www.journals.unam.mx/index.php/ciencia/article/view/121130

González, G., Alemán, S. and Infante, D., 2003. Asexual genetic variability in Agave fourcroydes: Selection among individuals in a clonally propagated population. Plant Sciences, 165, pp. 595/601. https://doi.org/10.1016/S0168-9452(03)00227-9

Hashmi, G., Huetel, R., Meyer, R., Krusberg, L. and Hammerschlag, F., 1997. RAPD analysis of somaclonal variants derived from embryo callus cultures of peach. Plant Cell Reports, 16(9), pp. 624–627. https://doi.org/10.1007/BF01275503

Hedrick, P.W. and Miller, P.S., 1992. Conservation Genetics: Techniques and Fundamentals. Ecological Applications, 2(1), pp. 30–46. https://doi.org/10.1037/A10125519304

Infante, D., Molina, S., Demey, J.R. and Gámez, E., 2006. Asexual genetic variability in Agavaceae determined with inverse sequence-tagged repeats and amplification fragment length polymorphism analysis. Plant Molecular Biology Reporter, 24(2), pp. 205–217. https://doi.org/10.1007/BF02914059

Kajla, S., Poonia, A.K., Brar, B., Sciences, A., Duhan, J.S., 2015. Molecular assessment of genetic stability using ISSR and RAPD markers in commercial banana cultivar cv. Robusta. Indian Journal of Biotechnology, 14(2015), pp. 420–424. Available at: http://nopr.niscair.res.in/handle/123456789/33421

Karp, A. and Bright, S.W.J., 1985. On the causes and origins of somaclonal variation. Oxford surveys of plant molecular and cell biology 2, pp. 199–234. Available at: https://agris.fao.org/agrisearch/search.do?recordID=US201301432481

Kunakh, A.V.I., Mel’nyk, V.M., Drobyk, N.M., Andreev, I.O., Spiridonova, K.V., Maryana, O.T., Konvalyuc, I.I. and Volodymyr, I.A., 2005. Genetic Variation Induced by Tissue and Organ Culture in Gentiana Species. In: Jan A.M.J Rybczynski, Michael RD eds. The Gentianaceae - Volume 2: Biotechnology and Applications, Springer, Berlin, Heidelberg, pp. 199–238. https://doi.org/10.1007/978-3-642-54102-5_9

Kuznetsova, O., Ash, O.A. and Gostimsky, S.A., 2006. The effect of the duration of callus culture on the accumulation of genetic alterations in pea Pisum sativum L. Russian Journal of Genetics, 42, pp. 555–562. https://doi.org/10.1134/S1022795406050139

Ladyżyński, M., Burza, W. and Malepszy, S., 2002. Relationship between somaclonal variation and type of culture in cucumber. Euphytica, 125(3), pp. 349–356. https://doi.org/10.1023/A:1016017825907

Larkin, P.J. and Scowcroft, W.R., 1981. Somaclonal variation - a novel source of variability from cell cultures for plant improvement. Theoretical and Applied Genetics, 60(4), pp. 197–214. https://doi.org/10.1007/BF02342540

Martinez-Palacios, A., Ortega-Larrocea, M.P. and Chávez, V.M., 2003. Somatic embryogenesis and organogenesis of Agave victoriae-reginae, Plant Cell, Tissue and Organ Culture, 74, pp. 135–142. https://doi.org/10.1023/A:1023933123131

Méndez-Gallegos, S.J., Rössel, D., Amante-Orozco, A., Talavera-Magaña, D., García-Herrera, J. and Velez-Jiménez, A., 2011. Biocombustibles a
base de nopal y maguey. *Revista Salud Pública y Nutrición*, Edición Especial No.5, pp. 83–93.

Mohanty, S., Panda, M.K., Subudhi, E. and Nayak, S., 2008. Plant regeneration from callus culture of Curcuma aromatic and analysis in vitro detection of somaclonal variation through cytophotometric anlaysis. *Biologica Plantarum*, 52(4), pp.783–786. https://doi.org/10.1007/s10535-008-0153-x

Navarro, W. and Perea, M. 1996. Técnicas en vitro para la producción y mejoramiento de plantas (2a EUNA; Heredia, Ed.). Costa Rica.

