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CRYOPRESERVATION OF CHAYOTE (*Sechium edule* JACQ. SW.) ZYGOTIC EMBRYOS AND SHOOT-TIPS FROM *IN VITRO* PLANTLETS

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Abstract

This paper presents the development of cryopreservation protocols for zygotic embryos and apices of chayote (*Sechium edule* Jacq. Sw.), a tropical plant species with recalcitrant seeds. Zygotic embryos of two cultivars, Ccocro negro (CN) and Claudio (Cl) could withstand cryopreservation, with survival percentages of 10 and 30 %, after desiccation to 23 and 19 % moisture content (fresh weight basis), respectively. Apices sampled on *in vitro* plantlets of cultivars Cl, 13 and JM were successfully cryopreserved using a vitrification technique. Optimal conditions included the culture of mother-plants for 22 days on medium containing 0.3 M sucrose, culture of excised apices on the same medium for 1 day, loading of apices for 20 min with 2M glycerol + 0.4M glycerol, treatment with a series of diluted PVS2 solution (60 % PVS2 followed by 80 % PVS2 solution for 15 min (cultivar Cocoro Blanco [CB]) or 30 min (cultivars CN and Cl) at each concentration), rapid freezing and thawing, washing of shoot-tips with a 1.2 M sucrose solution, followed by recovery on media with progressively decreasing sucrose concentrations until the standard concentration of 0.1 M was reached. The highest survival percentages achieved ranged between 17 and 38 %, depending on the cultivar.

Keywords: chayote; *Sechium edule*; cryopreservation; zygotic embryos; shoot tips; vitrification; desiccation.

INTRODUCTION

Chayote (*Sechium edule* Jacq. Sw.) seeds have been classified recalcitrant (15) and therefore cannot be conserved under the reduced seed moisture content and low temperature conditions employed for storing orthodox seeds. Chayote is a viviparous species; the seeds do not show any dormancy period and germinate within the fruit. They are damaged if they are extracted from the fruit and die rapidly due to desiccation because of the total or partial absence of lignified testa (13, 17). Newstrom (17) mentions that storing seeds at 5 °C under low humidity can delay germination by a maximum of 6 weeks. Chayote genetic resources are

thus traditionally conserved as whole plants in field collections, which leaves the plants exposed to pests, diseases and other natural hazards such as drought, weather damage, human error and vandalism. In addition, they are not in a condition that is readily conducive to germplasm exchange because of the great risks of disease transfer through the exchange of vegetative material. Field genebanks are costly to maintain and, as a consequence, are prone to economic decisions that may limit the level of replication of accessions, the quality of maintenance and even their very survival in times of economic stringency (10). In Costa Rica, the largest collection of chayote germplasm, which includes 58 traditional Costarrican varieties, is maintained by the School of Agricultural Sciences of the National University in Heredia.

Cryopreservation represents the only safe and cost-effective option for the long-term conservation of genetic resources of this species. Cryopreservation protocols have been established for over 200 different species using various explants including seeds, zygotic embryos, shoot-tips, pollen, somatic embryos, calluses and cell suspensions (3, 11, 27). However, no cryopreservation protocol is currently available for chayote.

The research presented in this paper aimed at developing cryopreservation protocols for chayote, using zygotic embryos and shoot-tips sampled from *in vitro* plantlets of several cultivars.

MATERIALS AND METHODS

Plant material

Mature fruits of five different cultivars, cocoro blanco (CB), cocoro negro (CN) (average length of fruit 5.5 cm), Claudio (Cl), JM and 13 (average length of fruit 12 cm) were employed in this study, depending on their availability. The fruits (Fig. 1) were provided by chayote producers from the Cartago province and brought to the Centro de Investigacion en Biotecnologia of the Instituto Tecnologico de Cartago, where the experiments were performed.

