

Cryopreservation of Cat's Claw (Uncaria tomentosa)

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Abstract

Uncaria tomentosa presents tomentum that resembles cat's claws, hence its common name, is a plant that produces various secondary metabolites that are traditionally used in alternative medicine. The natural distribution of this species has been affected by indiscriminate harvesting from its habitat. In the present research, cryopreservation (liquid nitrogen, LN, -196° C) was evaluated as an option for *ex situ* conservation of this species. The following techniques were evaluated: vitrification and encapsulation-dehydration of apices, vitrification of cell suspensions, and seed desiccation and vitrification. Preculture conditions and exposure times to LS and PVS2 were evaluated. Apex survival was the highest (82%) with preculture in 0.25 M sucrose followed by incubation for 20 and 30 min in LS and PVS2, respectively, prior to cooling in LN. The encapsulation-dehydration technique was evaluated by using sucrose preculture and different capsule moisture contents. Survival of apices cooled in LN was not significantly different between treatments and varied from 31.8% to 52.9% for capsule moisture contents between 22.7% and 20.3%. For cell suspensions precultured in 0.5 M sucrose, cell multiplication and formation of calli with very good appearance were observed in 61.1% of the cultures following vitrification. For cryopreservation of seeds, germination was 89.5% using the desiccation technique and 67.6% to78.1% using vitrification.

Keywords

Uncaria tomentosa, Vitrification, Encapsulation-Dehydration, Conservation of Plant Genetic Resources, PVS2, Preculture, Regeneration Media

1. Introduction

Uncaria tomentosa, commonly known as cat's claw, is widely used in traditional medicine in some countries *Corresponding author.

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Among the various procedures for germplasm conservation, cryopreservation allows storage of live organisms in a state of suspended animation for extended periods at ultralow temperature, generally using liquid nitrogen (LN, -196° C). This technique is especially useful for the preservation of plant material with recalcitrant seeds and of laboratory-generated material such as meristems, apices and cells [12].

The encapsulation-dehydration procedure consists of coating the explants to be conserved in calcium alginate to form artificial seeds. This facilitates the manipulation of delicate tissues, provides physical protection during the dehydration, cooling and warming processes [13] [14], protects against the toxic effects of cryoprotectants in vitrification solutions and does not require expensive programmable freezers [15] [16].

The vitrification technique consists of dehydration of the tissues of interest by exposure to solutions with high osmotic potential. These are very concentrated cryoprotectant mixtures known as PVS formulations (*Plant Vitrification Solution*) composed of substances such as sucrose, glycerol, polyethylene glycol and DMSO which facilitate the vitreous transition when samples are plunged into LN. Programmable freezers are not required for this technique [17]-[19].

This is the first report of protocols for the cryopreservation of *U. tomentosa* seeds and cell suspensions using vitrification and for apex preservation using encapsulation-dehydration and vitrification.

2. Materials and Methods

2.1. Plant Material

U. tomentosa was established *in vitro* by using methods developed by Alvarenga (2010). Seeds were disinfected in a solution of 5 g·L⁻¹ Benlate[®] and 5 g·L⁻¹ Agri-mycin[®] for 40 min with agitation, washed three times with distilled water, then immersed in a 3.14% sodium hypochlorite solution for 15 min followed by three washes in sterile distilled water. Five or six seeds per jar were inoculated onto 20 ml MS culture medium [20] with 30 g·L⁻¹ sucrose, pH 5.7 and 3.3 g·L⁻¹ Phytagel[®]. Cultures were maintained at 25°C ± 2°C, with a photoperiod of 16 h (low diffuse light, 500 luxintensity)/8h dark. After seeds germinated and shoots reached a length of 2 to 3 cm, the multiplication stage was initiated on recovery medium (the culture medium described for seed germination enriched with 1 mg·L⁻¹ gibberellic acid (AG₃) and 1 mg·L⁻¹ calcium pantothenate), under a light intensity of 2000 lux. In vitrification and encapsulation-dehydration experiments, apices were excised from developed plants 2 or 3 weeks after the last multiplication.

2.2. Apex Vitrification

Vitrification protocols developed by Sakai *et al.* [21] were used. Apices were isolated and precultured in increasing concentrations of sucrose, followed by incubation for 20 min in Loading Solution (LS, liquid MS medium supplemented with 0.4 M sucrose and 2 M glycerol), then incubated in 100% PVS2 vitrification solution (30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose in culture medium) for several periods.

In the present study, two preculture treatments were evaluated: 0.25 M sucrose for 24 h and 0.25 M sucrose for 24 h followed by 0.5 M sucrose for 24 h on solid multiplication medium. For each preculture treatment, apex survival was evaluated after 4 weeks on recovery medium. To evaluate the effect of incubation time in LS, after each period of preculture in sucrose, apices were transferred to 1.2 mL polypropylene cryotubes and incubated in 1 mL of LS for 0, 10, 20 or 30 min at 0°C, then cultured on recovery medium (described previously) on filter paper for 1 week in the dark, then transferred to diffuse light (500 lux) at $25^{\circ}C \pm 2^{\circ}C$. For evaluation of the subsequent steps in the cryopreservation process, apices were incubated in LS for 10 and 20 min, after which LS was eliminated using a sterile dropper and 1 mL PVS2 vitrification solution was added. Apices were incubated for 10, 30 and 50 min at 0°C. After each incubation period, PVS2 was replaced with 1 mL of fresh PVS2 and

apices were immersed in LN for 1 h. Cryovials containing apices were warmed in a water bath at 40°C for 2 min. The PVS2 was then eliminated from the cryovials using a sterile dropper and apices were washed twice for two min with MS medium supplemented with 1.2 M sucrose. Apices were placed in Petri plates on filter paper to eliminate excess washing medium and then placed on recovery medium enriched with 0.5 M sucrose for 48 h in darkness. After this period, apices were transferred to recovery medium and cultured at $25^{\circ}C \pm 2^{\circ}C$ in darkness for 1week, then under diffuse light until shoot formation. Survival was evaluated after 2 weeks. All experiments consisted of 10 experimental units (apices) with three repetitions per treatment. Data were analyzed using means comparison by multiple factor ANOVA using the R program.

