

Callus induction in papaya (*Carica papaya* L.) and synseed production for low temperature storage and cryopreservation

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ABSTRACT

The mid- to long-term preservation of papaya (*Carica papaya* L.) would allow for the safeguarding of important germplasm. In this study, soft friable callus (SFC) and hard callus (HC) were induced from the first two true leaves of 10-day-old seedlings containing a midrib derived from the germinated seed of two cultivars ('Rainbow' and 'Sunrise Solo'). Following germination on a Murashige and Skoog (MS) medium that contained 3% sucrose and was free of plant growth regulators (PGRs), sections of the first true leaves from 10-day-old seedlings were exposed to seven published callus or somatic embryogenesis protocols for zygotic embryos, leaves or hypocotyls. Optimal SFC and HC induction was carried out on a half-strength MS medium following the Fitch (1993) or the Ascêncio-Cabral et al. (2008) protocol, respectively. SFC formed shoots that could then convert to plants when transferred to a full-strength MS medium devoid of PGRs. Plantlets 10-cm tall were acclimatised in two steps: first by *in vitro* acclimatisation in aerated vessels, the Vitron, under CO₂-enriched (3000 ppm CO₂), then by the transfer of individually rooted plantlets in Rockwool® blocks to a substrate of soil: pine bark : perlite (1:1:1, v/v/v). SFC and HC were then encapsulated in alginate beads, which were exposed to low temperature storage (LTS) at 10°C and 15°C, and also cryopreserved for 30 days. All encapsulated alginate beads that contained SFC, HC or leaf tissue that had been stored under LTS or cryopreserved were able to regenerate callus when placed on an optimal callus induction medium. Plants derived from the control, LTS and cryopreservation protocols, either from SFC or HC, were successfully acclimatised.

Key words: acclimatisation, callus, encapsulated alginate bead, Murashige and Skoog, paw-paw

Abbreviations:

2,4-D – 2,4-dichlorophenoxyacetic acid, 2,4,5-T – 2,4,5-trichlorophenoxyacetic acid, ABA – (±)-*cis,trans*-abscisic acid, AdS – adenine sulphate, BA – 6-benzyladenine, CIM – callus induction medium, EAB – encapsulated alginate bead, EIM – ELS induction medium, ELS – embryo-like structure, GA₃ – gibberellic acid, HC – hard callus, IBA – indole-3-butyric acid, LED – light-emitting diode, LN – liquid nitrogen, LTS – low temperature storage, MS – Murashige and Skoog, NAA – α-naphthaleneacetic acid, PGR – plant growth regulator, PPF – photosynthetic photon flux density, RH – relative humidity, SDW – sterile distilled water, SFC – soft, friable callus, TDZ – *N*-phenyl-*N'*-1,2,3-thiadiazol-5-yl-urea or thidiazuron

INTRODUCTION

The most common form of propagating papaya (*Carica papaya* L.; Caricaceae), a tropical fruit, is by seed (Teixeira da Silva et al. 2007, Jiménez et al. 2014). Papaya fruit contains papain, an enzyme, while its leaves contain secondary metabolites such as flavonoids and coumarins (Canini et al. 2007). Somatic embryogenesis is already quite well established in papaya (Teixeira da Silva et al. 2007), which is extremely responsive *in vitro* and serves as an important receptive tissue for genetic transformation (Tennant 2010). Furthermore, papaya growth and development show great elasticity when grown under different red: blue ratios of light-emitting diodes or LEDs, and are highly responsive to photoautotrophic micropropagation (Teixeira da Silva 2014). Somatic embryos are useful propagules as they serve as

uniform, clonal units for mass propagation, for example in bioreactors or for the establishment of suspension cultures (Anandan et al. 2012). In this study, using two F₁ hybrid cultivars, 'Rainbow' and 'Sunrise Solo', seven previously established protocols (Tab. 1) were followed for the induction of soft, friable callus (SFC) and hard callus (HC) from young leaves. The literature indicates that the former leads to the formation of shoots while the latter leads to the formation of embryo-like structures (ELs). Using HC, protocols were established for the development of encapsulated alginate beads (EABs; syn. synthetic seed or synseed) for the encapsulation of embryogenic callus. The objective of using EABs is to establish a mid-term (low temperature storage, LTS) or long-term (cryopreservation) preservation protocol for important germplasm (Sharma et al. 2013). Several cryopreservation studies in papaya exist. For

Table 1. Seven published protocols tested in this study used for the establishment of callus and somatic embryos in papaya (*Carica papaya* L.)

