RESEARCH ARTICLE

Micropropagación de plantas de lechosa en recipientes de inmersión temporal a partir de brotes axilares

Micropropagation of papaya plants in temporary immersion recipients from axilary shoots

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Resumen

Se estandarizaron las condiciones de iniciación, multiplicación, enraizamiento y aclimatización de plantas hermafroditas de lechosa cv Maradol provenientes de brotes axilares, producidos en recipientes de inmersión temporal RITA®. En cada envase, contentivo de 200 ml de medio de cultivo líquido de Fitch, se colocaron cuatro brotes de 2 a 3 cm de longitud. Los biorreactores se conectaron a tres líneas de inmersión de 5, 2 y 1 min cada 4h y se colocaron 6 envases en promedio por línea, en condiciones de fotoperíodo de 16 h. Transcurridos 30 a 45 días, se cuantificaron los brotes y se clasificaron de acuerdo al tamaño: < 2 cm (pequeños), entre 2 a 3 cm (medianos), > 3 cm con y sin raíz (grandes). Los dos primeros tipos de brotes se continuaron multiplicando en los mismos medios; y los más elongados se aclimatizaron utilizando el Sistema Autotrófico Hidropónico (SAH). Se determinó la sanidad y la fidelidad de las plantas producidas mediante pruebas de ELISA y RAPD, respectivamente. Durante un periodo de 6 meses se reciclaron un total de 47 recipientes, los cuales produjeron 1.091 brotes: 377 pequeños; 482 medianos; 175 grandes sin raíz y 57 con raíz. Usando el SAH se obtuvo 89,5% de plantas aclimatizadas cuando se usaron brotes enraizados, y 41,6% a partir de brotes sin raíces. Con la combinación de las técnicas RITA y SAH se logró un sistema continuo y eficiente de producción de plantas sanas y fieles al tipo, en comparación con los métodos convencionales de micropropagación y aclimatización.

Palabras clave: *Carica papaya*, RITA®, sistema autotrófico, estabilidad genética.

Abstract

We standardized initiation, multiplication, rooting and acclimatization conditions of papaya cv Maradol hermaphrodite plants from axillary buds produced in temporary immersion reactor RITA®. Recipients contained 200 ml of Fitch liquid culture medium, and four shoots of 2 to 3 cm. in length were placed in each. The bioreactors were connected to three different immersion lines of 5, 2, and 1 min each 4h, with 6 containers per line on average, in 16 h photoperiod. After 30 to 45 days, the shoots produced were quantified and classified according to size: <2 cm (small), from 2 to 3 cm (medium), >3 cm with or without roots (large). The first two types of shoots were multiplied in the same culture media, and more elongated shoots were acclimatized using Autotrophic Hydroponic System (AHS). The sanity and fidelity of the produced plants were determined using ELISA and RAPD, respectively. For a period of six months 47 vessels were recycled and 1,091 shoots were produced: 377 small; 482 medium; 175 large without roots and 57 rooted shoots. Using AHS, 89.5% acclimatized plants were obtained when rooted shoots were used, and 41.6% from rootless shoots. With the combination of RITA and AHS techniques we achieved a continuous and efficient production of healthy and true to type papaya plants, in comparison to conventional micropropagation and acclimatization procedures.

Key words: *Carica papaya*, RITA®, autotrophic system, genetic stability.

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Introduction

Papaya (*Carica papaya* L.) is one of the traditional fruits of greatest importance in Venezuela with a planted surface area of 10,614.01 ha and a production of 199,002 MT (FAO, 2013). To date, great technological progress has been achieved in this field and the tendency is to replace the low-technology, native Venezuelan cultivars with hybrids of the Maradol group dependent on certified imported seeds, which produce uniform plants of smaller size, and greater productivity and quality of fruits (Aular and Casares, 2011).

Papaya is conventionally propagated by seeds. However, latency problems have been identified, which affect germination and conservation in the medium and long term. Therefore, it must be planted as close as possible to the harvest season. Only a percentage of the plants are andromonoecious with hermaphroditic flowers and they produce elongated commercial fruits, so three plants are sown per point. Heterogeneous plants are produced when they come from unselected and hybrid seeds. Furthermore, the health of the seeds must be ensured, because some diseases, such as bacteriosis produced by *Erwinia* sp., are transmitted by this channel (Guevara *et al.* 1993; Posada, 2005).

