REPORT

Somatic embryogenesis in papaya cultivar 'Maradol Roja' as an alternative for its propagation and genetic improvement

✍Laisyn Posada Pérez[1], Rafael Gómez Kosky[1], Yenny Padrón Montesinos[1], Justo González Olmedo[2], Romelio Rodríguez Sánchez[2]

[1] Instituto de Biotecnología de las Plantas, Universidad Central "Marta Abreu" de Las Villas Carretera a Camajuaní km 5.5, Santa Clara, CP 54830, Cuba.

[2] Centro de Bioplantas, Universidad de Ciego de Ávila Carretera a Morón km 9, Ciego de Ávila, Cuba

✍laisyn@ibp.co.cu

ABSTRACT

In papaya, the low rooting capacity of *in vitro* cultivated plants increases their mortality rates during *ex vitro* acclimatization, further limiting the exploitation of this plant regeneration system, especially for the elite cultivar 'Maradol Roja'. Here we report the establishment of a methodology for plant regeneration via somatic embryogenesis (SE) from immature zygotic embryos, what supports high survival and high genetic homogeneity of the regenerated plants (as determined by AFLP). It was demonstrated the expression of *AUX/LAX* and *PIN* genes coding for auxin polar transporters at different stages of SE, a novel aspect for this crop. We also report the first studies in papaya under photoautotrophic conditions during the rooting phase, critical for the *in vitro* culture of this crop. The use of growth regulators Pectimorf® and phloroglucinol, zeolite as support, and culture flasks with increased ventilation for rooting and *in vitro* acclimatization, derived in positive effects on *ex vitro* acclimatization. The incorporation of the RITA® temporary immersion system to the methodology guarantees the high efficiency of the SE system. These are novel and promising findings which make more efficient the *in vitro* regeneration system. The results are also unprecedented in papaya, particularly for the 'Maradol Roja' cultivar of high commercial value. This work granted the Annual Award of the National Academy of Sciences of Cuba for the year 2016.

Keywords: Somatic embryos, rooting, Floroglucinol, photoautotrophism, *in vitro* acclimatization, zeolite

Biotecnología Aplicada 2017;34:3501-3504

RESUMEN

Embriogénesis somática en papaya cultivar 'Maradol Roja': una alternativa para la propagación y el mejoramiento genético. La baja capacidad de enraizamiento de las plantas de papaya durante su cultivo *in vitro* incrementa la mortalidad durante su aclimatización *ex vitro*, y ha limitado la explotación de este sistema de regeneración con el cultivar élite 'Maradol Roja'. En este trabajo se estableció una metodología de regeneración vía embriogénesis somática (ES) a partir de embriones cigóticos inmaduros, con la cual se logra una elevada supervivencia y una alta homogeneidad genética de las plantas regeneradas, determinada esta última por AFLP. Se demostró la expresión de los genes *AUX/LAX* y *PIN* que codifican para transportadores polares de auxinas en las diferentes etapas de la ES, aspecto novedoso para este cultivo. Se informa además sobre los primeros estudios del cultivo en condiciones fotoautotróficas en la fase de enraizamiento, considerada crítica para el cultivo *in vitro* de papaya. La metodología propuesta incluyó el tratamiento con los reguladores del crecimiento Pectimorf® y floroglucinol, el uso de zeolita como soporte, frascos de cultivo con incremento de la ventilación para el enraizamiento y la aclimatización *in vitro*, lo cual aumentó la supervivencia durante la aclimatización *ex vitro*. La incorporación de los sistemas de inmersión temporal tipo RITA® incrementan la eficiencia del sistema de ES. Tales procederes son novedosos para este cultivo y garantizan sistemas de regeneración *in vitro* más eficientes. Estos resultados no tienen equivalentes en el mundo, y en particular para el cultivar 'Maradol Roja', de interés comercial. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2016.

Palabras clave: Embriones somáticos, enraizamiento, Floroglucinol, fotoautotrófismo, aclimatización *in vitro*, zeolita

*I*ntroduction

Papaya (*Carica papaya* L.) is a plant widely distributed among geographic tropical and tropical regions, and it is one of the main fruit crops for human consumption and a profitable source of exports in India, Brazil, Nigeria and Mexico. The high nutritional value of papaya is the basis for a variety of food and medicinal applications [1].