Reference	Explants and cultivar(s) used	Basal medium	Hormones (concentration) and other additives	Abiotic culture conditions
Chen et al. 1987	Shoot tip, stem, leaf, cotyledon and root explants of Solo and Sunrise	Half-strength MS	160 mg l ⁻¹ AdS, 1.0 mg l ⁻¹ NAA + 0.5 mg l ⁻¹ kinetin + 1.0 mg l ⁻¹ GA ₃	Unknown
Fitch 1993 (inspired or based on Fitch and Manshardt 1990 and Litz and Conover 1982)	Hypocotyl sections from 10-day-old seedlings of Kapoho, Sunset, Sunrise, Waimanalo	Half-strength MS	4.52 µM 2,4-D; 400 mg l ⁻¹ glutamine; 6.0% sucrose; 0.7% agar	Continual darkness; 27°C
Bhattacharya and Khuspe 2000	Immature zygotic embryos of Honey Dew and CO-2	Full-strength MS	3 mg l ⁻¹ 2,4,5-T; 0.7% agar	Continual darkness; 25°C
Fernando et al. 2001	Mature zygotic embryos of Sunrise Solo	Full-strength MS	2 mg l ⁻¹ 2,4-D; 0.7% agar	Continual darkness; 25°C
Ascêncio-Cabral et al. 2008	Zygotic embryos of Maradol	Half-strength MS + Chen et al. (1987) vitamins	0.75% Difco® Bacto agar	16-h photoperiod; 68 µmol m ⁻² s ⁻¹ ; 25°C
Farzana et al. 2008	Zygotic embryos, hypocotyls, mature and immature leaves of Rathana	Full-strength MS	3 mg l ⁻¹ NAA (first 2 explants) or 3 mg l ⁻¹ NAA + 3 mg l ⁻¹ IBA + 1 mg l ⁻¹ 2,4-D (latter 2 explants); 3.0% sucrose, 0.8% agar	16-h photoperiod; 55 µmol m ⁻² s ⁻¹ ; 27°C
Malabadi et al. 2011	Immature zygotic embryos of Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, Co-3	Full-strength MS	4.52 µM 2,4-D + 2.27 µM TDZ; 3.0% sucrose, 0.7% agar	16-h photoperiod; 100 µmol m ⁻² s ⁻¹ ; 25 ± 3°C; RH = 55-60%

2,4-D – 2,4-dichlorophenoxyacetic acid; 2,4,5-T – 2,4,5-trichlorophenoxyacetic acid; AdS – adenine sulphate; GA₃ – gibberellic acid; IBA – indole-3-butyric acid; MS, –Murashige and Skoog (1962); NAA – α -naphthaleneacetic acid; RH – relative humidity; TDZ – thidiazuron

example, Castillo et al. (1998) encapsulated somatic embryos in alginate beads, while Ashmore et al. (2007) and Tsai et al. (2009) used a vitrification-based protocol. Even though LTS is a good way to preserve vegetative tissues for short- or medium-term periods of time (Cha-um and Kirdmanee 2007, Engelmann 2011, Teixeira da Silva et al. 2014), no LTS study of papaya tissues or plantlets exists.

This study had two key objectives: 1) to compare seven established protocols in the papaya literature for the induction of SFC and HC; 2) to create EABs from three explants (SFC, HC or leaf tissue), encapsulate them and subject them to LTS and cryopreservation.

MATERIAL AND METHODS

Chemicals and reagents

All chemicals and reagents, including those of the medium constituents used in the seven protocols indicated in Table 1, were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), at the highest available purity, and whichever was cheapest. Toxic chemicals such as mercuric chloride were treated with maximum caution and disposed of professionally to avoid user injury or environmental contamination.

