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Original article

Induction of somatic embryogenesis in hermaphrodite papaya from cotyledon leaves of *in vitro* seedlings or adult plant-derived explants

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Summary

Introduction – **The clonal propagation of hermaphrodite *Carica papaya* L. would reduce the costs of papaya production. The objective of this study was to induce somatic embryogenesis on cotyledon leaves from *in vitro* seedlings and from adult plant-derived explants of hermaphrodite papaya.** *Materials and methods* – **The papaya hybrid 'Uenf/Caliman 01' was used. Cotyledonary segments from seedlings germinated *in vitro* were treated with 4-chlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (2,4-D). In parallel, leaf segments of adult plants were treated with 2,4-D and abscisic acid (ABA). The relative frequencies of callogenesis, oxidation, embryogenesis, number of somatic embryos per callus, rhizogenesis and regeneration of normal or abnormal plantlets were evaluated.** *Results and discussion* – **With cotyledon explants, a medium supplemented with 36 µM 2,4-D was shown effective in forming a high number of somatic embryos (46.7 embryos callus^{-1}). Using 48 µM 2,4-D in the culture medium increased the recovery of normal plantlets (between 16 and 30%). With adult leaf explants, the induction treatments with ≥ 36 µM 2,4-D produced low callus formation and high explant oxidation. Somatic embryos *in vitro* (1.62 embryos callus^{-1}) of leaf plants were obtained with an initial concentration of 9 µM 2,4-D but did not recover into plant.** *Conclusion* – **Cotyledon explants of 'Uenf/Caliman 01' were amenable to let induce, generate and recover somatic embryos; but not adult explants. Further studies are needed to improve the viability of these somatic embryos and make this method efficient in the propagation of elite papaya material.**

Keywords

Brazil, pawpaw, *Carica papaya*, *in vitro* propagation, plant tissue culture, auxin

Significance of this study

What is already known on this subject?

- Only the fruits of the hermaphrodite papaya plants are commercialized.
- Hermaphroditic plants represent only 2/3 of the hybrid progeny.
- Somatic embryogenesis from adult tissues as the initial explant is difficult.
- It is possible to produce papaya seedlings by somatic embryogenesis from juvenile explants (zygotic embryos or cotyledonary leaves, for example) for which the sexual status is not checked.

What are the new findings?

- Somatic embryogenesis was induced from hermaphrodite plants of papaya; explant-specific responses were observed.

What is the expected impact on horticulture?

- The vegetative propagation of hermaphrodite plants by somatic embryogenesis could reduce the costs of papaya production.

Résumé

Induction de l'embryogenèse somatique chez le papayer hermaphrodite à partir des cotylédons issus de semis *in vitro* ou de feuilles de plants adultes.

Introduction – **La multiplication clonale du papayer (*Carica papaya* L.) hermaphrodite réduirait les coûts de production de la papaye. L'objectif de cette étude était d'induire l'embryogenèse somatique à partir des cotylédons de semis *in vitro* et d'explants foliaires adultes de papayers hermaphrodites.** *Matériel et méthodes* – **Le papayer hybride 'Uenf/Caliman 01' a été utilisé. Des segments cotylédonaires de plantules germées *in vitro* ont été traités à l'acide 4-chlorophénoxyacétique et à l'acide 2,4-dichlorophénoxyacétique (2,4-D). Des segments de feuille de plantes adultes ont également été traités au 2,4-D et à l'acide abscisique (ABA). Les fréquences relatives de callogenèse, d'oxydation, d'embryogenèse, du nombre d'embryons somatiques par cal, de rhizogenèse et des régénération de plantules normales ou anormales ont été évaluées.** *Résultats et discussion* – **A partir des cotylédons, l'apport de 2,4-D à 36 µM dans le milieu de culture s'est montré efficace pour former un nombre élevé d'embryons somatiques (46,7 em-**

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bryons cal^{-1}). La concentration en 2,4-D de 48 µM dans le milieu de culture a amélioré la régénération de plantules normales (entre 16 et 30%). A partir d'explants foliaires adultes, les traitements d'induction au 2,4-D ≥ 2,4 µM ont produit un faible formation de cal et une forte oxydation des explants. Des embryons somatiques (1,62 embryons cal^{-1}) issus d'explants foliaires ont été obtenus *in vitro* avec une concentration initiale en 2,4-D de 9 µM, mais n'ont généré aucune plante. *Conclusion* **– Les explants cotylédonaires de 'Uenf/Caliman 01' sont capables d'induction, de génération et de régénération d'embryons somatiques; contrairement aux explants adultes. De nouvelles études sont nécessaires pour améliorer la viabilité de ces embryons somatiques et rendre cette méthode efficace en vue de la propagation de matériel élite de papayers.**

Mots-clés
Brésil, papayer, *Carica papaya*, multiplication *in vitro*, culture de tissu végétal, auxines

Introduction

The propagation of papaya (*Carica papaya* L.) is troublesome due to the characteristics of its reproductive biology and cultivation. The species is polygamous, presenting male, female and hermaphrodite plants, but only fruits of hermaphrodite plants are of commercial interest (Teixeira da Silva *et al*., 2007).

