Cryopreservation of rhizome buds of *Asparagus officinalis* L. (cv. Morado de Huétor) and evaluation of their genetic stability

E. Carmona-Martín[1], J.J. Regalado[2], R. Perán-Quesada[3], C.L. Encina[1]

[1] Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", CSIC-UMA, 29750 Algarrobo-Costa, Málaga, Spain ecarmona@eelm.csic.es

[2] Departamento de Biodiversidad y Biología Experimental,
Facultad de Ciencias Exactas y Naturales, Instituto de Micología y Botánica, UBA-CONICET, CABA, Buenos Aires, Argentina

[3] Departamento de Biología Vegetal, Facultad de Ciencias, Campus de Teatinos s/n, Málaga, Spain

Abstract

We describe an encapsulation–dehydration procedure with prefreezing steps for the cryopreservation of rhizome bud explants of *Asparagus officinalis* L. cv. Morado de Huétor. With this procedure, survival of Rhizome buds was at least 84 and 42% developed to complete plantlets at 8 weeks. Flow cytometry and EST-SSR molecular markers were used to assess genetic stability of the regenerated material. Effects of preculture time in a medium rich in sucrose and prefreezing treatments (0 °C or/and − 20 °C) on plant recovery were evaluated. Rhizome Buds of the "Morado de Huétor" landrace were incubated in preculture medium (MS + 0.3 M sucrose) for 48 h, encapsulated in alginate beads and desiccated until a water content of 35%, prefrozen for one hour at 0 °C plus one hour at − 20 °C, followed by cryopreservation in liquid nitrogen, and then were rewarmed and recovered in ARBM medium for 6 weeks and finally incubated in ARBM-0 for 4 weeks. Analyses of ploidy and molecular stability of plantlets recovered from cryopreserved rhizome buds of two selected genotypes showed no differences compared with the mother plants. Cryopreservation of RB explants of *A. officinalis* with this Encapsulation–Dehydration procedure will be useful in long-term preservation programs.

Keywords Encapsulation–dehydration, Asparagus, Rhizome bud, Cryopreservation, Molecular markers, Flow cytometry

Introduction

The cultivated asparagus (*Asparagus officinalis* L.) is an economically important species worldwide. The genetic base of asparagus is narrow (Caruso et al. 2008) but can be widened in crossings with autochthonous landraces with different genetic backgrounds. The cultivar Morado de Huétor (MH) is an autochthonous Spanish landrace originated from the natural crossing between *A. officinalis and A. maritimus.* (Moreno et al. 2008a). MH shows high genetic variability due to the hybrid origin, including different ploidy levels (triploid, pentaploid, hexaploid and octoploid), although most MH genotypes are tetraploid (Moreno et al. 2006). This high heterozygosity results in highly heterogeneous plantations with limited productivity. The cultivated area of this landrace has decreased drastically in recent years (Moreno et al. 2008b) because of competition with more productive commercial varieties, resulting in a high risk that this autochthonous landrace could disappear. Some mechanisms have been initiated to preserve this unique asparagus. Our research team has developed clonal selection for outstanding agronomic characteristics and methods for *in vitro* micropropagation and established a germplasm bank of MH elite genotypes (Carmona-Martín et al. 2014; Regalado et al. 2015a). The maintenance of this germplasm bank requires an important investment in time, material and storage space. Cryopreservation under LN offers a better strategy for the long-term preservation of elite clones, because this method prolongs storage life, minimizes storage space requirements and reduces the costs of maintenance (Bouman and De Klerk 1990; Panis and Lambardi 2005).

Different methods of cryopreservation have been tested in asparagus, including the "Droplet" method (Mix-Wagner et al. 2000), controlled cooling (Suzuki et al. 1997, 1998; Jitsuyama et al. 2002), vitrification (Kohmura et al. 1992), and preculture and desiccation (Uragami et al. 1990). In these protocols, different explant materials have been tested, including shoot tips (Mix-Wagner et al. 2000), meristems (Suzuki et al. 1997, 1998; Jitsuyama et al. 2002), bud clusters (Kohmura et al. 1992), embryogenic cells (Nishizawa et al. 1992, 1993) and stem segments (Uragami et al. 1990). The primary problem in asparagus micropropagation protocols is rooting, and these problems increase when nodal segment explants are subjected to cold treatments during the cryopreservation procedure (Yang and Cloré 1974). Kumu et al. (1983) found that six or more months are required to develop a whole plant of asparagus from shoot tips after freezing in LN. Kohmura et al. (1992) obtained high levels of shoot formation from bud clusters cryopreserved using a vitrification method, but an additional step of rooting was necessary after the recovery of explants from LN. Finally, Mix-Wagner et al. (2000) obtained well-developed shoots from shoot tips cryopreserved using the Droplet-method, but rooting was not described in this publication. With the micropropagation of asparagus through RB explants (Carmona- Martín et al. 2014), the rooting problem is corrected with rooting rates of 74%. Thus, the use of RBs as explants for cryopreservation appears a promising option to obtain good rooting results in the cryopreservation procedure.

Encapsulation–dehydration (E–D) is a method of cryopreservation developed for pear and potato shoot tips (Dereuddre et al. 1990; Fabre and Dereuddre 1990). This method is based on the technology developed to produce synthetic seeds, i.e., the encapsulation of explants in alginate calcium beads (Redenbaugh et al. 1986). This procedure has been used with good results in different species, including *Quercus* sp. (Fernandes et al. 2008), *Chrysanthemum* sp. (Martín et al. 2011) and species of mint (González-Benito et al. 2016), and with different explants, including somatic embryo clusters (Fernandes et al. 2008), shoots (Martín et al. 2011) and nodal segments (González-Benito et al. 2016). For asparagus, this method of cryopreservation has not been tested.