Plant material, seed germination culture conditions

Seeds of two hybrid papaya (*Carica papaya* L. 'Rainbow' and 'Sunrise Solo') cultivars were purchased from a local supermarket with guaranteed import quality and with no (or few) apparent surface infection or markings and were surface sterilised and germinated using the protocol of Giang et al. (2011). Seeds were briefly removed from ripe fruits, soaked for 48 h and washed in running tap water to remove the sarcotesta. Only sinking seeds following a floatation test (i.e., viable seeds) were used. Seeds were surface sterilised in 0.1% mercuric chloride + 2-3 drops of Tween-20 for 5 min, rinsed three times in sterilised distilled water (SDW), sprayed with 80% ethanol for 1 min then rinsed three times in SDW. Surface-sterilised seeds were slightly embedded (5 per Petri dish; As-One, Osaka, Japan) in an autoclaved (100 KPa; 21 min) full-strength (macro- and micronutrients) Murashige and Skoog (MS; 1962) medium (pH 5.8 following adjustment with 1N NaOH or HCl) containing 3% sucrose and 2 g l⁻¹ gellan gum (Gelrite®, Merck, USA). The MS medium contained no PGRs. Petri dishes were sealed with Parafilm®

(Pechiney Plastic Packaging Co., Chicago, USA) and were incubated at 25°C, at first in the dark for seven days. Seedlings were then transferred to 250-ml Erlenmeyer flasks (five plantlets per flask) containing 50 ml of Hyponex® (N:P:K = 6.5:6:19; Hyponex Japan Corp., Tokyo, Japan) containing 3% sucrose and 2 g l⁻¹ Gelrite® and placed under a 16-h photoperiod with a photosynthetic photon flux density (PPFD) of 45 μmol m⁻² s⁻¹ provided by plant growth fluorescent lamps (Plant Lux, Toshiba Co., Hiroshima, Japan). Young leaves of 10-day-old seedlings were used for EABs and LTS and the cryopreservation experiments that follow.

Callus induction and shoot and plantlet development

Seven previously published protocols, whose details are provided in Table 1, were compared. The objective was to induce SFC and HC, the former being white or pale and able to form shoots while the latter being brown and able to form ELSs. Based on these trials, the assumption was made that the optimal callus induction medium (CIM) and ELS induction medium (EIM) would correspond to and be defined by SFC and HC, respectively, based on the best callus and ELS induction. This was assessed from the literature, indicated in Table 2 (the Fitch [1993] and Ascêncio-Cabral et al. [2008] methods, respectively). The first two true leaves of 10-day-old seedlings containing a midrib were used as the explant, independent of the original explant used in the seven published protocols (Tab. 1). SFC and HC production was quantified after 30 days. Shoot formation from SFC and plantlet (shoots with a root system) induction from HC was possible when sub-cultured onto fresh CIM and EIM, respectively. After an additional 30 days (i.e., 60 days after treatment), shoots that formed with at least two full leaves were cultured on a Hyponex® medium containing 3% sucrose and 2 g l⁻¹ Gelrite® and cultured under the conditions defined above for seedlings, except that low PPFD (10 μmol m⁻² s⁻¹) was used.

Cryopreservation by encapsulation

1. Explant preparation

A protocol that was originally optimised for hybrid *Cymbidium* (Teixeira da Silva 2013a) was used, but was modified and adapted to papaya tissue (optimization trials and data not shown). Three explant types (all 2 mm², independent of the fresh mass) were tested: young leaf segments of 10-day-old seedlings with mid-rib tissue, SFC and HC.

Table 2. Scoring, fresh weight and survival of soft, friable callus (SFC) or hard callus (HC) induction of two papaya (*Carica papaya* L.) cultivars using 7 established protocols from the literature after 30 days ($n = 30/\text{treatment}$) (see also Fig. 1B-G)

Protocol	Rainbow				Sunrise Solo			
	SFC Score	HC Score	FW (mg)	%S	SFC Score	HC Score	FW (mg)	%S
Chen et al. 1987	1	0	137 c	73 b	1	0	108 c	66 b
Fitch 1993	3	0	284 a	100 a	3	0	238 a	98 a
Bhattacharya and Khuspe 2000	0	2	63 d	91 ab	0	1	39 d	96 a
Fernando et al. 2001	0	2	57 d	71 b	0	2	46 d	74 b
Ascêncio-Cabral et al. 2008	0	3	66 d	89 ab	0	3	41 d	86 ab
Farzana et al. 2008	2	1	193 b	100 a	1	1	151 b	100 a
Malabadi et al. 2011	1	2	126 c	100 a	1	1	137 bc	100 a