Efficient micropropagation protocols of hermaphroditic cultivars of papaya would reduce the number of plants needed to plant a hectare from 6,000 to 2,000. Field trials have demonstrated that the plants obtained from micropropagation of buds were uniform and conserved 100% hermaphroditism and their elongated fruits, while the plants from seeds conserved only 75% and therefore, 25% bore round fruits that are less commercial (Talavera *et al*. 2009). The micropropagation protocols for this fruit tree need to be improved to take them to the commercial phase. The phases of initiation from field plants, elongation and multiplication of cultivars, hybrids and transgenic plants are considered overcome, specifically, high multiplication coefficients have been achieved (from 2.5 to 12 times). However the low percentages of *in vitro* and *ex vitro* rooting of the shoots and the high mortality of the plants obtained *in vitro* during the 'transfer from culture' stage have been identified as the main bottlenecks, and in over a decade, diverse work has been generated that addresses these issues (Chan *et al*. 2003; Rohman *et al*. 2007; Talavera *et al*. 2009).

Additionally, the presence of viruses in the initial *in vitro* tissues should be monitored in a commercial propagation system. This aspect has not been addressed in the reviewed work on micropropagation. The viruses that frequently affect the papaya crop are papaya ringspot virus (PRSV), papaya mosaic virus (PapMV) and cucumber mosaic virus (CMV). With the aim to detect them, the use of the enzyme-linked immunosorbent assay (ELISA) is the most simple, economical and effective strategy, because these viruses are very good immunogens, allowing polyclonal antibodies to be obtained with very high values. Specific polyclonal antibodies against the protein of the viral capsid have been obtained by experiment in the country (Marys *et al*. 2000). However, there are many commercial shells with proven efficiency, such as Agdia®, used extensively in the world for the diagnosis of these and other viruses.

The development of temporary immersion systems (TIS) has permitted the optimization of the multiplication of plant species and propagation on a commercial scale, because the operating costs are reduced by the use of liquid media with greater ease of handling; the establishment times of the initial *in vitro* explants decrease; the explants' multiplication rates increase; plants with greater vigor are obtained compared to the conventional forms of *in vitro* propagation in semi-solid media or semi-submerged or submerged liquid media: the plants behave better during the 'transfer from culture stage' than the material obtained from semi-solid and liquid media; and the automation of some of the stages of *in vitro* cultivation is made possible, which reduces manual work for commercial plant production (Colmenares and Giménez, 2003; Berthouly and Etienne, 2005; Niemenak *et al*. 2008). However, physiological abnormalities may appear, especially hyperhydricity, which is reduced by intermittently exposing the explants to the liquid medium. Therefore, it is considered crucial to adjust the immersion times to prevent the manifestation of hyperhydricity (Berthouly and Etienne, 2005).

The TIS can be used in the mass propagation of elite plants through the multiplication of axillary shoots, microtubers and somatic embryos. Successful experiences have been reported of micropropagation of fruit trees, such as grape vines, *Amelanchier* x grandifolia, banana, plantain, citrus fruit and pineapple trees using different temporary immersion systems, and some of them are produced on a commercial scale (Colmenares and Giménez, 2003; Jiménez 2005; Berthouly and Etienne, 2005; Niemenak *et al.* 2008).

Some previous experiences of micropropagation through temporary immersion systems in papaya are known. Using the manually operated double-container system, Damiano et al. (2003) obtained greater rates of propagation of papaya shoots when comparing it to semi-solid and stationary liquid media. Posada *et al.* (2003) used TIS for the multiplication and germination of somatic embryos of Red Maradol papaya, using 'Sartorius' filtering systems with 250 ml capacity. The frequency of immersion was three times a day for one minute. As a result, it was possible to multiply the somatic embryos, achieving multiplication coefficients of 40 in the culture medium supplemented with 1 $mg.l^{-1}$ of 2.4-D, after 45 days, and 87% germination. There is a previous study about liquid cultures of somatic embryos in agitation for escalation of the mass production of papaya plants (Castillo *et al.* 1998) in which the pro-

duction potential of somatic embryos was quantified and assessed though explants of hypocotyls of papaya. Only in liquid media in agitation at 120 rpm. According to this research, 455 somatic embryos were produced for every 200 mg of callus in the liquid maturity medium after 112 days, compared to 174 days in the solidified medium. According to the authors, the use of the liquid systems for the production of secondary embryogenesis resulted in a more efficient strategy for the large-scale proliferation of immature embryos, because shorter cultivation periods are required to obtain greater yields. It indicates that the liquid system is adequate for the automation of the rapid production of somatic embryos and continuous propagation in bioreactors.