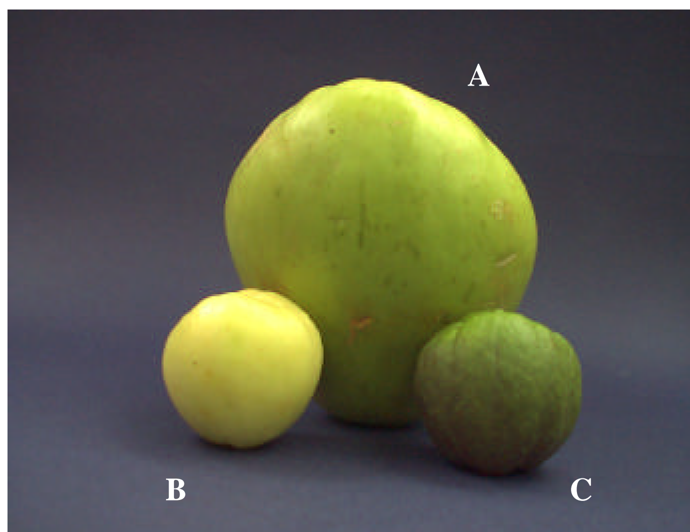

Figure 1. Fruits of chayote cultivars Claudio (A), Cocoro blanco (B) and Cocoro negro (C).

In vitro culture of embryos and plantlets

Fruits and seeds were disinfected according to the method established by Alvarenga *et al.* (2). Fruits were washed thoroughly with running water and detergent. Seeds were then extracted and immersed in a calcium hypochlorite solution (4 % active chlorine) for 10 min, and rinsed three times with sterile water under the laminar airflow cabinet.

Embryos (Fig. 2) were extracted from the seeds and placed on semi-solid Murashige and Skoog medium (16) with 0.5 mg l^{-1} benzyladenine (BA), 30 g l^{-1} sucrose and 2 g l^{-1} Phytagel in the dark at 24±1 °C for 24 h before being used for freezing experiments.

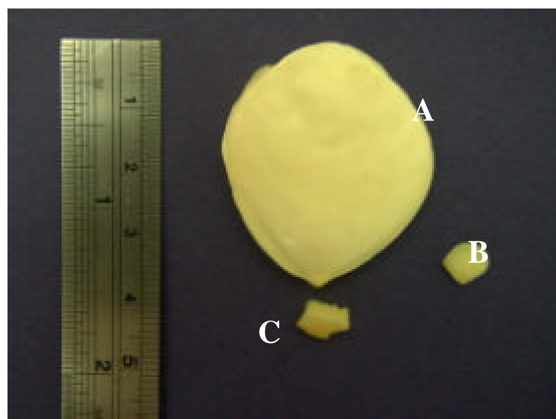

Figure 2. Excised embryos of cultivars Claudio (A) and Cocoro negro (B). C: embryos of cultivar Claudio after excision of the major part of the cotyledons.

For freezing experiments using shoot-tips, embryos were germinated *in vitro* on MS medium with 0.5 mg l^{-1} BA. The plantlets obtained were multiplied by node cuttings following the protocol of Abdelnour *et al*. (1). Shoot-tips were sampled from *in vitro* plantlets 4 weeks after the last subculture

Cryopreservation of zygotic embryos

For cryopreservation, embryos were placed in Petri dishes under the laminar air flow cabinet for 0 to 5 h, thereby reaching moisture contents (MCs, fresh weight basis) between 92 (no desiccation) and 19 % (5 h desiccation). The MC of embryos was measured gravimetrically after each dehydration period by placing 3 individual embryos in an oven at 103 °C for 24 h. Large sections of the cotyledons were removed from embryos of cultivar Cl before desiccation to obtain explants of comparable size and to avoid over-dehydration of cotyledons before dehydration of axes started (Fig. 2). After desiccation, embryos were placed in 1.5 ml sterile polypropylene cryotubes (10 embryos per cryotube) and frozen rapidly by direct immersion in liquid nitrogen. After 1 h storage at –196 °C, embryos were rewarmed by immersing the cryotubes for 60-90 s in a water bath at 40 °C. For recovery, embryos were cultivated on MS medium with 30 g l^{-1} sucrose, 2 g l^{-1} Phytagel and 0.5 mg l^{-1} BA at 24±1 °C in the dark during the first 2 days, then under a 16 h light/8 h dark photoperiod, with a light intensity of 34 µE m^{-2} s^{-1}. Survival was measured after 3 weeks by assessing the percentage of germinated embryos.