FW – fresh weight of a single explant, HC – hard callus, %S – percentage survival, SFC – soft, friable callus. Scoring system: 0 = no HC/SFC, 1 = very small amount of HC/SFC, 2 = 30-70% of explant covered with HC/SFC, 3 = the entire explant covered with HC/SFC. All data presented as means. Different letters within each FW column indicate significant differences at $p = 0.05$ according to Tukey's multiple range test. Different letters within each %S column indicate significant differences at $p = 0.05$ following a χ^2 test

2. Encapsulated alginate bead development and re-culture conditions

Explants (SFC, HC and young leaf tissue with mid-vein) were encapsulated by immersing each explant with sterilised forceps into a 3.5% (w/v) sodium alginate solution made up in a full-strength MS medium. Using 5- or 10-ml glass pipettes with the terminal end sawn off, droplets containing a single explant per EAB were fed into a 100 mM CaCl_2 complexing solution. For SFC, a 4.0% sodium alginate solution was used. The complexing solution was stirred gently with a sterilised metal spoon once every 10 min to prevent the EABs from adhering to each other and/or fusing. After hardening, the EABs were rinsed gently for 10 min in SDW under sterile conditions on a clean bench to wash away any remaining CaCl_2 residue. EABs were immediately cultured on PGR-free full-strength MS medium to develop shoots.

3. Preculture and priming of encapsulated alginate beads for cryopreservation

EABs were pre-cultured in 100-mm diameter, 15-mm-deep Petri dishes (25 per dish) containing 50-ml full-strength MS medium supplemented with 2% (w/v) sucrose for 24 h and sealed with Parafilm®. Following preculture, EABs were plunged into liquid nitrogen (LN) (10 EABs/treatment, each within 2-ml polypropylene tubes [2.0 ml Conical Screw Cap Microtube; Quality Scientific Plastics Inc., Kansas, USA]), at five per tube. After storage in LN for one month, polypropylene tubes containing EABs were placed in a 35°C water bath for 3 min prior to re-culturing on CIM and EIM. Desiccation and moisture content were not determined.

Low temperature storage

Two experiments were performed: in the first experiment, uniform SFC-derived shoots from CIM that had rooted on a Hyponex® medium for 90 days and that had reached at least 6 cm in height, with at least eight fully developed leaves and a well developed root system, were placed in LTS. In the second experiment, once it was assessed that developed plantlets had died under LTS, EABs containing either young leaf segments of 10-day-old seedlings with mid-rib tissue, SFC or HC (all 2 mm², independent of the fresh mass) were preserved in LTS to assess whether regeneration ability (i.e., the ability to form shoots) would be lost or not. In both experiments, two cold temperatures (10°C and 15°C) were tested. Control treatments were cultured at 25°C on full-strength MS medium under a 16-h photoperiod at a PPFD of 45 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For the cold treatments, explants were placed under a 12-h photoperiod at a low light intensity (PPFD = 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$). For both experiments, and for both cultivars, LTS lasted 30 days.

Morphological parameters assessed

The percentage survival, fresh weight, regrowth and/or formation of SFC and HC from the seven established protocols (Tab. 1) was observed with the naked eye and by dissecting light microscopy, and quantified after 30 days, which was determined from trials as being the period of time sufficient to form SFC and HC without the initiation of other organs. For LTS and cryopreservation experiments, the fresh weight and percentage survival and amount of callus (SFC or HC) or number of shoots that formed from encapsulated SFC on CIM and

from encapsulated HC on EIM, respectively, was calculated at 60 days, which was determined from trials as being the period of time sufficient to form shoots after replanting onto a fresh CIM and EIM medium. The amount of HC and SFC formed was quantified using an arbitrary, relative scoring system in which: 0 = no HC/SFC, 1 = very small amount of HC/SFC, 2 = 30-70% of explant covered with HC/SFC, 3 = the entire explant covered with HC/SFC.

