In Vitro Long-Term Cultures of Papaya (*Carica Papaya* L.).

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Abstract

We present the data on proliferation corresponding to 10 years of continuous incubation *in vitro* of papaya shoots, and propose a reliable method for long-term micropropagation for papaya, using two types of explants: Microshoots from somatic embryos, and from axillary buds of papaya. Three different media were assayed. The proliferation medium (PPRM) allowed to maintain papaya shoots under continuous proliferation during 20 years, maintaining a consistent behaviour. Most of the shoots developed in PPRM rooted during the incubation, and after acclimated easily, maintaining the morphological characteristics of the parental plants, flowering and setting fruits normally. The PPRM medium consist in MS medium supplemented with NAA (0.1 mg l⁻¹), BA (0.5 mg l⁻¹), GA₃ (0.5 mg l⁻¹) and Adenine sulphate (40 mg l⁻¹). The average multiplication rate was higher than 20 shoots per explant along the long-term assay. The elongation medium (PELM), was designed to recover shoots with a poor growth, and allowed the development of high quality shoots ready for rooting, and consist in a MS basal medium supplemented with NAA (0.1 mg l⁻¹), Kin (0.5 mg l⁻¹) and GA₃ (1 mg l⁻¹). The rooting medium (PROM) was designed to induce high quality roots from non-rooted shoots and consist in a half strength MS medium plus IBA (1mg l⁻¹). On PROM, agar can be exchanged for expanded vermiculite. Acclimation took place inside an acclimatization tunnel under progressive hydric stress. After 4 weeks, the plant recovery rate was 90% for plants maintained under continuous proliferation during ten years.

Introduction

Papaya (*Carica papaya* L.) is an economically important fruit crop species belonging to family *Caricaceae* cultured worldwide in tropical and subtropical areas due to the nutritive and medicinal value of his fruits. World production is estimated in 1329320 tonnes, India, Brazil, Mexico, Dominican Republic, Indonesia and Nigeria being the major producers (FAOSTAT 2018).

Papaya plants can be propagated vegetatively by classic methods such as cutting (Reuveni and Shlesinger 1990) and grafting (Chandler 1958; cited by Allan et al 2010), but these methods are inappropriate for long scale production and normally papayas are propagated by seeds, but again this method is problematic due to the high heterozygosis of the seeds and the dioecious character of the papaya, resulting in strong differences in yield, fruit quality and pest and disease tolerance (Saker et al. 1999).

Micropropagation by tissue culture offer advantages over conventional propagation methods and it has been widely applied to papaya species. Different approaches have been follow such as axillary shoot culture (Drew 1988, 2003; De Winaar 1988; Yu et al. 2000; Panjaitan et al. 2007), and plantlet recovery after shoot regeneration from embryogenic callus or somatic embryos (Litz and Conover 1981; Jordan et al. 1983; Fitch 1993; Magdalita et al. 1997; Bhattacharya et al. 2003; Fitch 2005). Many times this regeneration and plant recovery occurs after procedures of plant genetic transformation in this species (Fitch et al. 1993, 1994; Cai et al. 1999; Magdalita et al. 2003, 2013; Kung et al. 2009, 2010; Fitch 2010; Jia et al. 2017). This fact increasing the importance of the development of reliable methods able to

preserve during long periods of time, and finally recover high quality plants from selected lines of genetically modified papaya.

In vitro reliable methods for papaya micropropagation adequate to be used during several years were also required for germplasm preservation *in vitro* of elite genotypes/mother plants, able to maintain elite lines of pathogen-free plants obtained through meristem cultures, after breeding processes or even to maintain an economically viable collection of mother plants, protected from environmental stresses or suitable for plant transport across frontiers.

All the methods developed until date have been successful in different degree, depending on papaya cultivars and physiological conditions of the plants, but it is usual that these protocols do not work properly when clonal lines must support continuous culture for long periods of time, as normally happen with transgenic or elite cell lines. After some months of incubation, it appears a progressive degeneration of the papaya *vitro*-shoots, resulting in limited or null growth, dwarfing, defoliation, abnormal callus proliferation, loose of rooting capabilities, apical necrosis, degeneration and finally the shoots died.

Studies (Larkin and Scowcroft, 1981; Renau-Morata et al., 2005; Jin et al., 2008) confirm that somaclonal variation always occurs in diverse degree with the increase of the number of subcultures *in vitro*, always depending on the plant genotype and the culture approach and conditions, these changes can preclude further stages of the *in vitro* plant development or even in the final product at the end of the *in vitro* culture, generating plants with undesirable or useful ploidy changes, somaclonal variations, epigenetic variations and morphological or functional changes at different levels (flowers, fruits, size, shape, color). Micropropagation through development of axillary buds is considered the *in vitro* plant regeneration system with the lower level of genetic instability and somaclonal variation (Clarindo et al. 2008).

There are few studies about *in vitro* long-term assays in plant species: Chaudhuri and Jha (2008) work during 12 years with Ipecac (*Cephaelis ipecacuanha*) to maintain mother plants under conditions of reduced growth, succeeding in the regeneration of viable and stable plants from this medicinal species. Other species such cherimoya (*Annona cherimola*) have been also maintained *in vitro* during 250 subcultures (more than 20 years) in our laboratory, maintaining the shoot multiplication rate but losing most of the rooting capabilities (Encina, unpublished results). To date, we do not found even in the last reviews (Al-Shara et al. 2018; Costa et al. 2019) any references involving long-term cultures focused to proliferation and preservation of papaya *in vitro* during long periods of time.

In this article we propose a reliable method which allowed the maintenance on active proliferation and full development for long time periods of *in vitro* cultures of papaya, overcoming the above indicated problems, with the final goal of recovery high quality plants of papaya.

Material And Methods

Plant material and disinfection.

Micropropagation of papaya (*Carica papaya* L.) was initiated from 2 years old mature papaya plants obtained from seeds of the type 'Solo', growing at glasshouse at IHSM "La Mayora" (CSIC-UMA), located in Malaga, Spain. Two types of explants were used: a) Regenerated shoots obtained from somatic embryos of papaya obtained following the procedures developed by Fitch et al. (1994), and b) *In vitro* micropropagated shoots obtained from axillary nodal sections.

This long-term micropropagation study starts at 1998, and the papaya cultures were maintained under continuous proliferation during 20 years, data were scored for 13 years (1998–2010) and a plant stock was maintained in proliferation until 2017 in PPRM medium.

The axillary nodal sections used as explants, were obtained from actively growing young shoots, induced by local application of a solution of BA (100 mg l^{-1}), as sticky gelled drops, over the axillary bud areas of the main shoot, after the elimination of the main apical meristem of the mother plant. Nodal explants were surface sterilized for 20 min by immersing in 0.5% sodium hypochlorite solution containing a few drops of Tween-20, rinsed three times with sterile distilled water (5 minutes each), and cultured under aseptic conditions.

Culture media and culture conditions.

