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EFFECT OF PRETREATMENT CONDITIONS ON THE CRYOPRESERVATION OF *IN VITRO*-CULTURED YAM (*DIOSCOREA ALATA* 'BRAZO FUERTE' AND *D. BULBIFERA* 'NOUMÉA IMBORO') SHOOT APICES BY ENCAPSULATION-DEHYDRATION.

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Summary. Apical shoot tips of *in vitro* plantlets of two edible yam species were subjected to a wide range of pretreatment conditions prior to cryopreservation using the encapsulation-dehydration technique. For *D. alata,* the highest survival rate (up to 67%) was obtained after a 3 to 10-day pretreatment with 0.9M and 1M sucrose followed by 11 to 16h. of silica gel-dehydration, rapid cooling in liquid nitrogen and, after rewarming, subculture for 35d. on medium supplemented with growth regulators. For *D. bulbifera*, optimal survival (up to 65%) was achieved following pretreatment with sucrose concentrations higher than 0.75M, regardless of the pretreatment duration, and 14 to 16h of dehydration. Recovery of frozen apices into plantlets was achieved after three months of culture on growth regulator-free medium, at a rate of 19% and 60% for *D. alata* and *D. bulbifera*, respectively.

Key words: cryopreservation; encapsulation-dehydration; recovery of frozen apices into plantlets, residual water content; silica gel-dehydration; sucrose-pretreatment; survival.

Abbreviations

ANOVA, analysis of variance; BA, 6-benzyladenine; cps, centipoise; DW, dry weight; FW, fresh weight; IPGRI : International Plant Genetic Resources Institute ; LN, liquid nitrogen; MS, Murashige and Skoog medium (16) ; NAA, naphthalene acetic acid; ORSTOM : Institut français de recherche scientifique pour le développement en coopération ; PAR, Photosynthesis Active Radiations.

Introduction

Cryopreservation represents an important tool for the safe long term conservation of genetic resources of recalcitrant seed and vegetatively propagated plant species. Various techniques have been set up for numerous temperate (20) and tropical (8) plant species. In the case of vegetatively propagated plant species, shoot apices represent the material of choice for cryopreservation.

In the case of yam, the first cryopreservation experiments were performed with cell suspensions of medicinal yam *D. deltoïdea* Wall. (4, 11). In 1994, Chulafich et al. (5) obtained regrowth of cryopreserved callus cultures of two *Dioscorea* species (*D. balcanica* Kosanin, *D. caucasica* Lipsky). More recently, cryopreservation of *in vitro* shoot apices of four yam species (*D. alata* L., *D. bulbifera* L., *D. wallichii* Hook. f, *D. floribunda* Mart.

& Gal.), using the encapsulation-dehydration technique (7, 10), was reported by Mandal et al. (14).

In this paper, the influence of various parameters in the pretreatment (sucrose concentration, duration of sucrose-pretreatment and dehydration) on the tolerance to rapid freezing in liquid nitrogen of encapsulated shoot apices of two yam species (*D. alata, D. bulbifera* cultivated type) is described. These two species belong to two different botanical sections of the genus *Dioscorea*, namely *Enantiophyllum* in the case of *D. alata* and *Opsophyton* in the case of *D. bulbifera*. The aim of this work was to define the experimental limits for the parameters studied which could then be used as a basis for the cryopreservation of other yam genotypes.

Material and Methods

Plant material

Two clones from the *in vitro* collection of yam germplasm maintained at ORSTOM Montpellier (12) were used. The cultivars used were 'Brazo fuerte' for *D. alata*, and 'Nouméa Imboro' for *D. bulbifera*. For the experiment, the mother microplants were obtained by nodal cuttings in 1l glass jars (5 to 10 nodal cuttings by jar) filled with 200ml 'MS standard medium' consisting of MS nutrient salts supplemented with Morel and Wetmore vitamins, and containing 5 % (w/v) sucrose, 0.2 % (w/v) activated charcoal and 7g.l^{-1} agar ; the jars were closed by a twist-off plastic cap (13). When mother microplants were 5 to 8 months old, they were then multiplied for massive shoot production (50 to 150 nodal cuttings per flask) ; after 18±4 days culture, apices were excised from the young growing shoots under a stereo microscope (Fig. 1A). All cultures were maintained under the following standard environmental conditions : at 27 ± 1°C, under a light intensity of 36 $\mu E.m^{-2}\ s^{-1}$ (PAR), with a 12h light/12h dark photoperiod.

Encapsulation and sucrose pretreatment

Excised apical shoot tips (2 to 5mm long) were placed overnight in Petri dishes on solidified 'MS standard medium' containing 5 % (w/v) sucrose.