Papaya hybrids are obtained from crossing between progenitors with distinct genetic pools, resulting in genotypes of higher vigor and superior yields. For instance, the hybrid 'Uenf/Caliman 01' was obtained from a crossing between 'Solo' and 'Formosa' parents and exhibits superior characteristics, such as elevated productivity and excellent fruit quality. Its fruit has intermediate size compared to the 'Formosa' and 'Solo' varieties, higher total soluble solids (in °Brix) and reddish-orange pulp color. However, seed propagation in hybrid papaya can only be accomplished via F_1 seeds, and generally varieties of 'Solo' group produce seeds in the proportion of hermaphrodite:female plants of 2:1. F_1 hybrids of the 'Formosa' group produce seeds in a 1:1 proportion of hermaphrodite:female plants (Schmildt *et al*., 2016). The impossibility of knowing the sex before the plants reach sexual maturity reflects on higher production costs, with greater demand for seeds and water and input consumption.

Thus, the clonal propagation of hermaphrodite hybrid papaya plants would reduce the costs of papaya production. In this context, biotechnological techniques such as somatic embryogenesis have been investigated as alternatives to seed propagation. Somatic embryogenesis allows the propagation of a large number of clones from a single initial explant. In papaya, somatic embryogenesis has been achieved using immature zygotic embryos, segments, roots, leaves and other parts of either juvenile or adult plants (Farzana *et al*., 2008; Heringer *et al*., 2013; Koehler *et al*., 2013; Vale *et al*., 2014; Roy *et al*., 2016; Cipriano *et al*., 2018) and triploid plants were obtained by immature endosperm culture (Sun *et al*., 2011). Hence, by inducing somatic embryogenesis in explants of adult plants of known gender, it would be possible to obtain hermaphrodite papaya clones.

The induction and modulation of somatic embryogenesis are controlled by physiological factors, such as the developmental stage of the tissue used as explant; environmental factors, for instance the season of explant collection; and chemical factors, such as the concentration of growth regulators (Roy *et al*., 2016; Cipriano *et al*., 2018; Gaj, 2004; Ascencio-Cabral *et al*., 2008; Bukhori *et al*., 2013) and the culture physical conditions (Posada-Pérez *et al*., 2017). In papaya, somatic embryogenesis was induced for the first time over 40 years ago (De Bruijne *et al*., 1974). Although many efforts have been made to optimize this protocol, the process is still complicated by several variables, including the endogenous levels of hormones and genetic and epigenetic factors (Vale *et al*., 2014; Jamaluddin *et al*., 2017).

Heringer *et al.* (2013) and Vale *et al.* (2014) described a protocol for somatic embryogenesis in the hybrid papaya 'Uenf/Caliman 01' using immature zygotic embryos as initial explant. However, the possibility of generating clones of hermaphrodite papaya plants requires somatic embryogenesis protocols that employ initial explants from adult plants of a particular sex. Our objective was to regenerate plants by somatic embryogenesis from the cotyledon leaves of papaya hybrids issued from *in vitro* germinated seeds, and to adapt the protocol for adult hermaphrodite plant-derived explants in order to regenerate seedlings that may have the pre-determined sex.

Materials and methods

Plant material

For somatic embryogenesis (SE) induction from cotyledon leaves of *in vitro* seedlings, seeds of the hybrid 'Uenf/Caliman 01' were washed in water and detergent solution and rinsed three times with distilled water; disinfested by immersion in 70% alcohol for 5 min, commercial sodium hypochlorite solution (1% active chloride) for 15 min, and 50% hydrogen peroxide for 10 min; and finally rinsed three consecutive times with autoclaved distilled water. The seeds were germinated *in vitro* on MS medium (Murashige and Skoog, 1962), and the first pair of cotyledon leaves were used as explants.

For SE induction from adult plant-derived explants of hermaphrodite papaya, six-months old plants of the hybrid 'Uenf/Caliman 01' were induced to emit lateral buds, after which the collection of young leaves was carried out. The leaves were disposed onto plastic trays and treated with fungicide solution (1 g L^{-1} Acrobat® + 10 mL L^{-1} Assist®), being kept inside a black plastic bag for 24 h. The leaves were washed with water and neutral detergent, rinsed with running water, and immersed in distilled water for 2 h to the fungicide dissipate. Disinfestation was accomplished under a flow hood using 70% alcohol for 20 s and 1.5% sodium hypochlorite for 20 min, then rinsing four times with autoclaved distilled water. The margins and main veins were removed, to obtain uniform parenchyma tissue, and explants were sectioned and placed on induction media.

Somatic embryogenesis induction

Segments of cotyledon leaves of *in vitro* seedlings (ca. 0.5 cm^2) were inoculated in test tubes with 10 mL of induction medium. Different induction treatments (T0-T8) were established with 2,4-D and 4-CPA concentrations (Table 1), where the explants remained for 60 days (0–60 d). Ten replicates were used per treatment. After 60 days, the relative frequencies of callogenesis (formation of friable callus) and

Table 1. Composition of the induction, maturity, germination and growth culture medium in somatic embryogenesis from cotyledon explants of papaya. T0 – T8 = different induction treatments (4-CPA = 4-chlorophenoxyacetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; ABA = abscisic acid; GA_3 = gibberellic acid).

