Verification of the Apomictic Origin of Cactus Pear (*Opuntia* spp Cactaceae) Seedlings of Open Pollinated and Crosses from Central Mexico

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ABSTRACT

Nopal is endemic to Mexico but it is becoming an interesting alternative fruit and forage crop for semiarid areas of the world. Few varieties originated from Mexican germplasm support the world market. Nopal is propagated asexually for commercial purposes, but seed propagation is essential for breeding. A main constraint to breeding is apomixis, which has been reported in numerous Opuntias including those of horticultural interest like *O. ficus-indica*. Apomixis makes difficult the screening of individuals obtained from crosses and complicates genetic studies. In this study 17 breeding populations of Mexican origin all showed the presence of putative apomictic seedlings, although with significant differences. Selfing induced a higher number of apomicts. When the accession CDO was used as a female the number of apomicts also increased, suggesting the presence of a maternal effect. The exploratory RAPD assay of nine seedlings of two populations, selected according to the former criteria revealed that late emergent seedlings showed a similar banding pattern as the maternal entries.

Keywords: Apomixis, cactus pear, nopal, seedlings, cactus breeding.

INTRODUCTION

Cactus pear is the main fruit crop in rain-fed conditions in the semiarid highlands of central Mexico. At present, more than 50,000 hectares are planted in this country, (SARH, 1993). Cactus pear is also important in Italy and Chile, and it is becoming an interesting alternative crop for several countries in North Africa and other semiarid areas of the world. Commercial plantations rely on a few varieties originated or selected from a common germplasm base of Mexican origin. New varieties with better fruit, vegetable, or forage quality, and adapted to local needs and climatic restrictions, are a common goal of cactus-pear breeders.

Cactus pear is propagated asexually for commercial purposes. However, seed propagation is essential for breeding. The plant has been reported to exhibit apomixis (Pimienta, 1990; Mondragon and Pimienta, 1995; Mizrahi et al., 1997), which means it is able to generate asexual seedlings from the maternal tissues. Apomixis has also been defined as the asexual reproductive process that, paradoxically, occurs in the ovule of flowering plants (Koltunow, 1993). Tisserat (1979) reported apomixis in several *Opuntias*: *O. aurantiaca* Lindl., *O. dillenii* Haw., O. *glaucophyla* Wendl., *O. leucantha* Link., *O. rafinesquii* Engelm, *O. tortispina* Engelm., *and O. ficus-indica* (L.) Mill.). According to Perez (1993), the presence of polyembryonic seeds in germination tests varied from 10.9% to 18.5 % for *O. streptacantha* and its hybrids, 3.6% to 24.7% *for O. robusta*, 0 to 14.3% for *O. cochinera*, 0 to 6.7% for *O. leucotricha*, and

0 to 50% for *O. rastrera*. From this list, *O. ficus-indica*, *O. robusta*, and *O. streptacantha* are sources of entries of horticultural interest.

The presence of fused polyembryonic seeds of unknown origin also has been observed. However, for propagation purposes, they can be separated readily because they are present in low numbers. Data from Mexican accessions show that they represent from 0.2% to 7% of the total seed number (Mondragon and Pimienta, 1995).

Artificial crossing may increase the presence of apomixis in those species prone to this phenomenon under natural circumstances, as reported by Brown (1975). Apomixis is found in many plants of hybrid origin, some of which never produce true seeds, as a result of fertilization of the ovules. Other plants are usually apomictic, but occasionally can produce sexual hybrids. In several species, polyembryony has been linked to genetic causes and appears to result from hybridization. In pines, occurrence of zygotic multiple embryos appears to be higher in large seeds than small seeds (Kozlowski, 1997).

In standard germination tests conducted with the American cultivar Andy Boy™, Mondragon (unpub. data) observed that when germinating seeds in a greenhouse, the number of apomicts increased, probably due to the improved growth conditions. This is an additional drawback for the breeding program, because the method of choice to germinate cactus-pear seeds includes high temperatures and steady moisture supply through the entire germination phase to ensure higher germination percentage and growth rates (Wang et al., 1996; Bunch, 1996).