Acclimatisation

Seedlings as well as plantlets that formed after LTS and cryopreservation treatments and that had reached at least 6 cm in height, with at least eight fully developed leaves and a well developed root system, were transferred to Rockwool® (Grodan, Denmark) containing Vitron (Otsuka, Tokushima, Japan) gas-permeable vessels and placed under *in vitro* photoautotrophic conditions (with 3000 ppm CO₂), as described by Teixeira da Silva (2014). After 60 days, 10-cm tall plantlets with a well developed root system left intact on individual Rockwool® blocks were transferred to a greenhouse into a substrate of gardening soil : pine bark : perlite (1:1:1, v/v/v) held in 2-liter clay pots and covered with a plastic box for 2-3 days. Thereafter, plants were left uncovered and were well watered with tap water once a day. Greenhouse conditions included natural light conditions with no supplemental lighting, a mean temperature of 29°C and a relative humidity of 70-80%.

Statistical analyses

Experiments were organised according to a randomised complete block design with three blocks of 10 replicates per treatment. All experiments were repeated three times. Data was subjected to analysis of variance with mean separation by Tukey's multiple range test using SAS® ver. 6.12 (SAS Institute, Cary, NC, USA). Percentage data was arc-sin transformed and significant differences between treatments determined with a χ^2 test. Significant differences between means were presented at the level of $p = 0.05$.

RESULTS AND DISCUSSION

The most effective way to establish *in vitro* cultures for papaya is initially from seeds. In this study, a previously defined protocol was used (Giang et al. 2011) with an almost 100% successful seed germination rate (Fig. 1A). The first two true leaves

of 10-day-old seedlings with mid-vein tissue were used for SFC and HC induction experiments. Both SFC and ELs could be induced from most of the seven established protocols (Tab. 2, Figs 1B-G), indicating the ease and responsiveness of papaya to *in vitro* culture, but the quantitative and qualitative outcomes differed. SFC and HC were best (or most) induced from the Fitch (1993) and Ascêncio-Cabral et al. (2008) methods, respectively, for both cultivars, based on a score of 3, with significantly higher fresh weight and significantly higher percentage survival (relative to all other protocols) (Tab. 2). These two protocols thus served as the CIM and EIM, respectively. HC was always significantly heavier than SFC (Tab. 2). The use of first two true leaves of 10-day-old seedlings containing a midrib as the explant rather than the original explant used in the seven protocols may explain why the level of SFC and HC differed from the original studies. The use of different cultivars in this study may also explain the variation observed in optimal regeneration performance, i.e., SFC and HC formation. Interestingly, both cultivars cultured in the Farzana et al. (2008) and Malabadi et al. (2011) protocols resulted in mixed organogenesis (callus + shoots) while all other protocols resulted in a clear developmental pattern, either callus or shoots. The *in vitro* development of papaya is already well established (reviewed in Teixeira da Silva et al. 2007, Jiménez et al. 2014). Effective *in vitro* protocols are important for the successful regeneration of transgenic tissue (Tennant 2010). Incidentally, Teixeira da Silva (2014) could micropropagate both cultivars used in this study under photoautotrophic and heterotrophic conditions while root-directed organogenesis (rhizogenesis) could be strictly controlled using an array of auxins (Teixeira da Silva 2013b).

Only a single study has examined the use of synseeds in papaya (Castillo et al. 1998), although other studies on cryopreservation exist (e.g., Ashmore et al. 2007, Tsai et al. 2009). In that study, somatic embryos 2 mm in diameter were directly encapsulated but the frequency of regeneration was significantly affected by "(1) the concentration of sodium alginate, (2) the presence or absence of nutrient salts in the capsule, and (3) the duration of exposure to calcium chloride". The best germination resulted when Castillo et al. (1998) used 2.5% sodium alginate in half-strength MS medium and, after 10 min of exposure to CaCl₂ for uniform encapsulation of somatic embryos, a maximum of 75% germination was achieved