Phases of somatic embryogenesis		Induction	Maturity	Germination	Growth
Time (days)		0–60	61–120	121–180	181–210
	T0	0	-	-	-
4-CPA (μM)	T1	12	-	-	-
	T2	24	-	-	-
	T3	36	-	-	-
	T4	48	-	-	-
2,4-D (μM)	T5	12	-	-	-
	T6	24	-	-	-
	T7	36	-	-	-
	T8	48	-	-	-
MS basal salt (g L^{-1})		2.15	4.30	4.30	4.30
Glutamine (mg L^{-1})		400	400	400	400
Myo-inositol (mg L^{-1})		-	100	100	100
Vitamins (mL L^{-1})		1	1	1	1
Sucrose (g L^{-1})		30	30	30	30
Agar (g L^{-1})		7	7	7	7
Light per day (h)		-	8	8	8
ABA (μM)		-	0.5	-	-
Activated charcoal (mg L^{-1})		-	15	-	-
GA_3 (μM)		-	-	0.5	-
Temperature (°C)		25	25	25	25

Table 2. Composition of the induction, maturity, germination and growth culture medium for somatic embryogenesis from hermaphrodite plants of the papaya hybrid 'Uenf/Caliman 01'. iT1, iT2 and iT3 = different induction treatment; mT1, mT2, mT3 and mT4 = different maturation treatments (2,4-D = 2,4-dichlorophenoxyacetic acid; ABA = abscisic acid; GA_3 = gibberellic acid).

Phases of somatic embryogenesis		Induction						Maturity			Germination	Growth
Time (days)		0	30	60	90	120	150	180	210	240	270	300
2,4-D (μM)	iT1	9.0	4.5	2.3	1.1	0.6	0.3	-	-	-	-	-
	iT2	36	18	9.0	4.5	2.3	1.1	-	-	-	-	-
	iT3	48	24	12	6.0	3.0	1.5	-	-	-	-	-
MS basal salt (g L^{-1})	mT1/mT2	2.15	2.15	2.15	2.15	2.15	2.15	4.30	4.30	4.30	2.15	4.30
	mT3/mT4							2.15	2.15	2.15		
ABA (μM)	mT1/mT3	-	-	-	-	-	-	0.5	0.5	0.5	-	-
	mT2/mT4							5	5	5		
Glutamine (mg L^{-1})		400	400	400	400	400	400	400	400	400	400	400
Myo-inositol (mg L^{-1})		100	100	100	100	100	100	100	100	100	100	100
Vitamins (mL L^{-1})		1	1	1	1	1	1	1	1	1	1	1
Sucrose (g L^{-1})		30	30	30	30	30	30	30	30	30	30	30
Phytagel (g L^{-1})		2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	-
Activated charcoal (mg L^{-1})		-	-	-	-	-	-	15	15	15	-	-
GA_3 (μM)		-	-	-	-	-	-	-	-	-	0.5	-
Light per day (h)		-	-	-	-	-	-		8	8	8	8
Vermiculite		-	-	-	-	-	-	-	-	-	-	ok
Temperature (°C)		27	27	27	27	27	27	27	27	27	27	27

embryogenesis (presence of embryogenic callus) were assessed.

Friable callus (0.15 g) were transferred to maturation medium (Table 1), where they remained for 60 days (61–120 days old). By 120 days, the mean number of somatic embryos per callus was determined.

Embryogenic callus (0.005 g) were then transferred to Petri dishes containing germination medium (Table 1), where they remained for 60 more days (121–180 days old). After 150 to 180 days, the relative frequencies of normal and abnormal plantlets as well as root emission (rhizogenesis) were evaluated.

The normal and abnormal plantlets were then transferred to growth medium, where they remained for 30 days (181–210 days old), totaling 210 days of *in vitro* culture. The plants were then finally acclimatized.

Since 2,4-D was the best auxin for induction of SE from cotyledon explants, it was also used for explants of hermaphrodite plants.

Leaf segments from adult plant-derived explants of hermaphrodite papaya (ca. 0.5 cm^2) of hermaphrodite plants were inoculated in 10 mL of induction medium (Table 2), where they remained for 150 days (0–150 days old). All three induction treatments consisted of different concentrations of 2,4-D with 15 replicates each. During SE induction, the explants were transferred every 30 days to culture media containing a 2,4-D concentration reduced by half (Table 2). Thus, the induction media series were: iT1 = 9 – 4.4 – 2.25 – 1.15 – 0.6 – 0.3 µM 2,4-D; iT2 = 36 – 18 – 9 – 4.5 – 2.25 – 1.15 µM 2,4-D; and iT3 = 48 – 24 – 12 – 6 – 3 – 1.5 µM 2,4-D. After 90 and 150 days, the relative frequencies of callogenesis, oxidation and embryogenesis were determined.

After 150 days, the friable callus were transferred to maturation media (Table 2), where they remained for another 90 days (151–240 days old). These maturation media consisted of full or half strength MS-salts with two concentrations of ABA, as follows: mT1 = MS + 0.5 µM ABA; mT2 = MS + 5 µM ABA; mT3 = ½ MS + 0.5 µM ABA; mT4 = ½ MS + 5 µM ABA. Thirteen replications were used per treatment. By 180 and 240 days, the relative frequencies of embryogenesis and mean number of somatic embryos per callus were determined.

At 240 days, the embryogenic callus were transferred to germination medium for 30 days (between 241 and 270 days old). At 270 days, the development stage of the embryos was determined. The embryos in the different development phases were transferred to growth medium for 30 days (271–300 days old), totaling 300 days of *in vitro* culture. Acclimatization experiments were not performed due to the low number of normal embryos formed.

Photographic record and ultrastructural characterization

Photographic record was accomplished using a magnifier with coupled camera and the software Motic Images Plus 2.0 during the somatic embryo induction, maturation and germination phases, as well as plantlet formation.

In SE induction from adult plant-derived explants of hermaphrodite papaya, ultrastructural characterization by scanning electron microscopy was performed after 300 days of culture. For this, fixation was done in Karnovsky solution (glutaraldehyde [2.5%] and paraformaldehyde [4.0%] in monobasic potassium phosphate buffer 0.1 M [pH 7.2] containing 5 mM calcium chloride) (Karnovsky, 1965). The fixed samples were dehydrated in ethylic series up to absolute alcohol; dried at critical point with CO_2 (Autosamdri 815, Tousimis®); and placed in stubs and subjected to metallic deposition with gold by cathodic pulverization process (Desk V, DentonVacuum®). The analysis and photo-documentation were carried out using a scanning electron microscope (JSM – 6610LV, Jeol®), and all images were digitally processed.