An important consideration in cryopreservation is that somaclonal variation has occasionally been reported in cryopreserved plant species (Harding 2004; Martín and González-Benito 2005; Panis and Lambardi 2005). The origin of this variation is usually attributed to the application of aggressive treatments in vitro before or during the cryopreservation process, rather than the cryopreservation treatment itself (Harding 1997). In cryopreserved asparagus, only phenotypical studies have been conducted, and in these studies, no morphological abnormalities are observed in plants recovered from cryopreserved segments of bud cluster explants (Kohmura et al. 1992), shoot tips or embryogenic cell suspensions (Nishizawa et al. 1993). However, morphological variations (Kunitake and Mii 1998), changes in ploidy level (Kunitake and Mii 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005) and somaclonal variations (Raimondi et al. 2001; Pontaroli and Camadro 2005) have been detected in micropropagated asparagus plants, apparently linked to the different methods involved in micropropagation/ regeneration. These variations can appear with high probability in plants recovered from cryopreserved explants when the micropropagation protocol applied throughout the freezing procedure has the capability itself to induce variations. By contrast, asparagus micropropagated through an RB explant protocol (Carmona-Martín et al. 2014; Regalado et al. 2015a) did not show variations and were true-to-type. Therefore, this micropropagation protocol is a suitable starting point to develop a cryopreservation method in asparagus.

The aim of this study was to develop a cryopreservation method based on the E–D method that uses RBs as the explant to obtain a high percentage of rooted asparagus plants in a short period of time. Additionally, the genetic stability of regenerated asparagus was assessed following this procedure. The use of this cryopreservation method will result in important reductions in economic costs and manipulation time and requirements for laboratory materials and storage space, which are all inherent to the maintenance of an asparagus germplasm bank.

Materials and methods

Plant material

Asparagus plants, *A. officinalis* var. "Morado de Huétor," were provided by CESURCA (Centro Sur C.A.) from plantations located in Huétor-Tájar (Granada, Spain) and were the source of RB explants used in dehydration and cryopreservation assays. RBs of two elite genotypes of the "MH" landrace (HT-089 and HT-177) were used to study possible somaclonal variations and changes in the ploidy levels in plants recovered from cryopreserved RBs. The RB explants were collected from potted plants of both genotypes maintained in a glasshouse located in the IHSM "La Mayora" (Málaga, Spain).

Dissection and disinfection of rhizome buds

The RBs were extracted, dissected and disinfected following the protocol developed by Carmona-Martín et al. (2014). Dissected RBs were treated for 15 min with a fungicide (Benomyl 3 g l^{-1}) under shaking. After washing in sterile distilled water, RBs were disinfected in a 20 g l^{-1} sodium hypochlorite solution for 15 min under vacuum and then rinsed three times with sterile distilled water in aseptic conditions.

Cryopreservation

Preculture

Sterile buds were preincubated for 24 or 48 h in MS (Murashige and Skoog 1962) medium supplemented with 0.3 M sucrose at 25 °C and under 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes.

Encapsulation

After preincubation, buds were encapsulated by insertion into a sterile solution of alginate (3%), sucrose (0.4 M) and glycerol (2 M). Each calcium-alginate bead corresponded to a drop of alginate solution 5 mm in diameter and contained one RB explant. A sterile micropipette with the tip trimmed was used to shape these calcium-alginate beads. To harden the calcium-alginate beads, beads were treated with a solution of $CaCl_2$ (100 mM), sucrose (0.4 M) and glycerol (2 M) for 30 min.

Dehydration

In preliminary studies, we observed that the capacity for RB growth remained when dehydrated to a moisture content (MC) of 35% of their fresh weight. To obtain this level of dehydration, the beads, with RBs inside, were dried over 24 h in a 1000 ml jar containing 100 g of silica gel covered with a sterile disc of filter paper.

Cryopreservation of RBs

Encapsulated-dehydrated RBs were placed in cryovials, ten beads in each one. The effects of precooling and prefreezing before immersion in LN were studied by subjecting cryovials to precooling at 0 °C (crushed ice) and/or prefreezing (− 20 °C) for one hour before their immersion in LN. As controls, cryovials with RBs, preincubated for 24 or 48 h, were immersed in LN without precooling or prefreezing. Sixty RBs, in three repetitions of 20 buds each, were cryopreserved for each combination (Table 1). The immersion in LN was always for 24 h.

Table 1 Scheme of the procedure for testing encapsulation–dehydration

Preculture (MS + 0.3M sucrose) 24 H	E–D	–	LN	Rewarming and rehydration	Plant recovery
		0 °C			
		− 20 °C			
		0 °C + − 20 °C			
Preculture (MS + 0.3M Sucrose) 48 H	E–D	–	LN	Rewarming and rehydration	Plant recovery
		0 °C			

E–D, encapsulation and dehydration of the explants for 24 h over silica gel; –, no prefreezing treatment; 0 °C, cryovials immersed for one hour in crushed ice at 0 °C; − 20 °C, cryovials immersed for one hour at − 20 °C; 0 °C + − 20 °C, cryovials immersed for one hour in crushed ice at 0 °C plus an immersion for one hour at − 20 °C; LN, immersion in liquid nitrogen for 24 h

Culture of cryopreserved buds

After cryopreservation, buds were thawed and rehydrated in distilled water at room temperature in sterile conditions. To decrease the contamination rate, RBs were sterilized a second time. This sterilization consisted of immersion in a solution of 1% sodium hypochlorite for 10 min, followed by three rinses in distilled sterile water.