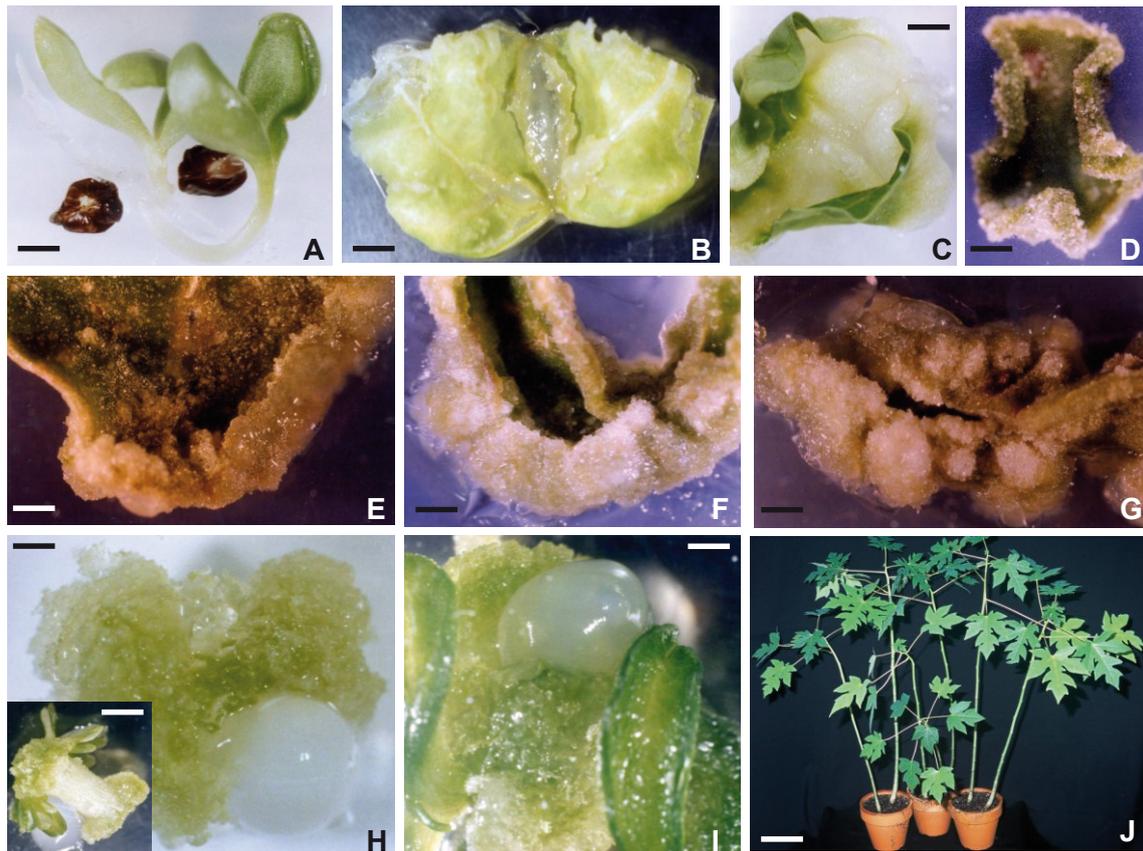


Figure 1. The response of papaya (*Carica papaya* L. ‘Sunrise Solo’ and ‘Rainbow’) to different established protocols from the literature, to cryostorage and to LTS. (A) Sterilised seed germination and one-week-old ‘Sunrise Solo’ epicotyls (first true leaves) used for the study. (B) ‘Rainbow’ SFC on Chen et al. (1987) medium. (C) ‘Sunrise Solo’ SFC on Farzana et al. (2008) medium. (D) ‘Sunrise Solo’ SFC on Malabadi et al. (2011) medium. (E) ‘Rainbow’ HC on Malabadi et al. (2011) medium. (F) ‘Sunrise Solo’ SFC on Fitch (1993) medium. (G) ‘Sunrise Solo’ HC on Ascêncio-Cabral et al. (2008) medium. (H) ‘Sunrise Solo’ SFC on CIM from encapsulated SFC cryopreserved for one month and shoot growth (inset) after a further 30 days on CIM. (I) ‘Sunrise Solo’ SFC on CIM from encapsulated SFC at LTS (15°C) for one month and shoot growth after a further 30 days on EIM. (J) Acclimatised plants approximately 1 m tall from the control (derived from Fitch [1993], no LTS, no cryopreservation, no alginate encapsulation; centre), LTS (right) or cryopreservation (left) 90 days after introduction into the greenhouse. B-G = response after 60 days in culture. Abbreviations: CIM – callus induction medium (optimised as Fitch 1993; Table 2). HC – hard callus, LTS – low-temperature storage, EIM – ELS induction medium (optimised as Ascêncio-Cabral et al. 2008; Tab. 2), SFC – soft, friable callus. Scale bars: (A) 2.5 mm; (B-I) 1 mm; (H, inset) 5 mm; (J) 20 cm