It has been demonstrated that the micropropagated plants are genetically stable through the proliferation of axillary shoots and meristems, because of their morphological, agronomic and industrial characteristics and the cytological, isoenzymatic and molecular analyses, and it is considered to be the most adequate system of papaya clone multiplication (Lopez *et al*. 2006). However, small differences have been observed between micropropagated plants and those produced from seeds, the former having a slightly smaller size and being slightly premature, which is advantageous for the yield (Fitch *et al*. 2005, May *et al*. 2007, Talavera *et al*. 2009). Additionally, when somatic embryogenesis has been used as a micropropagation process, morphological variations have been achieved such as plant height, the number and length of flowers, as well as the number of fruits and the dimensions and shape of them, above all when high concentrations of 2.4-D (15 $mg.l^{-1}$) were used. These differences could be associated with the polymorphic patterns obtained in the molecular analysis of the DNA using arbitrary initiators (RAPD) (Homhuan *et al*. 2008). In another study with low concentrations of 2.4-D of 2 $mg.l^{-1}$, there was a high percentage of ploidy variations of 14% in the regenerated *in vitro* plants (Clarindo *et al*. 2008). The rates of multiplication could be significantly increased by 20 times or more by using somatic embryogenesis, but there is the risk of increasing the variability of the plants produced (Posada, 2005; Clarindo *et al*. 2008; Homhuan *et al*. 2008).

In this work, the initiation, multiplication and pretransplant conditions of hermaphroditic papaya plants from axillary shoots were standardized in Recipients for Automated Temporary Immersion (RITA®); and the acclimatization of the micropropagated plants. Additionally, the genetic stability and health of them was evaluated usingRAPD markers and the ELISA technique, respectively.

Materials and Methods

Micropropagation from axillary shoots of hermaphroditic, type IV or elongata plants of the cv. Maradol in Temporary Immersion Recipients

Planting of Axillary Shoots in Liquid and Semi-Solid Media

Plant Material. Axillary shoots of hermaphroditic Maradol cultivar plants obtained from semi-solid cultures were used, which were placed in Recipients for Automated Temporary Immersion (RITA®, CIRAD, France) with 200 ml of liquid culture medium. The media were comprised of Murashige and Skoog (1962) salts and vitamins, with the addition of benzylaminopurine (BAP) (0.2 $mg.^{l-1}$) and kinetin (0.1 $mg.^{l-1}$) (Fitch, 1993). Four shoots were planted per recipient, each measuring 2 to 3 cm in aseptic conditions. The recipients were connected to three lines with the following immersion times: Line 1: 5 minutes / 4 $hours^{-1}$; Line 2: 2 minutes / 4 $hours^{-1}$; and Line 3: 1 minute / 4 $^{-1}$hours. An average of six containers were placed per line in photoperiod conditions of 16 hours of light a day at an intensity of 32 $\mu molm^{-2}s^{-1}$ of fluorescent white light and a temperature between 23.2 °C and 25 °C, and HR from 50.9% to 55.4%. After 30 to 45 days, the new shoots obtained with or without roots were counted and classified according to size for a period of six months. Shoots of less than 2 to 3 cm continued to multiply in the same culture media; the elongated shoots > 3 cm with or without roots were acclimatized using the Autotrophic Hydroponic System (AHS) (Rigato *et al*. 2001). The rate of proliferation of the production lines (number of shoots produced - number of shoots planted / number of shoots planted) was also calculated.

In the case of those planted in semi-solid culture, 200 ml glass containers were used, containing 30 ml of Fitch (1993) medium, solidified with agar of 7 gl^{-1}, in which four medium, 2 to 3 cm-long shoots were planted in a total of 15 containers, and they were recycled after 30 to 45 days. The planted containers remained in the same light and temperature conditions specified above. This test was repeated once. The rate of proliferation of shoots in the semi-solid media was calculated as stated in the previous paragraph.