Each bead containing a RB was incubated in 25 ml of shoot regeneration medium ARBM, which consisted of MS (Murashige and Skoog 1962) salts modified with EDDHAFe at 85.7 mg l^{-1} instead of EDTA and vitamins supplemented with 0.5 mg l^{-1} NAA, 0.7 mg l^{-1} KIN, 2 mg l^{-1} Ancymidol and 6% sucrose at a pH of 5.7 and solidified with 0.8% agar (Table 2). The medium was supplemented with 200 mg l^{-1} of the antibiotic Cefotaxime (filter sterilized) to decrease the bacterial proliferation observed in preliminary assays. The culture medium was added into 150 mm × 25 mm test tubes covered with polypropylene tops (Bellco Corp.), which were autoclaved for 20 min at 121 °C and 1.05 kg cm^{-2}. The cryopreserved RB explants were incubated for 6 weeks in a culture room at 25 ± 1 °C under a 16 h photoperiod provided by cool white fluorescent tubes (F40 tubes Gro-lux; Sylvania) with 45 µmol m^{-2} s^{-1} (400–700 nm) photosynthetic active radiation. After 6 weeks, the explants were transferred to new test tubes with 25 ml of ARBM-0 medium consisting of MS salts modified by adding EDDHA-Fe at 85.7 mg l^{-1} instead of EDTA and vitamins and supplemented with 0.1 mg l^{-1} NAA (naphthaleneacetic acid), 0.1 mg l^{-1} KIN (kinetin) and 0.8% agar (Regalado et al. 2015a). The cryopreserved RB explants were incubated for 4 weeks under the same environmental conditions. The percentages of growth and rooting of the cryopreserved RB explants were recorded after 10 weeks of incubation.

Table 2 Composition of culture media used in cryopreservation process and recovery. NAA: naphthaleneacetic acid

Culture medium	Growth regulator (mg l^{-1})			Sucrose (g l^{-1})	Agar (g l^{-1})	Goal
	NAA	KIN	Ancymidol			
MS + 0.3M SUC	–	–	–	103	6	RB preculture and osmoprotection
ARBM[a]	0.5	0.7	2	60	8	ARBM regrowth and rooting
ARBM-0[a]	0.1	0.1	–	30	8	Plantlet development

KIN, kinetin

[a]ARBM media consisting in MS salts, modified with EDDHA-Fe (85.7 mg l−1) instead of EDTA-Fe and vitamins (Murashige and Skoog 1962)

Acclimatization of recovered plants

The plantlets recovered from cryopreserved rhizome buds (PRCRBs) were acclimatized following the method reported by Carmona-Martín et al. (2014).

Cryopreservation of buds of HT-089 and HT-177

Thirty RBs of HT-089 and HT-177 genotypes were cryopreserved to analyze the genetic stability of the PRCRBs. These buds were cryopreserved following the protocol 8 (Table 3), which provided the best results using the RBs of the MH landrace.

Table 3 Percentage of regrowth and rooting of cryogenized asparagus rhizome bud explants applying different modifications of the encapsulation– dehydration method

E-D protocol	Regroth (%)	Rooting (%)
(1) 24 h P+ED+LN+RR+ARBM	34.5±6.2 b	40.0±11 a
(2) 24 h P+ED+0 °C+LN+RR+ARBM	43.8±6.2 b	42.9±9.4 a
(3) 24 h P+ED+−20 °C+LN+RR+ARBM	53.3±6.4 ab	41.3±8.7 a
(4) 24 h P+ED+0 °C+−20 °C+LN+RR+ARBM	62.7±6.3 ab	37.8±8.0 a
(5) 48 h P+ED+LN+RR+ARBM	48.3±6.5 b	48.3±9.3 a
(6) 48 h P+ED+0 °C+LN+RR+ARBM	68.4±6.2 a	46.2±8.0 a
(7) 48 h P+ED+−20 °C+LN+RR+ARBM	75±5.8 a	52.4±7.7 a
(8) 48 h P+ED+0 °C+−20 °C+LN+RR+ARBM	84±5.3 a	42.2±7.4 a

24 h P/48 h P, preculture for 24 or 48 h in MS medium enriched with 0.3 M sucrose; ED, encapsulation of explants and dehydration for 24 h over silica gel; LN, introduction of cryovials in liquid nitrogen, 0 °C, cryovials immersed for 1 h in crushed ice at 0 °C; −20 °C, cryovials frozen for 1 h at −20 °C, RR, rewarming and rehydration of beads. ARBM, asparagus rhizome bud medium. Different letters indicate groups that are significantly different by LSD at $\alpha = 0.05$

Genetic stability check

The genetic stability of PRCRB plants of HT-089 and HT-177 genotypes was checked. The studies were conducted with eight recuperated plantlets of both genotypes after 10 weeks of the cryopreservation treatment, plus their respective controls. Control plants were obtained from the mother plants from which the RB explants of both genotypes HT-089 and HT-177 were extracted.