All media consisted of MS salts formulation (Murashige and Skoog, 1962) supplemented with (mg l^{-1}): thiamine-HCl (100), pyridoxine-HCl (50), nicotinic acid (50), glycine (200), myo-inositol (100), sucrose (30000) and agar (8000) (Sigma, A-1296). The rooting medium also consist on MS salts but at half strength. The pH of all the culture media was adjusted to 5.7 before autoclaving. For all experiments, 25 ml aliquots of medium were distributed into 150x 25 mm test tubes, covered with polypropylene tops and autoclaved for 20 min at 121°C and 1.05 Kg cm^{-2} . One explant was cultured per tube. Cultures were incubated at $25 \pm 1^\circ\text{C}$ under a 16-h photoperiod with a light intensity of $45 \mu\text{mol m}^{-2} \text{ s}^{-1}$ (400–700 nm) PAR (Photosynthetic Active Radiation) provided by cool white fluorescent tubes (F40 tubes Gro-lux, Sylvania).

Culture initiation.

Both types of shoot explants, obtained from somatic embryos and from axillary nodal sections, were cultured during two years on five media based on MS mineral formulation differing on the supplements added (C1, C2, PPRM, PELM, PROM). 100 explants of each type were cultured in each medium. These media were designed for active growth of the papaya vitro-shoots, searching for different balances between axillary proliferation, shoot elongation and rooting. Shoot proliferation and elongation were regarded as quality parameters being our main objective the production of high quality plantlets and/or shoots adequate for rooting, acclimatization and recovery of a high number of normal papaya plantlets.

Media composition

Control 1: MS + NAA (0.2 mg l^{-1}) + (BA (0.2 mg l^{-1})). (Yu et al 2000; Fitch et al 2005)

Control 2: MS + NAA (0.1 mg l^{-1}) + Kinetin (0.5 mg l^{-1}). (Mondal 1990)

PPRM: MS + NAA (0.1 mg l^{-1}) + BA (0.5 mg l^{-1}) + GA₃ (0.5 mg l^{-1}) + ADE (40 mg l^{-1})

PELM: MS + NAA (0.1 mg l^{-1}) + Kinetin (0.5 mg l^{-1}) + GA₃ (1 mg l^{-1})

PROM: 0.5xMS + NAA (0.2 mg l^{-1}) + IBA (1 mg l^{-1}) + SUC (40 mg l^{-1}) + ADE (10 mg l^{-1})

Shoot micropropagation.

100 apical shoots (1.5-2 cm long) obtained from axillary shoots and 100 apical shoots regenerated from somatic embryos were subcultured repeatedly, every two months during 10 years, on the same media used in culture initiation: C1, C2, PPRM, PELM, PROM. Data, including main shoot length, number of leaves, number and length of axillary shoots, number and length of roots and rooting percentage, were collected from shoot cultures in each subculture, 6 times per year. The results obtained by both types of shoot explants and each media were compared.

After evaluation of the results obtained during the two first years, and never detecting significant differences for growth parameters between both types of explants we decided to select as source of plant material the shoots obtained from nodal sections in order to extend the assays for a longer period of time. Also, shoots explants regenerated from somatic embryos were maintained in culture for three more years in PPRM medium, to obtain data about their development *in vitro* (data not showed). Data obtained during the last 8 years correspond exclusively to shoot explants developed from nodal section explants.

Shoot proliferation.

The preliminary assays allowed us to design a proliferation medium PPRM able to generate an active growth of multiple shoots or rooted shoots, in a pattern of rosette.

Shoot elongation.

After preliminary assays using as explants small shoots of transgenic papaya (already in culture for 3–4 years) sometimes these shoots were unable to develop correctly, showing dwarf shoots without roots growing in clusters or individual non-rooted shoots with null growth, we design a medium in order to induce shoot elongation and produce shoots or plantlets with enough quality to be rooted or acclimated. This medium includes gibberellic acid and a softer cytokinin such as KIN instead BA to modulate the sprouting of axillary shoots.

Shoot rooting.

Shoots for *in vitro* rooting experiments were obtained from actively growing shoots from clusters or elongated good quality shoots. Apical shoots (2 cm long) with a minimum of 3 fully expanded leaves

were used for rooting. We studied the effects on rooting when MS basal medium was supplemented with 1mg l^{-1} IBA, a dose obtained after some preliminary tests.

The last rooting assays for root development include the substitution of agar by inert substrates such as expanded vermiculite, able to act as physical support for the shoots under rooting. The percentage of rooted shoots and the number and length of roots were recorded every 8 weeks.

Foliar mineral composition analysis.

Values for mineral composition of chlorotic and non-chlorotic papaya leaves, were determined following the protocols of MAPA (1986). Phosphorus total through spectrophotometry with Vanadate-Molybdate reactive. Boron through spectrophotometry using Azomethine H. Nitrogen total through digestion and distillation. Calcium, Magnesium, Potassium, Iron, Copper, Zinc and Manganese through atomic absorption spectrophotometry. The leaves, chlorotic and non-chlorotic, were collected from shoots growing in PPRM medium. The values obtained for non-chlorotic and chlorotic leaves were compared with adequate values for mineral composition of papaya leaves *in vivo*.

Root system morphology analysis.

The root system morphology of plantlets *in vitro* rooted in each media (PPRM, PELM, PROM) was analysed using a developmental model (Rose, 1983). Roots produced directly from the base of the plants were referred to as primary or first-order roots, those arising from the first-order as second-order roots, and so on. Roots were removed from the substrate by soaking them in water, after which the roots were gently washed and excess water was removed with paper towels. Detailed measurements of root length were made for five plantlets rooted in each media. Roots were separated into branching orders and the length of the roots in each order measured using video images (Digital Image Analysis system IBAS-2000 (IPS)). The following morphometric parameters were determined: number of adventitious roots, number of roots of each order, and length of the roots present in each order, and compared between media. The plantlets used in this analysis belonged to subculture number 62, beyond the 60 subcultures which micropropagation data are shown in this work due to the destructive nature of the method applied to analyse the root structure.

Plantlets acclimation.

Micropropagated plantlets were thoroughly washed in tap water and transplanted into polyethylene seedbed trays with 51 cells (4 cm wide x 4 cm deep) containing a mixture of autoclaved peat: vermiculite (1:1). Potted plantlets were maintained inside a polyethylene tunnel with 90% initial relative humidity. The average temperature was 25°C. Afterwards, the plastic cover was temporary and gradually removed every day, in an open-close cycle increasing daily the time with the plastic cover open, reducing drastically the RH for a limited period of time and recovering the initial RH afterwards by closing the tunnel. In this acclimatization system plantlets were subject to controlled shocks of hydric stress to activate growth and development of roots and shoots. At the end of the experiment the %RH was about 70%. During the

experiment, plants were periodically watered and fertilized with ®Ficote (Scott O.M., Spain) containing 15%N, 6%P, 12%K, 2%Mg, 0.22%Fe, 0.08%Mn, 0.03Zn, 0.01Cu. During this period the PAR (400–700 nm) at the top of the plants was in average 200–300 $\mu\text{molm}^{-2}\text{s}^{-1}$ and the photoperiod of 12/12. A total of 30 plantlets were acclimatized, ten obtained from each media used during ten years (PPRM, PELM, PROM). Survival rate, shoot length, number of leaves were recorded after 2 months of acclimation and compared between the plantlets obtained from each media.