The origin of apomictic embryos of cactus pear was elucidated by Velez and Rodriguez (1996). Using seeds from mature and immature fruits germinated *in vitro* and observed in the light and scanning electron microscopes, they found that the tissue originating the apomictic seedlings in cactus pear is the nucella. Up to 15 embryos in all stages of development were observed, but only those with suspensors germinated. All somatic embryos were confined at the micropilar end of the ovule. This study is in agreement with Yeung and Meinke (1993) who reported that in many apomictic species the presence of well-developed suspensors may allow the maturation and germination of the largest and oldest nucellar embryos because suspensors may play an active role in early embryo development.

The apomictic embryos have, disregarding the possibility of mutations, a genetic constitution identical to that of the female parent (Koltunow, 1993). Therefore, the presence of somatic embryos growing along the zygotic ones can make difficult the screening of individuals obtained from crosses and can complicate genetic studies. Costs increase due to the number of extra individuals that have to be maintained in the nurseries. They also diminish the output of the breeding program.

According to Hanna and Bashaw (1987), useful indicators of apomixis are:

- Uniform progeny or some identical maternal progeny from plants of cross-pollinated species
- Distinct maternal types among the progeny of F_1 crosses
- Limited or no genetic variation in an F_2 progeny of a cross between distinct parents
- Unusually high fertility of aneuploids, triploids, wide crosses, or other plants expected to be sterile
- Multiple seedlings per seed, among other morphological traits

DNA fingerprinting is the most accurate tool to elucidate the genetic origin of an individual, but it can be cumbersome. Therefore, when possible, it is more practical to rely on phenotypic markers. Proper molecular verification of the potential phenotypic markers is needed prior to extensive utilization. Morphological traits expressed during the seedling stage would be preferred to speed up the screening process and reduce costs.

Identification of maternal seedlings in *Citrus* has been practiced by means of simple morphological markers such as leaf shape (Weber, 1932, cited by Anderson et al., 1991). Isozymes and leaf shape were used to confirm zygotic seedlings of *Citrus* that present undesirable variability in the production of rootstocks in the nursery industry of this plant (Anderson et al., 1991).

Morphological identification is difficult if the parents are closely related or share the same phenotype. A combination of a phenotypic marker (red stem) and RAPD profiles were used by Ur-Rahman et al. (1997) to evaluate the genetic similarity between apomictic seedlings and their female parents of two *Malus* species. The random primers OPA-01, 08, 09, 10, 12, 13, 14, 16, 18, and 20 were the most effective in separating these individuals. Lopez et al. (1997) reported the identification of mango cultivars and embryo type, polyembryonic or monoembryonic, using RAPD. One of the RAPD markers was present only in polyembryonic cultivars. The fragment was 550 bp and was produced by the OPM-12 primer.

Considering the findings of Velez and Rodriguez (1996), zygotic embryos of cactus pear may have a developmental advantage that allows them to emerge earlier than the apomictic seedlings. The extent to which this advantage in growth is maintained is not known. Complete identification of apomictic individuals of cactus pear based only on phenotypic features is difficult due to the long duration of the juvenile phase in this plant, estimated to be 4 to 6 years.

Our objectives in this study were: to determine the extent of apomixis in several commercial cactus-pear cultivars representative of the different edible *Opuntia* genotypes of horticultural interest and some crosses, a preliminary verification of their somatic origin, and the development of useful criteria for early separation of somatic seedlings.

MATERIALS AND METHODS

Plant Material

Seeds were obtained from crosses performed in summer 1997 at the Northern Guanajuato Research Station (Instituto Nacional de Investigaciones Forestales y Agropecuarias, INIFAP) in central Mexico. They were stored in a dry cabinet for four months before planting. The seeds were screened visually for fused seeds. Seed scarification involved dipping the seeds in hot water (90°C) and rinsing, followed by a second dipping and overnight soaking. Seeds were disinfected by immersion in 10% sodium hypochlorite for 10 minutes, then rinsing thoroughly with sterile water. A seed dressing of the fungicide Banrot (Etridiazole + Thiophanate-methyl) at planting was used as a preventative of soft root rot (*Fusarium* spp. and *Pythium* spp.), a common problem found in cacti. The seeds were planted individually in standard plug trays. Seeds for the experiment were planted in the greenhouse during the last week of February, using 200 seeds per entry. The seeds were maintained under irrigated conditions, with 14 hours of light provided by sodium-vapor lamps. The plants were maintained in trays for three and one-half months, then transplanted individually in 4x4 plastic pots. Supernumerary seedlings were labeled as soon as they were evident. Both types of seedlings were transplanted to individual pots. Table 11 presents the list of traits scored. The first seedling emerging from the seeds was considered to be of sexual origin, and, under this assumption, the percentage of apomictic seedlings represented the number of seeds bearing more than one seedling. The same criterion was applied when selecting seedlings for the RAPD study.