A data matrix was prepared, categorized by line and month with the following variables: number of containers planted, type of shoots, number of shoots and number of shoots contaminated with bacteria and fungi. The matrix was entered into a Microsoft® Excel 2007 spreadsheet and exported to the Infostat v. 1.0/P (InfoStat, 2002) application. The data were modeled under a 3x4 factorial arrangement (number of lines x

types of shoot) with four repetitions under a completely randomized design for each month of production. Additionally, the production set was analyzed at six months through an analysis of variance combined with the time (month, line and type of shoot). The assumptions of normality were previously tested for both models of Infostat (InfoStat, 2002). For the 'line x type of shoot' analysis of interaction, the Chi-squared test was used for the level p = 0.05. The multiple correspondence factor analysis was used to observe the patterns of association between the three lines of production and the four kinds of shoot (Agresti, 1990; Greenacre and Hastie, 1987).

Acclimatization: Two methods were used for acclimatization of the shoots.

Conventional method: Using the conventional method, 304 shoots with or without roots from RITA® were acclimatized: A mixture of commercial Sunshine® N°5 substrate and sieved soil was prepared in the ratio 3:2. Then it was sterilized and previously perforated 270 ml cups were filled. The shoots with roots were washed with sufficient distilled water to fully remove the agar, taking care not to damage them. The shoots without roots were treated with an AIB solution of 8 $mg.L^{-1}$ (Posada, 2005). The shoots were individually transplanted in plastic cups and covered with another transparent cup of a smaller capacity with the aim to create a humid chamber with an adequate microclimate for their adaption. They were watered with a solution comprised of 50% Murashige and Skoog (1962) salts every two to three days. From the second week, the cups that covered the plants were gradually removed until the plants' acclimatization was achieved. Later they were transplanted to black bags or flowerpots.

Autotrophic Hydroponic System (AHS): Twenty-four shoots with roots and 24 without roots were planted, each type separately, in transparent disposable plastic boxes measuring 32 x 25 cm, containing commercial Sunshine® N°5 substrate, to which a solution of 50% Murashige and Skoog (1962) salts in the ratio 1:1 (weight/volume) was previously applied. In the boxes, 400 g of substrate was applied and they were watered once a week with the previously mentioned solution. This was repeated once.

In both methods, the transplanted shoots were kept at a temperature of 23.2 to 25 °C and a relative humidity of 50.9 to 55.4% in growth chambers with 16 hours of light a day.

The survival percentage (number of rooted acclimatized shoots / number of shoots planted x 100) during acclimatization was measured. A binomial test was applied to the survival percentages obtained. The plants were suitable for planting in the field in a period of three to four months and they were transplanted in four different areas of the Aragua State where they were morphologically evaluated.

Extraction and Amplification of Papaya DNA by RAPD

Twenty shoots taken at random from RITA® were sampled in the production lines. For the extraction of the DNA from leaf tissue, the Dellaporta Protocol modified by CIAT (1999) was used.

Once the genomic DNA was extracted, its quality and concentration was verified. The 20 starters used were the 20 sequences of the OPA series of Operon technologies, according to the protocol of Chen *et al.* (2011). The visualization and analysis was conducted with the Chemi –Doc Bio Rad, Software Quantity One, Version 4-2, equipment. The RAPD products were compared with the pattern of molecular weight of the DNA of the Lambda phage digested with BST E2.

Application of Diagnostic Tests on Plant Pathogenic Viruses in Regenerated Plants

Leaves from shoots of the Maradol papaya cultivar of mass propagation in RITA® were used for the detection of plant pathonogenic viruses. Four samples were taken at random from the multiplied sample in three lines of immersion.

The direct enzyme linked immunosorbent assay (ELISA) technique was used with a double layer of polyclonal antibodies and detection antibody conjugated with alkaline phosphatase AGDIA®, which was specifically designed to detect the viruses that most affect papaya, such as papaya ringspot virus (PRSV), papaya mosaic virus (PapMV) and cucumber mosaic virus, (CMV). The protocol described by Noa-Carrazana *et al.* (2007) was used for its application. Negative or healthy and positive controls (supplied with shell and available in the laboratory) were used. The readings were taken at a wavelength of 405 nm to measure absorbency with a spectrophotometer or ELISA plate readers, LAB SYSTEM brand, MultiSkan EX model. For the calculation of the cutting point, double the average absorbency value of the healthy control was used.

