Effect of antioxidants on the genetic stability of cryopreserved mint shoot tips by encapsulation–dehydration

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Abstract The effect of antioxidants applied in one step of a cryopreservation protocol by encapsulation–dehydration on recovery and genetic stability of mint shoot tips has been studied. Glutathione (0.16 or 0.24 mM), ascorbic acid (0.28 or 0.43 mM) and α -tocopherol (vitamin E) were added to the preculture medium (0.3 M sucrose). DNA was extracted from three different types of samples: leaves from shoots, callus at the base of shoots and callus. RAPD and AFLP markers were used to assess the genetic stability. The use of antioxidants did not improve recovery after cryopreservation. One of the genotypes, ‘MEN 198’, showed higher percentage of stable samples than the other one, ‘MEN 186’ (56 vs. 37%; considering all treatments and types of explant). The use of vitamin E improved the percentage of stable samples with respect to control treatment (no antioxidant) in ‘MEN 186’. No differences in the percentages of stable samples were observed among cryopreserved and non-cryopreserved (treated similarly without immersion in liquid nitrogen) plant material. Recovered shoots of both genotypes showed higher stability (76–80% stable samples) than callus samples (14–22%).

Keywords Antioxidants · Cryopreservation · Genetic stability · Recovery

Introduction

Cryopreservation (conservation of living material at temperature below -150°C , usually in liquid nitrogen, LN), has been applied to several plant species and types of plant material. Cryopreservation is a reliable method for long-term storage of plant germplasm and biotechnological plant material (e.g. cell cultures). Several cryopreservation protocols based on the vitrification of both intra- and extracellular solutions have been developed since the 1990s (Sakai 2004). Encapsulation–dehydration is one possible procedure. It is based on the synthetic seed technology and vitrification is achieved after dehydration and fast cooling (Fabre and Dereuddre 1990). One advantage of this technique, compared to other possible ones to cryopreserve plant material, is that plant material handling inside the alginate bead is usually easier, and it is a simple technique to implement. This technique has been applied to several species and genotypes (Engelmann et al. 2008).

The poor response to cryopreservation of some species or genotypes has been related, in some cases, to oxidative damage (Johnston et al. 2007). Several treatments applied to plant tissues prior immersion in liquid nitrogen to avoid ice crystal formation impose cellular stress that result in excessive reactive oxygen species (ROS) formation. ROS are formed during cellular metabolic reactions as by products of redox reactions, but also play a role in cell signaling, immunity and apoptosis (Kovalchuk 2011). Exposure to stresses, both biotic and abiotic, increases the levels of free radicals in cells. Oxidative damage has been found in several steps of cryopreservation protocols, as early as explant excision (Whitaker et al. 2010). Osmotic pretreatments can also impose oxidative stress, as shown with olive somatic embryos treated with sorbitol (Lynch et al. 2011). In addition, partial dehydration and cooling increased

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oxidative damage in zygotic embryos of recalcitrant seeds (Seršen et al. 2012). When the response after cryopreservation by encapsulation–dehydration of two *Ribes* species was compared, the species that showed higher recovery after cryopreservation displayed higher antioxidant activity (Johnston et al. 2007). Furthermore, differential response to cryopreservation (by vitrification with PVS2) of *Arabidopsis thaliana* 48- and 72 h-seedlings (the latter being more sensitive) was related to the expression of oxidative stress response genes (Ren et al. 2013). However, ROS, at low concentrations, regulate cellular functions and act as signalling molecules and, in this line, an increased ROS generation was found to be related to improved viability after cryopreservation of *Lilium × siberia* pollen (Xu et al. 2014).

ROS are highly reactive and can disturb the normal cellular activity through oxidative damage to membrane lipids, proteins and nucleic acids (Imlay 2003; Bhattacharjee 2011). DNA damage is produced mainly by $\text{OH}^\cdot$ and $^1\text{O}_2$: $\text{OH}^\cdot$ is the most damaging as affects purine and pyrimidine bases as well as deoxyribose; $^1\text{O}_2$ attacks guanine (Gill and Tuteja 2010). The damages produced include deletions, mutations, base modifications and strand breaks. Besides, $\text{O}_2^{\cdot-}$ has been reported to induce lipid peroxidation, inactivation of enzymes and changes in DNA, causing mutations in plants (Arora et al. 2002).