2.3. Apex Encapsulation-Dehydration

The encapsulation-dehydration technique was evaluated for apex cryopreservation using the protocol developed by Fabre & Dereuddre [22], which consists of encapuslation in calcium alginate, preculture in liquid medium with high sucrose concentration and partial dehydration of capsules prior to cooling in LN. In this study, apices were isolated and cultured on recovery medium for 24 h in darkness, then encapsulated in calcium alginate. Apices were placed in liquid MS culture medium with 3% sodium alginate without calcium. Using a sterile transfer pipette, apices were transferred to liquid MS medium with 100 mM CaCl₂, where they remained for 20 min for alginate polymerization and capsule formation. Encapsulated apices were transferred to sterile filter paper in Petri plates to drain excess moisture, and then incubated in liquid MS culture media with increasing concentrations of sucrose (0.3 M; 0.4 M; 0.5 M; 0.6 M; 0.7 M and 0.8 M) for 24 h each with agitation at 100 rpm at $25^{\circ}C \pm 2^{\circ}C$ in darkness. After each incubation period, capsules were transferred to sterile filter paper in Petri plates to eliminate excess moisture before culturing on recovery medium for 1 week in darkness, then in diffuse light (500 lux) at $25^{\circ}C \pm 2^{\circ}C$. Survival was evaluated after 4 weeks.

Capsule moisture content was determined after drying under the sterile air flow in a transfer chamber (data not shown) and a dehydration curve was established. Using the dehydration curve, two drying periods (3 and 3.5 h) corresponding to capsule moisture content of approximately 20% were selected. Encapsulated apices were dehydrated. One half of the encapsulated apices were cultured on recovery medium (–LN) and the remaining capsules were placed in 2 mL polypropylene cryotubes and immersed in LN (+LN) for 1h. Capsules were rewarmed quickly by plunging cryotubes in a 40°C water bath for 2 min. After each stage in the process, encapsulated apices were cultured on recovery medium at $25^{\circ}C \pm 2^{\circ}C$ in darkness for the first week, then in diffuse light for shoot induction. Survival was evaluated after 4 weeks. All treatments were repeated three times with 10 experimental units in each treatment. The experiment was repeated twice. Statistical analyses were performed using the general linear model procedure in the R program.

2.4. Vitrification of Cell Suspensions

Calli were established using the protocol developed by Alvarenga [9]. Leaf segments (1 cm²) from *in vitro* plants were placed on Gamborg B5 base medium [23] supplemented with 2 mg·L⁻¹ 2,4-D, 1 mg·L⁻¹ IBA, 30 g·L⁻¹ sucrose, 8.4 g·L⁻¹ agar, pH 5.7 (callus induction medium). Cultures were maintained in darkness at 25°C \pm 2°C for four weeks until friablecalli were obtained.

To initiate cell suspensions, 2.5 g callus were placed in 125 mL Erlenmeyer flasks containing 25 mL Gamborg medium complemented with 1 mg·L⁻¹ 2,4-D, 0.5 mg·L⁻¹ IBA, 20 g·L⁻¹ sucrose, pH 5.7 (cell suspension medium). Cultures were maintained at 25°C \pm 2°C for one week with a photoperiod of 16 h light (2000 lux) in an orbital shaker at 90 rpm. For maintenance and multiplication, suspensions were subcultured every 7 days, based on the growth curve (data not shown). A packed cell volume (PCV) of 7.5 mL was subcultured in 17.5 mL of fresh cell suspension medium in 125 mL Erlenmeyer flasks under the conditions described previously for suspension induction.

Once conditions for cell suspension culture and maintenance were established, cryopreservation treatments were tested. Suspensions were precultured for 24 h with agitation in cell suspension culture medium enriched with sucrose (0.15 M or 0.50 M). After preculturing, 950 μ L aliquots of the suspension were transferred to 1.5 ml polypropylene cryovials at 0°C and 50 μ L DMSO was added to each cryovial. Suspensions were incubated for 1 h, then immersed rapidly in LN and held in LN for 1 h. Cryotubes were removed from LN and warmed in a 40°C water bath for 2 min.

For recovery, suspensions were placedoncallus induction medium on sterile filter paper. The paper and cells

were transferred to fresh culture medium after 1 h, 24 h and 48 h to eliminate DMSO residues, then subcultured on fresh medium every 4weeks. Cultures were maintained at $25^{\circ}C \pm 2^{\circ}C$ in darkness. Survival was evaluated after one month as the percentage of cultures showing re-growth of cells.

Cell viability was analyzed using Evans blue stain [24] [25]. Immediately after each treatment, 500 μ L treated cells were placed in 1 mL Eppendorf tubes and incubated with 50 μ L Evans blue for 10 min, then centrifuged at 1000 rpm for 10 min. The supernatant was discarded and 500 μ L sterile distilled water was added and manually agitated. A 15 μ L sample was placed on a microscope slide and observed using a 10X light microscope to determine viability. Unstained cells were considered viable. Regeneration and viability tests were applied at each stage of the cryopreservation process with three repetitions.

2.5. Cryopreservation of Seeds

Cat's claw fruits were collected in order to extract seeds were extracted and seeds stored in a hermetically sealed glass bottle at 5°C for 2 months. Two methods of cryopreservation of seeds were evaluated: dehydration and vitrification. For the first method, seed moisture content was determined by the method recommended by ISTA [26], using 18 samples of 1000 seeds (approximately 0.1 g of seeds per sample). Seeds were immersed rapidly in LN and survival was determined as percent germination after 4 weeks. For vitrification experiments, 70 seeds were used per treatment. Incubation times in LS (0, 10 and 20 min) and PVS2 vitrification solution (0, 25 and 50 min) were evaluated. All treatments included a non-cryopreserved control. Seeds of U. tomentosa were placed in 1 mL polypropylene cryovials at 0°C and 1 mL LS was added. After each incubation period the solution was replaced with 1 mL PVS2. Seeds were cryopreserved by immersing the cryotubes in LN for 1 h, and then warmed by incubating in a 40°C water bath for 5 min. After warming, the vitrification solution was eliminated and seeds were washed three times for 2 min each with basic MS culture medium with 1.2 M sucrose at ambient temperature. Finally, seeds were placed in Petri plates for germination on three layers of paper towel with abundant water with a photoperiod of 16 h light (diffuse light 500 lux intensity)/8h dark at $25^{\circ}C \pm 2^{\circ}C$. Survival was evaluated after 6 weeks as the number of germinated seeds per plate. Each seed was an experimental unit and each treatment consisted of 70 experimental units with three repetitions. Data were compared using the General Linear model and multiple factor ANOVA.

