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Genetic Uniformity of the MSXJ papaya hybrid (*Carica papaya* L.) during Micropropagation

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ABSTRACT

Objective: To analyze the genetic uniformity of MSXJ hybrid papaya *in vitro* plants, obtained by direct organogenesis.

Design/Methodology/Approach: The MSXJ papaya hybrid demonstrates quality characteristics for the national and exports market. *In vitro* culture of plant tissues represents a useful tool for their multiplication and conservation, but somaclonal variation can diminish their genetic and agronomic uniformity. In order to analyze the genetic uniformity of in vitro plants of this hybrid, ten ISSR primers were used for *in vitro* plants micropropagated during nine subcultures. DNA was extracted using the CTAB method. Data were analyzed using the program PopGene v 1.3.1.

Results: Eighty-five loci of 200 to up to 2000 pb were generated, with 37 polymorphic loci. In the cluster analysis, three groups were observed which separate subculture one, subcultures two to eight, and subculture nine; the Gst value of 0.87 indicated genetic uniformity as far as subculture eight.

Study Limitations/Implications: Papaya is one of the most important tropical fruits worldwide; however, these plants need to be healthy and genetically uniform to guarantee commercial success. *In vitro* propagation allows obtaining healthy and uniform plants, but it is necessary to study genetic uniformity during their micropropagation.

Findings/Conclusions: The *in vitro* multiplication of the MSXJ papaya hybrid permitted the regeneration of vigorous plants in 30 d. Molecular profiles indicate that as far as subculture eight, there is genetic uniformity. As such, no more than eight subcultures are recommended during micropropagation.

Keywords: *In vitro* plants, genetic uniformity, papaya, ISSR.

INTRODUCTION

Papaya (*Carica papaya* L.), along with mango (*Mangifera indica* L.), avocado (*Persea americana* L.) and pineapple (*Ananas comosus* (L.) Merr), is one of four tropical fruits with the highest volume in exports. Globally, it is among the most well-known and consumed fruits (Altendorf, 2018). It is cultivated extensively in more than 60 countries in the tropics and subtropics (FAO, 2017). In 2017, Mexico positioned itself as the third top producer in the world and the fifth top exporter with 961,768 tons (SIAP, 2018).

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The cultivation of papaya faces significant problems, such as the ringspot virus (Valencia *et al*., 2017), which affects its production and productivity (Vincent *et al*., 2019). For this reason, the development of cultivars with better characteristics is of great importance. The MSXJ papaya hybrid developed in the Experimental Camp of Huimanguillo, Tabasco (INIFAP) demonstrates quality characteristics for the national and exports market: it has red flesh and is tolerant to high temperatures during its flowering and fruiting stage (Mirafuentes and Santamaría, 2014). However, efficient propagation methods are needed to ensure its genetic characteristics, sex, safety, and agronomic uniformity. One of these methods is *in vitro* cultivation, which is useful for obtaining plants with high genetic and phytosanitary quality, as well as facilitating their use in plant-breeding efforts (Olivera, 2009). In the case of the papaya, there are various reports on its *in vitro* propagation (Ascencio *et al*., 2008; Setargie *et al*., 2015; Teixeira, 2016; Bindu and Bindu, 2017; Solórzano *et al*., 2018; Chaudhary and Prakash, 2019). Posada *et al*. (2004) reported a propagation protocol using apexes and Solis *et al*. (2011) using meristems. Recently, Al-Shara *et al*. (2018) published a review of the methods and limitations in papaya micropropagation where they refer to the difficulties they have found in commercial *in vitro* propagation of papaya; among these, they cite the small margin of certainty in reproducing and ensuring the production of healthy plants without genetic changes. Regarding this, it is necessary to confirm the genetic uniformity of the plants obtained using *in vitro* cultivation techniques (Posada, 2005). Genetic uniformity studies constitute a valid and fast technique for the detection of variability, since it allows distinguishing genotypes (Dávila and Castillo, 2007). Genetic markers can be successfully employed to search for differences in the expression patterns between cultivars, varieties, and plants. The ISSR markers are dominant and highly reproducible, therefore very useful for DNA profiling (González and Aguirre, 2007). Thus, the genetic uniformity of *in vitro* plants of the MSXJ papaya hybrid obtained via direct organogenesis was analyzed using ten ISSR primers during nine subcultures.