Cryopreservation of shoot-tips

Shoot-tips (of around 1 mm in length) were cryopreserved using the vitrification technique. Apices were sampled from *in vitro* plantlets cultured on standard medium with 0.1 M sucrose or which had been placed for 22 days on medium with 0.3 M sucrose. After excision, apices were cultured for 1-2 days on medium supplemented with 5 % dimethylsulfoxide (DMSO) or 0.3 M sucrose. Apices were incubated for 20 min at 0 °C in a loading solution containing 2 M glycerol + 0.4 M sucrose, then for 0 to 90 min at 0 °C in the PVS2 vitrification solution (30 % (v/v) glycerol + 15 % (v/v) ethyleneglycol + 15 % (v/v) DMSO in culture medium with 0.4 M sucrose [24], pH 5.8), employed either at full strength or diluted at 60 % or 80 % (v/v) in standard medium. Apices were frozen after direct treatment with either full strength or diluted vitrification solution, or after a sequence of treatments with 60 %/80 % PVS2 solutions. Shoot-tips (10 apices in 0.5 ml PVS2 solution per 1.5 ml cryotube) were frozen rapidly by direct immersion in liquid nitrogen. After 1 h storage

at –196 °C, the apices were rewarmed by plunging the cryotubes for 90 s in a water bath at 40 °C. The PVS2 solution was eliminated by washing the apices twice (2 min/washing) with medium containing 1.2 M sucrose. Apices were then transferred on media containing 0.1 mg l^{-1} BA with daily decreasing sucrose concentrations (0.5 M for 24 h/0.3 M for 24 h) until the concentration of 0.1 M sucrose was reached. Survival was measured after 6 weeks by assessing the percentage of apices having produced a shoot. In the Tables, mean percentages are given with their standard error.

RESULTS

Cryopreservation of zygotic embryos

Excision and *in vitro* inoculation had no detrimental effect on embryos since 100 % of the embryos of cultivars CB, CN and Cl germinated and developed into plantlets (Table 1). At the beginning of the desiccation treatment, the desiccation of embryos of cultivar Cl, from which large sections of the cotyledons had been removed, was slower than that of whole embryos of cultivars CB and CN. However, from 2 h of desiccation onwards, embryos of all 3 cultivars displayed the same desiccation pattern. Survival of embryos of cultivars CB and CN decreased rapidly with increasing desiccation periods, reaching 0 % for MCs of 23 % and 21 % for CB and CN, respectively. Survival of control embryos of cultivar Cl remained much higher for all MCs tested, ranging from 100 % for MCs between 91 % (no desiccation) and 21 % (3 h desiccation), to 50 % for 19 % MC (5 h desiccation). After cryopreservation, no survival was achieved with embryos of cultivar CB, whatever their MC, whereas 10 % survival was obtained with cultivar CN for embryos desiccated to 23%. With cultivar Cl, survival of 20-30 % was achieved with embryos desiccated to 19 % MC.

Table 1. Effect of the dehydration duration (h) on the moisture content (%, fresh weight basis) and survival of control (-LN) and cryopreserved (+LN) embryos of chayote cultivars Cocoro Blanco (CB), Cocoro Negro (CN) and Claudio (Cl). The experiment was replicated 3 times, with 10-12 embryos per treatment. *-: not tested.

Dehydration duration (h)	Embryo moisture content (%)		Survival (%)			
	CB, CN	Cl		CB	CN	Cl
0	87±3	91±3	–LN	100	100	100
			+LN	0	0	0
1	65±2	83±3	–LN	75±3	75±3	100
			+LN	0	0	0
2	34±1	31±4	–LN	40±3	50± 0	89±3
			+LN	0	0	0
3	23±2	21±1	–LN	0	20± 0	100
			+LN	0	10± 0	0
4	21±1	19±1	–LN	-*	0	70±5
			+LN	-	0	30±3
5	20±1	19±1	–LN	-	-	50±3
			+LN	-	-	20±3

Cryopreservation of shoot-tips

No positive results were achieved when freezing chayote shoot-tips using the encapsulation-dehydration technique, due to the very high sensitivity of encapsulated apices

to even relatively low sucrose concentrations employed during pretreatment (data not shown). Culture of mother-plants on medium with 0.3 M sucrose for 1 to 21 days had no detrimental effect on the survival of excised shoot-tips of cultivars Cl and 13 (Table 2). Only with variety JM did the longest culture period tested have a slightly negative effect on shoot-tip survival.