In Cuba, the main marketable papaya cultivar is 'Maradol Roja', which is also cultivated in several Latin American countries as in Mexico, Nicaragua and Venezuela. In Cuba, in 2015 this crop provided yields of 105 562 tons, with a cultivated surface of 5396 ha [2].

This species is essentially obtained by cross-polynization and propagated by seeds. However, previous treatment of seeds is required for its direct use, since they are not able to keep some of the characteristics of starting genetic material (for instance, genetic segregation properties). Such changes generate variations in the quality and size of fruits, influencing crop yields.

Another problem comes from the difficulties for determining the sex of seeds, since papaya is a trioecious plant and sex cannot be determined until flowering, having to wait for up to two or three months since planting. There are three types of plants according to the sex of the inflorescence: female, male and

1. Zhang J, Shen W, Yan P, Li X, Zhou P. Factors that influence the yield and viability of protoplasts from *Carica papaya* L. Afr J Biotechnol. 2011;10:5137-42.

2. Ministerio de la Agricultura. Estadísticas oficiales del Ministerio de la Agricultura. Cierre de 2015. La Habana: MINAG; 2016.

hermaphrodite, the last one providing the fruits of the highest marketable quality. In this sense, two or three seeds have to be planted per planting spot in order to generate a full hermaphrodite plantation, also having to eliminate the other two undesired phenotypes at flowering. Such practice normally raises production costs by increasing the number of planted seedlings and their cultivation until removal [3].

One strategy effective to circumvent these problems is the use of *in vitro* culture techniques, either to produce a high number of plants (high quality vegetal material to be planted) or to support genetic improvement programs and the commercial introduction of new or hybrid cultivars. In fact, the massive propagation by organogenesis or somatic embryogenesis (SE) is justified for this crop due to its superior quality and uniformity of the plants obtained, despite its associated costs in comparison to seed propagation techniques [4].

Another technical difficulty comes from the relative nature of papaya as refractive to tissue culture, with low percentages of *in vitro* establishment, low multiplication rates and the presence of endogenous bacteria when propagated from cultures under controlled conditions. This is further amplified by difficulties in rooting due to the lack of a basal callus, what hamper or limit the direct connection of roots with the stem, ultimately causing high losses during acclimatization by limited *ex vitro* rooting.

All this has led to the preferential use of SE for papaya propagation and its genetic improvement. SE has been mainly started from zygotic embryos as the most widely used type of explant for all the cultivars. Nevertheless, a high number of works report callus formation prior to the generation of the somatic embryos, even in the case of using tissues with embryogenic-predetermined cells (zygotic embryos), thereby increasing the risks of genetic variability in the plants obtained [5]. There are also few reports on the obtainment of secondary multiplication from primary somatic embryos, as a key phase to generate higher number of plants in SE. Besides, somatic embryo germination is affected by hyperhydricity and callus formation at the base of the explant, leading to incomplete germination [6], and having the *in vitro* shoots to be passed to a culture medium for rooting. Ultimately, this reduces root formation as in organogenesis, something that increases mortality rates of the explants during the *ex vitro* acclimatization phase, above 70 %. All this constitutes the main problem of tissue culture for this species [7].

Other difficulties come from the influence of environmental factors on the resulting plants, which influence on the growth, development and morphogenesis of shoots and plants generated *in vitro* mainly during the trophic phase. According to Kozai [8], *in vitro* photoautotrophism can be induced by excluding carbohydrates from the culture medium and by increasing gas exchange in the culture vessel. Photoautotrophic culture provides several advantages, including growth and photosynthesis stimulation, high percentage of survival during the *in vitro* to *ex vitro* transition, the correction of physiological and morphological disorders in the cultured plants, avoids callus formation at the base of the explant what increases rooting, and reduces the losses due to microbial contamination. All these could provide an alternative for papaya cultivation, in order to circumvent the plant losses during the *ex vitro* acclimatization phase.