Seed survival was also evaluated using 2,3,5-triphenyl tetrazoliumchloride (TTC) staining [27], in which red-stained seeds were considered viable. Ten seeds of each treatment described previously were placed on three layers of paper towel moistened with distilled water in a Petri plate for 24 h. Seeds were then transferred to a new plate with distilled water with 3% sucrose (m/v) for an additional 48 h at 30°C in darkness. Seeds were incubated in a 1% solution of TTC for 72 h under the same conditions, then observed using a stereomicroscope to count the number of viable stained seeds. Staining experiments were repeated five times.

2.6. Evaluation of Survival

Apex survival was evaluated as the percentage of green apices showing signs of growth at the time of evaluation, as recommended by Pence *et al.* [28]. Brown or darkened apices were considered dead. Two methods were used to evaluate survival of cell suspensions: re-growth of callus, and cell viability as determined by the presence of unstained cells after exposure to Evans blue. Seed survival was evaluated as percent germination and percent viability as determined by TTC staining (viable seeds were stained red).

3. Results

3.1. Apex Vitrification

Sucrose preculture treatments had no negative effect and apex survival after preculture was 100%. After incubation in LS for 10, 20 or 30 min, survival of apices precultured in 0.25 M sucrose for 24 h was 100, 95.5 and 95.5%, respectively, and approximately 90% for apices precultured in 0.5 M sucrose for an additional 24 h (Data not shown). Significant differences between means were not observed, and interactions between LS exposure time and preculture conditions were not significant (p = 0.33) using multiple factor ANOVA.

Using combined data for apices incubated in LS for 10 and 20 min followed by incubation for 10, 30 and 50 min in PVS2 vitrification solution, apex survival was observed to decrease with increasing exposure durations to PVS2, with a more marked decrease in survival when apices were precultured in 0.25 M sucrose for 24 h (Figure 1).



Figure 1. Effect of vitrification treatments on survival of cat's claw apices (*U. tomentosa*) with (+LN) and without (-LN) cooling in liquid nitrogen. Survival evaluated after 1 week of culture on recovery medium.

The most important interactions affecting apex survival were incubation in PVS2 ($p = 4.30 \times 10^{-5}$) and preculture in sucrose ($p = 4.76 \times 10^{-4}$). Differences were highly significant with respect to other factors (p < 0.05). Survival of cryopreserved apices (+LN) was highest (82.2%) for apices precultured in 0.25 M sucrose for 24 h, followed by incubation in LS for 20 min and PVS2 for 30 min (Figure 1).

3.2. Apex Encapsulation-Dehydration

Apex survival was not significantly different after isolation and encapsulation (92.0% and 94.2%, respectively). Survival of encapsulated apices precultured in increasing concentrations of sucrose (MS with 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 M sucrose) for 24 at each concentration, was greatest with treatments PT4 (MS + 0.3, 0.4, 0.5 and 0.6 M sucrose for 24 h at each concentration) and PT1 (0.3 M for 24 h), (78.0% and 71.4%) respectively. Differences in survival were not significant between PT2 (MS + 0.3 and 0.4 M sucrose for 24 h each) and PT3 (MS + 0.3, 0.4 and 0.5 M sucrose for 24 h each) (58.1% and 59.5% respectively) (Data not shown).

The moisture content of encapsulated apices was 22.7% and 20.3% after drying for 3.0 and 3.5 h, respectively (moisture curve not shown). These drying periods were evaluated with the preculture treatments which had previously shown the highest survival (PT4, PT1 and PT2, 47%, 64% and 60% respectively). In general, a decrease in apex survival was observed after dehydration. Survival was lowest for dehydrated encapsulated controls without sucrose pretreatments (PT0, 47%) (Figure 2).

After LN exposure, survival was statistically similar among all treatments and moisture contents evaluated. Survival ranged from 31.8% for PT0 (encapsulated apices without sucrose pretreatment and dehydrated to 22.7%) to 52.9% for PT2 (MS + 0.3 M and 0.4 M sucrose, 24 h at each concentration and the same water content) (Figure 3 & Figure 4).

3.3. Vitrification of Cell Suspensions

Both sucrose pretreatments (0.15 M and 0.5 M) alone or in combination with DMSO resulted in high survival and cell multiplication (**Table 1**). After cooling in LN, regrowth of cells and callus formation were observed in 61.1% of the cultures pretreated in 0.15 M and 0.50 M sucrose for 24 h and 5% DMSO for 1 h; however, calli pretreated with 0.5 M sucrose showed better cell growth (**Figure 5**).

Analysis of cell viability using Evans blue stain (**Table 1**) showed that preculture with different sucrose concentrations had little effect on viability; however, the subsequent steps of the cryopreservation process (pretreatment with 5% DMSO and LN exposure) resulted in a drastic reduction in viability. Although cell regrowth and viability were similar, linear regression analysis showed a 40.7% correlation between the two data groups,



Figure 2. Survival of encapsulated cat's claw apices (*Uncaria tomentosa*) without LN exposure (–NL): PT0 (no sucrose preculture), PT1 (precultured in 0.3 M sucrose for 24 h), PT2 (precultured in 0.3 M sucrose + 0.4 M sucrose for 24 h at each concentration) and PT4 (preculture in 0.3 M sucrose + 0.4 M sucrose + 0.4 M sucrose for 24 h at each concentration). Survival was evaluated after 4 weeks on recovery medium. Means with the same letter are not significantly different (p < 0.05).