Table 2. Effect of the culture duration of mother-plants on medium with 0.3 M sucrose on the survival of excised shoot-tips of three chayote varieties. The experiment was replicated 3 times, with 20 shoot-tips per treatment.

Duration of culture of mother-plants on medium with 0.3 M sucrose (days)	Survival of shoot-tips (%)		
	CI	13	JM
1	97±1	92±1	85±2
7	98±1	97±2	87±3
14	97±1	93±3	83±3
21	95±2	92±3	78±4

Table 3. Effect of various treatments on the survival (%) of apices of three chayote cultivars (CI, 13 and JM). The experiment was replicated 3 times, with 30 apices per treatment. LS: loading solution (2 M glycerol + 0.4 M sucrose), applied at room temperature. PVS2: vitrification solution (30 % glycerol + 15 % ethylene glycol + 15 % DMSO in culture medium with 0.4 M sucrose), applied at 0 °C. LN: liquid nitrogen.

Treatment	Survival of apices (%)		
	CI	13	JM
Excision	100	98±1	87±1
Apices on medium with 5 % DMSO (1 day)	0	0	0
Apices on medium with 0.3 M sucrose for 1 day	100	96±1	80±2
Apices on medium with 0.3 M sucrose (1 day) + LS (20 min)	91±1	90±2	80±2
Apices on medium with 0.3 M sucrose (1 day) + LS (20 min) + PVS2 (15 min)	41±4	37±2	18±4
Apices on medium with 0.3 M sucrose (1 day) + LS (20 min) + PVS2 (15 min) + LN	0	0	0
Apices on medium with 0.3 M sucrose (2 days)	82±4	78±2	67±4
Apices on medium with 0.3 M sucrose (2 days) + LS (20 min)	78±2	76±2	64±1
Apices on medium with 0.3 M sucrose (2 days) + LS (20 min) + PVS2 (15 min)	28±2	23±2	20±2
Apices on medium with 0.3 M sucrose (2 days) + LS (20 min) + PVS2 (15 min) + LN	0	0	0
Mother-plants on medium with 0.3 M sucrose (22 days)	96±3	92±3	78±5
Mother-plants on medium with 0.3 M sucrose (22 days) + LS (20 min) + PVS2 (15 min)	35±2	21±2	22±6
Mother-plants on medium with 0.3 M sucrose (22 days) + LS (20 min) + PVS2 (15 min) + LN	0	0	0

Excision of apices had no (cultivars Cl and 13) or limited (cultivar JM) detrimental effect on their survival (Table 3). Culture of excised apices for 1 day on medium supplemented with 5 % DMSO was lethal for all three cultivars studied. Culture of excised shoot-tips on medium with 0.3 M sucrose for 1 day had no effect on their survival, whereas extending this culture period for one additional day resulted in a decrease of survival for all three cultivars Treatment of apices cultured on 0.3 M sucrose for 1 and 2 days with the loading solution had no detrimental effect on their survival. By contrast, a sharp decrease in survival was noted after treatment with the PVS2 solution. Similar observations were made with apices sampled from *in vitro* mother-plants cultured on medium with 0.3 M sucrose for 22 days: treatment with the loading solution had no detrimental effect on the survival whereas survival dropped after treatment with the PVS2 solution. However, no survival of apices was achieved after freezing in liquid nitrogen whatever the experimental conditions tested.