So far, there was found a single report on the papaya tissue culture *in vitro* by using photoautotrophic conditions [9], but no details were referred about the most critical phase: rooting. Specifically for SE, there were no reports on the use of zeolite as substrate for shoots or *in vitro* plants, or by using the culture flask with increase aeration. Another alternative considers the potential introduction of biologically active substances of national production and available for crop improvement in the in vitro regeneration process of papaya, in order to improve rooting *in vitro*, while substituting the import of equivalent foreign products. One of such substances is the bioregulator Pectimorf®, a mix of pectic oligosaccharides of 9-16 polymerization degree. It is produced by the enzymatic hydrolysis of pectic acid extracted from Persian lime (*Citrus latifolia* Tanaka) [10]. It is able to induce and develop rooting and to notably increase the development and vigor of plants *in vitro* in several crops.

Another product to be considered is phloroglucinol (PG), a phenolic compound obtained by degrading floridcin and a precursor in the synthetic pathway of lignin. It has been demonstrated to display vegetal growth promotion activity, with positive effects in the lignification of *in vitro plants*, the elimination of callus formation at the base of explants obtained from somatic embryos, and in the rooting phase [11].

In addition, current morphologic, cytological, isoenzymatic and phenol contents analysis techniques are unable to determine plant sex in papaya seeds or seedlings [11]. Most systems do not distinguish hermaphrodite plants and are not suitable for screening a high number of samples. This has led to the implementation of strategies using molecular markers for sex determination in this species, particularly techniques based on PCR amplification of molecular markers to determine the sex at early stages of development under field conditions [12]. So far, neither had been implemented for in vitro cultured plants, nor tested in the 'Maradol Roja' cultivar.

Hence, the aim of this work was to develop a study for obtaining 100 % hermaphrodite papaya plants of the 'Maradol Roja' cultivar as the main papaya cultivar in Cuba, through a SE plant regeneration methodology of high survival under *ex vitro* conditions. This work was granted with the Annual Award of the National Academy of Sciences of Cuba for the year 2016.

Results and discussion

Methodology for the regeneration of 100 % hermaphrodite papaya plants of the Cuban cultivar 'Maradol Roja' obtained by SE

Here we described the development of a methodology for the regeneration by SE of papaya plants cultivar 'Maradol Roja' from immature zygotic embryos (Figure). Consistent with an strategy aimed to circumvent the high mortality rates *in vitro* during the *ex vitro* acclimatization phase due to difficulties for

3. Vegas A, Sandrea Y, González O, Díaz A, Albarrán J, Schmidt A, *et al*. Micropropagación de plantas de lechosa en recipientes de inmersión temporal a partir de brotes axilares. Rev Colombiana Biotecnol. 2015;XVII(1):70-8.

4. Homhuan S, Kijwijan B, Wangsomnuk P, Bodhipadma K, Leung DWM. Variation of plants derived from indirect somatic embryogenesis in cotyledon explants of papaya. Science Asia. 2008;34:347-52.

5. Bukhori M, Song Ch, Khalid N, Pillai V, Abd Rahman N. Improved protocol for high frequency plant regeneration through somatic embryogenesis in *Carica papaya*. Res Biotechnol. 2013;4(5):9-19.

6. Sekeli R, Abdullah JO, Namasivayam P, Muda P, Abu Bakar UK. Better rooting procedure to enhance survival of field growth Malaysian Eksotika papaya transformed with 1-aminocyclopropane-1-carboxylic acid oxidase gene. ISRN Biotechnology. 2013;1-10.

7. Dhekney SA, Kandel R, Bergey DR, Sitther V, Soorianathasundaram Litz RE Advances in papaya biotechnology. Biocatal Agric Biotechnol. 2016;5:133-42.

8. Kozai T. Photoautotrophic micropropagation-environmental control for promoting photosynthesis. Propag Ornamental Plants. 2010;10:88-204.

9. Teixeira da Silva JA. Photoauto-,Photohetero-and Photomixotrophic in vitro propagation of papaya (*Carica papaya* L.) and response of seed and seedlings to light-emitting diodes. Thammasat Int J Sci Technol. 2014;19(1):57-71.