Figure 3. Survival of encapsulated apices of cat's claw (*Uncaria tomentosa*) precultured in sucrose, desiccated under sterile air flow and immersed in LN (+LN): PT0 (without sucrose preculture), PT1 (preculture in 0.3 M sucrose for 24 h), PT2 (preculture in 0.3 M sucrose + 0.4 M sucrose for 24 h at each concentration) and PT4 (preculture in 0.3 M sucrose + 0.4 M sucrose + 0.5 M sucrose + 0.6 M sucrose for 24 h at each concentration). Survival was evaluated after 4 weeks on recovery medium. Means with the same letter are not significantly different (p < 0.05).



Figure 4. Regeneration of encapsulated cat's claw apices (*Uncaria tomentosa*) precultured in (a) PT1 (MS + 0.3 M sucrose for 24 h), (b) PT2 (MS + 0.3 M + 0.4 M sucrose for 24 h at each concentration), (c) PT4 (MS + 0.3 M, 0.4 M, 0.5 M, 0.6 M sucrose for 24 h at each concentration). Recovery after LN exposure evaluated after 4 weeks on recovery medium.



Figure 5. Growth of cat's claw cells (*Uncaria tomentosa*) on recovery media after preculture in sucrose for 24 h, pretreatment with 5% DMSO for 1 h and cooling in LN. Preculture in (a) 0.15 M sucrose, (b) 0.50 M sucrose. Growth evaluated after 4weeks on callus induction medium.

 Table 1. Callus formation and cell viability in cat's claw (Uncaria tomentosa) cultures after preculture in sucrose, pretreatment with 5% DMSO without (-LN) or with (+LN) LN exposure.

Preculture in sucrose (M)	Incubation in DMSO(%)	Re-growth of cells/culture (%) \pm S.E.		Viable cells/field of view (%) \pm S.E.	
		-NL	+NL	-NL	+NL
0.15	0	72.2 ± 2.8	-	92.3 ± 6.5	-
	5	72.2 ± 2.8	61.1 ± 3.9	73.7 ± 13.5	30.1 ± 7.3
0.50	0	61.1 ± 3.9	-	75.8 ± 1.0	-
	5	72.2 ± 2.8	61.1 ± 3.9	58.4 ± 7.6	22.0 ± 5.8

as shown by the Pearson coefficient ($R^2 = 0.407$).

3.4. Cryopreservation of Seeds

Germination of seeds with a moisture content of $8.7\% \pm 1.84\%$ was 92.5%. After LN exposure, germination was 89.5%. Using the vitrification method for seed storage, the factor with the greatest significantly positive effect on seed survival was exposure to LS for 20 min (p = 0.0131). Exposure to LS for 10 min was not as effective unless followed by incubation in PVS2 for 50 min (p = 0.0290) (Figure 6). Survival of seeds incubated in LS for 20 min increased with incubation in PVS2 (70% and 78% survival after incubation in PVS2 for 0 or 25 min, respectively). However, survival decreased with exposure to PVS2 for 50 min (Figure 6 & Figure 7). Although seed germination after vitrification was high, values were lower than those observed with desiccation and rapid cooling in LN (Table 2). No correlation was observed between seed germination and viability as determined by TTC staining (R² = 0.0).

4. Discussion

For cryopreservation of apices, preculture in sucrose and incubation in PVS2 were the most important factors. As shown by Suzuki *et al.* [29], osmotic compounds are able to transmit signals that induce tolerance to dehydration, thereby providing greater tolerance to LN exposure. The sucrose concentrations evaluated in this study (0.25 and 0.50 M for 24 h each) coincide with those used by Ching *et al.* [30] for storage of protocorms of *Dendrobium* spp. in LN. Sucrose is the most frequently used carbon source in culture media for plant growth and is also widely used as an osmotic in cryoprotection to stabilize cell membranes and maintain tissue turgor [31].

In this study, incubation in PVS2 had a positive effect on survival of cryopreserved apices; however, survival decreased with increased exposure duration to the solution. When the vitrification technique is used, control of exposure times and concentrations of the compounds in vitrification solutions is recommended [32], since long incubation times may lead to tissue deterioration due to excessive dehydration and toxicity. PVS2 solution consists of a mixture of cryoprotectants such as glycerol, sucrose, ethylene glycol and DMSO which prevent the formation of ice crystals in tissues during rapid cooling [33]. However, depending on the explant and the exposure



Figure 6. Response of control (-LN) and cryopreserved (+LN) cat's claw (*Uncaria tomentosa*) seeds using the vitrification technique. Germination was evaluated 6 weeks after warming.



Figure 7. Germination of cat's claw (*Uncaria tomentosa*) seeds cryopreserved in liquid nitrogen (NL) using the desiccation technique. Germination was evaluated after 6 weeks on germination culturemedium.

Table 2. Viability and regeneration of cat's claw (*Uncaria tomentosa*) seeds without (-LN) or with (+LN) LN exposure, as determined by triphenyltetrazolium chloride (TTC) staining.

Cryopreservation treatments		Viability (%) ± S.E. (%)		Germination (%) \pm S.E.	
LS (min)	PVS2 (min)	-NL	+NL	-NL	+NL
0	0	74.0 ± 4.9	64.0 ± 4.9	92.5 ± 1.6	89.5 ± 2.6
10	0	78.0 ± 4.9	54.0 ± 4.9	81.5 ± 2.9	71.4 ± 3.9
	25	68.0 ± 4.9	60.0 ± 4.9	72.4 ± 4.8	67.6 ± 4.2
	50	72.0 ± 4.9	54.0 ± 4.9	64.0 ± 5.2	73.8 ± 3.1
20	0	48.0 ± 4.9	64.0 ± 4.9	73.3 ± 5.5	71.9 ± 4.6
	25	64.0 ± 4.9	70.0 ± 4.9	71.9 ± 4.6	78.1 ± 1.7
	50	62.0 ± 4.9	58.0 ± 4.9	77.6 ± 7.1	70.8 ± 3.2

^{*}S.E. Standard Error. Means with the same letter are not significantly different (p < 0.05).

time, cytotoxic effects may be encountered. Modification of the composition of the vitrification solution by increasing or reducing concentrations of its components should be based on the response of the tissues to be preserved [34]-[36].

For encapsulation-dehydration, an optimum range of 20% - 30% moisture has been recommended by many authors for explants encapsulated for cryopreservation, regardless of the species [16] [18]. Moisture content within this range allows vitrification of cell contents during LN exposure without the formation of lethal ice crystals [37]. The moisture contents obtained in the present study were within this optimum range and based on the results, the vitrification process was adequate.