DNA-Extraction Method

We adapted a CTAB (hexadecyltrimethylammonium bromide) protocol used previously for DNA extraction of *Clarkia* (Gottlieb et al., unpub. data). Samples of 8 to 9 grams of tender tissue from the youngest cladodes were obtained, washed, and blotted dry. The tissue was ground into fine powder in a mortar filled with liquid nitrogen. The frozen powder was transferred to a flask containing 25 ml of extraction buffer (100 mM Tris HCl pH 8; 1.4 M NaCl; 20 mM EDTA disodium and 2% CTAB w/v; 0.25% mercaptoethanol was added immediately before use). The samples were then incubated in a circulating water bath at 60°C for 25 minutes. They were removed and left to cool to room temperature (RT). Ten ml of chloroform/isoamyl alcohol were added and mixed by inverting. The mixture was allowed to incubate for an additional 10 minutes at RT. Then, the samples were decanted into 30-ml centrifuge tubes and centrifuged for 20 minutes at 5,000 × g at 18°C to 20°C. The aqueous phase was decanted into a graduated cylinder with Miracloth™ and diluted with two volumes of precipitation buffer (100 mM Tris HCl pH 8; 20 mM EDTA disodium, and 2% CTAB) w/v; 0.25% mercaptoethanol was added immediately before use) and incubated at RT for 30 minutes. The samples were centrifuged for 20 minutes at 15,000 × g at 18°C to 20°C. The supernatant was discarded and the pellet resuspended in 2 to 3 ml of NaCl 1M and incubated for 20 minutes. The pellets were fully dissolved by briefly warming the tubes in a water bath. Then, 2.5 volumes of cold 100% ethanol were added to the samples to promote DNA precipitation. The DNA was spooled with a glass pipette and allowed to dry for 3 to 5 minutes. Finally, the pellets were resuspended in deionized distilled water and stored at 4°C overnight. Treatment with 5 µL of RNAse (10 mg/mL) and incubation at 37°C for 40 minutes eliminated the RNA present in the samples. The A_{280}:A_{260} ratio and its suitability for PCR amplification assessed DNA quality. OD absorbance and DNA concentration were measured on a spectrophotometer.

RAPD Protocol

A modification of the protocol reported by Williams et al. (1993) and Rafalski, et al. (1994) to study apple cultivars by Conner et al. (1997) was followed for the cacti samples. Each reaction included 2.5 µL of 10X PCR buffer, 1.25 µL of 2.5 mM dinucleotide mix, one unit of Taq polymerase, 1.5 µL of 10 mM primer, and 1.5 µL of 25 mM $MgCl_2$, and completed to a total volume of 25 mL with deionized distilled water. We used 20 to 40 ng of genomic DNA. PCR reactions were performed according to the following conditions: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 45°C for 1 minute, extension at 72°C for 1 minute, final extension at 72°C for 5 minutes. DNA amplification was carried out using seven arbitrary primers (OPG-03, OPG-05, OPG-07, OPG-13, OPC-06, OPA-01, OPM-12) obtained from Operon Technologies, Alameda California. The reaction ran 40 cycles. PCR amplifications were performed on a PTC100 Programmable Thermal Controller (MJ Research Inc.). The amplification products were separated on 1.6% agarose gels in TBE buffer, followed by staining with ethidium bromide. Two of the PCR reactions were repeated three times using the same conditions to check repeatability of amplification products both within and between reactions. We selected a sample of five putative apomictic seedlings from the cross CDO × ROS and four from the cross CDO × REY for the DNA fingerprinting study. They were selected from those seedlings that emerged later, but were large enough to provide the amount of tissue required for DNA extraction.