Nei, M., 1972. Genetic Distance between Populations. *The American Naturalist*, 106(949), pp. 283–292. https://doi.org/10.1086/282771

Nieto, R., Vargas, J., Rodríguez, A. and Jiménez, V., 2016. El cultivo de maguey pulquero (Agave salmiana) en el valle del mezquital (Primera Ed). Universidad Politécnica de Francisco I. Madero. Hidalgo, México.

Oliveira, S.A., Machado, M.F.P.S. and Priolli, A.J., 1995. In vitro propagation of Cereus peruvianus Mill (Cactaceae). *In Vitro Cellular Developmental Biology – Plant*, 31, pp. 47–50. https://doi.org/10.1007/BF02632226

Ooms, G., Burrell, M.M., Karp, A., Bevan, M. and Hille, J., 1987. Genetic transformation in two potato cultivars with T-DNA from disarmed Agrobacterium. *Theoretical and Applied Genetics*, 73(5), pp. 744–750. https://doi.org/10.1007/BF00260785

Osorio Zambrano, M.A. and Infante, D.M.S., 2006. Study of asexual genetic variability in Agave cocui Trelease by molecular markers. *Biologia Nakari*, 17, pp. 1–7.

Palombi, M. and Damiano, C., 2002. Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (Actinidia delicosa A. Chev). *Plant Cell Reports*, 20(11), pp. 1061–1066. https://doi.org/10.1007/s00299-001-0430-z

Peng, X., Zhang, T. and Zhang, J., 2015. Effect of subculture times on genetic fidelity, endogenous hormone level and pharmaceutical potential of Tetrastigma hemsleyanum callus. *Plant Cell, Tissue and Organ Culture*, 122(1), pp. 67–77. https://doi.org/10.1007/s11240-015-0750-2

Pérez Ponce, J.N., 1998. *Propagación y mejora genética de plantas por biotecnología*. Instituto de biotecnología de las plantas: Santa Clara, Cuba.

Peschke, V.M., Phillips, R.L., 1992. Genetic implications of somaclonal variation in plants.

Advances in Genetics, 30, pp. 41–75. https://doi.org/10.1016/S00065-2660(08)60318-1

Pierik, R.L.M., 1997. In vitro culture of higuera plants. Springer science & business media, 4rt ed. The Netherlands.

Pradeep Reddy, M., Sarla, N. and Siddiqi, E., 2002. Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, 128, pp. 9–17. https://doi.org/10.1023/A:1020691618797

Rahman, M. and Rajora, O., 2001. Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). *Plant Cell Reports*, 20(6), pp. 531–536. https://doi.org/10.1007/A:1020691618797

Rathore, M.S., Chikara, J., Mastan, S., Rahman, H., Gopalakrishnan, V.A. and Shekhwat, N., 2011. Assessment of Genetic Stability and Instability of Tissue Culture-Propagated Plantlets of Aloe vera L. by RAPD and ISSR Markers. *Applied Biochemistry and Biotechnology*, 165, pp. 1356–1365. https://doi.org/10.1007/s12010-011-9352-6

Regalado, J., Carmona, E., Castro, P., Moreno, R., Gil, J. and Encina, C., 2015. Study of the somaclonal variation produced by different methods of polyploidization in Asparagus officinalis L. *Plant Cell Tissular Organ Culture*, 122, pp. 31–44. https://doi.org/10.1007/s11240-015-0747-x

Rodríguez-Garay, B., Lomelí-Sención, J.A., Tapia-Campos, E., Gutiérrez-Mora, A., García-Galindo, J., Rodríguez-Domínguez, J.M., Urbina-López, D. and Vicente-Ramírez, I., 2008. Morphological and molecular diversity of Agave tequilana Weber var. Azul and Agave angustifolia Haw. var. Lineño. *Industrial Crop and Products*, 29, pp. 220–228. https://doi.org/10.1016/j.indcrop.2008.05.007

Rodríguez, R., Macías, E.S., Silva, D., Vargas, L.A., 2014. El pulque: bebida e identidad. *Ciencias*, pp. 66–69. Available through: https://www.revistaciencias.unam.mx/en/161-revistas/revista-ciencias-111-112/1423-el-pulque-bebida-e-identidad.html