Osmotic dehydration has a basic cryoprotective function as it allows gradual tissue dehydration by concentrating solutes in the cell cytoplasm without drastic water losses over a short time period [38]. During the process, samples are placed in a solution with greater osmotic pressure, which simultaneously causes diffusion of water from the sample towards the solution and solutes towards the sample [39] [40]. The most commonly used osmotic agents are glucose, inositol and sucrose; however, sucrose is considered to be the best cryoprotectant agent [15] [41] [42]. Although sucrose has an important function in cryopreservation, some treatments can be harmful, either because of the concentration or the exposure time in different preculture media. High concentrations of sucrose can increase the formation of phenolic compounds in explants and promote wilting through oxidation, thereby reducing survival [19] [43]. This appears to have occurred in the present study, as survival decreased with additional incubation at higher sucrose concentrations (PT4, 0.3, 0.4, 0.5 and 0.6 m sucrose for 24 h each), with respect to the treatment that included only preculture at 0.3 and 0.4 M sucrose for 24 h at each concentration and dehydration to 22.7% moisture prior to LN exposure.

Moisture content is important at the cellular level for phospholipid arrangement and protein shape. However, physiological moisture content can produce lethal ice crystals during cooling in LN as well as during warming [41] [44]. The moisture contents evaluated in this study were adequate for survival of cat's claw apices. These results are consistent with research obtained by Gupta and Reed [45] for cryopreservation of blackberry and raspberry. These authors observed a correlation between moisture content and survival of encapsulated apices, with maximum survival at moisture content near 20%.

Quiaochum *et al.* [42] showed that the increase in survival of tissues precultured at high concentrations of an osmoticum was due to the increase in the concentration of soluble proteins and sugars in treated tissues, which facilitated vitrification of the intracellular contents when the temperature was lowered. Optimization of exposure periods and concentrations of sucrose or other osmoticums used as additives to stimulate adaptation and resistance to cryopreservation must be genotype-specific [41]. Multiple variations of preculture conditions should be evaluated for each species.

Survival of cells cryopreserved in the elongation stage is unlikely because the highly hydrated state favors intracellular freezing and the formation of lethal ice crystals. Ice crystals cause mechanical damage to the cells, while osmotic stress produces an imbalance of available water in the cytoplasm [46] [47]. For these reasons, the use of cells in the early exponential phase, when intracellular solutes are more concentrated and cells are smaller, is recommended for cryopreservation of cell suspensions [48] [49].

Survival and proliferation of cryoperserved cells varies depending on the mixture and concentrations of cryoprotectants used in pretreatments. In this study, preculture in 0.15 and 0.5 M sucrose for 24 h followed by incubation in 5% DMSO resulted in a high rate of survival, as evaluated by cell regrowth and staining with Evans blue. However, cells precultured in 0.5 M sucrose showed faster and more vigorous growth during recovery after LN exposure, which indicated that cells that withstood this treatment were better prepared for cooling and warming.

According to Aguilar *et al.* [48], a combination of two cryoprotectants is more effective than only one. A nonpenetrating cryoprotectant such as sucrose tends to be used in association with a penetrating cryoprotectant such as DMSO [50], and various studies have shown that the mixture of these two cryoprotectants is very efficient [49]. Sucrose promotes rapid cell dehydration and DMSO inhibits the formation of ice crystals that could rupture the membrane. Results obtained in the present study are similar to those reported by Hermoso and Méndez [51] for *Coffea arabica*, which belongs to the same family as *U. tomentosa* (Rubiaceae). These authors found that the use of 0.15 M sucrose and 5% DMSO was the best treatment for cryopreservation of suspensions of coffee. Similar results were obtained by Aguilar *et al.* [48] with this combination of cryoprotectants for *Citrus deliciosa*.

Dehydration is the simplest procedure for cryopreservation since it consists of dehydrating explants and then

cooling them rapidly by direct immersion in LN. This technique is mainly used with seeds, zygotic embryos or embryonic axes extracted from seeds, and has been applied to a large number of recalcitrant and intermediate species [18] [52]. In the present study, the moisture content of cat's claw seeds used for cryopreservation was 8.7% and germination was 89.5% after cooling in LN. These results indicate that the seeds with stand drying to relatively low moisture content which allows the seed to survive after LN exposure. These results are similar to those obtained with coffee seeds and embryos [53]-[55].

Using the vitrification technique for cryopreservation of seeds, exposure to LS for 20 min was the main factor affecting survival in the present study. Seeds subjected to vitrification survived cooling in LN, but vitrification was not more effective than desiccation. Germination was lower using vitrification and the requirements for labor and materials were much higher. These results coincide with those obtained by Prada *et al.* [56] for the cryopreservation of seeds and embryos of *Jatropha curcas* in which survival and development were better for seeds that were subjected to desiccation only.

Staining methods are frequently used to determine viability of cells and tissues that have been subjected to various stress factors, including LN exposure. Staining is fast and inexpensive and results can be used to predict the success of treatments [57] [58]. Staining with triphenyltetrazolium chloride (TTC), a colorless compound that is reduced to red-colored formazan by dehydrogenase enzymes in the mitochondria of living cells, is used mainly in seeds, embryos and meristems; however, the reliability of this test can be affected by other inorganic reactions that increase the production of formazan [58] [59]. The viability test using Evans blue allows differentiation of live and dead cells based on the selective permeability of the cellular membrane. As the stain cannot cross the membrane of living cells, only damaged or dead cells take on the blue color [24] [25] [60]. In this study, staining to determine viability showed survival of both cells and seeds cryopreserved in LN; however, in all cases, viability as determined by germination and re-growth of cells was higher than that shown by staining, confirming that the staining tests can only be used as a guideline.

According with results obtained on this study, cryopreservation of apex, seeds and cells is possible, and techniques usually used in other species are effective on *Uncaria tomentosa*.

