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 consecuente 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 plántulas 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 asexual 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* production 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 sequences 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)

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 mgL^{-1})	Shoot regeneration (BA mgL^{-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*				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*				5.0
	E3ASIO	*A. salmiana*		IO	1.0/15.0	10.0
	E3AMIO	*A. marmorata*			5.0/3.0	5.0

NA= not applicable

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 1µL 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 $MgCl_2$, 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.

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

Primers	Primer sequence
RAPD Y24	AACCGCGCTC
RAPD Y29	TTCGGGCCGT
RAPD Y37	TAACCGCGCC
RAPD Y38	TAACCGCGCC
RAPD Y41	GCGTCCTGGG
3'-ASSR02	5´-$(CT)_7$ ATC-3´
3'-ASSR15	5´- $(CT)_7$ ATG-3´
3'-ASSR20	5´- $(CT)_7$ GCA-3´
3'-ASSR29	5´- $(CT)_7$ GTA-3´
3'-ASSR35	5´- $(CT)_7$ TGA-3´

For the RAPD primers, the thermal cycles were 94ºC for 5 min, 54ºC for 1 min, 72ºC for 2 min for the first cycle, followed by 41 cycles of the thermal profile: 94ºC for 1 min, 54ºC for 1 min, 72ºC, all for 2 min, and a final cycle of 72ºC for 10 min, 54ºC for 1 min and 72ºC 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: 94ºC, 46ºC, 72ºC, 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 *Minihot esculenta*, 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 rom 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 (%)
Primer		RAPD Y24			RAPD Y41	
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			3´-ASSR29	
E1ASIO	13	12	92.30	12	12	100
E1AMIO	7	3	42.85	2	1	50
E2ASDO	3	0	0	4	0	0
E2AMDO	3	0	0	5	0	0
E2ASIO	8	5	62.5	6	0	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 deliciosa* 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 (Martınez-Palacios *et al*., 2003), while the culture of meristematic zones that do not undergo a state of