MATERIALS AND METHODOLOGY

The *in vitro* plants used were MSXJ papaya hybrids, developed in the Agricultural Biotechnology Laboratory of the Innovation and Development Park in the state Veracruz, located in the Monterrey Institute of Technology and Higher Education (ITESM) facilities. The *in vitro* plants were obtained from two hermaphrodite plants of the MSXJ hybrid, eight months old (P2 and P3). The samples were collected in Cotaxtla, Veracruz, Mexico. They were then regenerated via direct organogenesis from apical meristems, in a Murashige and Skoog (1962) basal medium at 100%, supplemented with 100 mg L^{-1} of myo-inositol, 0.4 mg L^{-1} of thiamine, 30 g L^{-1} of sucrose, and 2.5 g L^{-1} Phytagel at pH 5.7±0.01, with a photoperiod of 16 h light.

Multiplication of MSXJ hybrid. The *in vitro* plants were multiplied in the Agricultural Biotechnology Laboratory, and sub-cultured every 30 d nine times. For the *in vitro* multiplication of the MSXJ papaya hybrid, a Murashige and Skoog 100% solid mineral salts medium was used, supplemented with adenine 10 mg L^{-1}, thiamine 40 mg L^{-1}, myo-inositol 100 mg L^{-1}, ascorbic acid 100 mg L^{-1}, sucrose 30 g L^{-1}, and Phytagel 3 g L^{-1}. The growth regulators used were naphthalene-acetic acid (NAA) 0.05 mg L^{-1}, benzyl aminopurine (BAP) 0.5 mg L^{-1}, and gibberellic acid (GA3) 0.1 mg L^{-1} with a pH of 5.8. Jars with 250 mL capacity with 30 mL of culture medium were sterilized in an autoclave (Felisa) at 120° C for 20 min and were left to rest for 24 h so that it could solidify.

Six shoots of MSXJ hybrid *in vitro* plants were placed in each jar with culture medium and kept at a controlled temperature (24 to 26 °C) for 30 d. After this time, each shoot was individualized and sub-cultured again in fresh culture medium. Additionally, remains of the gelling agent and callus that could accumulate on the seedlings were removed. A small sample of plant tissue was collected from each sub-culture of the *in vitro* plants and placed in an Eppendorf tube; the samples were frozen in a CRIOTEC freezer at −50 °C. This was carried out for each of the nine sub-cultures of the MSXJ hybrid micropropagated to analyze its uniformity.

Analysis of genetic uniformity. The genomic DNA extraction was done in the Teaching, Research and Services Laboratory (LADISER) of Plant Biotechnology and Cryobiology in the Molecular Biology department of the Faculty of Chemical Sciences at Universidad Veracruzana. The CTAB 2% method was used (Murray and Thomson, 1980) with modifications. Two samples of *in vitro* plants were used for each one of the nine sub-cultures, resulting in a total of 18 samples. The purity and amount of DNA extracted from each of the samples was quantified and confirmed by UV spectrophotometry;

purity was determined through optical density (OD 260 nm/OD 280 nm), and DNA integrity and quality were determined with 1.5% agarose gels.