10. Cabrera JC, Gómez RK, Diosdado E, Hormaza JV, Iglesias R, Gutiérrez AE, *et al*. Procedimiento de obtención de una mezcla de oligosacáridos pécticos estimuladora del enraizamiento vegetal. Oficina Cubana de la Propiedad Industrial. Patente de invención 22859, Instituto Nacional de Ciencias Agrícolas, Cuba. 2003.

11. Teixeira da Silva JA, J Dobránszki J, Ross S. Phloroglucinol in plant tissue culture. In Vitro Cell Dev Biol Plant. 2013;49:1-16.

12. Chaves G, Nuñez V. A SCAR marker for the sex determination in Colombian genotypes of *Carica papaya*. Euphytica. 2007;153:215-20.

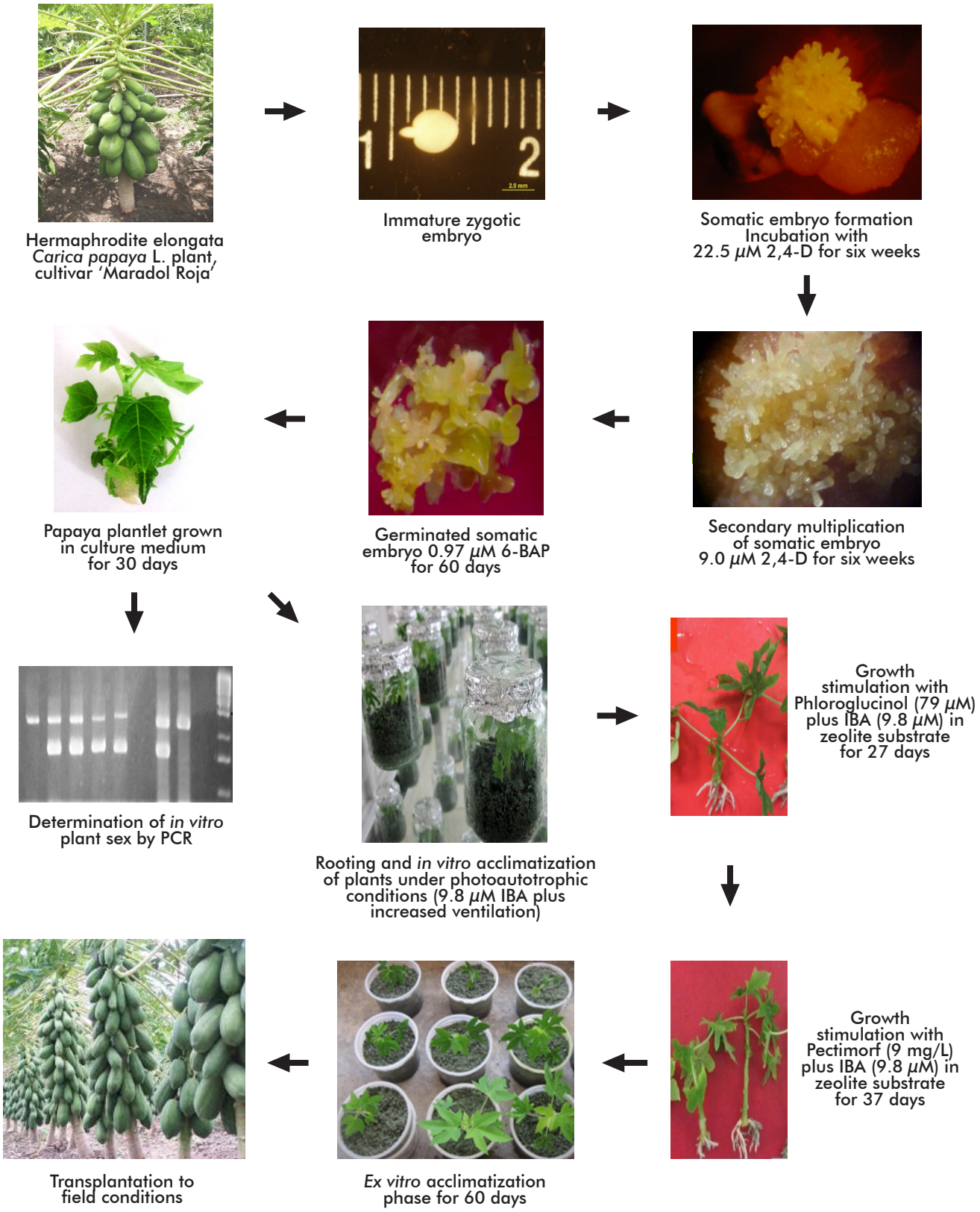

Figure. Methodology for the regeneration by somatic embryogenesis of papaya plants (*Carica papaya* L.) cultivar 'Maradol Roja' from immature zygotic embryos. 2,4-D: 2,4-Dichlorophenoxyacetic acid; 6-BAP: 6-Benzylaminopurine; IBA: indole butyric acid.

root development, a high *ex vitro* survival rate was obtained, and regenerated plants displayed a high genetic homology. Several work report callus formation for other papaya cultivars prior to the formation of somatic embryos, despite of starting from zygotic embryo tissues with embryogenic predetermined cells which ultimately lead to an increase in genetic variability risks. Conversely, few reports are available on the secondary multiplication of primary somatic embryos, a phase considered crucial for high multiplication rates in SE.

The methodology developed was supported by histology and electron microscopy studies, and provides a consistent tool to increase the efficiency of the propagation process. The incorporation of RITA® temporary immersion system for the germination of somatic embryos guarantee a high efficiency for the established SE system. It was demonstrated for the first time the expression of the genes *AUX/LAX* and *PIN* coding for the polar transporters of auxins in papaya somatic embryos. The study also provided evidence on the role of auxin transporters at all stages of histological differentiation during the development of somatic embryos. It was determined that there is a lower or higher expression of *PIN* genes and expression of *AUX/LAX*, except for LAX3 which was not expressed in somatic embryos at globular stage [13-16].

Photoautotrophic micropropagation for rooting and increased *ex vitro* survival rates