Somaclonal variation analysis

Due to the early loss of the original mother plant, and that at 1998 it was a lack of availability of reliable and sensitive molecular markers (EST-SSR) to detect somaclonal variations on papaya plants after multiple subcultures in vitro, we cannot offer any studies of verification of the “trueness-to-type” of the papaya progenies after 120 subcultures, we just evaluate the morphology and flowering of the acclimated plants, and the Nuclear DNA ploidy level at the end of assay.

Ploidy analysis by flow cytometry

The ploidy level of twenty micropropagated papayas was determined by estimating the relative DNA content using flow cytometry (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany). For analysis, 0.5 cm^2 of young leaves or tips of papaya shoots were chopped with a razor blade for 30–60 s to release nuclei (Galbraight et al., 1983) in a Petri dish containing 0.4 mL of nuclei isolation buffer (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50- μm nylon mesh (Partec 50- μm CellTrics disposable filter), and subsequently nuclei were stained with fluorescent dye (commercial Partec CyStain UV precise P, high resolution DNA staining kit 05-5002, staining buffer, about 1.6 mL). Finally, the samples were analysed after 30 s of incubation. *Carica papaya* type “Solo” ($2n = 2x = 18$) was used as an external standard. The nuclear DNA ploidy level of the samples was determined by using channel values corresponding to the average G_0/G_1 peaks of sample and standard plants. The peak relative to the standard nuclei was set to channel 50. Three independent repetitions were performed, with over 4,000 nuclei being analyzed in each.

Statistical analysis.

All data were analyzed using SPSS software package (version 19.0; SPSS INC., Chicago, IL, USA). Normally distributed variables were analyzed by one-way ANOVA, using a HSD-Tukey test in the post-hoc analysis for comparisons between different culture medium. Plant survival percentages were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution.

Results And Discussion

In our preliminary tests the best results on papaya shoot proliferation were obtained either when low levels of BA (0.5 mg l^{-1}) plus low levels of NAA (0.1 mg l^{-1}) or when high concentrations of Kinetin (1 mg l^{-1}) plus relatively high concentrations of NAA (2 mg l^{-1}), were present in the medium, but this last combination, used by Litz and Conover (1977) induce after few subcultures an excessive callus

proliferation and poor quality shoots. So, we opted by use 0.5 mg l^{-1} of BA plus with 0.1 mg l^{-1} of NAA in our proliferation media (PPRM). In our culture conditions papaya nodal explants were established *in vitro* without problems with a low level of contamination by microorganisms and bud sprouting of nodal explants was over the 85%.

Micropropagation.

The results obtained during the two first years of incubation showed minimal differences between both types of explants (Table 1), but also was detected a clear and progressive loss of quality in both media used as control, C1 and C2 (Fig. 1), treatments were eliminated after 2 and 3 years of incubation respectively, because the shoots and plantlets showed progressively clear signs of degeneration (defoliation, lack of growth, necrosis, death) and finally we were obliged to discard both controls due the minimal number of survival explants in culture. The other media PPRM, PELM and PROM do not show the problems of growth and development occurring in the control media (Table 1, Fig. 1).

Table 1

Comparison between micropropagation data obtained for explants issued from axillary shoots of mature papayas and shoots obtained from regenerated somatic embryos. Average data corresponding to the first 2 years (12 subcultures). Data recorded every 8 incubation weeks.

Medium	Initial explant	No. of axillary shoots	Length of axillary shoots (cm)	No. of leaves	No. of roots	Length of roots (cm)	Rooting (%)
Control 1	Nodal segment	1.4 ± 0.4 c	0.7 ± 0.1 c	0.6 ± 0.1 d	0.6 ± 0.1 c	1.2 ± 0.2 c	23.0 ± 14.5 c
	Somatic embryo	1.4 ± 0.4 c	0.7 ± 0.2 c	0.6 ± 0.1 d	0.6 ± 0.1 c	1.0 ± 0.1 c	23.6 ± 14.8 c
Control 2	Nodal segment	0.7 ± 0.3 c	0.8 ± 0.1 c	0.3 ± 0.1 d	0.9 ± 0.4 b	0.6 ± 0.2 d	15.9 ± 6.1 c
	Somatic embryo	0.7 ± 0.4 c	0.8 ± 0.2 c	0.4 ± 0.1 d	1.0 ± 0.3 b	0.6 ± 0.3 d	16.2 ± 7.2 c
PPRM	Nodal segment	24.4 ± 2.9 a	3.2 ± 0.2 b	4.1 ± 0.2 b	1.2 ± 0.3 b	3.1 ± 0.4 a	69.6 ± 7.2 a
	Somatic embryo	24.5 ± 3.2 a	3.2 ± 0.3 b	4.2 ± 0.2 b	1.2 ± 0.2 b	2.9 ± 0.5 ab	70.3 ± 7.1 a
PELM	Nodal segment	12.3 ± 2.5 b	4.1 ± 0.3 a	5.9 ± 0.3 a	0.5 ± 0.2 c	2.6 ± 0.2 b	38.3 ± 5.0 b
	Somatic embryo	12.9 ± 2.6 b	4.2 ± 0.3 a	6.1 ± 0.3 a	0.5 ± 0.1 c	2.7 ± 0.2 ab	38.6 ± 4.7 b
PROM	Nodal segment	2.0 ± 0.3 c	0.7 ± 0.1 c	3.0 ± 0.4 c	1.9 ± 0.3 a	2.7 ± 0.3 b	74.8 ± 3.9 a
	Somatic embryo	2.1 ± 0.3 c	0.7 ± 0.2 c	3.1 ± 0.4 c	2.0 ± 0.2 a	2.6 ± 0.3 b	75.3 ± 4.2 a
Different letters indicate significantly differences by HSD-Tukey at $\alpha = 0.05$ in each parameter between different culture media and initial explant.							