Apices were then encapsulated in 3 % (w/v) Ca^{2+}/alginate (7, 10) (Fig. 1B). Sodium alginate (Sigma, 250 cps) and 100mM Ca^{2+} solutions were prepared in liquid MS standard medium as above. Encapsulated apices were pretreated for 3 to 10d (*D. alata*) or to 13d (*D. bulbifera*) in liquid MS standard medium with various sucrose concentrations (0.75, 0.9, 1 and 1.1M), in 125ml Erlenmeyer flasks, on a rotary shaker set at 91 rpm, under standard environmental conditions.

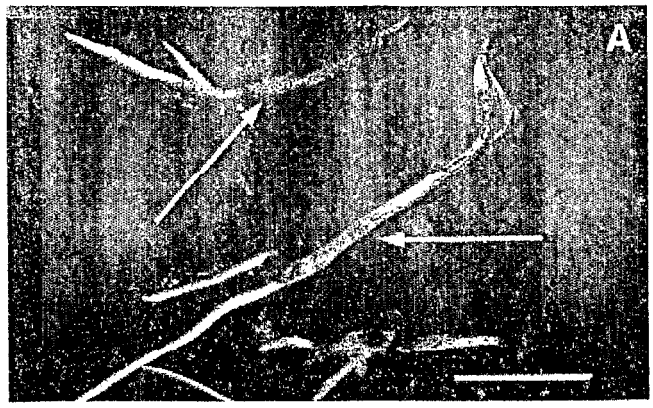
Figure 1 A-F: Shoot tips at different stages of the cryopreservation process.

Plant material production for excision process : (**A**) 3 week-old shoots of *D. alata*, before excision of the apices (arrow). *Bar 1 cm.*

Encapsulation and development of controls B-D : (**B**) Apical shoot-tips of *D. alata* encapsulated in alginate beads, before treatment (*Bar 1 mm*); (**C**) Shoot development of *D. alata* control (-LN), 5 weeks after a sucrose pretreatment (*Bar 1 mm*); (**D**) Swelling, callusing and bud development of *D. bulbifera* control (-LN), 5 weeks after dehydration. (*Bar 1 mm*).

Cryopreservation in liquid nitrogen (+LN): (**E**): Rooting and leaves production (*D. bulbifera*). *Bar 1 mm.*

Recovery after liquid nitrogen treatment (+LN) : (**F**): Recovery into leafy rooted shoots: *D. alata* (left) and *D. bulbifera* (right). *Bar 1 cm.*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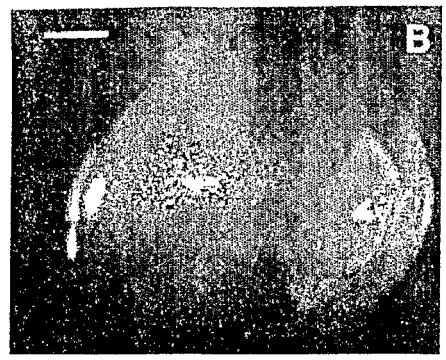
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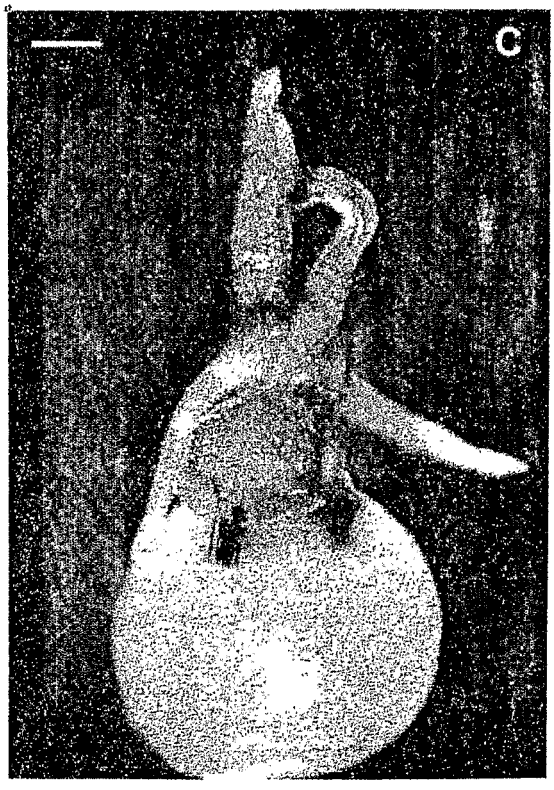
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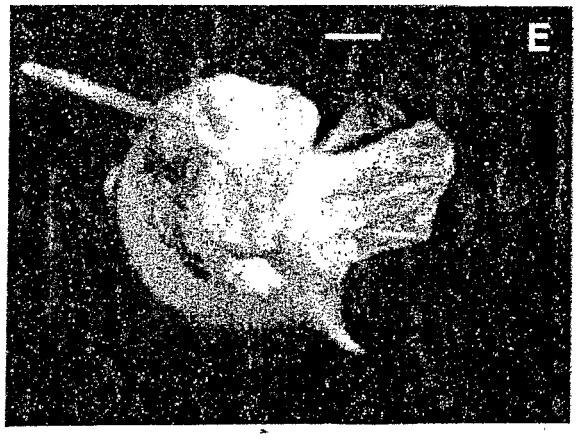
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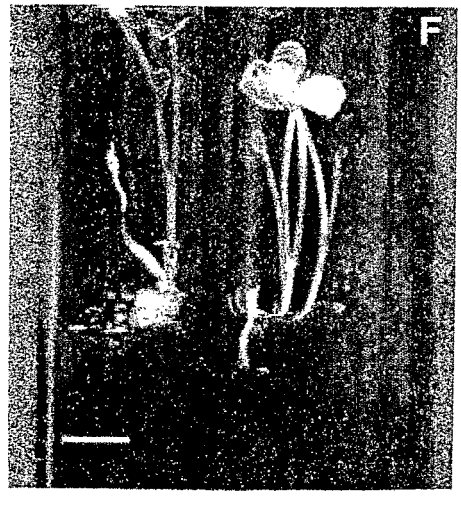
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Dehydration and freezing