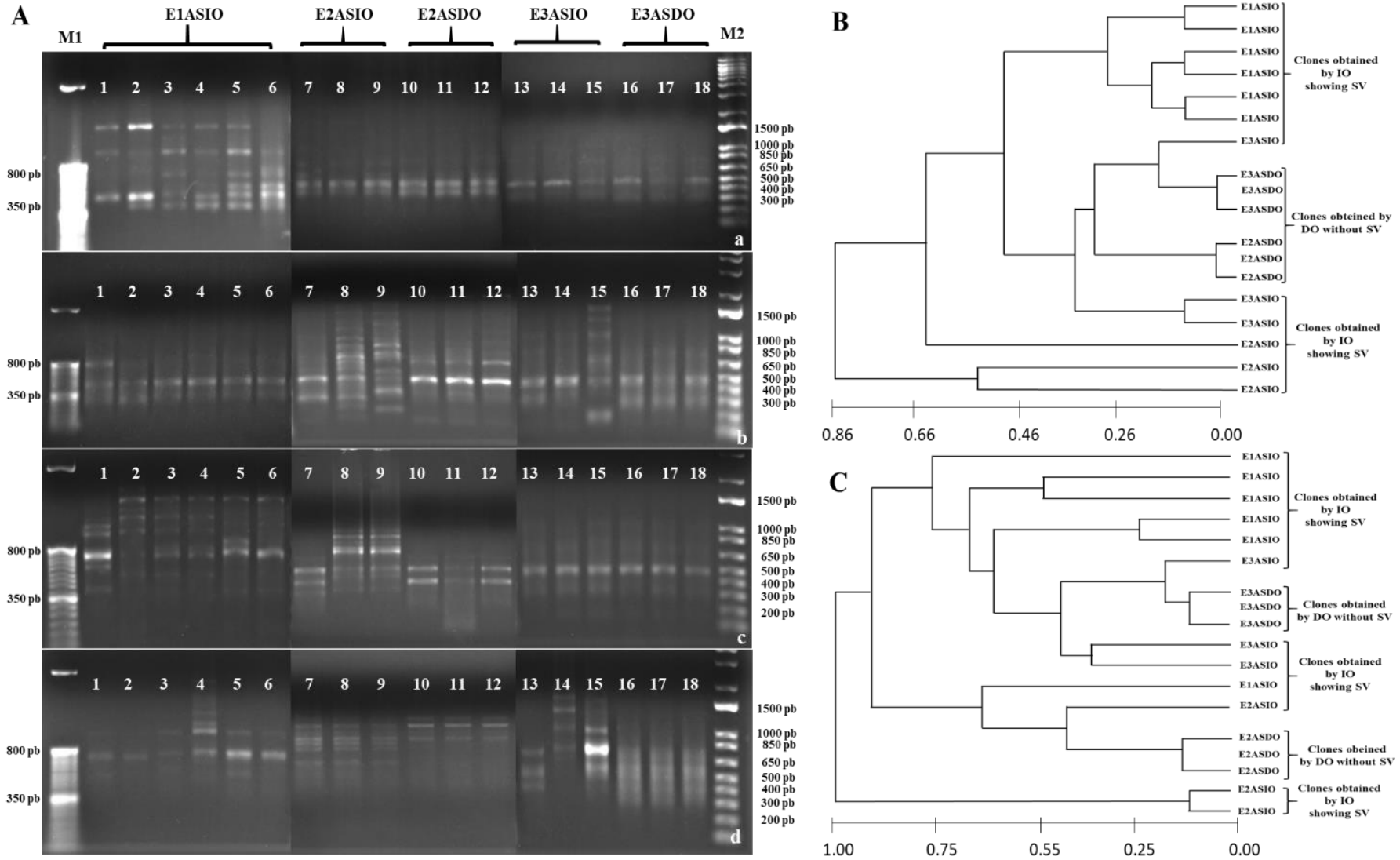

Figure 1. PCR amplification products using RAPD-type primers, Y24 (a) and Y41 (b), and ASSR-type primers, ASSR20 (c) and ASSR29 (d), in *A. salmiana* clones obtained from three different explants (E1=mature zygotic embryonic axis, E2=MZ of *in vitro* plantlets, E3=MZ of *ex vitro* plantlets), and two 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.

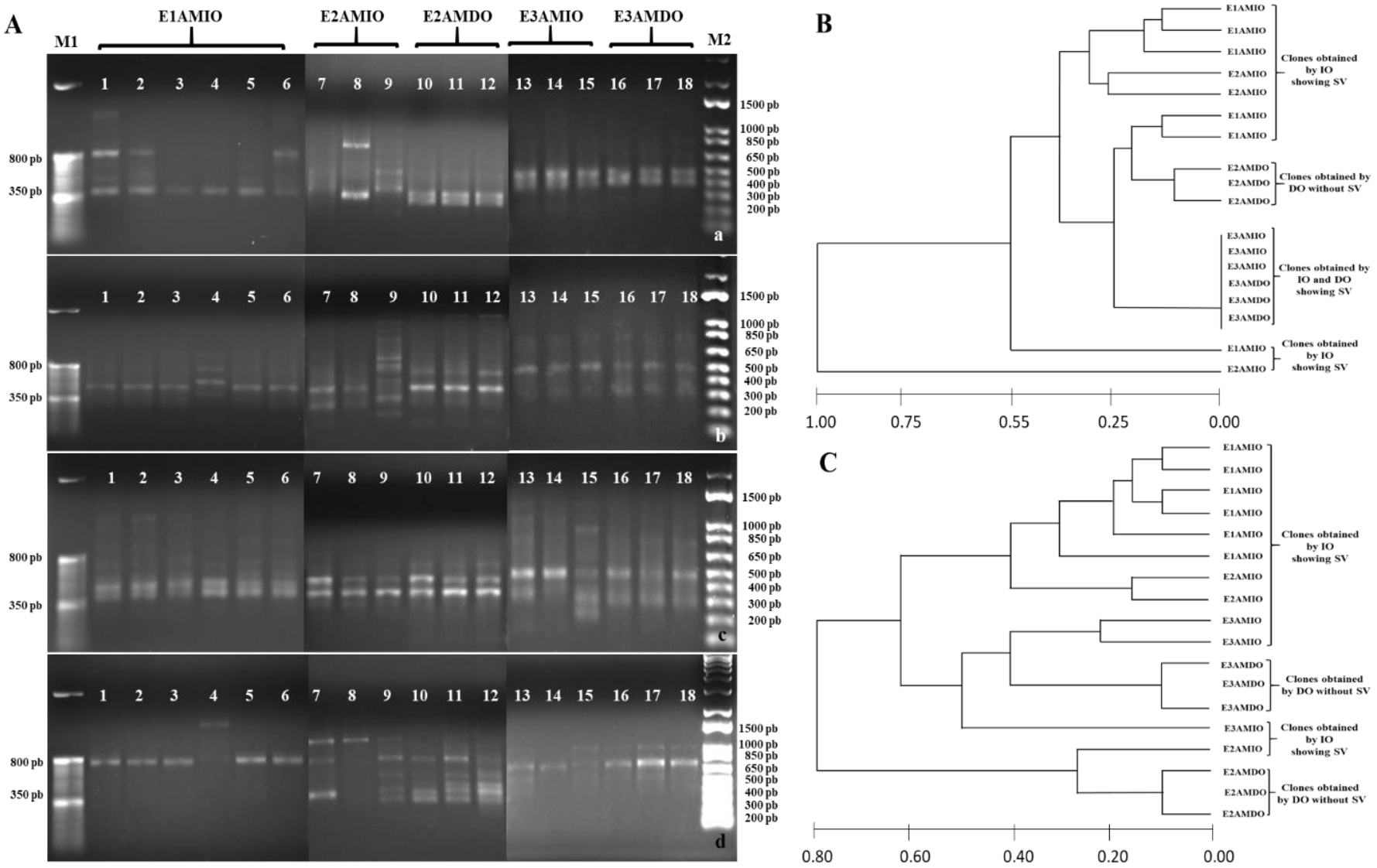

Figure 2. PCR amplification products using RAPD-type primers, Y24 (a) and Y41 (b), and ASSR-type primers, ASSR20 (c) and ASSR29 (d), in *A. marmorata* clones obtained from three different explants (E1=mature zygotic embryonic axis, E2=MZ of *in vitro* plantlets, E3=MZ of *ex vitro* plantlets), and two 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; Kaeppler *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 shooting 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 polymathy (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; Kaeppler *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. angustiofolia* 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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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

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