The photoautotrophic culture has several advantages, such as stimulating plant growth and photosynthesis, together with an increase in survival rates during the *in vitro-ex vitro* transition. Our results of *in vitro* cultivation of papaya showed that the methodology developed was able to preserve somatic embryos from death during the *ex vitro* acclimatization phase. This was possible due to the increased ventilation of culture vessels, by capping them with metallic foil paper with two holes. By these means, the relative humidity decreased inside the vessels to 68-72 %. Also, the flow of photosynthetic photons estabilized in the range of 48.0-62.5 $\mu mol/m^2 \cdot s^{-1}$ by placing the vessels in cultivation chambers under sunlight.

Simultaneously, the CO_2 concentration was stabilized to normal levels within the flasks to 350 µmol/mol. This was also complemented with the use of zeolite as substrate for plant in vitro cultivation, resulting in higher photosynthesis rates, the correction of morpho-physiological disorders, the prevention of basal callus formation, which combined with indole butyric acid treatment in the culture medium supported high percentages of rooting. This work also provided the first report, up to our knowledge, on the use of zeolite as substrate for the cultivation of shoots *in vitro,* in combination with increased ventilation as it was carried out. This procedure allowed us to improve the quality of the plants obtained with a better *ex vitro* acclimatization of in vitro obtained explants, and higher survival rates. These results also accounted for the pioneering studies on the photoautotrophic culture conditions in Cuba for this plant species [17].

Using plant growth regulators to improve rooting and explant *ex vitro* survival

The addition of Pectimorf® and PG promoted a positive effect in synergy with auxin IBA, when added to the culture medium under photoautotrophic conditions. Increased rooting to 84.2 % was achieved 37 days after the addition of Pectimorf® (9 mg/L) and IBA (9.8 µM), and 76 % of ex vitro survival. The effect attained with PG (79 µM) in combination with IBA (9.8 µM) was even higher, providing 100 % rooting in just 27 days and 96.5 % of survival under ex vitro acclimatization conditions. PG treatment also improved the morpho-physiological parameters of the plants obtained. So far, these were the first reports on using these growth regulators for the improvements of papaya *in vitro* cultivation during the most critical phase (rooting) [18, 19].