After sucrose pretreatment, beads were rapidly surface-dried on sterile filter paper, weighed, placed on sterile filter paper over 40g silica gel in 125ml airtight boxes and dehydrated for 4 to 23 h. Before use, boxes with silica gel were sterilised overnight at 150°C. Twenty beads were used per treatment. After each dehydration treatment, beads were first weighed; then 10 dried beads were placed in a 2ml polypropylene sterile cryotube and frozen rapidly by plunging the cryovials directly into liquid nitrogen, where they were kept for at least 2 h. The 10 remaining beads (desiccation controls) were placed in Petri dishes onto the Knop modified medium, so-called '2GGC', used for yam *in vitro* maintenance (12) without activated charcoal and maintained on $30g.l^{-1}$ sucrose for dehydration treatments up to 12h, without activated charcoal and supplemented with $50g.l^{-1}$ sucrose, $1mg.l^{-1}$ BA, $0.01mg.l^{-1}$ NAA for longer desiccation periods.

The residual water content of the alginate beads was expressed in g water per g dry weight. Dry matter was determined after desiccating 20 to 50 beads in 125ml air-tight boxes containing 40g of dry silica gel, for up to 30 days (DW_{30}).

Apices survival and shoot recovery

After freezing in liquid nitrogen, encapsulated apices were rewarmed for 40min in a laminar air flow cabinet and transferred in Petri dishes onto the '2GGC' medium without activated charcoal and supplemented with $50g.l^{-1}$ sucrose, $1mg.l^{-1}$ BA, $0.01mg.l^{-1}$ NAA. Assessment of apices survival was performed 5 weeks after rewarming, or after dehydration for controls. Survival rate was estimated by counting the number of apices showing any sign of regrowth, i.e. swelling, development of new leaf primordia and/or callusing.

For shoot recovery, most of frozen apices, showing any sign of regrowth after assessment, were transferred onto fresh '2GGC' medium ; their development into leafy rooted shoots was observed after one to six months.

In all cases, cultures were maintained under standard environmental conditions.

Data analysis

Three variables have been measured : survival, recovery, and residual water content of beads. In the present paper, only all the results from variable survival were statistically analysed and presented in Tables.

Experiments were conducted separetely for each clone, belonging one to *D. alata* 'Brazo fuerte' and the other to *D. bulbifera* 'Nouméa Imboro'. For each clone, results from freezing replicates on the one hand, and those from desiccation control replicates on the other hand, were separetely statistically analysed and recorded in distinct tables.

Data were subjected to analysis of variance and means separated by Newman-Keuls test at $p \leq 0.05$.

The main effects tested were : a) sucrose concentration for pretreatment, analysed by a 3-way ANOVA ; b) sucrose-pretreatment duration ; c) dehydration duration, the last two being analysed first by a 3-way ANOVA for means calculated over all sucrose concentrations and secondly by a 2-way ANOVA or a 1-way ANOVA for means calculated within each of the four sucrose concentrations used. For statistical analysis conveniences and presentation in Tables, data from sucrose-pretreatment duration and from dehydration duration were regrouped over 2 'durations' for most of the data.

Results and Discussion

Pretreatment effects

Sucrose-pretreatment duration

In the case of *D. alata* (Table 1), statistical analysis using 3-way ANOVA for pretreatment durations ranging between 3 to 7 d and for all sucrose concentrations used showed that survival of cryopreserved apices was significantly higher for longer pretreatment durations (6-7 d.). Significant differences (one-way ANOVA) in survival after pretreatment for 3 to 10 d. with different sucrose concentrations were noted for 1M only, between shorter (3 d.) and longer pretreatment durations'(6-10 d.).