It is clear that the proliferation media (PPRM) promotes a proliferation rate, with the axillary shoot growing in a cluster pattern, significantly higher (24 shoots/explant) than other media assayed, such as Shlesinger et al. (1987) use similar growth regulators than us for multiplication and rooting but the lack of data makes impossible to compare both methods. Later, the same authors Reuveni et al., 1990 working with MS 0.5 mg l⁻¹ BA + 1 mg l⁻¹ NAA + 160 mg l⁻¹ AdS; Rajeevan and Pandey 1983, MS + 2 µM BA; McCubbin and van Staden (2003) using DS salts and vitamins + 1mg l⁻¹ NAA + 1mg l⁻¹ BA + 3 g l⁻¹ activated charcoal; Shivayogi et al. (2019) using MS + 2.0 mg l⁻¹ BAP + 1.0 mg l⁻¹ NAA obtained a rate of shoot multiplication of 12 shoots/explant, 15.8 shoots/explant, 3.5 shoots/explant, 19.9 shoots/explant, respectively. PPRM results in average >24 shoots per explant are quite close the obtained by Anandan et al. (2011) and Mumo et al. (2013), that using MS supplemented with 1mg l⁻¹ BA plus 0.01 mg l⁻¹ NAA or

MS supplemented with 0.5 mg l^{-1} BA plus 0.1 mg l^{-1} NAA reached a multiplication rate of 22 and 25 shoots per explant respectively. Other authors, such as Roy et al. (2012) also obtain high rates of multiplication ranging between 22.4, 25.2 and 33.8 shoots per explant using MS supplemented with BA 1.5 , 1.0 , and 0.5 mg l^{-1} plus 0.1 mg l^{-1} ; and using MS supplemented with 1 mg l^{-1} Zeatin plus 0.2 mg l^{-1} NAA reach a multiplication rate of 34.2 shoots per explant, and shoot number reach the highest values (50) when organic addenda such as Casein Hydrolysate was added to medium, these micropropagation method also requires additional steps for shoot elongation and rooting to obtain full plantlets of papaya. Veena et al (2015) using MS medium + 2.0 mg l^{-1} + 0.1 mg l^{-1} NAA reached the highest number of shoots per explant (70).

The elongation medium PELM offer a good rate of axillary shoots development also ranking high in growth of leaves, which give to the cultures an aspect of very good quality. The rooting medium PROM give the best results on induction and development of roots (Table 1, Fig. 1).

Long-term micropropagation assays

Along the ten years of culture the data obtained for all proliferation parameters showed an acceptable homogeneity during the incubation in PPRM, PELM and PROM media, with the exception of the 4th and 8th years in which all the cultures and parameters especially the number of axillary shoots promoted in PPRM and PELM decreased notoriously, for unknown reasons. Figure 2 shows the results (No. of axillary shoots, Length of axillary shoots and No. of leaves) obtained along the long-term process of micropropagation.

We were able to confirm the ploidy stability of ours in vitro papayas progenies after 20 years incubating in PPRM medium. Through Flow Cytometry, we detect that the diploid level ($2n = 2x = 18$) of the mother plant remains diploid all along the long-term culture. The analyzed in vitro plantlets obtained at the end of micropropagation process are also diploid ($2n = 2x = 18$). (Fig. 3).

Proliferation

As we can see in Fig. 1 the best results in proliferation of axillary shoot correspond clearly to PPRM showing significant differences respect the PELM and PROM media, the average number of axillary shoot ranging from 21.2 in PPRM to 11.7 in PELM and 1.9 in PROM. The most notorious effect is the high number of axillary shoots obtained in PPRM and the almost lack of shoot proliferation in PROM. Respect the average length of shoots, best values corresponding to PELM (3.9 cm) and to PROM (3.1cm) and the worse values to PROM (0.7 cm). The number of leaves offer average values higher for PELM (5.9) and PPRM (3.8) and lower for PROM (3.0). The PROM medium show the highest average values for rooting rate (70.3%) respect the PPRM (63.1%) and PELM (35.7%). Respect the root length, small differences can be detected between treatments, best values in average corresponding to PPRM medium (2.9 cm) and identical values for PELM and PROM media (2.6 cm).

In general, PROM medium is better for root induction and development, and PPRM and PELM media are better for shoot development (Fig. 4). The advantage of PPRM is that in just one step can regenerate plantlets of good quality, reducing costs and shortening the time *in vitro*. These results make this medium PPRM an excellent option for maintenance of active collections of elite cell lines of papaya (transgenic new genotypes, selected breeding lines, mother plants, classic or endangered genotypes).

Additionally, we maintain shoot explants regenerated from somatic embryos in incubation for three more years in PPRM medium, and the final data scored of five-year-old cultures still showed similar developmental values (data not shown) to the values recorded for shoots developed from nodal sections after 5 years of incubation.

Foliar mineral composition analysis.

Along the long-term assays, some chlorosis problems with an unknown origin, were detected in the leaves of papaya when were incubated in PPRM medium (Fig. 5). Some authors as Hidaka et al. (2009) also detected chlorosis in leaves when papaya explants were incubated in media with high doses of BA and NAA (4 mg l^{-1}), origin that we must discard in our studies, due the low levels ($\leq 0.5 \text{ mg l}^{-1}$) of these growth regulators supplementing the media PPRM. Trying to explain this chlorosis, we carried out foliar analysis over non-chlorotic and chlorotic leaves collected from shoots growing in PPRM medium and compare the adequate values for mineral composition of papaya leaves *in vivo* with the *in vitro* chlorotic and non-chlorotic leaves (Table 2).

Table 2

Data obtained in foliar mineral composition analysis of chlorotic and non-chlorotic papaya leaves obtained from *in vitro* plantlets after 4 weeks growing in PPRM. As control, adequate values for mineral composition of *in vivo* papaya leaves (Tamimi et al. 1997) are showed.

Type of papaya leaves			
	Chlorotic <i>in vitro</i> leaves	Non-chlorotic <i>in vitro</i> leaves	<i>In vivo</i> leaves
Mineral			
N (%)	5.2	5.5	1.0-2.5
P (%)	0.3	0.3	0.2–0.4
K (%)	2.6	3.2	3.0–5.0
Ca (%)	0.4	0.7	1.0–3.0
Mg (%)	0.2	0.3	0.4–1.2
Na (%)	< 0.1	< 0.1	< 0.2
Fe (ppm)	38.0	113.0	25–100
Mn (ppm)	87.0	71.0	20–150
Zn (ppm)	31.0	176.0	15–40
Cu (ppm)	1.0	3.0	4–10
B (ppm)	46.0	34.0	20–50

The analysis showed as Manganese, Phosphorus, Boron and Sodium appears ranging the adequate values for papaya leaves, low levels of Calcium and Magnesium, and almost normal levels of Potassium for non-chlorotic and chlorotic papaya leaves, respect the values accepted as adequate for papaya leaves for Tamimi et al (1997). These low levels in Calcium and Magnesium are quite frequent in plants growing *in vitro*, and sometimes induced problems in micropropagation causing apical necrosis, as in avocado (Pliego-Alfaro et al. 1987). Copper also appears at low concentration in non-chlorotic papaya leaves and even at lower concentrations in chlorotic leaves. The high levels of Nitrogen are also frequent *in vitro*, probably due to the enormous amount of N available in the culture medium.