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References

- Pilarski, R., Filip, B., Wietrzyk, J., Kuras, M. and Gulewicz, K. (2010) Anticancer Activity of the Uncaria tomentosa (Willd.) DC. Preparations with Different Oxindole Alkaloid Composition. *Phytomedicine*, **17**, 1133-1139. http://dx.doi.org/10.1016/j.phymed.2010.04.013
- [2] Díaz, C. and Vargas, M. (2012) Estudio de validación preclínica del uso tradicional anti-tumoral de *Uncaria tomentosa* (Uña de gato). *Revista Médica de la Universidad de Costa Rica*, **6**, 24-31.
- [3] Dietrich, F., Kaiser, S., Rokenbach, L., Figueró, F., Scussel, L., Monte da Cunha, F. and Oliveira, A. (2014) Quinovic Acid Glycosides Purified Fraction from *Uncaria tomentosa* Produces Cell Death by Apoptosis in the T24 Human Bladder Cancer Cell Line. *Food and Chemical Toxicology*, 67, 222-229. <u>http://dx.doi.org/10.1016/j.fct.2014.02.037</u>
- [4] Xi, Y., Hongyi, S., Ye, L., Takashi, S., Syotarou, K., Shin, T. and Zhenya, Z. (2014) Anti-Cancer Activity Comparisons of Aqueous Extracts from *Inonotus Obliquus*, *Cordyceps militaris* and *Uncaria tomentosa in Vitro* and *in Vivo*. *Journal of Pharmacognosy and Phytochemistry*, 2, 19-25.
- [5] Aguilar, J., Rojas, P., Marcelo, A., Plaza, A., Bauer, R., Reininger, E. and Merfort, I. (2002) Anti-Inflamatory Activity of Two Different Extracts fo Uncaria tomentosa (Rubiaceae). *Journal of Ethnopharmacology*, 81, 271-276. <u>http://dx.doi.org/10.1016/S0378-8741(02)00093-4</u>
- [6] Feria, I., Lazo, E., Ponce, T., Cerda, C. and Ramos, A. (2005) Induced Accumulation of Oleanolic Acid and Ursolic Acid in Cell Suspension Cultures of *Uncaria tomentosa*. *Biotechnology Letters*, 27, 839-843. http://dx.doi.org/10.1007/s10529-005-6215-7
- [7] Caon, T., Kaiser, S., Feltrin, C., de Carvalho, A., Marques, T., González-Ortega, G. and Oliveira-Simoes, C. (2014) Antimutagenic and Antitherpetic Activities of Different Preparations from *Uncaria tomentosa* (Cat's Claw). *Food and*

Chemical Toxicology, 66, 30-55.

- [8] Canales-Springett, A., Ceroni, A., Domínguez, G. and Castillo, A. (2013) Respuesta de la regeneración natural de la Uncaria tomentosa (Willd) D.C. "uña de gato", al efecto de la luz en ecosistemas boscosos primarios intervenidos dentro del Bosque Nacional Alexander Von Humboldt, Pucallpa—Perú. Ecología Aplicada, 12, 99-109.
- [9] Alvarenga, S. (2010) Establecimiento *in Vitro* y cultivo de células de la Uña de Gato (Uncaria tomentosa)(Willd.) D.C. *Tecnología en Marcha*, 23, 24-33.
- [10] Huerta-Heredia, A., Marín-López, R., Ponce-Noyola, T., Cerda-García-Rojas, C., Trejo-Tapia, G. and Ramos-Valdivia, A. (2009) Oxidative Stress Induces Alkaloid Production in *Uncaria tomentosa* Root and Cell Cultures in Bioreactors. *Engineering in Life Sciences*, 9, 211-218. <u>http://dx.doi.org/10.1002/elsc.200800118</u>
- [11] Sonwa, D., Weise, S., Nkongmeneck, A., Nwaga, D., Zapfack, L., Nzooh, L. and Janssens, M. (2005) Potential Contributions of Biotechnologies in the Management and Conservation of Forest Resources of the Congo Basin. *International Forestry Review*, 7, 59-62. <u>http://dx.doi.org/10.1505/ifor.7.1.59.64162</u>
- [12] Keller, J. and Senula, A. (2010) Cryopreservation of Plant Germplasm. In: Davey, M. and Anthony, P., Eds., *Plant Cell Culture: Essential Methods*, Wiley-Blackwell, Oxford, 131-151. <u>http://dx.doi.org/10.1002/9780470686522.ch8</u>
- [13] Barnicoat, H., Cripps, R., Kendon, J. and Sarasan, V. (2011) Conservation in Vitro of Rare and Threatened Ferns— Case Studies of Biodiversity Hotspot and Island Species. In Vitro Cellular & Developmental Biology—Plant, 47, 37-45. <u>http://dx.doi.org/10.1007/s11627-010-9303-x</u>
- [14] Cruz-Cruz, C., González-Arnao, M. and Engelmann, F. (2013) Biotechnology and Conservation of Plant Biodiversity. *Resources*, 2, 73-95. <u>http://dx.doi.org/10.3390/resources2020073</u>
- [15] Burrit, D. (2008) Efficient Cryopreservation of Adventitious Shoots of Begonia X Erythrophylla Using Encapsulation-Dehydration Requires Pretreatment with Both ABA and Proline. *Plant Cell, Tissue and Organ Culture*, 95, 209-215. <u>http://dx.doi.org/10.1007/s11240-008-9434-5</u>
- [16] Ai, P.-F., Lu, L.-P. and Song, J.-J. (2011) Cryopreservation of in Vitro-Grown Shoot-Tips of *Rabdosia rubescens* by Encapsulation-Dehydration and Evaluation of Their Genetic Stability. *Plant Cell, Tissue and Organ Culture*, **108**, 381-387.
- [17] Wang, Z. and Deng, X. (2004) Cryopreservation of Shoot Tips of Citrus Using Vitrification: Effect of Reduced form of Glutathione. *CryoLetters*, 25, 51-58.
- [18] Engelmann, F. (2011) Use of Biotechnologies for the Conservation of Plant Biodiversity. In Vitro Cellular & Developmental Biology—Plant, 47, 5-16. <u>http://dx.doi.org/10.1007/s11627-010-9327-2</u>
- [19] González-Arnao, M.T. and Englemann, F. (2013) Introducción a la conservación ex situ de los recursos genéticos vegetales. En González-Arnao, M.T. and Englemann, F., Eds., *Crioconservación de plantas en América Latina y el Caribe*, Instituto Interamericano de Cooperación para la Agricultura, San José, 39-48.
- [20] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissues Cultures. *Physiologia Plantarum*, 15, 473-497. <u>http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x</u>
- [21] Sakai, A., Kobayashi, S. and Oiyama, I. (1990) Cryopreservation of Nucellar Cells of Navel Orange (*Citrus sinensis* Osb. Var. brasiliensis Tanaka) by Vitrification. Plant Cell Reports, 9, 30-33. <u>http://dx.doi.org/10.1007/BF00232130</u>
- [22] Fabre, J. and Dereuddre, J. (1990) Encapsulation Dehydration—A New Approach to Cryopreservation of *Solanum* Shoot-Tips. *CryoLetters*, **11**, 413-426.
- [23] Gamborg, O., Miller, R. and Ojima, K. (1968) Nutrients Requirements of Suspension Cultures of Soybean Root Cells. *Experimental Cell Research*, **50**, 151-168. <u>http://dx.doi.org/10.1016/0014-4827(68)90403-5</u>
- [24] Somer, J., Evans, G. and Shwartz, C. (1972) Influence of Experimental Aortic Coarctation on the Pattern of Aortic Evans Blue Uptake *in Vivo. Atherosclerosis*, 16, 127-133. <u>http://dx.doi.org/10.1016/0021-9150(72)90015-9</u>
- [25] Xi, H., von Rad, U. and Durner, J. (2002) Nitric Oxide Induces Transcriptional Activation of the Nitric Oxide-Tolerant Alternative Oxidase in *Arabidopsis* Suspension Cells. *Planta*, **215**, 914-923. http://dx.doi.org/10.1007/s00425-002-0828-z
- [26] International Seed Testing Association (ISTA) (2008) International Ruler for Seed Testing: The Germination Test. Seed Science and Technology, 7.
- [27] Stemponkus, P. and Lamphear, F. (1967) Refinement of the Triphenyl Tetrazolium Chloride Method of Determining Cold Injury. *Plant Physiology*, **42**, 1423-1426. <u>http://dx.doi.org/10.1104/pp.42.10.1423</u>
- [28] Pence, V., Winget, G., Lindsey, K., Plair, B. and Charls, S. (2009) In Vitro Propagation, Cryopreservation, and Genetic Analysis of the Endangered Hedeoma todsenii (Lamiaceae). Madroño, 56, 221-228. <u>http://dx.doi.org/10.3120/0024-9637-56.4.221</u>
- [29] Suzuki, M., Tandon, P., Ishikawa, M. and Toyomasu, T. (2008) Development of a New Vitrification Solution, VSL, and Its Application to the Cryopreservation of Gentian Axillary Buds. *Plant Biotechnology Reports*, **2**, 123-131.