Sahijram, L., Soneji, J.R. and Bollamma, K.T., 2003. Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro Cellular and Developmental Biology – Plant*, 39(6), pp. 551–556. https://doi.org/10.1079/IVP2003467

Soneji, J.R., Rao, P.S. and Mhatre, M., 2002. Suitability of RAPD for analyzing Spined and Spineless
variant regenerants of Pineapple (Ananas comosus L., Merr.). *Plant Molecular Biology Reporter*, 20, pp. 307a-307i. https://doi.org/10.1007/BF02782469

Torres-Morán, M.I., Escoto-Delgadillo, M., Molina-Moret, S., Rivera-Rodríguez, D.M., Velasco-Ramírez, A.P., Infante, D. and Portillo, L., 2010. Assessment of genetic fidelity among Agave tequilana plants propagated asexually via rhizomes versus in vitro culture. *Plant Cell, Tissue and Organ Culture*, 103(3), pp. 403–409. https://doi.org/10.1007/s11240-010-9777-6

Valenzuela Sánchez, K.K., 2006. Desarrollo de tecnologías para la regeneración in vitro y transformación genética del maguey (Agave tequilana Weber, var. azul). Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional. Available at: http://www.ira.cinvestav.mx:86/tesis/valenzuela_2006.pdf.

van den Bulk, R.W., Löffler, H.J.M., Lindhout, W.H. and Koorneef, M., 1990. Somaclonal variation in tomato: effect of explant source and a comparison with chemical mutagenesis. *Theoretical and Applied Genetics*, 80(6), pp. 817–825. https://doi.org/10.1007/BF00224199

Venkatachalam, L., Sreedhar, R.V. and Bhagyalakshmi, N., 2007. Micropropagation in banana using high levels of cytokinins does not involve any genetic changes as revealed by RAPD and ISSR markers. *Plant Growth Regulation*, 51(3), pp. 193–205. https://doi.org/10.1007/s10725-006-9154-y

Wang, C. and Tian, M., 2014. Callus-mediated and direct protocorm-like body formation of Bletilla striata and assessment of clonal fidelity using ISSR markers. *Acta Physiologiae Plantarum*, 36, pp. 2321–2330. https://doi.org/10.1007/s11738-014-1594-9

Williams, J., Kubelik, A., Livak, K., Rafalski, J. and Tingey, S., 1991. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids* 18, pp. 6531–6535. https://doi.org/10.1093/nar/18.22.6531

Yah-Chulim, J.E., Rodríguez-Buenfil, I.M., Reyes-Escogido, M.L. and López-Puc, G., 2012. Optimization of growth regulators in organogenesis of Bletia purpurea (Lam.) using response surface design and genetic evaluation. *African Journal of Biotechnology*, 11(57), pp. 12045–12052. DOI: 10.5897/AJB12.1028

Yamagishi, M., Abe, H., Nakano, M. and Nakatsuka, A., 2002. PCR-based molecular markers in Asiatic hybrid lily. *Scientia Horticulturae*, 96, pp. 225–234. https://doi.org/10.1016/S0304-4238(02)00095-X

Yamagishi, M., 1995. Detection of section-specific random amplified polymorphic DNA (RAPD) markers in Lilium. *Theoretical and Applied Genetics*, 91, pp. 830–835. https://doi.org/10.1007/BF00223888

Yeh, F.C. and Boyle, T.J.B., 1997. Population genetic analysis of codominant and dominant markers and quantitative traits. *Belgium Journal of Botany*, pp. 129-157.

Zambrano, Y.A., Demey, J.R., Martínez, G., Fuenmayor, F., Gutiérrez, Z., Saldaña, G. and Torrealba, M., 2002. Método rápido, económico y confiable de, mini preparación de ADN para amplificaciones por RAPD en bancos de germoplasm. *Agricultura Tropical*, 52(2), pp. 235-243.

Zietkiewicz, E., Rafalski, A. and Labuda, D., 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, 20, pp. 176–183. DOI: 10.1006/geno.1994.1151

Zhou, Z.M. and Miwa, T.H., 1999. Analysis of genetic structure of a Suillus grevillei population in a Laris kaem-feri stand by polymorphism of inter-simple sequence repeat (ISSR). *New Phytologist*, 144, pp. 55–63. Available at: https://doi.org/10.1046/j.1469-8137.1999.00504.x