Experimental design and statistical analysis

A completely randomized design (CRD) was adopted. The data were subjected to descriptive statistics. The means of the treatments were compared by the Kruskal-Wallis non-parametric test ($P \leq 0.05$) using the program R (Team R.C., 2015).

Results and discussion

Explant specific responses

During SE induction from cotyledon leaves of *in vitro* seedlings, all treatments except the control produced friable callus (Figures 1B and 1C). After 60 days in induction medium, treatments T3 and T7 (with 36 µM 4-CPA or 2,4-D, respectively) yielded 100% callogenesis (Figure 2A). Embryogenesis (Figure 1C) was also observed in all treatments, except T0 (control) and T1 (12 µM 4-CPA) (Figure 2A).

After 120 days (maturity phase), 100% of callus from induction treatments T4 (48 µM 4-CPA) and T7 (36 µM 2,4-D) were embryogenic, the latter yielding the highest mean number of somatic embryos (46.7) (Figure 2B). Induction of SE from cotyledon explants of the papaya 'Uenf/Caliman 01' took place under elevated auxin concentrations. Overall, the SE initiation process in papaya is dependent on the exposure of the initial explant to a relatively high concentration of auxin in the culture medium. In another study using immature zygotic embryos of 'Uenf/Caliman 01' as initial explants, higher SE rates were obtained in medium supplemented with an optimum auxin concentration (20 µM 2,4-D) (Heringer *et al.*, 2013; Vale *et al.*, 2014). This has been observed in other papaya cultivars. However, the auxin concentration and source were highly variable according to the genotype (Malabadi *et al.*, 2011).

After 150 days (germination phase), only induction treatments T4 (48 µM 4-CPA) and T8 (48 µM 2,4-D) produced normal plantlets, the latter yielding the highest relative frequency (Figures 2C and 2D). Thus, treatment T7 (36 µM 2,4-D) gave the highest mean number of somatic embryos (46.7 embryos $callus^{-1}$), and T8 (48 µM 2,4-D) the highest frequency of normal plants (0.16), thereby constituting the most efficient treatments for SE induction from juvenile plants of the hybrid papaya 'Uenf/Caliman 01' (Figure 1).

Overall, the most efficient treatments for SE induction from cotyledon leaves also led to formation of abnormal plantlets (Figures 1E and 2). The quality and viability of the somatic embryos are still a problem in the research of countless species. In order to reduce the frequency of abnormalities and promote higher germination rates, the treatment with the gradual reduction of the auxin concentration in the culture medium (Koehler *et al.*, 2013) was done in this study in the SE induction from adult plant-derived explants of hermaphrodite papaya.

In the SE induction from adult plant-derived explants of hermaphrodite papaya, only treatment iT1 (9 µM 2,4-D) displayed formation of friable callus (Figures 3C–E). Explant oxidation was observed in treatments iT2 and iT3 (initial concentration of 36 and 48 µM 2,4-D, respectively) (Figures 3B, 4A and 4B). SE induction had maximum callogenesis (32%)