Antioxidants are part of the mechanism that controls the accumulation of ROS in plants, to lessen their cytotoxic and reactive properties (Bhattacharjee 2011). Ascorbic acid is present in chloroplast, cytosol, vacuoles and apoplast; it is a direct scavenger of $\text{O}_2^{\cdot-}$ and has an essential role removing H_2O_2 through the Halliwell-Asada pathway. H_2O_2 is also removed thorough the ascorbate/glutathione cycle (Alscher and Hess 1993). Glutathione prevents lipid peroxidation and, by the oxidation of its thiol group, avoids the oxidation of the thiol group of enzymes. Vitamin E refers to a group of compounds that include both tocopherols and tocotrienols; α -tocopherol is a lipophilic antioxidant that protects membranes against lipid peroxidation and a scavenger of $^1\text{O}_2$, $\text{OH}^\cdot$ and $\text{O}_2^{\cdot-}$ (Bhattacharjee 2011).

Many antioxidants have proved to be effective increasing regrowth after cryopreservation in several species (Reed 2014). Survival of *Citrus* shoot tips after cryopreservation by vitrification was enhanced by the use of glutathione (GSH) in the preculture medium (containing glycerol and high sucrose concentration), in the loading and the vitrification solutions, and in the recovery medium (Wang and Deng 2004). Similarly, supplementation of both preculture and regrowth media with melatonin (0.1–0.5 μM) improved regrowth of shoot tips of American elm, St John's Wort and tobacco after cryopreservation by vitrification with PVS2 (Uchendu et al. 2013, 2014). When 0.28 mM ascorbic acid was added to several steps of the vitrification protocol, higher survival was observed in shoot tips of *Nephelium*

ramboutan-ake not subjected to LN exposure than without the addition of the antioxidant. Furthermore, survival after exposure to LN took place only with the addition of ascorbic acid during the loading step (Chua and Normah 2011). Uchendu et al. (2010a) compared the use of vitamin E and ascorbic acid, at several concentrations, in four steps (pretreatment, loading, rinsing, regrowth) of a vitrification protocol for the cryopreservation of shoot tips of two blackberry cultivars. Both substances improved regrowth after cryopreservation when applied in any of the steps tested; nevertheless ascorbic acid was more effective and at a wider range of concentrations (0.16–0.58 mM).

Although the relationship between antioxidant treatments and recovery improvement after cryopreservation has been well documented, the effect of the antioxidants on genetic fidelity of the conserved material has not been sufficiently explored.

Therefore, we hypothesize that antioxidants could play a role not only improving the recovery after cryopreservation, but also reducing genetic instability. A previous study from our group, in which the genetic instability of chrysanthemum shoot tips originated from several steps of a cryopreservation protocol was assessed, found an increased frequency of DNA variation after the high concentration (0.3 M) sucrose pre-treatment (Martín et al. 2011). Consequently, the aim of this work was to study the effect of several antioxidants on recovery and genetic stability of shoot tips of two mint genotypes after cryopreservation by encapsulation–dehydration. The antioxidants were applied during the step of the protocol where osmotic stress was firstly applied to plant material (culture on 0.3 M sucrose).

Materials and methods

Plant material

Two clones of *Mentha × piperita* L. ('MEN 186' and 'MEN 198') were received from the genebank of IPK (Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany). In vitro plants of each clone were micropropagated by monthly subculture of shoots on MS medium (Murashige and Skoog 1962) supplemented with 3 % sucrose and 0.7 % agar and incubated at 25 °C, with a 16 h photoperiod and a photosynthetic photon flux density (PPFD) of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from cool white fluorescent tubes.