http://dx.doi.org/10.1007/s11816-008-0056-5

- [30] Ching, L., Anthony, J., Poobathy, R. and Subramaniam, S. (2012) Encapsulation-Vitrification of Dendrobium Sonia-28 Supported by Histology. *Plant OMICS*, 5, 345-350.
- [31] Sharaf, S., Shibli, M., Kasrawi, S. and Baghdadi, S. (2011) Cryopreservation of Wild Shih (*Artemisa herba-alba* Asso.) Shoot-Tips by Encapsulation-Dehydration and Encapsulation-Vitrification. *Plant Cell, Tissue and Organ Culture*, **108**, 437-444.
- [32] García, L., De Feria, M. and Acosta, K. (2007) Aspectos básicos de la conservacion in vitro de germoplasma vegetal. *Biotecnología Vegetal*, 7, 67-79.
- [33] Scoochi, A. and Rey, H. (2010) Conservación de germoplasma in vitro. En: Levitus, G., Echenique, V., Rubinstein, C., Hoop, E. and Mroginski, L., Eds., *Biotecnología y Mejoramiento Vegetal II Argentina*, Ediciones Instituto Nacional de Tecnología Agropecuaria, Buenos Aires, 179-185.
- [34] Zhou, Q.-N., Sun, A.-H., Li, Z., Hua, Y.-W., Jiang, Z.-H., Huang, T.-D. and Huang, H.-S. (2012) Cryopreservation and Plant Regeneration of Anther Callus in Hevea by Vitrification. *African Journal of Biotechnology*, **11**, 7212-7217.
- [35] García-Rojas, T. and Abdelnour-Esquivel, A. (2013) Crioconservación de ápices y semillas de cedro (*Cedrela odorata* L.) mediante las técnicas de vitrificación y deshidratación. *Agronomía Costarricense*, 37, 113-126.
- [36] Harding, K. and Benson, E. (2015) Writing Standard Operating Procedures (SOPs) for Cryostorage Protocols: Using Shoot Meristem Cryopreservation as an Example. *Methods in Molecular Biology*, **1257**, 431-456. <u>http://dx.doi.org/10.1007/978-1-4939-2193-5_22</u>
- [37] Surenciski, M., Flachsland, E., Terada, G., Mroginski, L. and Rey, H. (2012) Cryopreservation of Cyrtopodium hatschbachii Pabst (Orchidaceae) Immature Seeds by Encapsulation-Dehydration. Biocell, 36, 31-36.
- [38] Mikula, A., Tomiczak, K. and Rybczyński, J. (2011) Cryopreservation Enhances Embryogenic Capacity of *Gentiana cruciata* (L.) Suspension Culture and Maintains (Epi)genetic Uniformity of Regenerants. *Plant Cell Reports*, **30**, 565-574. <u>http://dx.doi.org/10.1007/s00299-010-0970-1</u>
- [39] Abbasi, B., Ghaffari, A. and Bayat, Y. (2012) Mathematical Modeling of Moisture and Solute Diffusion in the Cylindrical Green Bean during Osmotic Dehydration in Salt Solution. *Food and Bioproducts Processing*, 90, 64-71. http://dx.doi.org/10.1016/j.fbp.2010.11.015
- [40] Kumar, V., Kumar, G. and Sharma, P. (2012) Osmotic Dehydration of Litchi Pulp as a Pretreatment for Drying Processes. *Agricultural Engineering International: CIGR Journal*, **14**, 146-151.
- [41] Day, J. and Stacey, G. (2007) Cryopreservation and Freeze-Drying Protocols. Humana Press, Totowa. http://dx.doi.org/10.1007/978-1-59745-362-2
- [42] Qiaochum, W., Laamanen, J., Uosukainen, M. and Valkonen, M. (2005) Cryopreservation of in Vitro-Grown Shoot Tips of Raspberry (*Rubus idaeus* L.) by Encapsulation-Vitrification and Encapsulation-Dehydration. *Plant Cell Reports*, 24, 280-288. <u>http://dx.doi.org/10.1007/s00299-005-0936-x</u>
- [43] Poobathy, R., Xavier, R., Sinniah, U. and Subramaniam, S. (2013) Molecular Stability of Protocorm-Like Bodies of Dendrobium Sonia-28 after Encapsulation-Dehydration and Vitrification. *Australian Journal of Crop Science*, 7, 189-195.
- [44] Gogoi, K., Kumaria, S. and Tandon, P. (2013) Cryopreservation of *Cymbidium eburneum* Lindl. and *C. Hookerianum* Rchb. f., Two Threatened and Vulnerable Orchids via Encapsulation Dehydration. *In Vitro Cellular & Developmental Biology—Plant*, 49, 248-254. <u>http://dx.doi.org/10.1007/s11627-013-9505-0</u>
- [45] Gupta, S. and Reed, B. (2006) Cryopreservation of Shoot Tips of Blackberry and Raspberry by Encapsulation-Dehydration and Vitrification. *CryoLetters*, **27**, 29-42.
- [46] Azcon, J. (2008) Fundamentos de Fisiología Vegetal. 2nd Edition, McGraw-Hill/Interamericana de España, S.A., España.
- [47] Woods, E., Benson, J., Agca, Y. and Crister, J. (2004) Fundamental Cryobiology of Reproductive Cells and Tissues. *Cryobiology*, 48, 146-156. <u>http://dx.doi.org/10.1016/j.cryobiol.2004.03.002</u>
- [48] Aguilar, M., Engelmann, F. and Michaux-Ferriere, N. (1993) Cryopreservation of Cell Suspensions of *Citrus deliciosa* Tan and Histological Study. *CryoLetters*, 14, 217-228.
- [49] Kobayashi, T., Niino, T. and Kobayashi, M. (2005) Simple Cryopreservation Protocol with an Encapsulation Technique for Tobacco BY-2 Suspension Cell Cultures. *Plant Biotechnology*, 22, 105-112. http://dx.doi.org/10.5511/plantbiotechnology.22.105
- [50] Ávila, L., Madero, J., López, C., León, M., Acosta, L., Gómez, C. and Delgado, L. (2006) Fundamentos de criopreservación. *Revista Colombiana de Obstetricia y Ginecología*, 54, 291-300.
- [51] Hermoso, L. and Méndez, A. (2000) Multiplicación masiva del café (*Coffea arabica* L. cv. Catimor) mediante el cultivo de suspensiones celulares embriogénicas. *Acta Científica Venezolana*, **51**, 90-95.