Table 1: Effect of sucrose concentration, sucrose pretreatment duration and dehydration duration on the survival rate (%) of encapsulated apices of *D. alata* 'Brazo Fuerte' after freezing in liquid nitrogen. Assessment of survival was performed 5 weeks after rewarming.

	Number of replicates [1]	Survival (%) after cryopreservation in liquid nitrogen				
	( ) Total number of replicates	Sucrose concentration for pretreatment:				
		0.75 M [2]	**0.9 M** [2]	**1.0 M** [2]	**1.1 M** [2]	**Mean** [3] [without 10d][3]
Pretreatment duration (d)						
3	10 (40)	24	41	23 b	14	25 c
4-5	20 (80)	20	53	36 ab	20	32 b
6-7	20 (80)	30	59	51 a	26	42 a
10	8 (24)	37	51	60 a		
p [4]		NS	NS	**	NS	***
Dehydration duration (h)						
7-8	10 (40)	13 b	26 b	20 b	22 ab	20 d
9-10	12 or 10(46)	30 b	49 a	39 ab	23 ab	34 bc
11-12	12 or 10(46)	45 a	66 a	52 a	32 a	50 a
14-16	12 or 10(46)	22 b	67 a	58 a	20 ab	40 b
18-23	12 or 10(46)	21 b	50 a	40 ab	9 b	29 cd
p [4]		***	***	**	*	***
Mean [7-23] [without 10d] [5]		25 c	51 a	37 b	20 c	***

[1] Number of freezing replicates (10 apices/freezing replicate) for each of the four sucrose concentrations. Pretreatment duration (d) parameter: results are averaged over 1 or 2 pretreatment days and over all the 10 dehydration durations used, except at 10d where no data for 7-8h and 1.1M treatments exist. Dehydration duration (h) parameter: results are averaged over 2 dehydration periods and over all the 6 pretreatment durations used, except for 7-8h and 1.1M treatments where no data exist for 10d.

[2] Means followed by same letter, within a column for each parameter, are not significantly different ($p<0.05$) according to Newman-Keuls test following up 1-way ANOVA.

[3] and [5] Mean separation, within 'Mean' column for each parameter ([3]) or within 'Mean' row ([5]), by Newman-Keuls test at $p<0.05$ following 3-way ANOVA. Data concerning 10d pretreatments were not taken into account for 'Mean' values and 3-way ANOVA.

[4] *, **, *** significant at 0.05, 0.01, 0.001 levels respectively. NS, not significant.

In the case of *D. bulbifera*, no significant differences in survival were observed between 5 to 10 d of pretreatment (Table 2). The optimal pregrowth duration for yam apices is thus longer those required for most other species, which are in the range of 2-3d (9).

No significant differences in survival (data not shown) were observed between sucrose-pretreatment durations for control apices of both species (Fig. 1C).

Table 2: Effect of sucrose concentration, sucrose pretreatment duration and dehydration duration on the survival rate (%) of encapsulated apices of *D. bulbifera* 'Nouméa Imboro' after freezing in liquid nitrogen for. Assessment of survival was performed 5 weeks after rewarming.

	Number of replicates [1] ( ) Total number of replicates	Survival (%) after cryopreservation in liquid nitrogen				
		Sucrose concentration for pretreatment:				
		0.75 M[2]	0.9 M[2]	1.0 M[2]	1.1 M[2]	**Mean** [without 3 and 13d & 4-7h] [3]
Pretreatment duration (d)						
3 [6]	7 to 8 (31)	16 [6]	29 [6]	24 [6]	21 [6]	23 [6]
5-6	13 to 17 (63)	34	51	54	52	49
7-8	21 to 28 (102)	19	30	37	36	43
9-10	10 to 17 (57)	25	45	55	46	45
13 [6]	6 to 11 (32)	11 [6]	50 [6]	32 [6]	40 [6]	39 [6]
p [4]						NS
Dehydration duration (h)						
4-7 [6]	1 to 19 (45)	0 [6]	4 [6]	10 [6]	19 [6]	13 [6]
8-10	17 to 19 (72)	12 b	28 b	39 b	36 b	31 b
11-12	10 to 12 (45)	15 ab	42 a	56 ab	43 ab	50 a
14-16	14 or 15 (59)	29 ab	58 a	65 a	64 a	55 a
18-23	15 to 17 (64)	31 a	55 a	54 ab	44 b	50 a
p [4]		*	**	*	**	***
Mean [8-23] [whithout 3 & 13d] [5]		25 b	49 c	59 a	51 ac	***

[1] Number of freezing replicates (10 apices/freezing replicate) for each of the four sucrose concentrations. Pretreatment duration (d) parameter: results are averaged over 1 or 2 pretreatment durations and over all the 10 dehydration durations used, except for 3d and 13d because of incomplete data. Dehydration duration (h) parameter: results are averaged over 2 dehydration durations and over all the 8 pretreatment periods used, except for 4-7h because of incomplete data.