Preconditioning treatments

Nodal segments (approximately 1 cm long) were excised, cultured on MS medium and incubated for 3 weeks at 25 °C (light)/–1 °C (dark) with a photoperiod of 16 h and 50 μmol

$\text{m}^{-2} \text{s}^{-1}$ PPFD. Subsequently, shoot tips (approx. 2 mm long, with 1–2 pairs of leaf primordia) developed from axillary buds of nodal segments were excised under dissecting microscope and cultured on liquid MS medium supplemented with 0.3 M sucrose, on top of two filter paper disks. Different antioxidants were used in this step of the protocol: 0.16 or 0.24 mM glutathione, 0.28 or 0.43 mM ascorbic acid, all of them added to the medium; or α -tocopherol (vitamin E) spread on filter paper and placed on the liquid medium. Shoot tips cultured on 0.3 M sucrose without any antioxidant were considered the control treatment. In all cases, tips were incubated at 25 °C for 1 day, with a PPFD of approximately $10 \mu\text{mol m}^{-2} \text{s}^{-1}$, with 16 h photoperiod.

Cryopreservation

Pre-treated tips were immersed in alginate solution (liquid MS + 0.35 M sucrose + 3 % sodium alginate medium viscosity). Drops of this solution, each one containing a shoot tip, were dispensed in MS liquid medium containing 100 mM CaCl_2 and 0.35 M sucrose, and maintained for 30 min in that solution. Subsequently, the already formed beads were cultured in MS liquid medium supplemented with 0.75 M sucrose, shaking at 120 rpm, for 18–20 h. After this period, the beads were removed from the medium, blotted on sterile filter paper and placed on open Petri dishes to be dried for 5 h under the flow of a laminar-flow bench (to 22 ± 1 % water content fresh weight basis, Teixeira et al. 2014). Once dried, five beads were placed in a cryovial and immersed for 1 day in liquid nitrogen (LN). Subsequently, the cryovials were reheated in a sterile water bath at 40 °C for 2 min and, afterwards, the beads were cultured in 6 cm diameter Petri dishes (5 beads per Petri dish) containing recovery medium [MS + 0.5 mg L^{-1} 6-dimethylallylamino-purine (2-iP) + 0.1 mg L^{-1} α -naphthalene acetic acid (NAA) + 3 % sucrose + 0.7 % agar; Senula et al. 2007]. Cultures were incubated at 25 °C, the first 24 h under darkness followed by 20 days with approximately $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD, and then transferred to $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, always with a 16 h photoperiod. 4 weeks after recovery (since the beads were cultured), all surviving plant material from one Petri dish was transferred to a culture jar with MS medium and cultured for further 4 weeks. After this time, data on survival and shoot recovery were registered. In genotype ‘MEN 186’ both non-cryopreserved (subjected to all treatments except immersion in LN) and cryopreserved shoot tips were studied, while only cryopreserved tips were studied in ‘MEN 198’.

Sampling and DNA isolation

From each treatment, different types of plant material were used for DNA extraction after the 8-week recovery period: shoots regenerated from tips (“L”), callus that appeared at

the base of shoots (“cL”), and explants that resulted only in callus growth after treatments (“C”). DNA was also extracted from 5 shoots from which nodal segments were excised to start the experiment and pooled to include them in the analysis as reference for stability; this DNA will be referred as in vitro samples (“VITRO” in the dendrograms). The samples were stored at –80 °C until DNA isolation. Genomic DNA was extracted from frozen tissue, according to Gawel and Jarret procedure (1991).

Seven to 11 samples were analyzed per antioxidant treatment for RAPD analysis, and 3 or 5 samples for AFLP (see Tables 1, 2).