with all germinated synseeds producing normal plantlets. This study showed similar successful results: three explant types (SFC, HC and young leaf tissue with mid-vein) could be successfully encapsulated and cryopreserved for one day (data not shown) or 30 days (Tab. 3). Cryopreservation of synseeds allows for the short-, medium- or long-term storage of important germplasm (Sharma et al. 2013). Replanting any of these explants onto CIM or EIM resulted in SFC or shoots, respectively (Figs 1H, 1I). SFC, presumably containing adventitious buds, when transferred to a plant growth regulator (PGR)-free MS medium, readily converted into shoots that could root equally easily on a Hyponex® medium. Plantlets 10 cm

tall, 10 each from the seven protocols, control or from the LTS or cryopreservation experiment, could be successfully acclimatised at 100% (Fig. 1J). Use of the Vitron and CO₂-enrichment eases acclimatisation without complicated steps that need to consider issues related to the build-up of ethylene, concerns about relative humidity, or hyperhydricity. Kaity et al. (2008) found epigenetic changes in plants derived from cryopreservation. In the plants that were acclimatised, no observable radical or aberrant morphological changes were observed, although plant height differed (data not shown). Future studies based on this protocol should envisage checking the plant material for somaclonal variation using molecular markers.

Table 3. Survival of EABs subjected either to cryostorage or to LTS at two temperatures for 30 days of two papaya (*Carica papaya* L.) cultivars, as assessed by re-growth of SFC on CIM or HC on EIM (pooled data) 60 days after treatment ($n = 30/\text{treatment}$)

Protocol	Rainbow		Sunrise Solo	
	FW (mg)*	%S	FW (mg)*	%S
Cryopreservation	206 f	44 c	241 e	59 bc
LTS 10°C	318 d	71 b	366 c	81 ab
LTS 15°C	401 b	88 a	447 a	91 a

CIM – callus induction medium (optimised; Fitch 1993; Table 2), EAB – encapsulated alginate bead; EIM – ELS induction medium (optimised; Ascêncio-Cabral et al. 2008; Table 2), ELS – embryo-like structure, FW – fresh weight of a single explant (*independent of whether HC or SFC formed), HC – hard callus, %S – percentage survival, SFC – soft, friable callus. All data presented as means. Different letters within each FW column (and across both cultivars) indicate significant differences at $p = 0.05$ according to Tukey's multiple range test. Different letters within each %S column (and across both cultivars) indicate significant differences at $p = 0.05$ following a χ^2 test

Despite the existence of LTS treatments for several tropical species, no protocols exist for papaya (Cha-um and Kirdmanee 2007). In this study, a conventional LTS protocol was not used, namely the LTS of well developed and rooted plantlets. Rather, using a somewhat unique approach, explants (SFC, HC and young leaf tissue with mid-vein) were first encapsulated as EABs. EABs were then subjected to LTS and stored for one day (data not shown) or 30 days at low temperature (10 or 15°C), which led to the successful regeneration of SFC through CIM or shoots (indirect) through EIM (Tab. 3).

CONCLUSIONS

This study presents a protocol for the alginate encapsulation of leaves or leaf-derived callus (soft or hard). This is the first such protocol for papaya and can serve to preserve important germplasm using LYS or cryopreservation (Teixeira da Silva et al. 2014). Using two cultivars, 'Rainbow' and 'Sunrise Solo', optimal soft, white and friable callus was obtained using the Fitch (1993) protocol while harder brown callus was obtained using the Ascêncio-Cabral et al. (2008) protocol. Most likely, if tweaked, the callus-induction protocol could also serve for other cultivars or even for other explant types, such as stem (node or internode) tissue. The ability to store these encapsulated alginate beads at low temperatures and to cryopreserve them (Sharma et al. 2013), followed by their successful regeneration, provides a useful and innovative mid- to long-term way to preserve *Carica* sp. germplasm. In addition, a fairly novel concept, *in vitro* acclimatisation using aerated vessels and CO₂-enrichment, already shown for *Spathiphyllum* (Teixeira da Silva et al. 2006), sweet potato (Teixeira da Silva et al. 2005a), *Epidendrum* (Teixeira da Silva et al. 2005b), banana and hybrid *Cymbidium*

(Teixeira da Silva et al. 2005c), and applied in this study to papaya, also ensures the survival of *in vitro*-derived material once acclimatised to the greenhouse.

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