Ploidy level analysis

The ploidy level of the plants was determined estimating the relative DNA content using flow cytometry (FCM) (Ploidy Analyser PA-I; Partec GmbH, Münster, Germany). For analysis, 0.5 cm pieces of shoot tips (of young in vitro shoots) were chopped with a razor blade for 30–60 s to release nuclei (Galbraight et al. 1983) in a Petri dish containing 0.4 ml of nuclei isolation buffer (commercial PartecCyStain UV precise P, high-resolution DNA staining kit 05-5002, extraction buffer). The homogenate was filtered through a 50 µm nylon mesh (Partec 50-lm CellTrics disposable filter), and subsequently, the nuclei were stained with fluorescent dye (commercial PartecCyStain UV precise P, high-resolution DNA staining kit 05-5002, staining buffer, approximately 1.6 ml). Finally, the samples were analyzed after 30 s of incubation. A diploid sample (*A. officinalis* cv. "Baitoru") ($2n = 2x = 20$) was always used as an external standard. The nuclear DNA ploidy levels of the samples were determined using channel values that corresponded to the average G0/ G1 peaks of the sample and standard plants. The peak relative to the standard nuclei was set to channel 50. Three independent repetitions were performed, with over 10,000 nuclei analyzed in each.

Genetic stability using EST-SSR markers

The genetic stability of the PRCRBs was studied using ESTSSR markers. Total genomic DNA was extracted from 1 g of *in vitro* spear tips of eight plantlets recovered from cryopreserved RB explants of both genotypes HT-089 and HT-177. Extraction was performed using a standard CTAB protocol that followed the protocols and methods of Torres et al. (1993). The quality and concentration of extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer- 8 (Thermo Scientific, Waltham, MA). DNA from mother plants of both genotypes was used as a control for amplification pattern and fragment size. All samples were characterized with a set of 12 EST-SSR markers (AAT1, AG3, AG6, AG7, AG8, AG10, AG12, TC1, TC3, TC5, TC7 and TC9) previously developed and published by Caruso et al. (2008) using the method described by Carmona-Martín et al. (2014). The amplified fragments in the characterization of the PRCRBs were compared with those obtained in the characterization of the mother plants to determine possible somaclonal variation.

Statistical analyses

All data were analyzed using the SPSS statistical software package (version 19.0; SPPS INC, Chicago, IL, USA). The growth and rooting rates obtained in different assays of cryopreservation developed with rhizome bud explants were analyzed by Generalized Linear Models using Logit as the link function and Binomial as the probability distribution. Fisher´s least significant difference (LSD) tests were used for pairwise comparisons between groups.

Results

Cryopreservation

To facilitate the analysis, the results obtained in the cryopreservation of the RB explants (Table 3) were analyzed using two different approaches. The first analysis examined the effect of the duration of RB preculture. We compared the results obtained in each assay of 24 h of preculture with those of the assay with 48 h of preculture that corresponded to the same precooling and prefreezing treatment (Fig. 1). Based on the data, the increase of preculture time from 24 to 48 h resulted in an important increase of the regrowth rate, independent of the precooling and prefreezing treatments. However, this increase was only 14% and was not statistically significant in the assays without steps of precooling and prefreezing, whereas in the assays with a step of precooling, prefreezing or both, the increase in the regrowth rate was over 20% and always statistically significant.

The second analysis examined the effect of the steps of precooling and prefreezing on the RB explants before the cryopreservation. We compared the results obtained in the assays with the same preculture time (24 or 48 h) with or without steps of precooling and prefreezing (Fig. 1). The percentage of regrowth increased significantly when beads were subjected to precooling (0 °C) plus prefreezing (− 20 °C) treatments before cryopreservation, compared with the protocols without cooling pretreatments (–). The increase of regrowth was higher in RB explants precultured for 48 h (35.7%) than that in RB explants pretreated for 24 h (28.2%), see Table 3. Combining the best results obtained for time of preculture in medium rich in sucrose and precooling studies, we selected the following as the optimum method of cryopreservation: the protocol number 8, consisting of 48 h of preculture, encapsulation and dehydration of the explants, prefreezing at 0 °C for 1 h, precooling at −20 °C for 1 h, and cryopreservation in LN using cryovials for 24 h, followed by rewarming and rehydration for 30 min and culture in ARBM for 6 weeks plus 4 weeks in ARBM-0. This protocol resulted in the highest rate of regrowth (84.0 ± 5.3%). Table 3 shows the rooting rates of cryogenized RB explants for all the protocols used in this work. No significant differences were observed among the different procedures for rooting rates. The average rooting rate (protocol 8) at 10 weeks after cryopreservation was 42.2 versus 75% rooting obtained for the non-cryogenized control.

Acclimatization rates of PRCRBs were high at approximately 95%, with similar rates obtained for non-cryogenized plants. Morphologically, plants cryogenized and non-cryogenized were identical. No differences in acclimatization rates were observed among the different protocols.

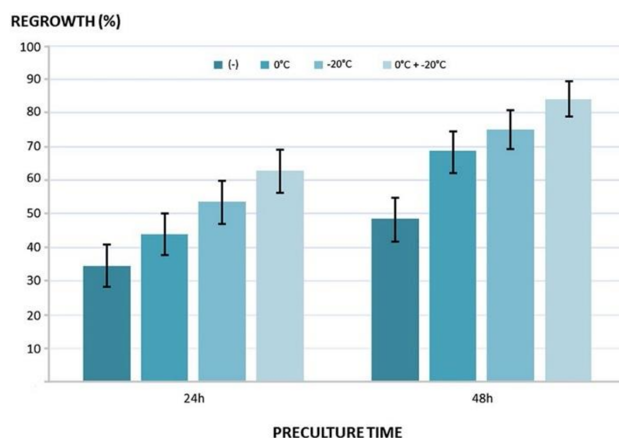

Fig. 1 Comparison between different precooling and prefreezing treatments for preculture times of 24 and 48 h. –, cryopreservation protocols without steps of precooling and prefreezing; 0 °C, cryopreservation protocols including RB explants in crushed ice for 1 h; − 20 °C, cryopreservation protocols including RB explants in a freezer at − 20 °C for 1 h; 0 °C + − 20 °C, cryopreservation proto- cols including RB explants in crushed ice for 1 h and in a freezer at − 20 °C for 1 h.