RAPD analysis

Eight primers from Operon Technologies Inc. (Alameda/CA, USA) were used for RAPD analysis: OPA-11 (5′ CAATCGCCGT 3′), OPE-19 (5′ ACGGCGTATG 3′), OPF-1 (5′ ACGGATCCTG 3′), OPF-3 (5′ CCTGATCACC 3′), OPF-10 (5′ GGAAGCTTGG 3′), OPO-7 (5′ CAG CACTGAC 3′), OPO-10 (5′ TCAGAGCGCC 3′) and OPO-20 (5′ ACACACGCTG 3′). DNA amplification reactions were performed according to Martin et al. (2015). The PCR amplifications were performed in a DNA Thermal Cycler (Eppendorf) using one cycle of 1 min at 94 °C, followed by 35 cycles of 45 s at 92 °C, 1 min at 37 °C and 2 min at 72 °C, and a final cycle of 3 min at 72 °C. Aliquots of 12 μL of amplification products were loaded onto 1.5 % (w/v) agarose gels for electrophoresis (carried out at 110 V) using 1 × TBE running buffer (Sambrook et al. 1989), followed by staining with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) for 7–10 min and photographed under UV light. The size of the amplified bands was related by reference to the molecular size marker (100 Base-Pair Ladder, GE Healthcare). All the amplifications were repeated at least twice, and only bands reproducible in several runs were considered for analysis.

AFLP analysis

AFLP analysis was performed according to Vos et al. (1995). The PCR reaction was performed in 20 μL containing 30 ng of each primer *Eco RI* + A and *Mse I* + C, 0.2 mM of each dNTP, 1.5 mM MgCl_2 , 0.4 U of Taq DNA polymerase (Biotaq, Bioline) and 3 μL of diluted fragments in the 1 × PCR buffer provided by the manufacturer (Bioline). PCR amplification consisted of 28 cycles of 30 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C, and was carried out in a MJ thermocycler. For the selective amplification, the *Eco RI* primers were labelled with FAM fluorochrome; the primer combinations used were: A2 (*Eco* + ACG/*Mse* + CCA); A3 (*Eco* + ACA/*Mse* + CAG); C1 (*Eco* + ACC/*Mse* + CCA); C2 (*Eco* + ACC/*Mse* + CAG); C5 (*Eco* + ACA/*Mse* + CAT); C6 (*Eco* + ACA/*Mse* + CCA); C7 (*Eco* + AGT/*Mse* + CAT).

Table 1 Percentage of stable samples (compared to the in vitro plant material) recovered from mint ‘MEN 186’ shoot tips, according to RAPD and AFLP markers

	Percentage of stable samples and number of samples analysed				
	RAPD analysis			AFLP analysis	
	No. of samples	100 % similarity (Jaccard coefficient = 1)	95 % similarity (Jaccard coefficient = 0.95)	No. of samples	95 % similarity (Jaccard coefficient = 0.95)
Pretreatment					
Control	10	20.0 ab	40.0 a	5	40.0
0.16 mM glutathione	9	0.0 a	44.4 ab	4	50.0
0.24 mM glutathione	11	27.0 bc	54.5 ab	4	50.0
0.28 mM ascorbic acid	8	62.5 cd	62.5 ab	5	60.0
0.43 mM ascorbic acid	10	40.0 bcd	80.0 bc	5	40.0
Vitamin E	11	72.7 d	100 c	5	60.0
Cryopreservation					
Non-cryopreserved	24	41.7	75.0	10	50.0
Cryopreserved	35	32.3	57.1	18	50.0
Type of plant material					
“L”	19	79.0 b	100 b	12	75.0 b
“cL”	19	21.0 a	57.9 a	9	44.4 ab
“C”	21	14.3 a	38.0 a	7	14.3 a
Total	59	37.3	64.4	28	50.0

Percentages with the same letter are not significantly different according to pair-wise comparison by LSD ($\alpha = 0.05$)

Type of plant material: “L” shoot, “cL” callus at the base of the shoot, “C” callus

Table 2 Percentage of stable samples (compared to the in vitro plant material) recovered after cryopreservation of mint ‘MEN 198’ shoot tips, according to RAPD and AFLP markers