Results and Discussion

Micropropagation from Axillary Shoots of Hermaphroditic, Type IV or Elongata Plants of Maradol Papaya Cultivar in Temporary Immersion Recipients

In a six-month period, a total of 47 recipients were harvested in three lines with different immersion times, which produced a total of 1,091 shoots. A monthly average of 182 shoots was produced in six containers harvested per month. The range of shoots harvested per month fluctuated between 68 and 298, like the harvested containers, which were between 4 and 12.

The shoots produced were classified in the following way: 377 small shoots (< 2 cm), 482 medium shoots (2 to 3 cm), 175 large shoots (> 3 cm) without roots and 57 large shoots with roots. The majority of the shoots was medium sized, close to half of the shoots produced (44.18%), and was used to be recycled and multiplied in RITA®. The large shoots without roots comprised 21.26% and they were directly acclimatized. Meanwhile, the small shoots (34.56%) were placed in Fitch (1993) semi-solid media for their maintenance and development. According to the analysis of variance (ANOVA), there were no significant differences with respect to the total number of shoots produced in the three lines of immersion evaluated. However, it was observed that they differ in terms of the kind of shoots produced. The greatest amount of shoots sized < 2 cm was produced in the frequencies of 1 and 2 minutes; the greatest proliferation of shoots sized 2-3 cm was produced in the frequency of 5 minutes / 4 hours^{-1}; and shoots sized > 3 cm with or without roots were predominantly produced in the line with a watering frequency of 2 minutes / 4 hours^{-1}. Therefore, in the lines of 1 and 5 minutes / 4 hours^{-1}, the greatest amount of small and medium shoots was produced, and the frequency of 2 minutes / 4^{-1} h served for the production of the four kinds of shoots, including the elongated shoots, with or without roots, suitable for the *ex vitro* phase (Table I). The above can be visualized in the multiple correspondence analysis between the kind of shoot and the frequency of immersion (Figure 1). In general, there were fluctuations in the average number of shoots/month in the production lines, with a greater amount of shoots in the first three months and the sixth month (data not shown).

According to the previous results, the rates of proliferation were calculated to be 5.3, 6.0 and 5.9 times for the lines 1, 2 and 3, respectively, and the average rate of proliferation to be 5.7. In the results of Damiano *et al*. (2003), this rate was significantly higher, up to 24±3.32 for papaya shoots micropropagated in the double-container TIS with an immersion period of 60 minutes/day in the Murashige and Skoog (1962) multiplication medium with 0.15 and 0.015 mg.l^{-1} of BAP and AIB, respectively, compared to the controls in semi-solid media, and there was inhibition in the multiplication of the shoots in the static liquid cultures. However, the proliferated shoots were not very elongated and difficult to separate for the following stage of *in vitro* pretransplant, which was not addressed in this work.

The rates of proliferation found in this research were similar to those obtained in coffee plants (*C. arabica* and *C. canephora*) during multiplication in microcuttings (Berthouly and Etienne, 2005) and in banana shoots (Alvard *et al*. 1993) with immersion times of