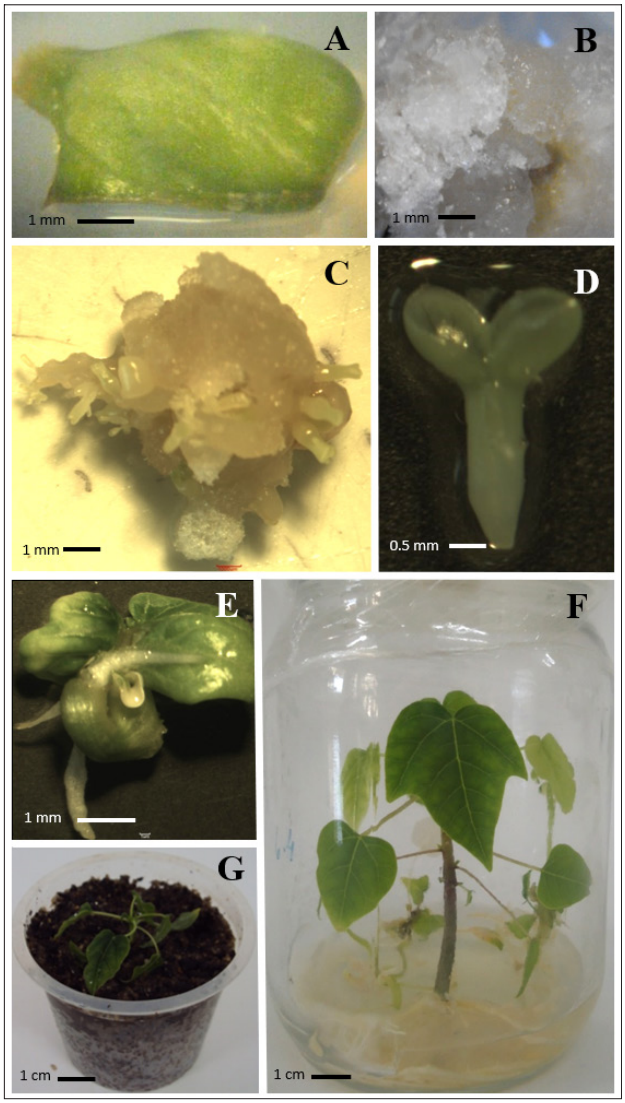

Figure 1. Somatic embryogenesis from cotyledon leaves of *in vitro* seedlings of the papaya hybrid 'Uenf/Caliman 01'. *Induction phase*: A) No response after 30 days (T0 - control); B) No embryogenic callus after 30 days (T3 – 4 μM 4-CPA in induction medium); C) Embryogenic callus after 60 days (T7 – 36 μM 2,4-D in induction medium), with embryos at different development stages; *Germination phase*: D) Cotyledon somatic embryo after 180 days (T8 – 48 μM 2,4-D in induction medium); E) Abnormal plantlet after 180 days (T4 – 48 μM 4-CPA in induction medium); *Growth phase*: F) Normal plantlet at 210 days (T8); G) Plantlet during acclimatization at 240 days.

and embryogenesis (17%) rates after 150 days (Figures 4A and 4B). In these explants, the induction rate was 83% lower than that obtained with cotyledon explants. Moreover, the number of somatic embryos obtained was 96.53% lower.

In addition, a more delayed response to SE induction was verified in explants from mature and hermaphrodite plants, with formation of somatic embryos only occurring after about 90–150 days (12–16 weeks). In comparison, somatic embryos were observed after 60 days (eight weeks) in cotyledon explants.

In another study using immature zygotic embryos of the hybrid papaya 'Uenf/Caliman 01', an embryogenesis rate of 46.7% was reported (Heringer *et al.*, 2013), and the presence of somatic embryos around 3 weeks after the onset of induction was verified. This is a rapid response compared to the observation in the present study (8 weeks), where segmented cotyledon leaves were used as initial explants. The timing difference might arise from the nature of the explant (zygotic embryo *vs.* cotyledon leaves). For other papaya genotypes, SE from immature zygotic embryos took longer, occurring after 10 weeks for the cv. Rathna (Farzana *et al.*, 2008) and about 8 weeks in other twelve papaya cultivars (Malabadi *et al.*, 2011).

These results provide evidence for an explant-specific response whereby each part of the plant, in a certain physiological stage of development, presents specificity regarding the morpho-anatomical constitution as well as the contents of endogenous hormones and secondary compounds (such as phenols), which may interfere with the response during *in vitro* cultivation (Gaj, 2004). Phenols are associated with explant oxidation, a response observed in the present study only in the SE induction from mature explants (treatments iT2 and iT3) (Figures 3 and 4).

Farzana *et al.* (2008) obtained 20% callus induction from segments of young papaya tree leaves from cv. Rathna grown *ex vitro.* However, such callus presented oxidation and very slow growth compared to those formed from cotyledons. Furthermore, a significant difference was observed in the maturation stage of young leaves: the apical leaves (immature) produced more calluses than mature leaves. As such, it can be observed that SE induction in papaya is influenced by the nature and maturity level of the initial explant.

Segments of plantlets germinated *in vitro* are excellent explant sources owing to their juvenility, exhibiting more responsive tissues. Conversely, mature papaya tissues may have an inhibitory effect on the capacity of the meristematic cells of forming embryogenic calluses (Farzana *et al.*, 2008). The process of transition between somatic and embryogenic cells takes place under the influence of the exogenous growth regulators, endogenous plant hormones and stress inflicted by the *in vitro* conditions. Thus, the success of SE relies on the balance between the imposed stress level and the physiological state of the explant cells. Different cells from distinct plant tissues may produce significantly different amounts of endogenous auxins. Some explants require the addition of regulators. Hence, the response, as regards the auxin concentration in SE induction, may be genotype/explant-specific (Cipriano *et al.*, 2018; Fehér *et al.*, 2003), in line with data in the present study.

Effects of exogenous phytohormones

After 180 to 240 days, when the effect of different ABA concentrations and MS salts on embryo maturation was evaluated, somatic embryos were only present in the treatments with 0.5 μM ABA (mT1 and mT3), independently of the concentration of MS salts (Figure 4D). ABA had influenced the SE process in the papaya cv. Golden THB (Cipriano *et al.*, 2018), cvs. Co-5, Pusa Dwarf and Washington (Murashige and Skoog, 1962), and in cv. Golden (Abreu *et al.*, 2014). The use of ABA promotes an increase in the frequency of produced somatic embryos and in their conversion into plants (Koehler *et al.*, 2013). In this study, whilst 5 μM ABA may have inhibited the formation of embryos, 0.5 μM ABA may have been too low to promote an increase in the frequency and in the conversion of somatic embryos into plantlets.

The SE process was asynchronous with embryos at different developmental stages (Figures 5C and 5J): 28.6% at