DNA was amplified with the polymerase chain reaction (PCR) technique in an AXYGEN MAXYGENE II thermocycler. A preliminary evaluation was carried out of 20 ISSR (Inter Simple Sequence Repeats) reported by Sudha *et al*. (2013). Only the primers with the greatest number of the clearest bands were selected. The reaction mixture final volume was fo 25 μL, which included 2 μL of genomic DNA of the MSXJ hybrid, 5 μL of 5x My Taq Reaction Buffer, 2 μL of the primer, 0.2 μL of My Taq DNA Polymerase, and 15.8 μL of ultrapure water. The DNA amplification program consisted of an initial denaturation at 94 °C for 5 min, followed by 45 one-minute denaturation cycles at a temperature of 52 °C, extension at 72 °C for 2 min, and final extension at 72 °C for 7 min. The amplified products were separated by electrophoresis in 3% agarose gel using 2 μL of Tri-color Buffer (5x DNA Loading), Gel Red Nucleic Acid Stain, and 8 μL of each PCR sample; 3 μL of MPM Hyper Ladder 1Kb molecular weight marker was used, suspended in TBE 1x (Tris/Borate/EDTA) at 90 V for 1:30 h. Finally, the amplified products were read in a UV transilluminator (Benchtop UV), capturing images for their subsequent analysis.

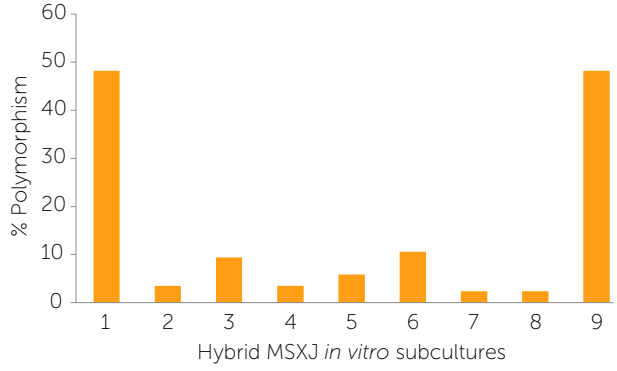

Figure 1. Percentaje of polymorphism for ten ISSR primers, in nine *in vitro* subcultures of *Carica papaya* MSXJ hybrid.

Data analysis. The number of bands per primer were calculated, as well as the percentage of polymorphism, genetic identity and distance, according to Nei (1972). In addition, a cluster analysis was carried out with the unweighted pair group method with arithmetic mean (UPGMA) using the PopGene program v. 1.3.1.

RESULTS AND DISCUSSION

Micropropagation. The *in vitro* propagation protocol for the MSXJ papaya hybrid allowed obtaining vigorous seedlings free of diseases, with enough shoots for their multiplication. This coincides with that reported by Solís *et al*. (2011), since when propagating *in vitro Carica papaya* var. PTM-331 from apical meristems at 35 d they obtained large seedlings with a multiplication coefficient of 3.42.

Molecular analysis. The first critical step to carry out a molecular study in plants is to obtain good-quality DNA (Castro-Gómez *et al*., 2012). The purity of extracted genomic DNA, determined by absorbency proportion at 260/280 nm, was 1.8 on average; which, according to Alejos-Velázquez (2014), is accepted as pure DNA. In addition, the integrity gels showed appropriate quality for their amplification with ISSR primers. The genomic DNA concentration of the 18 MSXJ hybrid samples was 30.9 ng μL^{-1} on average, allowing the amplification of clear DNA bands.

Genetic uniformity analysis. Once the preliminary analysis of 20 ISSR primers for the genetic uniformity analysis of *in vitro* plants of the MSXJ hybrid micropropagated for nine subcultures was completed, ten ISSR primers were selected: UBC- 857-ACA CAC ACA CAC ACA CYC; T05- CGT TGT GTG TGT GTG TGT; ICL3- DBD ACA CAC ACA CAC ACA; UBC 841- GAG AGA GAG AGA GAG AYC; UBC 836- AGA GAG AGA GAG AYA; ICL16- GAG AGA GAG AGA GAG AYG; UBC 807- AGA GAG AGA GAG AGA GT; UBC 842- GAG AGA GAG AGA GAG AG; UBC835- AGA GAG AGA GAG AGA GYC; UBC825- ACA CAC ACA CAC ACA CT. These primers amplified 7 to 12 bands in 3% agarose gel with an average of 8.5 bands (Figure 1).