[2] Means followed by same letter, within a column for each parameter, are not significantly different ($p<0.05$) according to Newman-Keuls test following 2-way ANOVA.

[3] and [5] Mean separation, within 'Mean' column for each parameter ([3]) or within 'Mean' row ([5]), by Newman-Keuls test at $p<0.05$ following 3-way ANOVA.

[6] Data concerning 4-7h, 3d and 13d pretreatments were not taken into account for 'Mean' values, 2 and 3-way ANOVA.

[4] *, **, *** significant at 0.05, 0.01, 0.001 levels respectively. NS, not significant.

Sucrose concentration

In the case of *D. alata* (Table 1), a 3-way ANOVA demonstrated that survival was significantly higher after pretreatment with 0.9M sucrose (51% survival) than with other

sucrose concentrations. For *D. bulbifera* (Table 2), the highest survival rate was obtained after pretreatment with 1M sucrose (59%), showing a highly significant difference compared to 0.9M and 0.75M (49 and 25% survival, respectively).

With *D. alata*, the 3-way ANOVA comparison of survival of desiccation controls after pretreatment with 0.9 and 1M sucrose (Table 3) showed, as had been previously observed with cryopreserved apices, a significantly higher survival rate for 0.9M (73%) than with 1M (64%). In the case of *D. bulbifera*, no significant effect of sucrose concentration on the survival of desiccation controls was noted (Table 4).

Table 3: Effect of sucrose concentration during pretreatment and of dehydration duration on the survival rate (%) of control encapsulated apices for *D. alata* 'Brazo Fuerte'. Assessment of survival was performed 5 weeks after dehydration.

	Number of control replicates [1]	Survival (%) after dehydration				
	( ) Total number of replicates	Sucrose concentration for pretreatment:				
		0.75 M [2]	**0.9 M** [2]	**1.0 M** [2]	**1.1 M** [2]	**Mean**{0.9M+1.0M} [3] [without 10d&0-6h]
Dehydration duration (h)						
0 [2]	6 to 13 (34)	98	97	80	70	
4-6 [2]	2 to 7 (19)	95	83	80	75	
7-8	6 to 11 (36)	92 a	83	82 a	61 a	82 a
9-10	5 to 11 (36)	86 a	76	68 ab	46 ab	73 b
11-12	10 to 14 (48)	81 a	72	57 b	34 ab	64 bc
14-16	8 to 14 (43)	56 b	66	63 ab	36 ab	64 bc
18-23	9 to 14 (45)	37 c	68	50 b	24 b	60 c
p [4]		***	NS	**	•	***
Mean{7-23h} [5] [without 10d]			73 a	64 b		*

[1] Number of control replicates (10 apices/control replicate) for each of the four sucrose concentrations. Results are averaged over 1 or 2 dehydration durations and all the 6 sucrose-pretreatment periods (3 to 10d) used, except for 7-8h and 1.1M treatment where no data exist for 10d and for 0h, 4-6h, 0.75M and 1.1M treatments because of incomplete data.

[2] Means followed by same letter within a column are not significantly different ($p<0.05$) according to Newman-Keuls test following 1-way ANOVA. Data concerning 0h and 4-6h were not taken into account for 1-way ANOVA.

[3] and [5] Mean separation, within 'Mean' column ([3]) and row ([5]), by Newman-Keuls test at $p<0.05$ following up 3-way ANOVA. Data concerning 10d, 0h, 4-6h, 0.75M and 1.1 M treatments were not taken into account for 'Mean' values and 3-way ANOVA.

[4] *, **, *** significant at 0.05, 0.01, 0.001 levels respectively. NS, not significant.

The sucrose concentrations allowing the attainment of optimal survival with the two yam species studied (0.9-1M for *D. alata*, 0.9 to 1.1M for *D. bulbifera*) were higher than those previously determined for numerous other species. Indeed, a sucrose concentration of 0.75M was optimal for apices of grape, sugarcane, potato and coffee (18, 17, 3, 15) as well as the four yam species studied by Mandal et al. (14). Higher sucrose concentrations have

generally been found to have detrimental effect on survival, as shown notably with apices of sugarcane and coffee (17, 15).

Dehydration duration

In the case of *D. alata* (Table 1), dehydration periods of 11-12h ensured significantly higher mean survival rates (50%; ***: 3-way ANOVA without 10d data). For all pretreatment conditions, statistical analysis (1-way ANOVA) revealed significantly higher survival rates for 11-12h dehydration. The highest survival rates were obtained with 0.9M sucrose for dehydration periods of between 9 and 23h, with an optimum for 11 to 16h (66 to 67%), and with 1M sucrose for dehydration periods of between 11 and 16h (52 to 58%).