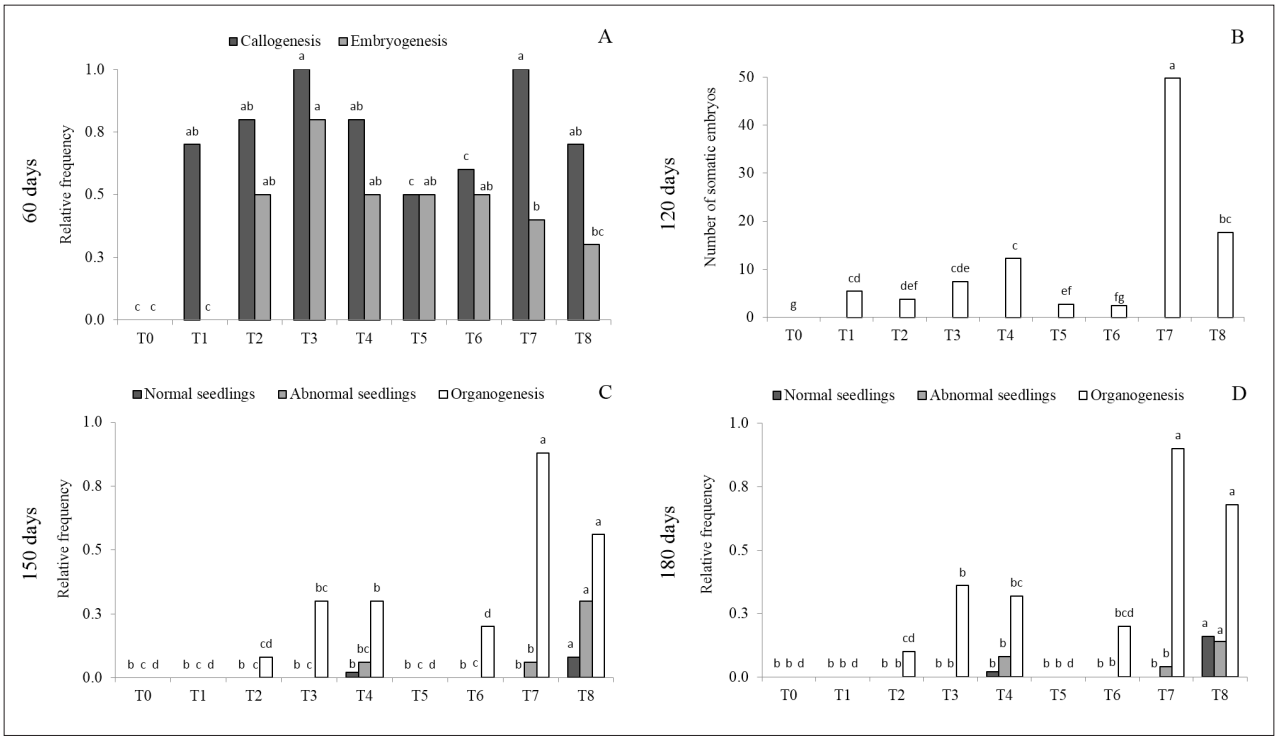

Figure 2. *Induction phase*: A) Relative frequency of callogenesis and somatic embryogenesis after 60 days; *Maturity phase*: B) Number of somatic embryos callus^{-1} after 120 days; *Germination phase*: C) Relative frequency of normal and abnormal seedlings and rhizogenesis after 150 days; and D) After 180 days. Treatments: T0 = control; T1 = 12 µM 4-CPA; T2 = 24 µM 4-CPA; T3 = 36 µM 4-CPA; T4 = 48 µM 4-CPA; T5 = 12 µM 2,4-D; T6 = 24 µM 2,4-D; T7 = 36 µM 2,4-D; T8 = 48 µM 2,4-D. Means marked with the same letter do not differ by Kruskal-Wallis test ($P \leq 0.05$; $n = 10$).