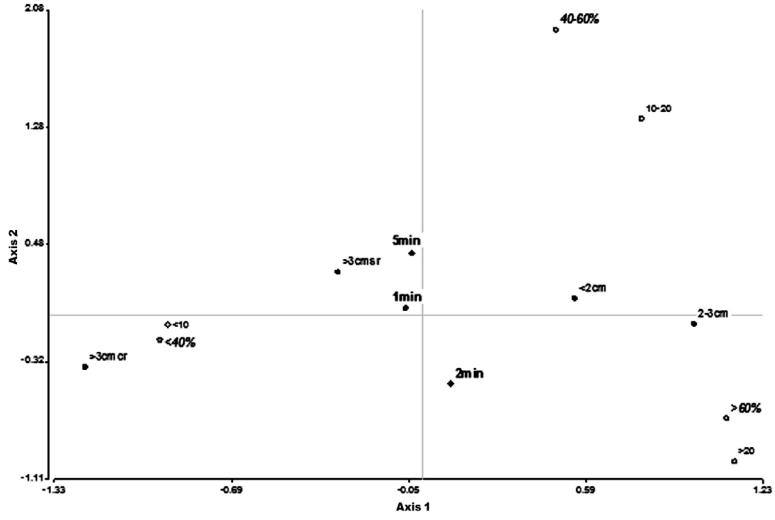

Figure 1. Multiple correspondence analysis of the three frequencies of immersion (1, 2 and 5 minutes every 4 hours) and the kinds of papaya shoots produced in the RITA at six months of micropropagation.

Contribution to the Chi-Squared Test

	Singular Value	Inertia	Chi-Squared	(%)	Cumulative %
1	0.80	0.63	272.20	28.10	28.10
2	0.65	0.42	179.20	18.50	46.60

Table 1. Cumulative number according to the type of Maradol papaya cultivar shoots produced in RITA® using three lines of immersion frequencies at six months of micropropagation.

Type of Shoot					
Line of Immersion (Minutes Every 4 Hours)	**< 2 cm**	**2-3 cm**	**> 3 cm without root**	**> 3 cm rooted**	**Overall Total**
1	151	164	47	18	380
2	137	143	68	21	369
5	89	175	60	18	342
Total	377	482	175	57	1091

Pearson's Chi-Squared Distribution	22.51	6	p: 0.0010
Chi-Squared MV-G2	23.16	6	p: 0.0007
Cramer's Contingency Coefficient	0.08		
Pearson's Contingency Coefficient	0.14		

20 minutes / 2 hours^{-1} with this same system. However, Colmenares and Giménez (2003) achieved much higher rates working with several species of Musaceae, 8.4 for the Williams banana cultivar and 11.2 for Hartón plantain with immersion times of 20 minutes / 4 hours^{-1}.

The shoots produced by the proliferation of the axillary buds of Maradol papaya cultivar in RITA® formed tillers around the initial shoots and they were longer than those of the semi-solid media. On the other hand, due to the impoverishment of the liquid media after the 30 days of cultivation, there was leaf senescence and the rooting of some of the shoots was induced. The roots produced had a normal fibrous aspect without callus formation at the base (Figure 2 b).

With the micropropagation of the papaya shoots in RITA®, it was not necessary to apply gibberelic acid (GA_3) to elongate the shoots, nor auxins to induce roots, as has been recommended by several authors (Drew *et al*. 1991; Solis *et al*. 2011). This process makes the changes to different culture media unnecessary, which reduces the operating costs of preparation and handling, as well as cutting the production times of full plants.

In this study, the rates of multiplication obtained in the RITA® system and the plants' vigor were greater, more than double, compared to the estimated 2 to 2.5 in the conventional semi-solid system every 30 to 45 days. In the latter case, the shoots were smaller and rarely rooted in the multiplication medium. Some authors have reported similar rates of proliferation of 3.42 (So-