Genetic stability of recovered genotypes HT-089 and HT-177

Ploidy level analysis

Asparagus officinalis cv. "Baitoru" (2n = 2x = 20) was used as the external diploid standard in all ploidy level analyses, establishing a value of 50 for the peak corresponding to the G1 somatic nuclei (2n = 2x = 20) and a value of 100 for the peak corresponding to the G2 somatic nuclei (2n = 4x = 40) in the flow cytometry histogram. The histogram of the mother plants HT-089 and HT-177 showed two peaks, with the primary peak situated at a value of 100 representing G1 somatic nuclei (2n = 4x = 40) and the secondary peak located at a value of 200 representing G2 somatic nuclei (2n = 8x = 80), indicating that the two mother plants were tetraploids (4x = 40). The flowcytometry histogram obtained for the PRCRBs from genotypes HT-089 and HT-177 showed two peaks with the same values as those obtained for mother plants. In Table 4 the values of the mean of 3 repetitions of G1 peaks in cryopreserved and mother plants have been presented. These results confirmed the stability of the ploidy level (2n = 4x = 40) of the PRCRBs for both selected genotypes.

Table 4 Genetic variability assessment of the cryopreservation process in genotypes HT-177 and HT-089 of *Asparagus officinalis* var. "Morado de Huétor" using flow cytometry

A. officinalis	Mean ± SD	CV (%) ± SD	DNA ploidy level
CV. Baitoru-5 (CONTROL)[a]	50.74[A]	3.45[A]	2N=2X=20[a]
MH genotype HT-177 mother plant	103.64 ± 1.49	2.54 ± 0.20	2N=4X=40
MH genotype HT-177 cryopreserved	102.32 ± 1.09	2.07 ± 0.18	2N=4X=40
MH genotype HT-089 mother plant	100.84 ± 1.63	4.03 ± 0.42	2N=4X=40
MH genotype HT-089 cryopreserved	101.09 ± 1.85	3.01 ± 0.23	2N=4X=40

Values are given as the mean of 3 repetitions and standard deviation of the mean of G_0/G_1 peaks, as well as the mean of 3 repetitions of coefficient of variation and standard deviation

[a]*A. officinalis* cv. Baitoru-5 is the diploid control sample

EST-SSR analyses

Amplification of the SSR loci used 12 primer pairs that produced 27 alleles that ranged from 148 to 234 bp in size for genotype HT-177 and 27 alleles that ranged from 145 to 234 bp for HT-089. Table 5 shows in detail the alleles for the 12 EST-SSRs used in the analysis of the eight PRCRB explants and their corresponding mother plants in both genotypes. Somaclonal variation was not detected for any of the 12 EST-SSRs analyzed in PRCRBs obtained from the E–D method.

Table 5 Allelic constitution of plants recovered from cryopreserved RB explants by encapsulation–dehydration protocol number 8 and the corresponding mother plant samples at 12 EST-SSR loci in *A. officinalis* L. cv. MH genotypes HT-177 and HT-089, showing no somaclonal variation

Marker name	Allele size (bp)			
	A. officinalis MH genotype HT-177		*A. officinalis* MH genotype HT-089	
	Mother plant (CONTROL)	Cryopreserved material	Mother plant (CONTROL)	Cryopreserved material
AAT1	213/215/219	213/215/219	216/218	216/218
AG3	212/213	212/213	213	213
AG6	181/189	181/189	187/189	187/189
AG7	173/174/175	173/174/175	173	173
TC1	218/220/228	218/220/228	220/222/226/234	220/222/226/234
TC3	148/158/162	148/158/162	145/146/147/148	145/146/147/148
AG8	220/222/226	220/222/226	210/220/222/226	210/220/222/226
AG10	160/177/185	160/177/185	183/185	183/185
AG12	222/228/230/234	222/228/230/234	228/229/230	228/229/230
TC5	167/169	167/169	164/167	164/167
TC7	195/197	195/197	196/199/210	196/199/210
TC9	165	165	161/162/165	161/162/165

Discussion

Cryopreservation protocols

RB explants of *A. officinalis* cv. MH were used to develop an E–D method. The influence of the preculture time in a medium rich in sucrose, the dehydration time, the precooling treatments and the genetic stability of the recovery material were assessed.

The first step in the use of RB explants from MH was to test the different cryopreservation methods previously described such as simple freezing, vitrification, and preculture and desiccation. These tests were unsuccessful, and bud sprouting was never recorded. Because of these negative results, a search for new and efficient protocols was required. The characteristics of an RB explant as a small, multilayered piece of tissue containing root and shoot primordia suggested that a method using encapsulation in alginate could be suitable. After some test assays, an E–D method, normally used for somatic embryos and shoot tips, was selected. To our knowledge, the encapsulation and germination of RB explants has not been attempted. An alginate capsule permits safe exposure to extreme treatments that would be highly damaging or lethal to non-encapsulated samples (Engelmann et al. 2008).