The amplifications generated 85 loci with a molecular weight of 200 to 2000 pb, and 37 polymorphic loci. The primers showing higher polymorphism were UBC841 with 12 bands, followed by UBC807 with 11, and UBC842 with six bands. The consistent and well-defined profiles of these ISSR primers showed that they can be used to determine the genetic uniformity of the MSXJ papaya hybrid during its *in vitro* micropropagation.

In the genetic uniformity analysis of the MSXJ papaya hybrid subcultures micropropagated *in vitro*, a 9.41% polymorphism was observed in subculture one. This polymorphism was greater than that of the next subcultures, and this can be explained by high heterozygosity due to the plant's cross-pollination. In subcultures two to eight, polymorphisms from 1.18 to 9.41 were present. The plants regenerated in these

subcultures, though not identical, are considered similar amongst themselves since the Gst value was 0.87, which indicates high genetic identity between subcultures. Because *in vitro* cultivation is a cloning technique, the variability in polymorphism in these subcultures is explained by the high heterozygosity that is reflected in variability among explants. This had already been reported by Rani and Raina (1998), who cited that the variability in the subcultures of plants regenerated using in vitro processes does not necessarily indicate that they are different from their phenotype. Additionally, López *et al*. (2006), after studying the genetic uniformity of papaya plants micropropagated from the proliferation of axillary buds and meristems, demonstrated that their morphological, agronomic, industrial, and molecular characteristics are stable.

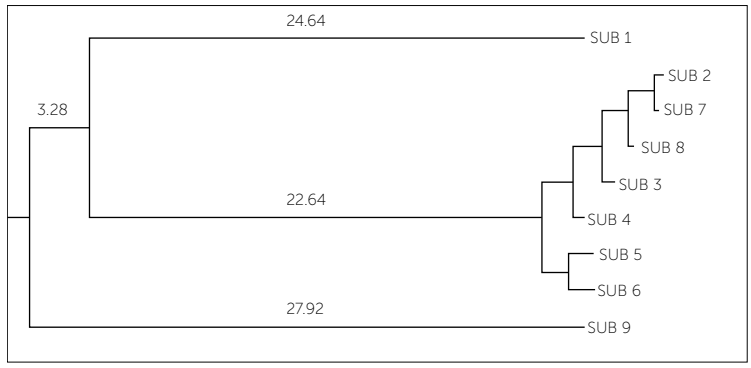

Figure 2. UPGMA dendrogram of nine *in vitro* subcultures for *Carica papaya* MSXJ hybrid, from DNA amplified with ISSR primers based on Nei's genetic distance.

The above is corroborated with the cluster analysis of the MSXJ papaya hybrid (Figure 2), stemming from the ISSR profiles obtained through Nei's (1972) similarity algorithm and the UPGMA grouping algorithm, which shows one group that includes subcultures two to eight and the separation of subcultures one and nine. Subculture one presented the highest values in genetic identity (0.6342 to 0.66001) according to Nei. In subcultures two to eight it decreases slightly (0.5122 to 0.5983), and the lowest value was observed in subculture nine. Subculture nine presented the highest values in genetic distance (0.5158 a 0.6690) from the other subcultures, representing the greatest distance from subculture one. These data explain the separation of subcultures one and nine. In addition, the decrease of the genetic identity when increasing the number of subcultures coincides with that reported on the increase in frequency of the appearance of somaclonal variants as propagation cycles increase. This study recommends not exceeding eight subcultures, with the aim of minimizing the risk of genetic variability in the propagated material.

CONCLUSIONS

The *in vitro* multiplication of MSXJ hybrid papaya plants permitted the regeneration of vigorous plants in 30 d, useful for establishing commercial orchards. The DNA obtained has sufficient quality to analyze genetic uniformity with ISSR primers in *in vitro* MSXJ hybrid papaya plants. The molecular profiles obtained indicate that genetic uniformity exists as far as the eighth subculture. Therefore, to reduce the risk of genetic variability in the propagated material, it is recommended that no more than eight subcultures during micropropagation.

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