RESULTS AND DISCUSSION

Seed Germination and Apomixes

Germination ranged from 10% obtained with the accession Reyna (REY) to 79% in the cross of Cristalina (CRI) to Rosalito (ROS). The average germination was 54%, comparable to values reported by Wang et al. (1996), who obtained a maximum of 55% using cactus soil mix and gibberellins on seeds from clones collected in Mexico, Texas, and Chile, and Nieddu and Chessa (1997), who reported an average of 49% germination with the Italian cultivar 'Gialla' (*O. ficus-indica*).

Seeds from selfed flowers germinated as efficiently as seeds obtained from open pollinated flowers, and seeds obtained from crosses, presented germination values near the average, with the exception of CRIS × Amarilla CN (ACN), which reported only 16%.

Germination started 7 to 10 days after planting. The time needed to reach the maximum germination percentage ranged from 32 days in the cross REY × ROS to 86 days for the selfed accession Rosalito, during the 105 days considered for the trial. The time needed to reach maximum germination did not affect the germination percentage ($r = -0.12$) nor the percentage of apomictic seedlings ($r = 0.002$). We did not find any correlation between the germination percentage and the number of apomictic seedlings ($r = -0.27$). Unlike other species, like pines (Kozlowski, 1997) the size of the seed of cactus pear was not associated with the percentage of apomictic seedlings, as shown in Table 1.

Late-emerging seedlings (assumed apomictic) were visible as early as two days after the zygotic seedlings, the former all germinated during the cotyledon stage. They were smaller and easy to identify. The differences in seedling size were less evident in the following six months when these seedlings attained growth rates similar to the putative zygotic seedlings. This observation supports the need for early screening of these individuals to avoid confusion and inaccurate identification.

Table 1. Germination and Apomictic Seedling Percentage

Accession	Seed Weight (g/100 seeds)	Germination (%)	Apomictic Seedlings (%)	Germination Span (days)
Cristalina (CRIS)	2.44	67 a	17 a	71
Copa de Oro (CDO)	2.23	70 a	20 a	42
Reyna (REY)	1.70	10 c	5 cd	26
Rosalito (ROS)	2.00	54 ab	2 d	70
CDO self	2.04	70 a	15 b	82
REY self	1.62	38 bc	13 b	49
ROS self	2.07	52 ab	3 d	86
CDO x ROS	2.25	50 b	18 a	74
ROS x CDO	2.20	47 b	1 d	47
CRIS x ROS	2.25	79 a	8 bc	37
ROS x CRIS	1.65	49 b	2 d	36
CDO x Amarilla CN	1.50	78 a	19 a	61
REY x ACNF	1.75	50 b	6 cd	61
CDO x REY	1.95	66 a	16 b	60
ROS x REY	1.80	55 b	2 d	61
REY x ROS	1.75	62 b	6 cd	32
CRIS x ACNF	2.50	16 c	2 d	74

* Significance values according to Tukey: 0.05

Late-Emergent Seedlings Possess the Same RAPD Profile as the Maternal Entries

From the seven primers tested, only one did not give any amplification products. Figure 1 shows the amplification products obtained with the decaprimers OPG-13 and OPM-12. The total number of bands among the maternal entries showed little variation, a probable indication that although they are phenotypically different, they still are closely related. An additional band was detected for the entry ROS when compared to CDO; likewise, two and one missing bands were detected when comparing CDO to REY.

Given that the three crosses share the same maternal entry, we compared the banding patterns across putative apomicts with that of the CDO entry. In general, the number of bands among apomicts and the maternal parent is similar. Slight differences in band brightness were observed, probably due to minor differences in the concentration of template DNA. This pattern was maintained when comparing among apomicts of the cross CDO x REY.