Adjustment of the pretreatments before cryopreservation in LN is crucial for plant recovery. The survival rate of explants increased with incubation before cryopreservation in a culture medium with a high dose of sucrose. When preculture time in high sucrose increased from 24 to 48 h, the percentage of shoot regrowth always increased (Fig. 1). Suzuki et al. (1997) reported that freezing resistance of shoot tips in *A. officinalis* increased after a 48 h preculture in a medium supplemented with a high concentration of sugar, consistent with the results obtained for our experimental conditions. Similar conclusions were reached by Jitsuyama et al. (2002) working with embryogenic cell suspensions of *A. officinalis* who reported that freezing tolerance increases with the accumulation of sugars and proteins in cells during preculture.

The composition of the alginate beads used in this study was adapted from Sakai et al. (2000) who successfully applied this method in the species *Wasabi japonica, Mentha spicata* and *Chrysanthemum morifolium*. With this encapsulation including sucrose and glycerol in the composition of beads, the asparagus RB explants are progressively dehydrated and simultaneously osmoprotected during the cryopreservation process, which increased the final viability of the explants.

Dehydration also provides ways to overcome the lack of natural tolerance to drying (Reed 1996). It is extremely important to calculate the MC of RB explants and to optimize the drying time and the method to dehydrate the RB explants to minimize the maximum formation of ice crystals. The multilayer structure of the RB explants requires a slow method of dehydration, and the alginate beads provided a protective cover for such a progressive and slow dehydration of the explants. Moreover, dehydration must be a repeatable process; therefore, dehydration was conducted in hermetically closed containers with silica gel, instead of in a laminar flux cabinet.

The use of precooling and prefreezing and particularly the combination of both pretreatments applied before the immersion in LN improved the plant recovery by 20.1, 26.7 and 35.7%, respectively (see Table 3). In a previous report on asparagus cryopreservation, Kohmura et al. (1992) working with bud clusters of cv. "Hiroshima green" applied a similar technique and gradually decreased the temperature from 0 to − 30 °C over 1 h before immersion in LN. However, the combination of these precooling treatments with a vitrification method resulted in a poor rate of plant recovery (3%). In this study, the use of precooling combined with the E–D method for similar explants of cv. MH dramatically improved the efficiency of the cryopreservation process, with 84% plant recovery.

The rooting problems of asparagus increase when explants are subjected to cooling conditions (Yang and Cloré 1974). Several methods used for asparagus cryopreservation (Kumu et al. 1983; Kohmura et al. 1992; Suzuki et al. 1997; Jitsuyama et al. 2002) with different explants such as shoot tips, bud clusters and meristems require a subsequent rooting induction phase after LN freezing, which delays the full recovery of plants over 6 months. Using RB explants of cv. MH with our E–D method, we obtained 42.2% rooted plantlets on average after only 8–10 weeks.

The acclimatization rates (95%) obtained for PRCRBs were identical to those obtained for non- cryogenized plants (Carmona-Martín et al. 2014), and the growth and development of PRCRBs were normal. No differences were observed for PRCRBs in acclimatization for the different E–D protocols tested. These results confirmed that plantlet acclimatization was not compromised by this cryogenic procedure.

Assessment of genetic variability

In different plant species subjected to E–D treatment, the genetic stability of regenerated plants has been confirmed (Fernandes et al. 2008; Martín and González-Benito 2005; Martín et al. 2011; Saha et al. 2015). In the genus Asparagus, genetic variability after cryopreservation has not been examined, and only the absence of morphological abnormalities have been reported (Kohmura et al. 1992; Nishizawa et al. 1993). Nevertheless, ploidy and somaclonal variations in *A. officinalis* are described in protocols involving callus induction and regeneration via organogenesis or via embryogenesis (Araki et al. 1992; Odake et al. 1993; Kunitake et al. 1998; Raimondi et al. 2001; Pontaroli and Camadro 2005), in addition to morphological variations in plantlets (Kohmura et al. 1992). In previous studies of asparagus micropropagation using RB explants, neither ploidy changes nor somaclonal variation was detected (Carmona-Martín et al. 2014; Regalado et al. 2015a).

Flow cytometry is routinely used to evaluate ploidy level (Ozaki et al. 2004; Moreno et al. 2008a; Carmona- Martín et al. 2014; Regalado et al. 2015a, b). Based on the ploidy level analysis of PRCRBs after the application of the E–D cryopreservation method, no major changes in ploidy occurred during the process for the two genotypes examined in this study.

However, although major changes in ploidy were not detected, other minor changes in DNA content could occur, requiring molecular studies to confirm the genetic stability of regenerants. The EST-SSR molecular markers were previously applied in asparagus to evaluate the occurrence of somaclonal variations during micropropagation from RB explants (Carmona-Martín et al. 2014; Regalado et al. 2015a). EST-SSR markers have also been used to study genetic stability in regenerants following various cryopreservation methods, including encapsulation–dehydration in *Pyrus* (Condello et al. 2009) and in *Quercus* sp. (Fernandes et al. 2008), vitrification for *Malus* (Liu et al. 2008) and slow-cooling for *Rubus* (Castillo et al. 2010). In a study of genetic integrity of cryopreserved shoot tips of *C. morifolium* (Wang et al. 2014), eight SSR primer pairs were used to detect somaclonal variation, whereas for shoot tips of cork oak, four EST-SSRs were used. To evaluate the genetic stability of the RB explants after the E–D procedure, we used 12 EST-SSRs, and no somaclonal variations were detected (see Table 5).