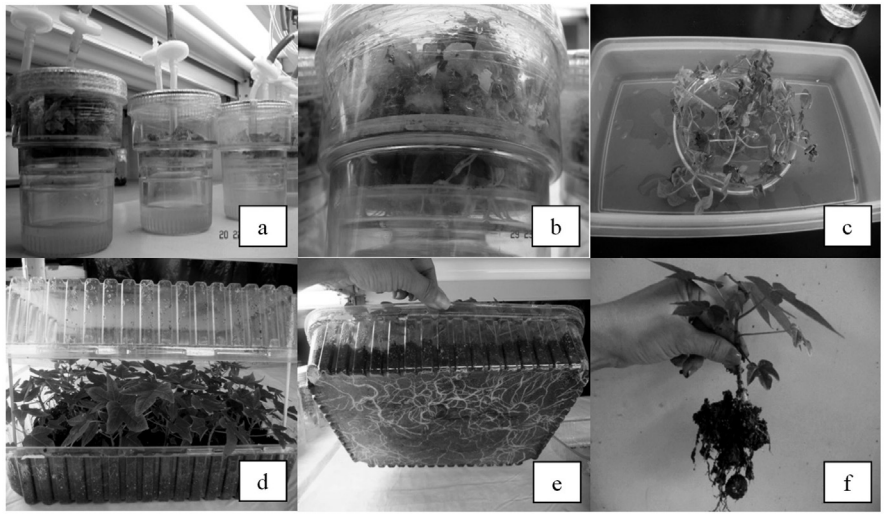

Figure 2. Micropropagation process of plants of the Maradol papaya cultivar from shoots in RITA® and acclimatization through the Autotrophic Hydroponic System (AHS): a) RITA showing growth of the shoots in controlled conditions; b) List of production of shoots and seedlings in one of the containers; c) Aspect of the shoots without roots from RITA treated with AIB; d) Gradual hardening of the plants at 26 days from transplant in the Autotrophic Hydroponic System (AHS); e) Breakdown of the development of the plant roots; and f) Development of the acclimatized plants at 26 days from transplant in the AHS.

lis *et al*. 2011). However, other authors have obtained much greater rates of 7.50, 9.87 and 12.73 times (Lai *et al*. 2003; Gallardo, 2002; Rohman *et al*. 2007) in semi-solid media.

In this research, the appearance of hyperhydric shoots was low (plants from three containers of the 47 harvested), which represented just 6% of the containers used. Similarly, Damiano *et al*. (2003) and Posada *et al*. (2003) did not find hyperhydricity problems in the micropropagated shoots or somatic embryos.

The contamination percentage was low (12.5%) in the first two months and predominantly of bacterial origin (5%), possibly due to the endogenous contamination of the tissues, which was not perceived in the semi-solid media. Several authors have indicated the presence of endogenous, non-plant pathogenic bacteria in the *in vitro* cultivation of papaya (Posada, 2005). This problem was easily detectable in the liquid media, which resulted in the contaminated shoots being discarded at the start of micropropagation. Additionally, during the production of shoots in the second, fifth and sixth months, there was low contamination from fungi (7%), possibly due to contamination of the environment.

Acclimatization of Papaya Shoots and Plants

Low survival percentages were achieved using the conventional method: 40.9% and 49.0% using > 3 cm shoots with or without roots, respectively, and there were no significant differences between the results (Table 2).

Using AHS, the percentage of acclimatized plants was increased, achieving 89.5% when > 3 cm shoots rooted *in vitro* were used. However, it was low when the shoots without roots were acclimatized and there were significant differences between these percentages (Table 2).

In vivo survival during acclimatization was greater with the shoots rooted *in vitro* compared to the results obtained by other authors, which oscillated between 65% and 85% (Posada, 2005; Tetsushi *et al*. 2008; Anandan *et al*. 2011). At approximately 26 days, the plants were very vigorous, presenting abundant fibrous roots and they were suitable for transplant to flowerpots (Figure 2 d, e and f). When shoots without roots were used, the appearance of roots was observed in some of the plants at 30 days from being transplanted in the substrate, and they took 30 more days to be suitable for transplant to flowerpots. In the 'transfer from culture' stage, the presence of roots seemed to be necessary for greater survival in the sterile substrate, as has been reported by other authors, who have observed that although the roots are not completely functional due to the scarcity of absorbent hairs, a greater size and number of roots induce the *ex vitro* formation of new roots and absorbent hairs, which will extract the nutrients necessary for the seedlings' development from the soil (Solis *et al*. 2011).

The trays had advantages compared to the use of individual cups, because they implied greater ease in handling the plant lots in the same container for weekly watering and humidity control and because of their light weight, these trays can be sent and distributed to producers. The plants were suitable for planting in the field in a period of three to four months and they were morphologically normal and hermaphrodite (data not shown).

Amplification of DNA of Hermaphrodite Maradol Papaya Cultivar Shoots Produced in RITA® by RAPD

The results indicated that there were no differences between the patterns of amplified fragments in the 20 samples from RITA® analyzed, because the fragments were distributed similarly for the 20 starters used, demonstrating genetic unity of the micropropagated shoots. These results ensure the loyalty of the plants produced from the multiplication of axillary buds (Figure 3). Similar results were obtained by López *et al*. (2006), micropropagating the same cultivar from nodal segments and using AFLP markers, as well as the morphological characteristics of plants in the field.