Table 4: Effect of sucrose concentration during pretreatment and of dehydration duration on the survival rate (%) of control encapsulated apices for *D. bulbifera* 'Nouméa Imboro'. Assessment of survival was performed 5 weeks after dehydration.

	Number of control replicates [1] ( ) Total number of replicates	Survival (%) after dehydration				
		Sucrose concentration for pretreatment:				
		0.75 M [2]	**0.9 M** [2]	**1.0 M** [2]	**1.1 M** [2]	**Mean** [4] [without 3 and 13d & 0h, 4-7h] [3]
Dehydration duration (h)						
0 [3]	7 to 12 (37)	99 [3]	100 [3]	96 [3]	83 [3]	95 [3]
4-7 [3]	1 to 18 (44)	100 [3]	97 [3]	89 [3]	75 [3]	87 [3]
8-10	14 to 17 (63)	92 a	92 a	88	67	89 a
11-12	9 to 11 (38)	77 a	76 b	65	62	82 b
14-16	11 (44)	93 a	88 b	85	76	87 ab
18-23	14 to 17 (62)	68 b	75 b	77	69	78 b
p [5]		**	*	NS	NS	**
Mean [8-23h] [whithout 3 & 13d]		87	86	83	79	

[1] Number of control replicates (10 apices/control replicate) for each of the four sucrose concentrations. Results are averaged over 1 or 2 dehydration durations and all the 8 sucrose-pretreatment periods (3 to 13d) used, except for 0h and 4-7h because of incomplete data.

[2] Means followed by same letter within a column are not significantly different ($p<0.05$) according to Newman-Keuls test following 2-way ANOVA.

[3] Data concerning 0h, 4-7h, 3d and 13d treatments were not taken into account for 'Mean' values, 2 and 3-way ANOVA.

[4] Mean separation, within 'Mean' column for each parameter ([3]), by Newman-Keuls test at $p<0.05$ following 3-way ANOVA.

[5] *, **, *** significant at 0.05, 0.01, 0.001 levels respectively. NS, not significant.

For *D. bulbifera* (Table 2), a wider range of dehydration period durations (from 11 to 23h) ensured optimal mean survival (50-55%). Statistical analysis (3-way ANOVA) performed for pretreatment durations between 5 to 10 d revealed that survival after shorter dehydration periods (8-10 h) was significantly lower (31%). For the three higher sucrose

concentrations (0.9; 1.0; 1.1M) employed during pretreatment, dehydration periods of 14-16h ensured optimal survival, with 58, 65 and 64%, respectively, which differed significantly from the shorter dehydration periods. The type of development observed with *D. bulbifera* after 5 weeks of dehydration is nearly the same on control (-LN) and treated apices (+LN) (Fig. 1D).

After a pretreatment of apices with 0.75M sucrose, Mandal et al. (14) obtained 64 and 26% survival with *D. alata* and *D. bulbifera*, respectively, compared to 45 and 31% in our best results. However, it is difficult to draw any conclusion from these results since desiccation was performed differently in these experiments : Mandal et al. (14) desiccated apices under the laminar air flow and silica gel was employed in the present study.

In previous experiments (data not shown), dehydration stress periods longer than 12h provoked an inhibition of the growth of encapsulated apices cultured on growth regulator-free medium. Thus, as a control, a medium with growth regulators was used after 12h dehydration, whereas an hormone-free medium was used up to 12h. For *D. alata* controls (Table 3, Fig. 2A), the change from one medium to the other at 12h did not affect the survival rate which remained significantly close from 11 to 23h when averaged over 0.9M plus 1M treatments; for this clone, 0.75M and 1.1M treatments produced the same decreasing and poor survival rate beyond 12h and 8h respectively, as most often observed on cryopreserved apices. Whereas, for *D. bulbifera* controls (Table 4, Fig. 2B), whatever the four sucrose concentrations and the dehydration duration used, a survival rate $\geq$ 62% were obtained.

The effect of dehydration duration on control apices showed that the longest dehydration periods employed (18-23h) still ensured high mean survival rates (Tables 3 and 4, Fig. 2) with 50% (1M) or 68% (0.9M) for *D. alata*, and 78% (0.75M-1.1M) for *D. bulbifera*, which differed from values obtained after rapid freezing in liquid nitrogen, viz 40% (1M) or 50% (0.9M) for *D. alata* and 50% (0.75M-1.1M), for *D. bulbifera*.

The three studied parameters of the pretreatment could be classified as follows according to their impact on the apices survival after freezing, in a decreasing order of importance: dehydration duration, sucrose concentration and sucrose-pretreatment duration.