According to Harding (1997), the origin of somaclonal variations is usually attributed more to the application of aggressive in vitro tissue culture procedures before or during the cryopreservation process than to the actual cryopreservati on treatment. The type of explant selected, the composition of the culture medium and also the cryopreservation method used helped to maintain the genetic stability of the RB explants under our experimental conditions.

In conclusion, we developed an E–D method for RB explants of *A. officinalis* L. cv. MH and demonstrated that the time of preculture in a medium rich in sucrose and the treatments of precooling before immersion in LN were important for the recovery of plant material after freezing in LN. The type of the explant and the culture medium were essential factors in obtaining full plantlets in 8–10 weeks. Flow cytometry and EST-SSR molecular markers were useful tools to confirm the genetic stability of asparagus explants during the cryopreservation process. Combining advantages of using RB explants and an efficient cryopreservation procedure, an advantage was gained in safeguarding biodiversity and in preserving elite asparagus genotypes that could be valuable for future breeding programs.

Author contributions All authors conceived and planned the experiments. E. C.M. performed the experiments and wrote the manuscript with input from all authors.

Compliance with ethical standards

Conflict of interest. The authors declare that the research review was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Araki H, Shimazaki H, Hirata Y, Oridate T, Harada T, Yakuwa T (1992) Chromosome number variation of callus cells and regenerated plants in *Asparagus officinalis* L. Plant Tissue Cult Lett 9:169–175

Bouman H, de Klerk GJ (1990) Cryopreservation of lily meristems. Acta Hortic 266:331–337

Carmona-Martín E, Regalado JJ, Padilla IMG, Westendorp N, Encina CL (2014) A new and efficient micropropagation method and its breedi ng applications in *Asparagus* genera. Plant Cell Tissue Organ Cult 119:479–488

Caruso M, Federici CT, Roose ML (2008) EST-SSR markers for asparagus genetic diversity evaluation and cultivar identification. Mol Breed 21:195–204. https://doi.org/10.1007/s11032-007-9120-z

Castillo NRF, Bassil NV, Wada S, Reed BM (2010) Genetic stability of cryopreserved shoot tips of Rubus germplasm. In Vitro Ce ll Dev Biol Plant 46:246–256. https://doi.org/10.1007/s1162 7-009-9265-z

Condello E, Palombi MA, Tonelli MG, Damiano C, Caboni E (2009) Genetic stability of wild pear (*Pyrus pyraster* Burgsd) after cryopreservation by encapsulation dehydration. Agric Food Sci 18:136–143

Dereuddre J, Scottez C, Arnaud Y, Duron M (1990) Effects of cold hardening on cryopreservation of axillary pear (*Pyrus communis* L. cv. Beurre Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen: effects of previous cold hardening. CR Acad Sci Paris 310, Ser III: 317–323

Engelmann F, Gonzalez-Arnao MT, Wu Y, Escobar R (2008) The development of encapsulation dehydration. In: Reed BM (ed) Plant cryopreservation: a practical guide. Springer, Corvallis, pp 59–75

Fabre J, Dereuddre J (1990) Encapsulation–dehydration: a new approach to cryopreservation of *Solanum* shoots tips. Cryo Lett 11:413–426

Fernandes P, Rodriguez E, Pinto G, Roldán-Ruiz I, De Loose M, Santos C (2008) Cryopreservation of *Quercus subers* omatic embryos by encapsulation–dehydration and evaluation of genetic stability. Tree Physiol 28:1841–1850

Galbraight DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle intact plant tissues. Science 220:1049–1051

González-Benito ME, Kremer C, Ibáñez MA, Martín C (2016) Effect of antioxidants on the genetic stability of cryopreserved mint shoot tips by encapsulation–dehydration. Plant Cell Tissue Organ Cult 127:359–368

Harding K (1997) Stability of the ribosomal RNA genes in Solanum tuberosum L. plants recovered from cryopreservation. Cryo Lett 18:217–230

Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. Cryo Lett 25:3–22

Jitsuyama Y, Suzuki T, Harada T, Fujikawa S (2002) Sucrose incubation increases freezing tolerance of Asparagus (*Asparagus officinalis* L.) embryogenic cell suspensions. Cryo Lett 23:103–112

Kohmura H, Sakai A, Chokyu S, Yakuwa T (1992) Cryopreservation of in vitro-cultured multiple bud clusters of asparagus (*Asparagus officinalis* L. cv. Hiroshima green (2n = 30) by the techniques of vitrification. Plant Cell Rep 11:433–437

Kumu Y, Harada T, Yakuwa T (1983) Development of a whole plant from a shoot tip of *Asparagus officinalis* L. frozen down to –196 °C. J Fac Agric Hokkaido Univ 61(3):285–294

Kunitake H, Mii M (1998) Somatic embryogenesis and its application for breeding and micropropagation in asparagus (*Asparagus officinalis* L.). Plant Biotechnol 15:51–61

Kunitake H, Nakashima T, Mori K, Tanaka M (1998) Somaclonal and chromosomal effects of genotype, ploidy and culture duration in *Asparagus officinalis* L. Euphytica 102:309–316

Liu YG, Liu LX, Wang L, Gao AY (2008) Determination of genetic stability in surviving apple shoots following cryopreservation by vitrification. Cryo Lett 29:7–14

Martín C, González-Benito ME (2005) Survival and genetic stability of *Dendranthema grandiflora* Tzevelev shoot apices after cryopreservation by vitrification and encapsulation–dehydration. Cryobiology 51:281–289