	Percentage of stable samples and number of samples analysed				
	RAPD analysis			AFLP analysis	
	No. of samples	100 % similarity (Jaccard coefficient = 1)	95 % similarity (Jaccard coefficient = 0.95)	No. of samples	95 % similarity (Jaccard coefficient = 0.95)
Pretreatment					
Control	7	28.6	85.7	4	100
0.16 mM glutathione	10	70.0	90.0	4	100
0.24 mM glutathione	7	42.9	85.7	3	66.7
0.28 mM ascorbic acid	8	62.5	87.5	4	100
0.43 mM ascorbic acid	8	50.0	87.5	5	80
Vitamin E	10	70.0	100	4	100
Type of plant material					
“L”	21	76.2 b	100 b	10	100 b
“cL”	20	50.0 ab	90.0 ab	10	100 b
“C”	9	22.2 a	66.7 a	4	50.0 a
Total	50	56.0	90.0	24	91.7

Percentages with the same letter are not significantly different according to pair-wise comparison by LSD ($\alpha = 0.05$)

Type of plant material: “L” shoot, “cL” callus at the base of the shoot, “C” callus

The PCR selective amplification was performed using 1 cycle of 30 s at 94 °C, 30 s at 65 °C, 1 min at 72 °C followed by 12 cycles in which the annealing temperature decreases 0.7 °C per cycle, followed by 23 cycles of 1 min at 94 °C, 30 s at 56 °C, and 1 min at 72 °C. Amplification products were analysed in an automated ABI3730 sequencer by the company SECUGEN S.L. (Madrid, Spain). The resulted electropherograms were analysed using GeneMarker® v 1.90 software (SoftGenetics, LLC).

Data analysis

Five beads were cultured on each Petri dish containing recovery medium; two and six replicates were used for non-cryopreserved (LN–) and cryopreserved (LN+) samples, respectively, for each of the treatments studied. Non-cryopreserved samples were subjected to the same treatments with the exception of the immersion in LN and in the warm water bath. Survival (callus formation or shoot development) and recovery (shoot development) were recorded after the 8-week recovery period. Data were analyzed by logistic regression for events data and pair-wise comparisons were carried out by LSD with a confident interval of 95 % (Agresti 2002). Analyses were carried out with the Generalized Lineal Models procedure of the program IBM SPSS Statistics 20.

Amplified fragments from the RAPD analyses were scored as present (1) or absent (0). Genetic similarities were calculated using the Jaccard similarity coefficient (Jaccard 1908): $GS(ij) = a/(a + b + c)$; where a is the number of polymorphic DNA fragments common to both individuals, b is the number of fragments present in i and absent in j , and c is the number of fragments present in j and absent in i . Jaccard coefficient ranged between 0 and 1. The resultant matrix was subjected to cluster analysis by the unweighted pair-group method analysis (UPGMA) and a dendrogram was constructed from the clustering results with the TREE program. These analyses were performed using the computer program NTSYS-PCv. 1.80 (Rohlf 1992). Similar analyses were carried out with AFLP results. Percentage of genetic stable samples (similarity of 100%, i.e. Jaccard coefficient = 1, or 95%, i.e. Jaccard coefficient ≥ 0.95 , with the “VITRO” samples) was analysed using a logistic regression method for binary data and pair-wise comparisons were carried out by LSD with a confident interval of 95 %. Analyses were carried out with the Generalized Lineal Models procedure of the program IBM SPSS Statistics 20.

Results

Survival and recovery were high (100–90 and 70–90%, respectively) in non-cryopreserved shoots of ‘MEN 186’, with no significant differences among treatments (Fig. 1a).