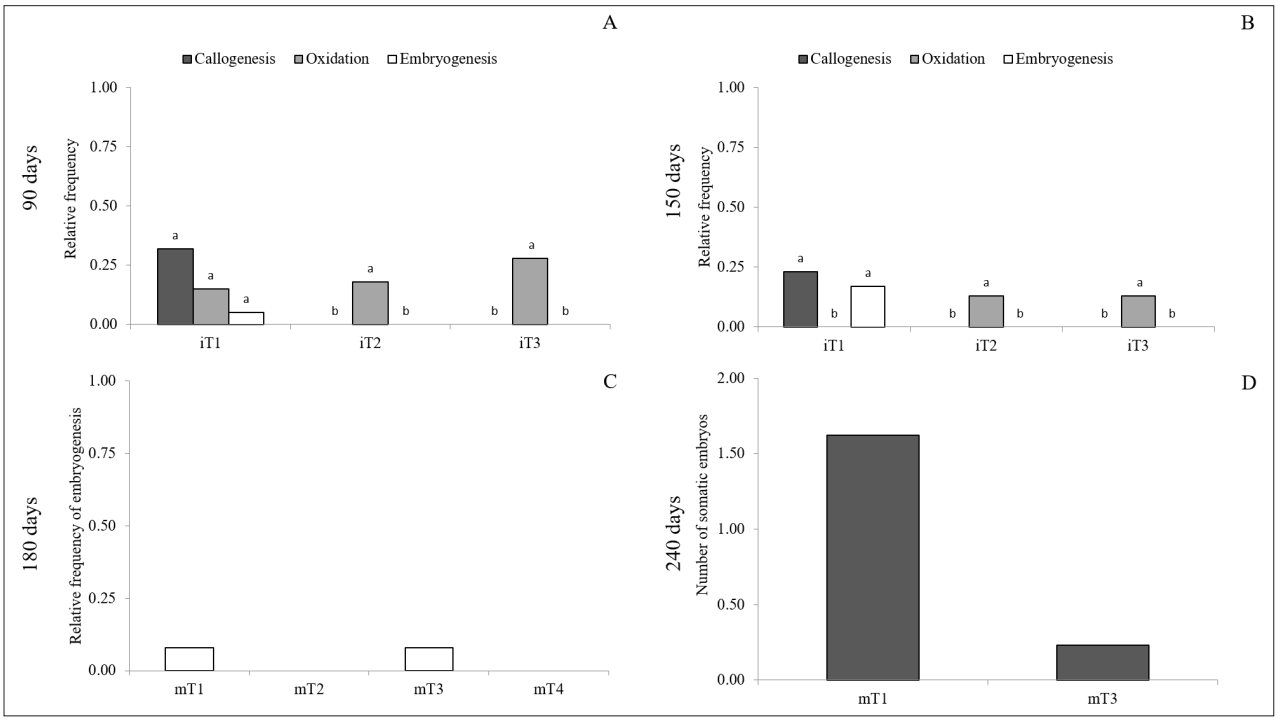

Figure 4. *Induction phase*: A) Relative frequency of callogenesis oxidation and embryogenesis after 90 days; and B) After 150 days; iT1 = 9 – 4.4 – 2.25 – 1.15 – 0.6 – 0.3 µM 2,4-D; iT2 = 36 – 18 – 9 – 4.5 – 2.25 – 1.15 µM 2,4-D; and iT3 = 48 – 24 – 12 – 6 – 3 – 1.5 µM 2,4-D). Means marked with the same letter do not differ by the Kruskal-Wallis test ($P \leq 0.05$; $n = 15$). *Maturity and germination*: C) Relative frequency of embryogenesis after 180 days; D) Number of somatic embryos callus^{-1} after 240 days; mT1 = MS + 0.5 µM ABA; mT2 = MS + 5 µM ABA; mT3 = ½ MS + 0.5 µM ABA; T4 = ½ MS + 5 µM ABA. Means marked with the same letter do not differ by the Kruskal-Wallis test ($P \leq 0.05$; $n = 13$).

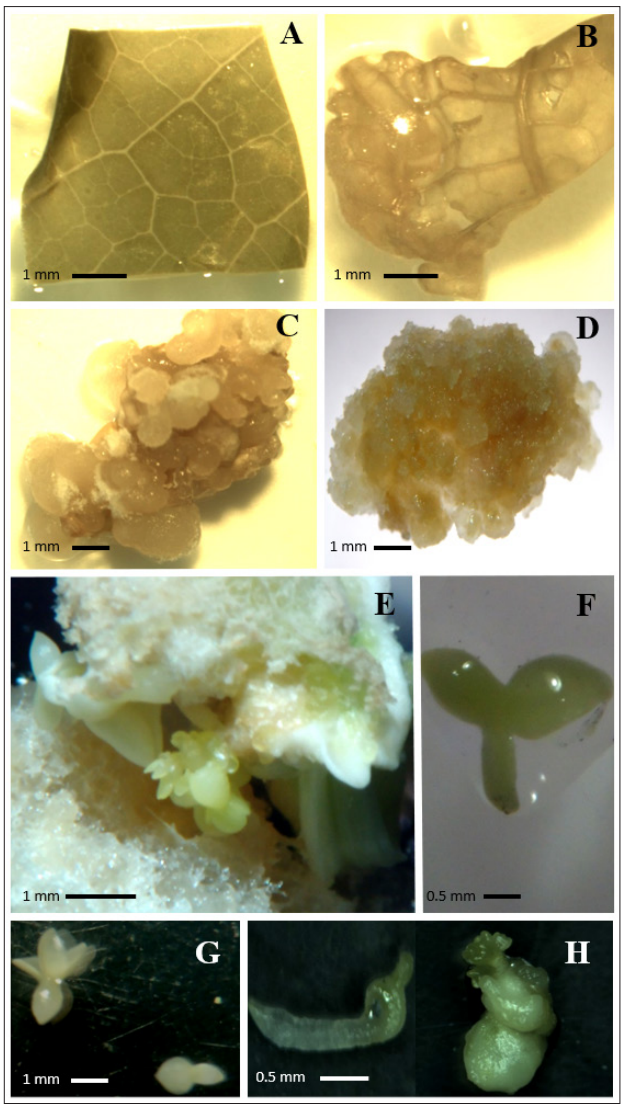

Figure 3. Somatic embryogenesis from hermaphrodite plants-derived explants of the papaya hybrid 'Uenf/Caliman 01'. *Induction phase*: A) No response after 90 days (iT2); B) Oxidized explants after 90 days (iT3 = 48 – 24 – 12 – 6 – 3 – 1.5 µM 2,4-D); C) Friable callus after 30 days (iT1 = 9 – 4.4 – 2.25 – 1.15 – 0.6 – 0.3 µM 2,4-D); *Maturity and germination phases*: D) Embryogenic callus on germination medium after 270 days; E) Embryogenic callus containing embryos at different development stages; F-G) Normal somatic embryos in torpedo and cotyledon stages at 270 days.); H) Abnormal somatic embryo and secondary embryogenesis (embryo in heart-shape stage).