The iron is present in leaves with high values at the beginning of the period of incubation and at the end of the incubation period decreased dramatically just when the leaves start to fade showing discoloration and chlorosis. We do not know the reason for this decrease of iron, but perhaps could be due to the exhaustion of the iron reserves in the culture medium, due to the huge uptake of iron for these fast growing clusters of papaya plantlets, or/and by the light degradation of the iron chelate making difficult the uptake by the plantlets, or could be due to some kind of interaction or inhibition caused by the very

high level of Zinc found in non-chlorotic *in vitro* leaves. Castillo et al. (1997), indicate that a mix of iron chelates (EDDHA plus EDTA) improved shoot proliferation in papaya, and after some assays changing the type of iron chelate we cannot confirm this increase in shoot proliferation, perhaps because we never supplement the culture medium with both types of iron chelates together (EDTA and EDDHA), we just detected that changing the standard iron chelate EDTA by EDDHA in the mineral formulation MS, that EDDHA-Ferric form delayed for some time the discoloration and chlorosis of papaya leaves respect the EDTA-Ferric form normally used in MS formulation. Another possibility for discoloration and chlorosis could be a deficiency of magnesium as can be inferred by the low level detected in non-chlorotic and chlorotic leaves.

Elongation.

The PELM medium was designed to recover and develop most of the axillary shoots until a size adequate for rooting. Small shoots obtained from proliferating clusters or regenerated from callus, normally too short to be useful for rooting, can be developed using this medium until reach the suitable size to be considered a high quality shoot adequate for rooting. In PELM a percentage of shoots develop roots (35.7%) producing high quality plantlets suitable for transplanting and acclimation. Roy et al. (2012) also develop an elongation method to improve the length of shoots before the rooting step supplementing with urea and activated charcoal the MS medium including Zeatin and NAA, but no data were showed in the article about the increase of shoot length or rooting rate obtained with this treatment. Other authors such Wu et al, (2012) use GA₃ to obtain shoot elongation but using a medium MS containing BA at low dose 0.25 mg l⁻¹.

Rooting.

The percentage of rooting in PPRM fluctuates along time between 84% and 38%, the lowest values corresponding to the years in which the general growth and development was poor (years 4th and 8th), in average a 63.1% of shoots develop roots (Fig. 6). In the case of PELM the rooting ranged between 60% and 15%, and in average a 35.7% of shoots rooted. In PROM medium, the rooting rate ranged between 84% and 52%, showing in average a 70.3% of success. In general, the root induction and development is better in PROM medium but the values corresponding to PPRM are also good enough to compete with the obtained with PROM.

For most authors, IBA was the auxin choice for rooting papaya shoots. Rajeevan and Pandey (1983), recorded a 90% rooting using MS + IBA 10μM, Shlesinger et al. (1987) Reuveni et al., (1990) using MS + 1 mg l⁻¹ IBA obtained a rooting rate closed to 100% De Winnaar (1988) and Roy et al. (2012) applied high concentrations of IBA (4 or 5 mg l⁻¹) reaching until a 90% rooting. McCubbin and van Staden, (2003) combining a 1hour pulse in a 5 mg l⁻¹ IBA solution with a second phase in DS salts and vitamins + 5.3g l⁻¹ PEG-6000 + 3g l⁻¹ Activated Charcoal in a vermiculite substrate with rooting rates of 80%. Mumo et al. (2013), applying 2.5 mg l⁻¹ of IBA obtain until an 83% of rooting success. Hidaka et al. (2008) and Van-Hong et al. (2018) applying 2 mg l⁻¹ IBA obtain rooting rates of 85% and 100% respectively. In PROM

medium we use lower doses of IBA (1 mg l^{-1}) and NAA (0.2 mg l^{-1}) at together, in an agar substrate and for our papaya line, the average rooting rate is a 70.3%, with picks of 84%, ranging at similar level to the achieved by the above mentioned authors. Perez et al (2016) using $\frac{1}{2}$ MS + $79 \mu\text{M}$ Phloroglucinol + $9.8 \mu\text{M}$ IBA + zeolite as substrate reached a 100% rooting with roots of good quality (1.76 root/plant, 2.4 cm in length). Caple and Cheah (2016) working with MS + 3 mg l^{-1} + 4% sucrose obtained a low rate of multiplication (3–4 shoots/explant). Shivayogi et al. (2019) using MS + 3 mg l^{-1} NAA reached an 80% rooting. But even in PPRM medium (supplemented exclusively with NAA) we can obtain in average a 63.1% rooting with picks reaching until an 84% rooting. Authors such as Fhaizal et al. (2006), Anandan et al. (2011) and Wu et al. (2012) also applied for rooting a low level of auxin (0.5 mg l^{-1} IBA) with results ranging from 75%, 60–90% respectively, also close to our rooting rates.

When the shoots were rooted in a simplified rooting medium consisting in MS medium liquid supplemented with 1 mg l^{-1} IBA added over a base of expanded vermiculite, the rooting induction reached the 100% in most of experiments carried out, in complete agreement with the results obtained for Yu et al. (2000) and Panjaitan et al. (2008) rating a 94.5% and 90% rooting, in a quasi-identical set of rooting assays, also using 1 mg l^{-1} IBA and vermiculite substrate but only with a shorter incubation period. Otherwise, Suksa-Ard et al. (1998) and Caple and Cheah (2016) only reached a limited rooting rate (56% and 54%, of good quality roots) respectively, using vermiculite as substrate.

These results suggest that an inert substrate such as expanded vermiculite is a very good option for growth and development of roots in papaya vitro shoots, but the rooting in PPRM in just one step, also appear as a very good method to obtain plantlets able to be acclimated with success, due to the shortening of the incubation time and the lower costs. The vermiculite protocol appears more interesting for rooting recalcitrant shoots or for shoots lacking roots after incubation in PPRM, both rooting systems are clearly complementary and can be used together to obtain the maximum percentage of plantlets ready for acclimation (Fig. 7).

Root system structure analysis.

As we can see in Table 3 the higher root number for first and second order roots correspond to proliferation medium (PPRM) with significant differences respect PELM, no differences between PPRM and PROM were detected on number of first and third order roots. Respect the root length the best results correspond to PROM, with a global growth for all orders of roots scoring clearly higher, compared with the results obtained for the rest of media assayed (PPRM and PELM).

Table 3

Results of analysis through Digital Image Analysis System on papaya root morphology and development *in vitro*. Data taken after 62 subcultures.

Culture medium	Root Number			Root Length (cm)		
	First order	Second order	Third order	First order	Second order	Third order
PPRM	4.0 ± 0.7 a	5.6 ± 0.5 a	2.4 ± 0.5 a	4.0 ± 0.7 b	3.0 ± 0.5 a	0.8 ± 0.4 ab
PELM	2.0 ± 0.0 b	1.4 ± 0.5 c	0.6 ± 0.5 b	3.0 ± 0.7 b	1.6 ± 0.5 b	0.4 ± 0.5 b
PROM	4.0 ± 1.0 a	4.0 ± 0.0 b	1.8 ± 0.8 a	6.6 ± 0.5 a	3.6 ± 0.5 a	1.6 ± 0.5 a
Different letters indicate significantly differences by HSD-Tukey at $\alpha = 0.05$ in each parameter between different culture media.						