Fig. 1 Survival and recovery of mint shoot tips (**a** genotype ‘MEN 186’ and **b** ‘MEN 198’) after cryopreservation by encapsulation–dehydration. LN–: non-cryopreserved tips; LN+: cryopreserved tips; Control: pretreatment on 0.3 M sucrose without antioxidants; 0.16 GHS and 0.24 GHS: 0.16 or 0.24 mM glutathione, respectively; 0.28 AA and 0.43 AA: 0.28 or 0.43 mM ascorbic acid, respectively; VitE: α -tocopherol. All antioxidants included in the 0.3 M sucrose medium. Bar standard error. For survival and recovery, means with the same letter are not significantly different according to pair-wise comparison by LSD ($\alpha=0.05$)

After cryopreservation, survival was maintained high (86–97%); however, recovery decreased significantly (13–23%), with no significant difference among treatments (Fig. 1a). Recovery rates in cryopreserved shoots of ‘MEN 198’ were higher than in ‘MEN 186’ (Fig. 1b). In this genotype, survival ranged from 37% (0.28 mM ascorbic acid) to 77% (0.16 mM GHS). Although there were significant differences (Wald’s $\chi^2 = 17.147$; $df = 5$; $sig. = 0.004$) among treatments, none of the antioxidants significantly increased survival compared to the control treatment (no antioxidant). Similar results were obtained from the statistical analysis of recovery (Wald’s $\chi^2 = 20.594$; $df = 5$; $sig. = 0.001$); however, recovery was not improved by the antioxidant treatments and was significantly decreased in the case of 0.24 mM GHS.

The eight RAPD primers used yielded 75 and 94 scorable bands for the ‘MEN 198’ and ‘MEN 186’ genotypes, respectively, ranging from 2500 to 300 bp. From these results, one divergence in the presence or absence of a single amplified fragment represented a dissimilarity ranging from 0.015

to 0.016 (Jaccard coefficient between 0 and 1). In 'MEN 186', 39 RAPD markers (41.5%) resulted monomorphic. Thirty-seven out of 59 samples (63%) presented polymorphic markers. However, a lower percentage of samples with polymorphisms was detected in 'MEN 198', 44% (22 non stable samples out of 50), with a total of 29 polymorphic RAPD markers.

The AFLP profiles were generated from some of the same samples analyzed with RAPD markers: 29 samples in 'MEN 186' and 25 for 'MEN 198', including the in vitro plant material. The used primer combinations produced 126 and 139 scorable fragments, respectively for each genotype. From these results, one divergence in the presence or absence of a single amplified fragment represented a dissimilarity ranging from 0.011 to 0.015. Monomorphic markers represented 38.9% of the total in 'MEN 186' and a higher value, 59.7%, was observed in 'MEN 198'. A representation of polymorphic samples of 'MEN 186' is shown in Fig. 2. The first and second electropherograms belong to the in vitro (VITRO) and G2+L60 samples, respectively, both with the maximum similarity coefficient (1). Some differences, i.e. absence or presence of new fragments, are observed in samples VE+cL120, G1+C37 and VE+C118 (Fig. 2).

According to the RAPD analysis of genotype 'MEN 186', the minimum similarity observed among the 60 samples, including the in vitro plant material, was 0.62 (Fig. 3). Most of the 22 identical (Jaccard coefficient=1) samples to the in vitro one corresponded to vitamin E treatment (Table 1). The percentage of stable samples recovered from the vitamin E treatment (72.7%) was significantly different to the percentages obtained with control and glutathione treatments (0–27%; Table 1). There was no significant difference when non-cryopreserved or cryopreserved samples were considered (41.7 and 32.3%, respectively). When data were analysed according to the type of plant material, 80.0% of shoots were stable, while for callus at the base of shoots ("cL") and callus ("C") this figure decreased significantly to 16.7 and 14.3%, respectively (Table 1).

Considering 0.95 similarity level of the RAPD markers to the in vitro plant material (Table 1; Fig. 3), 38 samples (64.4%) were stable in 'MEN 186'. Treatments analysed showed similar behaviour to the one observed at Jaccard coefficient similarity of 1: best results were obtained with vitamin E (100% stable samples), which shows significant differences with the other treatments except with 0.43 mM ascorbic acid (80% stable samples). On the other hand, the lowest percentages of stable plant material were obtained from the control and 0.16 mM glutathione treated samples (40 and 44.4%, respectively). However, in this analysis at 0.95 similarity, differences in the number of stable samples were observed between non-cryopreserved and cryopreserved samples (75 and 57.1%, respectively), although

there were not significant. When the data were compared according to the type of sample, 100% of shoots were stable, and this value was significantly different to the percentage obtained from both types of callus samples.