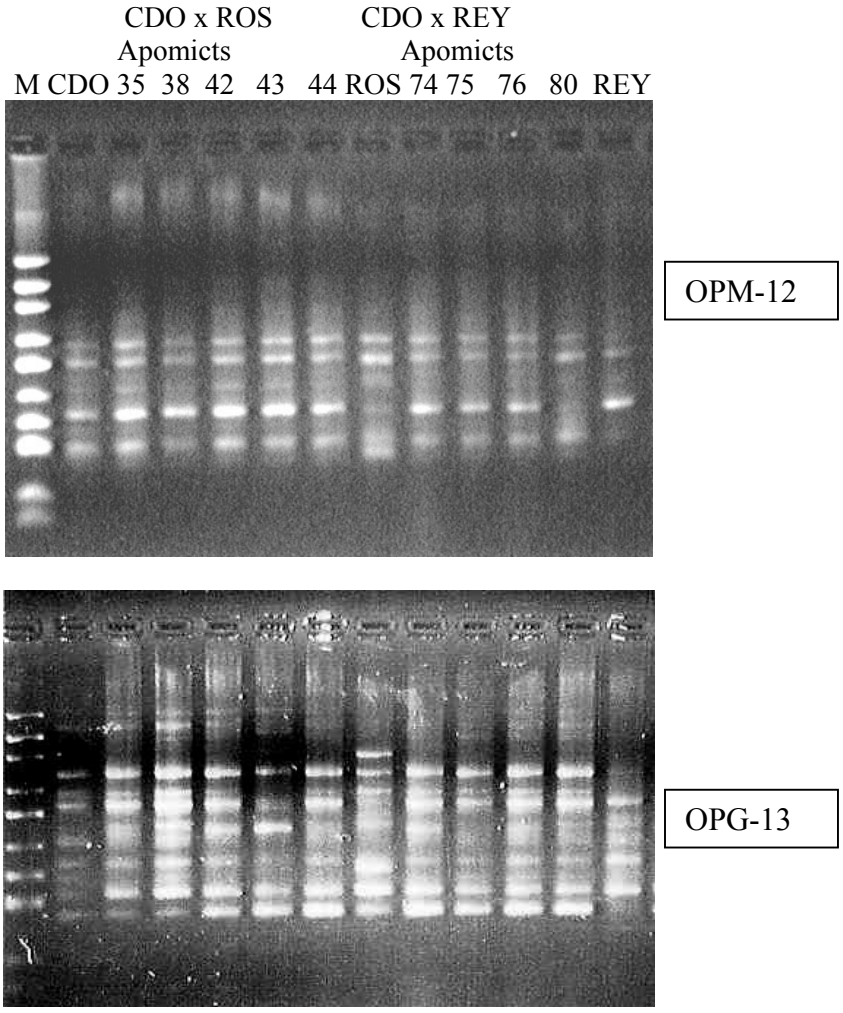

Figure 1. Amplification Profiles of Apomictic Seedlings of Cactus Pear With Primers OPG-13 (CTCTCCGCCA) and OPM-12 (GGGACGTTGG). Lane M; uPC 19 DNA marker (Biosynthesis). (The numbers represent the seedling identification).

Effect of Selfing on the Expression of Apomixes

Selfing induced a higher number of apomicts in low-apomict accessions (ROS and REY in Table 1). On the other hand, selfing did not modify significantly the value observed in CDO ("highly-apomictic"); which presented 20% of somatic seedlings as open pollinated versus 18% in the seeds originated from a selfed individual.

Pollen Source Affects the Percentage of Apomictic Seedlings

The presence of apomictic seedlings was observed in all the entries tested. However, higher values were consistently found associated with particular accessions. The highest value was observed with the open-pollinated accession Copa de Oro, which presented up to 20% of apomictic seedlings. High numbers of somatic seedlings were also observed in all crosses involving CDO as a female.

When we crossed this accession with Rosalito, an entry that presented very low incidence of maternal seedlings (1%), the number of apomicts increased significantly, up to 18%. Similar effect was observed when this entry was crossed with Amarilla CN, and Reyna. These crosses reached 19% and 16% of somatic seedlings, respectively.

If two entries that have a low incidence of apomicts were crossed, the number of somatic seedlings increased, but remained low. As observed in the cross ROS × REY, which presented 2% and 5% as open pollinated, this value increased to 6% in the cross. These results suggest the possibility of a maternal effect as well as different levels of dominance. However, further studies are needed to ascertain the inheritance mode of this trait.

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