Rooting in PPRM induce a mix of stumpy and normal roots, in complete agreement with the previous results obtained for Yu et al. (2000) rooting papaya shoots in MS culture media gelled with agar. The abnormal roots apparently appear as result of the negative effect of the gelling agent over the root development. Rooting on PROM even in a gelled support result in better quality roots. The results showed small differences respect the root number between PPRM and PROM media. In PPRM the number of second order root is higher than in PROM, but considering the root length the PROM medium is clearly superior than the PPRM medium. Even if the rooting induction rate is not too different for both media, the differences in root quality (root length) are important on plant recovery, but surprisingly we do not detect differences on acclimation and survival rate at transplanting between these two treatments. Apparently, both types of roots are functional and allow the plantlets growth and develop normally after transplanting. Only during the first month of growth at glasshouse the plantlets obtained from PROM seems to develop quicker than the originated from PPRM but at the end of the second month these differences disappear and both types of plants showed similar size and development.

The morphology of the shoot in explants incubated and developed in PELM is also different from the obtained when explants are incubated in PPRM, in general PELM shoots *in vitro* are longer and harder, mostly without axillary development and growth generally as individual shoots, in contrast with the rosette pattern of growth showed by the shoots incubated in PPRM.

Plantlets acclimatization.

No differences were detected in survival rate, ranging in the 90% in all the media assayed. Best results in development correspond to plantlets obtained from explants obtained from PPRM and PROM media. These plants showed a significant difference in the average shoot length and leaf number respect the plants incubated in PELM (Table 4).

Table 4

Average data corresponding to 10 plantlets obtained from *in vitro* plants 5 cm long developed on PPRM, PELM and PROM after 2-month acclimation. Data on survival rate, average length and leaf number were scored.

Culture medium	Survival rate (%)	Average shoot length (cm)	Leaf No.
PPRM	90 ± 10 a	20.5 ± 1.2 a	19.2 ± 2.0 a
PELM	90 ± 10 a	11.1 ± 0.7 b	11.3 ± 1.5 b
PROM	90 ± 10 a	17.9 ± 1.2 c	17.5 ± 2.3 a
Different letters indicate significantly differences by HSD-Tukey at $\alpha = 0.05$ in each parameter between different culture media.			

Although early reports of acclimation Rajeevan and Pandey (1983), recorded just a 77.7 % survival, Shlesinger et al. (1987) and Reuveni et al., (1990) reached a rate of acclimation of 85%, or poor levels of survival (De Winnaar 1988), later reports claim a 100% acclimation success (Drew 1988; Manshardt and Drew 1998). Thick, short, stumpy roots and yellowing of leaves have frequently been reported on agar-supplemented medium (Drew 1987; Kataoka and Inoue 1987; Drew and Miller 1989; Drew et al. 1993; Teo and Chan 1994; Yu et al. 2000). We also reported some thick roots, but in general for all media studied normal roots well branched are frequent (see Table 3) even in a medium with agar substrate and when plants with these roots were acclimated and transplanted the survival rate was high (90%), higher than Anandan et al. (2011), than Hidaka et al. (2008) and Setargie et al. (2015) records, with 72%, 80% and 40% survival respectively, and similar to the survival values recorded by Wu et al. (2012 and Yu et al. (2000) ranging between 87% and 94.5%. Some authors. The good quality of roots obtained by Perez et al. (2016) allows a high rate of survival (96.5%). McCubbin and van Staden, (2003) and Shivayogi et al. (2019) indicate success on acclimation but without data on percentage.

When we change the rooting medium and the substrate to MS liquid plus 1 mgL⁻¹ IBA in expanded vermiculite we reach a 100% success in acclimation, even working with shoots maintained for years *in vitro*.

Acclimation allowed finally the recovery of plants after long-term incubation *in vitro*, showing a normal morphology and development, flowering and fruiting normally after one year (Fig. 8).

Conclusion

We present a reliable method for maintenance and development of papaya plantlets *in vitro* starting from different sources of explants, able to preserve during more than twenty years *in vitro* actively growing papaya cell lines, maintaining the original ploidy level of progenies, just incubating the shoot explants in PPRM medium, and also we offer alternative methods to control efficiently morphogenesis (elongation, rooting, acclimation) of papaya plants during long periods of time. We suggest the use of PPRM medium

to obtain a continuous proliferation of papayas and also as one-step method to produce full plantlets with good rates of success.

Abbreviations

ADE

adenine hemisulfate

BA

benzyladenine

NAA

naphthalene acetic acid

IBA

indole butyric acid

Kin

kinetin

GA₃

gibberellic acid

PPRM

papaya proliferation medium

PELM

papaya elongation medium

PROM

papaya rooting medium

C1

Control 1

C2

Control 2

Declarations

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References

1. Allan P, Clark C, Laing M (2010) Grafting papayas (*Carica papaya* L.). Acta Hort 851:253-258. <https://doi.org/10.17660/ActaHortic.2010.851.38>.

2. Al-Shara B, Mat Taha R, Rashid K (2018). Biotechnological methods and limitations of micropropagation in papaya (*Carica papaya* L.) production: A review. Journal of Animal & Plant Sciences 28:1208-1226
3. Anandan R, Thirugnanakumar S, Sudhakar D, Balasubramanian P (2011). *In vitro* organogenesis and plantlet regeneration of *Carica papaya* L. J Agr Tech 7:1339-1348.
4. Bhattacharya J, Khuspe SS, Renukdas NN, Rawal SK (2003) Somatic embryogenesis and plant regeneration from immature embryo explants of papaya cv. Washington and Honey Dew. Indian J Exp Biol 40:624-627.
5. Cai W, Gonsalves C, Tennant P, Fermin G, Souza MJr, Sarindu N, Jan FJ, Zhu HY, Gonsalves D (1999) A protocol for efficient transformation and regeneration of *Carica papaya* L. In Vitro Cell Dev Biol Plant 35:61-69. <https://doi.org/10.1007/s11627-999-0011-3>
6. Caple AD, Cheah KT (2016) Micropropagation of Hermaphrodite *Carica papaya* L. 'Rainbow' Seedlings via Axillary Bud Pathway. Biotechnology.12:1-5.
7. Castillo B, Smith MAL, Madhavi DL (1997) Interaction of irradiance level and iron chelate source during shoot tip culture of *Carica papaya* L. HortScience 32: 1120-1123. <https://doi.org/10.21273/HORTSCI.32.6.1120>
8. Chaudhuri RK, Jha, TB (2008) Conservation and Production of Ipecac (*Cephaelis ipecacuanha* Rich.) Plants from Long Term Shoot Cultures. Plant Tiss Cult & Biotech 18:157-164. <https://doi.org/10.3329/ptcb.v18i2.3646>
9. Clarindo WR, Carvalho CR de, Araujo FS, Abreu IS de, Otoni WC (2008) Recovering polyploid papaya in vitro regenerants as screened by Flow cytometry. Plant Cell, Tissue and Organ Culture, 92:207-214.
10. Costa A., Abreu E., Schmildt E., Costa A., Schmildt O (2019). Advances observed in papaya tree propagation. Rev. Bras. Frutic. 41 (5):1-15. <http://dx.doi.org/10.1590/0100-29452019036>
11. De Winaar W (1988) Clonal propagation of papaya *in vitro*. Plant Cell Tiss Organ Cult 12:305-310. <https://doi.org/10.1007/BF00034373>
12. Drew RA (1987) The effect of medium composition and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya* L.). J Hort Sci 62:551-556. <https://doi.org/10.1080/14620316.1987.11515820>
13. Drew RA (1988) Rapid clonal propagation of papaya *in vitro* from mature field-grown trees. HortScience 23:609-611.
14. Drew RA (2003) Micropropagation of *Carica papaya* and Related Species. In: Jain S.M., Ishii K. (eds) Micropropagation of Woody Trees and Fruits. Forestry Sciences, vol 75. Springer, Dordrecht, pp 543-564
15. Drew RA (2006) The effects of medium composition and cultural conditions on *in vitro* root initiation and growth of papaya (*Carica papaya* L.). J Hort Sci 62:551-556. <https://doi.org/10.1080/14620316.1987.11515820>
16. FAOSTAT Database (2018). Food and Agricultural Organization, United Nations, Roma. <http://www.fao.org/faostat/en/#data/QC>