According to AFLP analysis, the minimum similarity level observed among the 28 samples analysed of genotype 'MEN 186' was 0.75 (Fig. 3). Only three samples were completely similar to the in vitro plant material, and 14 samples were stable at 0.95 similarity level. The treatments from which more stable samples were recovered were vitamin E and 0.28 mM ascorbic acid (60.0%), although there were no significant differences with the percentages obtained with the other treatments (Table 1). Non-cryopreserved and cryopreserved plant material showed the same results (50% stability). Again, higher stability percentage was found among shoots (75.0%), although it was not significantly different to the stability of callus at the base of shoots (44.4%).

In genotype 'MEN 198', the minimum similarity observed was 0.9, using both RAPD and AFLP markers (Fig. 4). Twenty-eight samples (56%) were stable at the maximum similarity coefficient using RAPD markers. This percentage increased to 90% when considering 0.95 similarity level; nearly the same value was obtained in the AFLP analysis at this level (91.7%; Table 2). In this genotype, AFLP markers detected three samples (12.5%) identical to the in vitro plant material (G2+L60, A1+L70, A1+cL71). There was not a significant difference among the percentages of stable recovered plant material regardless the antioxidant treatment used in any of the analyses. Both types of markers revealed significant differences in the stability according to the type of material (Table 2).

Discussion

The influence of antioxidant substances used during an encapsulation–dehydration cryopreservation protocol on the recovery of mint shoot tips and the genetic stability of the recovered material has been assessed. In the present study, the use of antioxidant substances in the preculture medium (with 0.3 M sucrose) did not improve recovery of mint shoot tips after cryopreservation. However, there are several works in which antioxidants have improved recovery after cryopreservation when the vitrification procedure was used (Wang and Deng 2004; Chua and Normah 2011; Uchendu et al. 2010a, b, 2013, 2014). In particular, Uchendu et al. (2010a, b) found that glutathione and vitamin E improved recovery after cryopreservation by vitrification of two *Rubus* genotypes, when applied in any of the protocol steps: pretreatment, loading, rinsing, or regrowth. Vitamin C improved recovery when used in the first three steps, and also when added in recovery medium lacking iron (Uchendu et al. 2010a). On the contrary, not only we have

Fig. 2 AFLP electropherograms of some samples from accession ‘MEN 186’ amplified with the primers Eco + ACA/Mse + CAG. The *x*-axis represents the size (bp) of the DNA fragments and the *y*-axis

indicates the amount of the amplified products. See legend of Fig. 3 for explanation of the sample codes

not observed a beneficial effect, but the addition of 0.24 mM glutathione resulted detrimental for recovery in one of the mint genotypes (‘MEN 198’) studied in this work. However, it must be taken into account the fact that, in the present work, the antioxidants were added only in one step of the protocol. Besides, another difference with the studies mentioned above is that in our experiment the protocol analysed was encapsulation–dehydration instead of vitrification. For these reasons, further investigation could be necessary to establish if the stresses imposed by the vitrification procedure could differ to those of the encapsulation–dehydration protocol. Furthermore, ROS scavenging properties of

sucrose have been demonstrated (Van den Ende and Valurru 2009). The addition of antioxidants in our experiment was carried out in medium containing 0.3 M sucrose and the next step of the protocol used implies the incubation of apices in 0.75 M sucrose; therefore, the step of the protocol in which antioxidants are applied, and their possible interaction with sucrose, could influence the results.