Table 2. Survival percentages of the shoots with or without roots acclimatized in the Maradol papaya cultivar produced in RITA®.

Acclimatization Method Used	Conventional Method (%)	AHS Method (%)
Shoots > 3 cm without root	40.9	41.6
Shoots > 3 cm with root	49.0	89.5
Averages	44.95	65.55

Conventional method: Shoots acclimatized in plastic cups containing a mixture of commercial Sunshine® N°5 substrate and sieved soil.

AHS Method: Acclimatized in trays of 32 x 25 cm containing the commercial Sunshine® N°5 substrate.

There were only significant differences between the survival percentages of the shoots rooted *in vitro* acclimatized using the AHS method compared to the other treatments.

Considering both methods, the conventional method has the lower survival percentage at a level of $p < 0.01$

Diagnostic Test of Viral Diseases in the Shoots of Hermaphrodite Maradol Papaya Cultivar Shoots Produced in RITA®

The leaf samples analyzed from the micropropagation were negative for papaya ringspot virus (PRSV), papaya mosaic virus (PapMV) and cucumber mosaic virus (CMV) with similar or lower readings than the negative controls for each test, from 0.0 to 0.03, while the positive controls gave readings of 3.08, 2.6 and 2.1, respectively. These analyses ensured the health of said shoots.

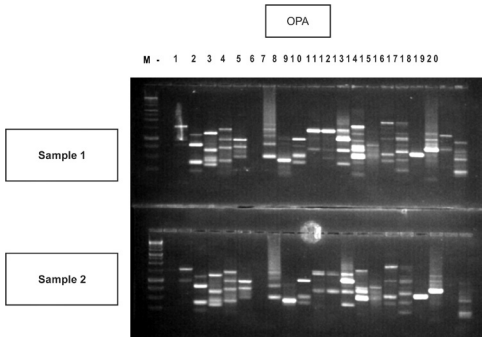

Figure 3. Amplified DNA of two leaf samples of the Maradol cultivar vitroplants with 20 OPERON® starters, showing similar amplified fragments. The first lane shows the molecular weight pattern of the DNA of the Lambda phage digested with BST E2, and lanes 1 to 20 show the amplified fragments with the initiators.

Conclusions

This work achieved a continuous production system of hermaphrodite, healthy, true-to-type, Maradol elongata cultivar papaya plants through the standardization of the establishment, multiplication, pretransplant and 'transfer from culture' procedures of plants produced through the RITA® system from *in vitro* axillary shoots. It was demonstrated that the temporary immersion system was the most efficient, because the rate of multiplication increased to an average of 5.7 compared to the conventional method in semi-solid media under the same environmental conditions. The shoots were not sensitive to the liquid medium in the frequencies of immersion used.

Lines with frequencies of immersion of 1 or 5 minutes / 4 hours-1 produced small and medium shoots, and the frequency of 2 minutes 4-1 hours was used for the multiplication of the four kinds of shoot, including the elongated shoots suitable for the 'transfer from culture' or *ex vitro* stage.

The use of AHS with *in vitro* rooted plants increased efficiency in the 'transfer from culture' process, because it improved the survival percentage and facilitated management of them.

The application of the two techniques together, TIS and AHS, was the most efficient for plant multiplication and transfer from culture.

The genetic stability and health of the produced plants were ensured with molecular and immunological tests, respectively, through this micropropagation process.

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Through the project: "Desarrollo de un Referencial Tecnológico para la Producción Masiva de Frutales Requeridos por la Industria mediante Sistemas de Biorreactores de Inmersión Temporal" (Development of a Technological Reference for the Mass Production of Fruit Trees Required by the Industry through Temporary Immersion Bioreactor Systems) (FCI 09PS38-05) it was possible to adapt and optimize a mass asexual propagation system. With the project "Mejoramiento Genético de la Lechosa en Zonas Productoras de la Región Central, mediante Técnicas Convencionales y Biotecnológicas" (Genetic improvement of Papaya in Production Areas of the Central Region through Conventional and Biotechnological Techniques) (Code 10-03-02-01) financed by the National Seed Plan of the Instituto Nacional de Investigaciones Agrícolas, this propagation system was restarted with the aim to produce mother plants to obtain good quality seeds, acclimatization was improved and the field trials were conducted.

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