17. Fitch MMM, Manshardt RM, Gonsalves D, Slightom JL (1993) Transgenic papaya plants from *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep 12:245-249. <https://doi.org/10.1007/BF00237128>
18. Fitch MMM et al. (1994) Genetic Transformation in *Carica papaya* L. (Papaya). In: Bajaj YPS (eds) Plant Protoplasts and Genetic Engineering V. Biotechnology in Agriculture and Forestry, vol 29. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-662-09366-5_17
19. Fitch MMM (2005) *Carica papaya* Papaya. In: Biotechnology of fruits and nut crops. R.E. Litz, (Ed.) N° 29 CAB International Wallingford. UK, pp. 17-207 <https://doi.org/10.1079/9780851996622.0174>
20. Fitch, MMM (2010) Papaya ringspot virus (PRSV) resistance in papaya: update on progress worldwide. Transgenic Plant J 4:16-28.
21. Fitch MMM (2020) *Carica papaya* Papaya. In: Biotechnology of fruits and nut crops RE Litz, F Pliego-Alfaro, JI Hormaza (Eds.) CABI Wallingford. UK, pp. 131-153
22. Galbraight DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051.
23. Hidaka T, Komori S, Yamada M, Fukamachi H (2008). Mass-production of papaya (*Carica papaya* L.) cv Shahi samplings using shoot-tip culture for commercial use. South Pac Stud 28: 87 – 95.
24. Jia R, Zhao H, Huang J, Kong H, Zhang Y et al. (2017) Use of RNAi technology to develop a PRSV-resistant transgenic papaya. Sci Rep 7: 12636. <https://doi.org/10.1038/s41598-017-13049-0>
25. Jin, S., Mushke, R., Zhu, H., Tu, L., Lin, Z., Zhang, Y. and Zhang, X. 2008. Detection of somaclonal variation of cotton (*Gossypium hirsutum*) using cytogenetics, flow cytometry and molecular markers. Plant Cell Rep. **27**: 1303–1316.
26. Jordan M, Cortes I, Montenegro G (1983) Regeneration of plantlets by embryogenesis from callus cultures of *Carica candamarcensis*. Plant Sci Lett. 28:321-326. [https://doi.org/10.1016/S0304-4211\(83\)80025-X](https://doi.org/10.1016/S0304-4211(83)80025-X)
27. Kabir H, Rahman Z, Mamun NZK (2016) Somatic embryogenesis and plant regeneration from zygotic embryo in *Carica papaya* L., Red-Lady. Plant 4: 45-50. <https://doi.org/10.11648/j.planr.20160406.11>.
28. Kataoka I, Inoue H (1987) Studies on the clonal propagation for tropical and subtropical fruit trees by tissue culture. In: *In Vitro Propagation of Papaya*. Vol. 38. Technical Bulletin of Faculty of Agriculture, Kagawa University, pp. 7–10.
29. Kung YJ, Bau HJ, Wu YL, Huang CH, Chen TM et al. (2009) Generation of transgenic papaya with double resistance to Papaya ringspot virus and Papaya leaf distortion mosaic virus. Phytopathology 99:132-1320. <https://doi.org/10.1094/PHYTO-99-11-1312>
30. Kung YJ, Yu TA, Huang CH, Yeh SD (2010) Generation of hermaphrodite transgenic papaya lines with virus resistance via transformation of somatic embryos derived from adventitious roots of in vitro shoots. Transgenic Res 19:621-635. <https://doi.org/10.1007/s11248-009-9344-2>
31. Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214.

32. Litz RE, Conover RA (1977) Tissue culture propagation of papaya. Proc Fla State Hortic Soc 90: 245-246.
33. Litz RE, Conover RA (1981) Effect of sex type, season and other factors on *in vitro* establishment and other culture of *Carica papaya* L. explants. J Amer Soc Hortic Sci 106:792-794.
34. Magdalita PM, Persley DM, Godwin ID, Drew RA, Adkins SW (1997) Screening *C. papaya* x *C. cauliflora* hybrids for resistance to papaya ringspot virus-type P. Plant Pathology 46:837-841. <https://doi.org/10.1046/j.1365-3059.1997.d01-90.x>
35. Magdalita PM, Laurena AC, Yabut-Perez BM, Zaporteza MM, Tecson-Mendoza EM, Villegas VN, Botella JR (2003) Towards transformation, regeneration and screening of papaya containing antisense ACC synthase gene. In: Plant Biotechnology 2002 and Beyond, Vasil IK (ed), Netherlands, Kluwer Academic Publishers, pp. 323–327. 1 https://doi.org/0.1007/978-94-017-2679-5_67
36. Magdalita PM, Laurena AC, Perea MTM (2013) Cloning and characterization of partial 1-aminocyclopropane-1-carboxylate oxidase-gene and anti-sense transformation into yellow ‘Solo’ papaya via *Agrobacterium tumefaciens*. Journal of the International Service for the Acquisition of Agri-Biotech Applications 19: 63-76.
37. MAPA (1986). Métodos oficiales de análisis de suelos, III. Madrid. 166 p
38. McCubbin, J, van Staden, J (2003) A modified technique for in vitro propagation of papaya (*Carica papaya* L.). South African Journal of Botany 2003, 69:287-291
39. Mishra M, Shukla N, Chandra R (2007) Micropropagation of papaya (*Carica papaya* L.) In: Protocols for Micropropagation of Woody Trees and Fruits. SM Jain and H. Haggman (Eds.) Springer, Dordrecht, The Netherlands pp.437-441
40. Mohamad Fhaizal MB, Vilasini P, Noorsaadah AR, Norzulaani K (2006) Effect of carbenicillin on somatic embryos formation of papaya (*Carica papaya* L. var Eksotika I). Malaysian J Sci 25:47-54.
41. Mondal M, Mukherjee BB (1990) *In vitro* propagation of shoot buds of *Carica papaya* L. (Caricaceae) var. Honey Dew. Plant Cell Reports 8:609-612. <https://doi.org/10.1007/BF00270065>
42. Mumo NN, Rimberia FK, Mamati GE, Kihurani AW (2013) *In vitro* regeneration of selected Kenyan papaya (*Carica papaya* L.) lines through shoot tip culture. Afr J Biotechnol 12:6826-6832. <https://doi.org/10.5897/AJB2013.12902>
43. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473-497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
44. Panjaitan SB, Aziz MA, Rashid AA, Saleh NM (2007) *In-Vitro* Plantlet Regeneration from Shoot Tip of Field-grown Hermaphrodite Papaya (*Carica papaya* L. cv. Eksotika). Int J Agric Biol 6:827-832.
45. Pérez LP, Montesinos YP, Olmedo JG, Barbon Rodriguez R, Sánchez RR, Montenegro ON, Escriba RCR, Daniels D, Gómez-Kosky R (2016) Effect of phloroglucinol on rooting and in vitro acclimatization of papaya (*Carica papaya* L. var. Maradol Roja) In Vitro Cell.Dev.Biol.—Plant (2016) 52:196–203. DOI 10.1007/s11627-015-9733-6.
46. Pliego-Alfaro F, López-Encina C, Barceló-Muñoz A. (1987). Propagation of avocado rootstocks by tissue culture. South African Avocado Growers’ Association Yearbook. 10: 36–39