Evaluation of residual water content of beads

We obtained a strong linear correlation between dry mass (DW_{30}) and sucrose molarity for sucrose-pretreated alginate beads. During the whole experiment, we used DW_{30} values estimated by linear regression; water content of beads before dehydration ranged from 1.54 (0.75M) to 2.47$g.g^{-1}$ DW (1.1M) (Table 5).

Table 5: Dry mass and water content of sucrose-pretreated alginate beads, determined after 30d of drying with silica gel in airtight boxes at room temperature.

Sucrose concentration	DW_{30} (% FW) estimated by linear regression(1)	Water content before dehydration ($g.g^{-1}$ DW)
0.75M	28.8	2.47
0.9M	33.3	2.00
1.0M	36.3	1.76
1.1M	39.3	1.54

(1) From mean values over 13 to 15 replicates for each of the four sucrose concentrations (y= 6.4319 + 29.872x; N= 4; r= 0.999). Similar results were obtained from replicate data (y= 6.4177 + 29.883x; N= 55; r= 0.960). Data not shown.

For *D. alata*, mean residual water content values over 0.9 + 1M treatments decreased from 1.88g H2O g^{-1} without dehydration to 0.47, 0.15 and 0.12g H2O g^{-1} DW for 4, 12 and 23h dehydration, respectively (Fig. 2A). For *D. bulbifera*, mean water content values for 1 +

1.1M sucrose treatments decreased from 1.76g H2O g^{-1} without dehydration to 0.41, 0.13 and 0.11g H2O g^{-1} DW for 4, 12 and 23h dehydration, respectively (Fig. 1.B). As already noted by Fabre and Dereuddre (10), we observed that water content values for the different sucrose concentrations employed became similar after 7-8h of dehydration. Standard deviations were then lower than 0.03 (Fig. 2).