Scientific relevance of the study

A new methodology using SE for plant regeneration was obtained, for the cultivation of the Cuban cultivar 'Maradol Roja', the morphoagronomic characterization of somatic embryos at different ontogenic developmental phases and a high survival for the plants obtained under *ex vitro* conditions. Moreover, the application of the PCR technique to determine plant sex from leaves samples in vitro guaranteed the selection of a 100 % hermaphrodite population. Molecular studies demonstrated for the first time the expression behavior of auxin polar transport for *AUX/LAX* and *PIN* at the different developmental stages for somatic embryos. These were also the first studies in Cuba assessing the cultivation of the 'Maradol Roja' cultivar under photoautotrophic conditions, also including the use of plant growth regulators (Pectimorf® and PG) and zeolite as planting substrate during rooting and acclimatization in vitro and acclimatization *ex vitro*. Particularly, the inclusion of Pectimorf® treatment is an economically attractive alternative due to its national production.

The methodology established can be implemented in commercial laboratories (biofactories) in Cuba and abroad. It is expected that these results could have a positive impact on the propagation of this commercially relevant crop in Cuba in the short time, also setting guidelines for future research on the genetic regulation during SE in papaya species.

Acknowledgements

The authors thanks the contribution and colaboration during the conduction of the study to the following colleagues: Maritza Reyes Vega, Alexis Rodríguez Concepción, Ortelio Hurtado Ribalta, Raúl Barbón Rodríguez, Luis Rojas Martínez and Milady León Quintana, from the Instituto de Biotecnología de las Plantas Universidad Central "Marta Abreu" de Las Villas, Cuba; Rene Carlos Rodríguez Escriba, from Centro de Bioplantas, Universidad de Ciego de Ávila; Osvaldo Norman Montenegro, Centro de Bioactivos Químicos (CBQ), also at the UCLV; and Diosdada Gálvez Guerra from the Instituto Nacional de Investigaciones en Viandas Tropicales (INIVIT).

13. Posada-Pérez L, Gómez-Kosky R, Reyes VM Embriogénesis somática en *Carica papaya* L. var. 'Maradol Roja'. Biotecnol Veg. 2007;7(3):131-38.

14. Rodríguez AG, Posada-Pérez L, Gómez-Kosky R, Reyes VM, Tejeda MR. Aclimatización de plantas de papaya var. `Maradol roja´ obtenidas por embriogénesis somática. Biotecnol Veg. 2009;9(2):91-7.

15. Posada-Pérez L, Rodríguez AG, Gómez-Kosky R, Reyes VM, Tejeda MR. Influencia de la época del año y el tipo de frasco en la embriogénesis somática en papaya var. 'Maradol roja'. Biotecnol Veg. 2009;9(1):33-40.

16. Posada-Pérez L, Gómez-Kosky R, León MR, Rojas LS, Padrón YM Identificación mediante PCR del sexo de plantas de *Carica papaya* L. variedad `Maradol Roja' obtenidas vía embriogénesis somática. Biotecnol Veg. 2015;15(1):47-51.

17. Posada-Pérez L, Padrón YM, González JO, Barbón RR, Hurtado OR, Rodríguez RS, et al. Effects of different culture conditions (photoautotrophic, photomixotrophic) and the auxin indole-butyric acid on the in vitro acclimatization of papaya (*Carica papaya* L. var. Red Maradol) plants using zeolite as support. Afr J Biotechnol. 2015;14(35):2622-35.

18. Posada-Pérez L, Padrón YM, González JO, Barbón RR, Rodríguez RS, Norman OM, et al. Efecto del Pectimorf en el enraizamiento y la aclimatización *in vitro* de brotes de papaya (*Carica papaya* L.) cultivar Maradol Roja. Cult Trop. 2016;37(3):50-9.

19. Posada-Pérez L, Padrón YM, González JO, Barbón RR, Rodríguez RS, Norman OM, et al. Effect of phloroglucinol on rooting and in vitro acclimatization of papaya (*Carica papaya* L. var. Maradol Roja). In Vitro Cell Develop Biol Plant. 2016;52(2):196-203.