Martín C, Cervera MT, González-Benito MT (2011) Genetic stability analysis of chrysanthemum (*Chrysanthemum x morifolium* Ramat) after different stages of an encapsulation–dehydration cryopreservation protocol. J Plant Physiol 168:158–166

Mix-Wagner G, Conner AJ, Cross RJ (2000) Survival and recovery of asparagus shoot tips after cryopreservation using the droplet method. N Z J Crop Hortic Sci 28:283–287

Moreno R, Espejo JA, Cabrera A, Millan T, Gil J (2006) Ploidic and molecular analysis of 'Morado de Huetor' Asparagus (*Asparagus officinalis* L.) population: a Spanish Tetraploid Landrace. Genet Resour Crop Evol 53:729–736. https ://doi.org/10.1007/s1072 2-004-4717-0

Moreno R, Espejo JA, Cabrera A, Gil J (2008a) Origin of tetraploid cultivated asparagus landraces inferred from nrDNA ITS polymorphisms. Ann Appl Biol 153:233–241. https ://doi.org/10.111 1/j.1744-348.2008.00254 .x

Moreno R, Espejo JA, Moreno MT, Gil J (2008b) Collection and conservation of 'Morado de Huetor' Spanish tetraploid asparagus landrace. Genet Resour Crop Evol 55:773–777. https ://doi. org/10.1007/s1072 2-008-9358-2

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1992) Cryopreservation of Asparagus (*Asparagus officinalis* L.) embryogenic cells and subsequent plant regeneration by a simple freezing method. Cryo Lett 13:379–388

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of Asparagus (*Asparagus officinalis* L.) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci 91:67–73

Odake Y, Udagawa A, Saga H, Mii M (1993) Somatic embryogenesis of tetraploid plants from intermodal segments of a diploid cultivar of *Asparagus officinalis* L. grown in liquid culture. Plant Sci 94:173–177

Ozaki Y, Narikiyo K, Fujita C, Okubo H (2004) Ploidy variation of progenies from intra- and inter-ploidy crosses with regard to trisomic production in asparagus (*Asparagus officinalis* L.). Sex Plant Reprod 17:157–164

Panis B, Lambardi M (2005) Status of cryopreservation technologies in plants (crops and forest trees). In: The role of biotechnology for the characterization and conservation of crops, forest, animal and fishery genetic resources in developing countries. FAO, Turin, Italy, pp 43–54

Pontaroli AC, Camadro EL (2005) Somaclonal variation in *Asparagus officinalis* L. plants regenerated by organogenesis from long-term callus cultures. Genet Mol Biol 28:423–430

Raimondi JP, Camadro EL, Masuelli RW (2001) Assesment of somaclonal variation in Asparagus by RAPD fingerprinting and cytogenetic analyses. Sci Hortic 90:19–29

Redenbaugh K, Paasch BD, Nichol JW, Kossler ME, Viss PR, Walker KA (1986) Somatic Seeds: Encapsulation of Asexual plant Embryos. Nat Biotechnol 4:791–801

Reed BM (1996) Pretreatment strategies for the cryopreservation of plant tissues. In: Normah MN, Narimah MK, Clyde NM (eds) *In Vitro* conservation of plant genetic resources. Plant Biotechnology Laboratory, Faculty of Life Sciences, University Kebangsaan, Bangi

Regalado JJ, Carmona-Martín E, Castro P, Moreno R, Gil J, Encina CL (2015a) Micropropagation of wild species of the genus *Asparagus* L. and their interspecific hybrids with cultivated *A. officinalis* L., and verification of genetic stability using EST-SSRs. Plant Cell Tissue Organ Cult 121:501–510

Regalado JJ, Carmona-Martín E, Moreno R, Gil J, Encina CL (2015b) Study of the somaclonal variation produced by different methods of polyploidization with colchicine in *Asparagus officinalis* l. Plant Cell Tissue Organ Cult 122:31–44

Saha S, Sengupta C, Ghosh P (2015) Encapsulation, short-term storage, conservation and molecular analysis to genetic stability in alginate-encapsulate microshoots of *Ocimum kilimandscharicum* Guerke. Plant Cell Tissue Organ Cult 120:519–530

Sakai A, Matsumoto T, Hirai D, Niino T (2000) Newly developed encapsulation-dehydration protocol for plant cryopreservation. Cryo Lett 21:53–62

Suzuki T, Kaneko M, Harada T (1997) Increase in freezing resistance of excised shoots tips of *Asparagus officinalis* L. by preculture on sugar-rich media. Cryobiology 34:264–275

Suzuki T, Kaneko M, Harada T, Yakuwa T (1998) Enhanced formation of roots and subsequent promotion of growth of shoots on cryopreserved nodal segments of *Asparagus officinalis* L. Cryobiology 36:194–205

Torres AM, Weeden NF, Martin A (1993) Linkage among isozyme, RFLP and RAPD markers in Vicia Faba. Theor Appl Genet 85:935–945

Uragami A, Sakai A, Nagai M (1990) Cryopreservation of dried axillary buds plantlets of *Asparagus officinalis* L. grown in vitro. Plant Cell Rep 9:328–331

Wang R, Gao X, Chen L, Huo L, Li M (2014) Shoot recovery and genetic integrity of *Chrysantemum morifolium* shoot tips following cryopreservation by droplet-method. Sci Hortic 176 (2014):330–339

Yang HJ, Cloré WJ (1974) Development of complet plantlets from moderately vigorous shoot of stocks of *Asparagus in vitro*. HortScience 9:138–139