Increasing the duration of treatment with the diluted PVS2 solutions progressively decreased the survival of non-cryopreserved shoot-tips of the three cultivars studied, from 42-80 % after 15 min with 60 % PVS2 to 0-27 % after 45 min with both 60 % and 80 % PVS2 solutions (Table 4). The three cultivars tested differed in their survival after cryopreservation. Apices of cultivar Cl displayed survival for treatment durations with 60 %/80 % PVS2 solutions between 15/15 min and 30/45 min, with the highest survival percentage (38 %) for 30/30 min. Apices of cultivar 13 withstood freezing after two treatments with the diluted PVS2 solutions (15/15 min and 30/30 min) and the highest survival percentage achieved was lower (24 %). In the case of cultivar JM, survival of shoot-tips could be achieved under four experimental conditions, from 15 min with 60 % PVS2 to the sequence 30/45 min with 60 %/80 % PVS2 solution. However, the highest survival noted was even lower (17 % only) and callusing was observed under the extreme treatment conditions ensuring survival (*i.e.* 60 % PVS2/15 min and 60 % PVS2/30 min + 80 % PVS2/45 min).

Table 4. Effect of treatment with diluted PVS2 solutions on survival of control (-LN) and cryopreserved (+LN) apices of three chayote cultivars. Apices were sampled on mother-plants which had been cultured for 22 days on medium with 0.3 M sucrose. The experiment was replicated 3 times, with 30 apices per treatment for cultivars Cl and 13, and 20 apices per treatment for cultivar JM. Treatment with the diluted PVS2 solutions was performed at 0 °C.

Treatment		Survival (%)		
		Cl	13	JM
0.3 M sucrose for 21 days (control)		93±2	89±2	70±7
60 % PVS2/15 min	-LN	80±4	42±5	65±6
	+LN	0	0	7±4 (callus)
60 % PVS2/15 min + 80 % PVS2/15 min	-LN	68±6	37±1	50±2
	+LN	11±2	24±4	13±1
60 % PVS2/30 min + 80 % PVS2/30 min	-LN	52±3	20±3	40±2
	+LN	38±2	13±2 (callus)	17±1
60 % PVS2/30 min + 80 % PVS2/45 min	-LN	34±2	12±1	38±1
	+LN	18±2	0	8±1 (callus)
60 % PVS2/45 min + 80 % PVS2/45 min	-LN	27±2	0	20±2
	+LN	0	0	0

DISCUSSION

In this paper, we show for the first time that cryopreservation of chayote zygotic embryos and shoot-tips from *in vitro* plantlets is possible. Zygotic embryos withstood cryopreservation, with survival percentages of 10-30 % after partial desiccation and rapid freezing. Apices were successfully cryopreserved using the vitrification technique, with survival percentages ranging between 17 and 38 %.

Embryos of recalcitrant seed species display various characteristics which make them difficult to cryopreserve (18). Embryos of some species are extremely sensitive to dehydration, and even minor reductions in their moisture content, to levels which are too high to ensure survival after freezing, lead to irreversible structural damages (5). Another characteristic is the size of the embryo, the smaller ones being generally more tolerant to desiccation. According to Berjak *et al.* (4), the majority of recalcitrant seeds are characterized by their relatively large size, with the cotyledons being responsible for this large size, while the embryonic axis usually represents only a small portion of the whole structure. It has thus been recommended to remove the cotyledons in order to achieve a more uniform dehydration and a higher survival after freezing. Freezing embryonic axes only instead of whole embryos is a relatively common procedure (12, 20); however, in cases where embryos prove extremely recalcitrant, as in the case of *Theobroma cacao*, recovery by means of neoformation of somatic embryos or adventitious buds is necessary (6, 19). Another difficulty with recalcitrant seeds is that there is no arrest in their development as is observed with orthodox seeds at the end of their maturation period (18). This makes it difficult to select seeds at a determined stage of embryo maturity, which can influence the reproducibility of results between seed lots, experiments and laboratories.