- [52] Abdelnour-Esquivel, A., Rojas, G. and Alfaro, U. (2007) Estudios preliminares para la crioconservación de especies forestales arbóreas. *Tecnología en marcha*, 20, 98-103.
- [53] Eira, M., da Silva, E.A., De Castro, R.D., Dussert, S., Walters, C., Bewley, D. and Hilhorst, H.W. (2006) Coffee Seed Physiology. *Brazilian Journal of Plant Physiology*, 18, 149-163. http://dx.doi.org/10.1590/S1677-04202006000100011
- [54] Dussert, S., Chabrillange, N., Engelmann, F., Anthony, F., Louarn, J. and Hamon, S. (1998) Cryopreservation of Four Coffee Species (*Coffeaarabica, C. costatifructa, C. racemosa* and *C. sessiliflora*): Importance of Water Content and Cooling Rate. Seed Science Research, 8, 9-15. <u>http://dx.doi.org/10.1017/S096025850000386X</u>
- [55] Abdelnour-Esquivel, A., Villalobos, V. and Engelmann, F. (1992) Cryopreservation of Zygotic Embryos of *Coffea spp. CryoLetters*, 13, 297-302.
- [56] Prada, J., Aguilar, M.E., Abdelnour-Esquivel, A. and Engelmann, F. (2015) Cryopreservation of Seeds and Embryos of Jatropha curcas L. American Journal of Plant Sciences, 6, 172-180. <u>http://dx.doi.org/10.4236/ajps.2015.61020</u>
- [57] De Souza, C.R., Ohlson, O.C., Araújo, M.I. and Panobianco, M. (2010) Tetrazolium Test for Evaluating Triticale Seed Viability. *Revista Brasileira de Sementes*, **32**, 163-169. <u>http://dx.doi.org/10.1590/S0101-31222010000300018</u>
- [58] Khoddamzadeh, A.A., Sinniah, U.R., Lynch, P., Kadir, M.A., Kadzimin, S.B. and Mahmood, M. (2011) Cryopreservation of Protocorm-Like Bodies (PLBs) of *Phalaenopsis bellina* (Rchb.f.) Christenson by Encapsulation-Dehydration. *Plant Cell, Tissue and Organ Culture*, **107**, 471-481. <u>http://dx.doi.org/10.1007/s11240-011-9997-4</u>
- [59] Verleysen, H., Samyn, G., Bockstaele, E.V. and Debergh, P. (2004) Evaluation of Analytical Techniques to Predict Viability after Cryopreservation. *Plant Cell, Tissue and Organ Culture*, **77**, 11-21. http://dx.doi.org/10.1023/B:TICU.0000016483.00158.a9
- [60] Hamer, P.W., McGeachie, J.M., Davies, M.J. and Grounds, M.D. (2002) Evans Blue Dye as an *in Vitro* Marker of Myofibre Damage: Optimising Parameters for Detecting Initial Myofibre Membrane Permeability. *Journal of Anatomy*, 200, 69-79. <u>http://dx.doi.org/10.1046/j.0021-8782.2001.00008.x</u>