47. Rajeevan MS, Pandey RM (1983). Propagation of papaya through tissue culture. *Acta Hort.* 131:131-140.
48. Renau-Morata B, Nebauer SG, Arrillaga et al (2005) Assessments of somaclonal variation in micropropagated shoots of *Cedrus*: consequences of axillary bud breaking. *Tree Genet Genomes* 1:3-10
49. Retuta AMO, Magdalita, PM, Aspuria ET, Espino RRC (2012) Evaluation of selected transgenic papaya (*Carica papaya* L.) lines for inheritance of resistance to papaya ringspot virus and horticultural traits *Plant Biotech* 29:339-349. <https://doi.org/10.551/plantbiotechnology.120215b>.
50. Reuveni O, Shlesinger DR (1990a) Rapid vegetative propagation of papaya plants by cuttings. *Acta Hort* 275:301-306. <https://doi.org/10.17660/ActaHortic.1990.275.36>
51. Reuveni O, Shlesinger DR, Lavi U (1990b) *In vitro* clonal propagation of dioecious *Carica papaya*. *Plant Cell Tiss Org* 20:41-46. <https://doi.org/10.1007/BF00034755>
52. Rose DA (1983) The description of the growth of root systems. *Plant Soil* 75:405-415.
53. <https://doi.org/10.1007/BF02369974>
54. Roy K, Roy SK, Hakim MDL (2012) Propagation of papaya (*Carica papaya* L.) cv. Shahi through *in vitro* culture. *Bangladesh J Bot* 41:191-195. <https://doi.org/10.3329/bjb.v41i2.13448>
55. Saker MM, Bekheet SA, Taha HS, Reda AA (1999) *In vitro* propagation of papaya (*Carica papaya* L.). *Arab J Biotech* 2:235-244.
56. Setargie A, Mekbib F, Abraha E (2015) *In vitro* propagation of papaya (*Carica papaya* L.). *World J Agric Res* 11:84-88. <https://doi.org/10.5829/idosi.wjas.2015.11.2.12619>
57. Shivayogi R, Malabasari, TA, Shantappa T, Uppar, DS, Biradar, BD, Mantur SM, (2019) Micropropagation Studies in Papaya (*Carica papaya* L.) cv. 'Surya' *Int. J. Curr. Microbiol. App. Sci.* 8: 2362-2367.
58. Shlesinger, D., Reuveni, O. and Lavi, U. (1987). Tissue culture propagation of papaya. *Acta Hort.* 212: 570-570
59. Suksa-Ard P, Kataoka I, Beppu K, Fujime Y, Subhadrabandhu S (1998) Root Development of Tissue-Cultured Papaya Shoots in Several Rooting Substrates. *Environ Control Biol* 36:115-120. <https://doi.org/10.2525/ecb1963.36.115>.
60. Tamimi YN, Silva JA, Yost RS, Hue NV (1997) Adequate Nutrient Levels in Soils and Plants in Hawaii (General Guide) *Agronomy & Soils* (CTAHR). AS-3, 2 pp.
61. Teo CKH, Chan LK (1994) The effects of agar content, nutrient concentration, genotype and light intensity on the *in vitro* rooting of papaya microcutting. *J Hortic Sci* 62:267–273. <https://doi.org/10.1080/14620316.1994.11516454>
62. Van-Hong N, Chen CH-R, Hsieh CHH (2018) Effects of nutritional and growth hormonal factors on *in vitro* regeneration of papaya (*Carica papaya* L. cv. Red Lady). *J Natn Sci Foundation Sri Lanka* 46:559 -568. <https://doi.org/10.4038/jnsfsr.v46i4.8631>
63. Veena GL, Dinesh MR, Kumar RA. (2015) Axillary bud culture in papaya. *Bioinfolet* 12:147-149.

64. Wu K, Zeng S, Chen Z, Duan J (2012). *In vitro* mass propagation of hermaphroditic *Carica papaya* cv. Meizhonghong. Pak J Bot. 44:1669-1676.
65. Yu TA, Yeh SD, Cheng YH, Yang JS (2000) Efficient rooting for establishment of papaya plantlets by micropropagation. Plant Cell Tiss Org 61:29-35. <https://doi.org/10.1023/A:1006475901439>

Figures

Figure 1

Average micropropagation data after an incubation of 2 years (12 subcultures every 8 weeks) obtained for the different media assayed (C1, C2, PPRM, PELM, PROM).

Figure 2

Average data after an incubation of 10 years (60 subcultures every 8 weeks) obtained for the different media assayed (PPRM, PELM, PROM): No. of axillary shoots, Length of axillary shoots, No. of leaves.

Figure 3

Flow cytometry traces for: A) *Carica papaya* type "Solo" used as mother plant ($2n = 2x = 18$). B) Micropropagated papaya after 120 subcultures ($2n = 2x = 18$).

Figure 4

In vitro papaya plantlets growing in different media: A) PPRM B) PELM C) PROM.

Figure 5

Chlorotic leaves on in vitro papaya plantlets after 4 weeks incubation in PPRM.

Figure 6

Average data after an incubation of 10 years (60 subcultures, subculturing every 8 weeks) obtained for the different media assayed (PPRM, PELM, PROM): Rooting percentage, No. of roots and length of roots.

Figure 7

Results of the rooting of papaya shoots over a physical support of expanded vermiculite with liquid MS medium supplemented with 1 mg l⁻¹ IBA: A) Test tube with rooted papaya shoot. B) Papaya plantlets obtained from test tubes. C) Detail of a plantlets of papaya rooted in expanded vermiculite.

Figure 8

Rooting and acclimation of papaya explants and plantlets. A) In vitro rooting of papaya shoots. B) Acclimation of potted vitro plantlets. C) Field growth of acclimated papaya vitroplant.