Although we have not found any reference relating genetic stability to the use of antioxidants in plant cryopreservation protocols, our hypothesis was that the improvement in regrowth produced by these substances would imply higher genetic stability. Nevertheless, the use of glutathione,

Fig. 3 Dendrograms generated by the UPGMA method using Jacard's similarity coefficient, based on AFLP or RAPD markers of samples from mint plant material recovered 8 weeks after cryopreservation of accession 'MEN 186', using the encapsulation–dehydration technique. First two letters/numbers represent the pretreatment: "CT"

control, "G1" 0.16 mM glutathione, "G2" 0.24 mM glutathione, "A1" 0.28 mM ascorbic acid, "A2" 0.43 mM ascorbic acid, "VE" vitamin E. Third number/symbol: "0" non-cryopreserved, "+" cryopreserved. The type and number of samples follow: "L" shoot, "cL" callus at the base of the shoot, "C" callus

ascorbic acid or vitamin E applied in the medium supplemented with 0.3 M sucrose (pretreatment previous to the encapsulation and incubation in 0.75 M sucrose), did not improved recovery in the two mint genotypes studied. However, according to RAPD markers' results, vitamin E seemed to improve stability in the less stable genotype ('MEN 186'). In a previous work of our group where the effect of cryopreservation technique, genotype and culture medium on genetic stability was studied, genotype 'MEN 198' showed higher percentage of stable samples than 'MEN 186' (Martín et al. 2015). These genotypes have been used again in the present work to compare the antioxidant treatments in plant material with different stability behaviour. Similar results have been obtained in the present study. The RAPD markers revealed 64% of stable samples (for 0.95 similarity, Table 1) in 'MEN 186', while that valued was 90% for 'MEN 198' (Table 2).

The negative effect of free radicals on genetic stability of cryopreserved animal cells has also been studied (Kopeika et al. 2015), and the use of antioxidants has been considered to minimize this effect. However, as it was observed in our results, the use of antioxidants is not always necessarily correlated with an improvement of DNA stability. Hosseini et al. (2009) supplemented the culture media for bovine embryos after cryopreservation with known antioxidants to avoid the observed DNA fragmentation; nonetheless, no significant improvement was observed, suggesting that either more than one mechanism for DNA fragmentation might exist or the antioxidant media were used in suboptimal quantities to achieve the desired effect.

The percentage of stable samples differed among the plant type materials recovered. In both genotypes, that percentage was always higher in the recovered shoots compared to the

Fig. 4 Dendrograms generated by the UPGMA method using Jac- card's similarity coefficient, based on AFLP or RAPD markers of samples from mint plant material recovered 8 weeks after cryopres- ervation of accession 'MEN 198', using the encapsulation–dehydration technique. First two letters/numbers represent the pretreatment: "CT"

control, "G1" 0.16 mM glutathione, "G2" 0.24 mM glutathione, "A1" 0.28 mM ascorbic acid, "A2" 0.43 mM ascorbic acid, "VE" vitamin E. Third number/symbol: "0" non-cryopreserved, "+" cryopreserved. The type and number of samples follow: "L" shoot, "cL" callus at the base of the shoot, "C" callus

callus tissues (either "cL" or "C"; Tables 1, 2). However, it was only significantly different to the percentage of stable samples of callus at the base of shoots ("cL") in 'MEN 186' when 100% similarity was considered. The percentage of stable callus ("C") was always significantly lower than that of recovered shoots ("L" samples). The dedifferentiation process in callus, followed by redifferentiation events when indirect shoots are developed, leads to frequent genetic modifications (Munthali et al. 1996; Al-Zahim et al. 1999; Salvi et al. 2001). A higher genetic stability in recovered shoots is an expected result even when they derived from instable callus (Miñano et al. 2014). This is not the case in our study since "cL" samples and their corresponding shoot ("L") were identical (e.g. samples G20L46 and G20cL47 in 'MEN 186') or closely related (e.g. CT+L9 and CT+cL10 in "MEN 186"; Fig. 3).

As we observed in our previous study (Martín et al. 2015), the results obtained in this work confirm that the detected instability could not be attributed to the extreme low temperature ("LN") as there were not differences between non cryopreserved (but treated) and cryopreserved apices of 'MEN 186'. Likewise, alternative treatments to

the antioxidants assayed should be considered to improve genetic fidelity and to understand the mechanisms affecting the loss of stability.

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