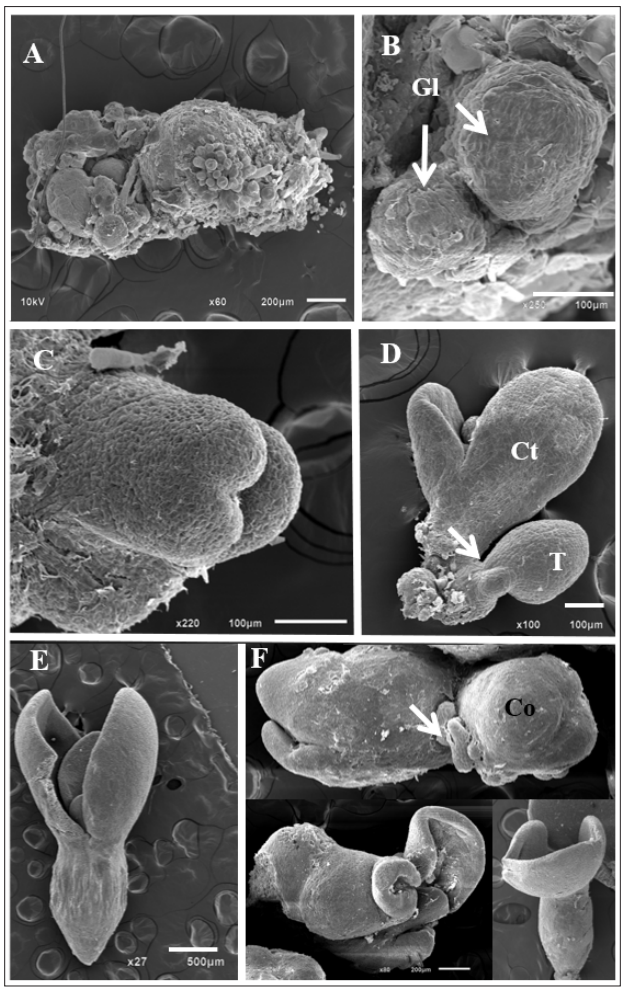

Figure 5. Ultrastructural analysis of somatic embryogenesis of hermaphrodite plants-derived explants of the papaya hybrid 'Uenf/Caliman 01' after 300 days: A) Embryogenic callus with pre-embryogenic regions (induction treatments iT1 and maturity treatment mT1); B) Globular; C) Late-heart; D) Cotyledon and torpedo, detail of the suspensor (arrow); E) Late-cotyledon; F) Abnormal embryos: early-heart embryo (the formation of the apical depression) and secondary embryogenesis process (arrow) (above); trumpet abnormal embryos (below) (Co = heart-shaped embryo; Ct = cotyledon embryo; Gl = globular embryo; T = torpedo embryo).

the globular phase (Figure 5E); 9.5% at the heart phase (Figures 5F and 5J); 19.1% at the torpedo phase (Figure 5I); and 42.8% at the cotyledonary phase (Figures 5G and 5H). Abnormal embryos were also observed, especially trumpet-like (Figures 5K and 5L). Due to paucity of normal embryos obtained from explants of hermaphrodite plants, it was not possible to obtain experimental material sufficient to perform acclimatization.

2,4-D plays a fundamental role in the induction of embryogenic cultures of papaya owing to its effects on division and cell differentiation, and in the modulation of DNA methylation patterns (epigenetic variations) (Fehér *et al.*, 2003; Smulders and De Klerk, 2011). However, synthetic auxins (such as 2,4-D and 4-CPA) may be related to physiological disturbances during and after the SE process. Moreover, the prolonged maintenance of embryogenic cultures in medium containing 2,4-D may cause a loss of maturation potential and affect the conversion of the embryo into a plant (Cipriano *et al.*, 2018; Gaj, 2004). In this study, even with the gradual reduction of the auxin concentration, its elevated content in the induction medium, though ideal for SE induction, may have induced the malformation of the embryos (especially trumpet type) and plantlets, as well as indirect rhizogenesis.

In the ultrastructural analyses embryos at different development stages were observed simultaneously. The presence of embryos at different development stages indicates

an asynchronous process, with embryonic induction and development occurring in different cell groups and at different moments. Koehler *et al.* (2013) and Cipriano *et al.* (2018) also observed somatic embryos of papaya at distinct development stages, further evidencing an asynchrony of SE for this species.

Research aiming at establishing and optimizing SE protocols for papaya is important to enable mass multiplication of plants. In this respect, adult plant tissues used as explants for SE induction may offer a solution to obtain plants of a known gender (Farzana *et al*., 2008). Hence, despite the low efficiency, asynchrony during the process and abnormalities seen in the embryos and plantlets, the information generated in this work should contribute to further studies enabling the clonal propagation of hermaphrodite papaya via SE.

Furthermore, other aspects of the clonal propagation should be considered in addition to the viability and efficiency of the tissue culture tool tested in the present study. Once determined the mass production of seedlings via SE, the confirmation if this material will result in hermaphrodite adult-plants still depends on them reaching the reproductive stage. Thus, parallel studies with the application of technologies of early sex detection (Ming *et al*., 2007), such as a genetic marker of sex (Chaves-Bedoya and Nuñez, 2007), are suggested. This will allow the early confirmation that the seedlings obtained from leaf explants of hermaphrodite plants will be hermaphrodite adult-plants. Such results would promote great impact to horticulture to get rapid and reliable propagation of hermaphrodite papaya plants.

Conclusion

A protocol for the propagation via somatic embryogenesis (ES) from cotyledon leaves of the hybrid papaya 'Uenf/Caliman 01' was successfully established. In parallel, low frequencies of embryos were obtained from adult hermaphrodite plant-derived explants, and it was not possible to regenerate viable seedlings. The process of somatic embryogenesis induction in the hybrid papaya 'Uenf/Caliman 01' was confirmed as an explant-specific response.

Cotyledon explants were more prone to form somatic embryos at higher concentrations of 2,4-D; thus, supplementing the medium with 36 µM 2,4-D led to formation of a higher number of somatic embryos and 48 µM 2,4-D permitted the highest frequency of normal plants. Differently, in adult explants the induction occurred at lower 2,4-D concentrations. Somatic embryos of hermaphrodite plants can be obtained in medium with a starting concentration of 9 µM 2,4-D.

Further studies are needed to obtain somatic embryos in a larger number and at a lower frequency of abnormality. Accordingly, it would be possible to perform plant regeneration and hermaphrodite sex confirmation.

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