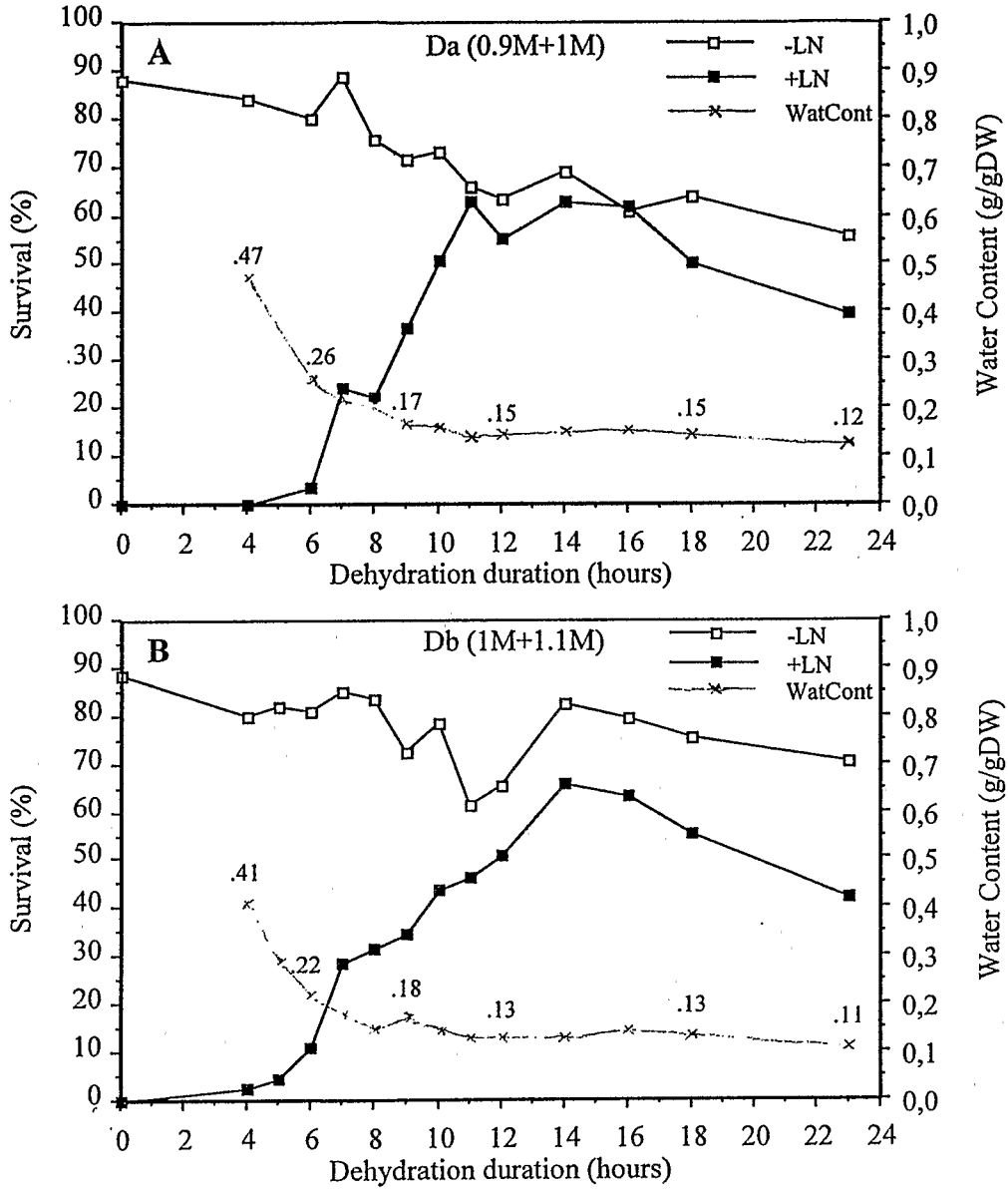

Figure 2: Effect of dehydration duration on the water content of beads and survival rate of control (-LN ☐) and cryopreserved (+LN ■) encapsulated apices of *D. alata* 'Brazo Fuerte' after pretreatment with sucrose (0.9M+1M)- (**A**), and *D. bulbifera* 'Nouméa Imboro' after pretreatment with sucrose (1M+1.1M)- pretreated ones (**B**). Each point corresponds to a mean over 2 sucrose concentrations and all the 6 or 8 pretreatment durations used depending on the clone.

In our experiments, the water content of encapsulated apices had to be decreased down to 0.15g H2O g^{-1} DW in order to obtain high survival after freezing. The percentage of water loss was of 67, 62, 58 and 55% FW (± 1%) for 0.75, 0.9, 1 and 1.1M sucrose pretreatments, respectively, thus lower than the 71-72% water loss obtained by Benson et al. (2) after pretreatment with 0.75M and 4h air-dehydration. Our results demonstrated that, in most cases, survival increased when dehydration was extended to a defined threshold, around 0.13-0.15g. H2O g^{-1} DW, which was obtained after desiccation periods from 10 to 18h.

A new and more accurate method for measuring of dry weight was used in our experiments, which consisted of desiccating alginate beads for 30 d in airtight boxes containing dry silica gel, to avoid mass loss due to caramelization of sugar when drying at a temperature higher than 100°C.

The results obtained with this new method were consistent with critical values reported for encapsulated *Vitis* shoot-tips (18) and encapsulated *Catharanthus* suspension cells (1).

Growth recovery

Recovery rates of frozen apices into leafy rooted shoots after one, two and three months of culture on medium without growth regulators were 31, 52 and 60%, for *D. bulbifera* and 9, 15 and 19% (up to 26% after six months) for *D. alata* (Fig. 1E, F). With *D. alata*, the slower development of apices into plantlets was due to the production of polyphenols, anthocyanic swelling, growth inhibition of apices, and callus production.

Conclusion

This study demonstrated that apices of *D. alata* cv 'Brazo fuerte' and *D. bulbifera* cv 'Nouméa Imboro' could be successfully cryopreserved using the encapsulation/dehydration technique. For both species, survival rates higher than 50% were obtained after pretreatment with 0.9M or 1M, -and also with 1.1M sucrose for *D. bulbifera* clone-, followed by a large range dehydration durations.

As previously said in discussion, with 0.75M sucrose pretreatments, the best survival results from freezing presented here do not differ a lot from those obtained by Mandal et al. (14) with the two same species, *D. alata* and *D. bulbifera*. Higher percentages on survival and recovery have been observed on the whole yam freezing experiments. For *D. alata*, over 64% survival and 21.8% recovery into shoots were obtained by Mandal et al. (14) with 0.75M sucrose pretreatments, and by us (present work) with 0.9M ones. In the case of *D. bulbifera*, over 65% survival and 60% recovery from freezing were obtained by us (present work) with 1M sucrose pretreatments, compared to 26% survival and 0% recovery obtained by Mandal et al. (14) with 0.75M ones. This discrepancy might be due to differences in the culture conditions of mother plants and in the cryopreservation protocols employed. In addition, the clones used although belonging to the same species, might be genetically different due to their different origins. Such intraspecific differences may be related to physiological differences between cultivars, which may accumulate and metabolize sucrose according to different rates and levels. In the encapsulation-dehydration technique, sucrose pretreatment has been reported as playing a major role in the tolerance of apices to dehydration and further freezing in LN [see review in Bachiri et al. (1)]. Sucrose induces osmotic dehydration but is also absorbed by cells, thus increasing the ability of intracellular solutes to vitrify and also by stabilizing membranes and proteins (6, 18, 19) during dehydration and freezing.

Experiments are under way to improve the recovery of plantlets from cryopreserved apices. In a near future, the optimized protocol will be applied to some genotypes more representative of the diversity, to insure a routinely use.

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