The results obtained in this study confirm that chayote embryos display the characteristics of embryos of recalcitrant seeds. Removal of the major part of the cotyledons in cultivar Cl, which has the largest embryos, seemed to ensure a more uniform dehydration of the embryonic axes, thus leading to higher survival percentages for a broader range of MCs. The slower desiccation rate during the first hour of embryos of cultivar Cl in comparison with the other two cultivars, even though a large section of the cotyledons had been removed might be due to their much larger size and less succulent consistency. According to Berjak *et al*. (4), embryonic axes of some recalcitrant species are not appropriate for cryopreservation and, even when trying to optimize all parameters involved in a cryopreservation protocol, no survival can be obtained because of the histological composition of the tissues and the cellular structure of the embryonic axis. In such situations, it is recommended to use other types of explants for cryopreservation, such as apices, buds or somatic embryos as material to conserve (9, 20, 21). This is the case with chayote, in view of the absence of survival after freezing noted with one cultivar and the low survival percentages obtained with the other two cultivars studied.

The conversion of water into a vitrified state, at least of the intracellular water, seems to be indispensable to achieve survival of biological materials subjected to natural or artificial freezing (14). The vitrification technique requires a highly concentrated vitrification solution which induces sufficient dehydration of the cytosol without causing damage, in order to achieve a stable vitrified state upon freezing of samples in liquid nitrogen. Cells must maintain a sufficiently high solute concentration to avoid the formation of ice crystals; however it must not be too high to have a detrimental effect on cells. Explants must thus acquire tolerance to freezing, which is obtained through pretreatment with progressively increasing sucrose concentrations and incubation in the loading and vitrification solutions for different periods (23, 26). It has been mentioned that, in addition to its osmotic effect, sucrose increases the stability of membranes under severe hydric stress (7). Even though its precise

mechanism of action is not fully understood, it is believed that treatment of explants with the loading solution has mainly an osmotic effect. A treatment for durations shorter than 20 min does not allow permeation of glycerol inside the cells; it instead accumulates in the periprotoplasmic space, thereby inducing dehydration and allowing the concentration of solutes in the cytosol, and at the same time mitigating the mechanical stress caused by severe dehydration (23).

Another strategy for inducing tolerance to dehydration without causing too severe toxicity is to initiate the incubation period of explants in diluted vitrification solutions and to progressively increase their concentration before freezing in liquid nitrogen (25). With potato, this vitrification procedure, which uses a progressive increase in the concentration of the vitrification solution, significantly increased the survival of apices, in comparison with apices incubated directly in 100 % PVS2 (25). Chayote apices survived only when diluted vitrification solutions were employed. It seems that chayote apices are sensitive to such solutions, and this sensitivity was observed with all three cultivars studied, not only regarding the survival percentages but also the regeneration pattern (direct regrowth *vs* callusing). Apices of cultivars 13 and JM were more sensitive than those of cultivar Cl.

The present study also highlighted the importance of the conditioning treatment of chayote apices in order to achieve survival after freezing. Due to the susceptibility of excised apices to an exposure to 0.3 M sucrose for longer than one day, culture of mother-plants for several weeks appeared as a good strategy, since apices sampled on such plants regrew up to 100% when cultured on standard chayote micropropagation medium. This procedure allowed both recovery of apices after treatment with the vitrification solution and freezing in liquid nitrogen. It is possible that culture of mother-plants for an extended period on medium with a sucrose concentration higher than the standard one ensures a certain degree of osmotic dehydration as well as the accumulation of sucrose and other sugars which allow cells to survive under conditions of hydric stress and at the same time act as vitrifying substances (8).

The sequential increase in the concentration of the PVS2 vitrification solution allowed to avoid the detrimental effect of incubation of apices in the full strength vitrification solution; it proved to be the only way to obtain survival of chayote apices after freezing. We recommend to continue the research on chayote cryopreservation, both for embryos and apices, since the survival achieved during this study is only intermediate and can certainly be improved. Higher recovery percentages after freezing should indeed be obtained in a reproducible manner in order to envisage using cryopreservation for the long-term conservation of chayote germplasm. The effect of other vitrification solutions such as PVS4 or PVS6, which have been recommended for sensitive species (23) should be tested. The encapsulation-vitrification technique could also be investigated (22). Finally, histological examination of apices during the different steps of the freezing protocol will be very valuable for improving recovery.

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