



Research article

Genetic and epigenetic stability of recovered mint apices after several steps of a cryopreservation protocol by encapsulation-dehydration. A new approach for epigenetic analysis

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ABSTRACT

The genetic and epigenetic stability (analysis of DNA methylation using MSAP markers) of mint (*Mentha x piperita* L.) apices was studied after each step of a cryopreservation protocol, by encapsulation-dehydration. The effect of the addition of an antioxidant (ascorbic acid) during one of the protocol steps was also evaluated. Eight-week old *in vitro* recovered shoots from apices after each step of the protocol were genetically stable when compared to control *in vitro* shoots, using RAPD and AFLP markers. The addition of ascorbic acid in the medium with the highest sucrose concentration did not improve recovery and did not have any effect on stability. Apices sampled immediately after each step showed increased epigenetic differences as the protocol advanced, compared to *in vitro* control apices, in particular related to *de novo* methylation events. However, after one-day *in vitro* recovery, methylation status was similar to control apices. To improve the quality of methylation data interpretation, a simple and fast method for MSAP markers analysis, based on R programming, has been developed which allows the statistical comparison of treatments to control samples and its graphical representation.

1. Introduction

Cryopreservation is considered one of the most attractive long-term plant germplasm conservation techniques, especially for species with vegetative propagation or recalcitrant seeds. Protocols have been developed for many species (Kulus and Zalewska, 2014; Reed, 2017; Normah et al., 2019); however, there is concern regarding the possible genetic variation induced in plants. During the application of cryopreservation procedures cells/tissues are exposed to extreme conditions (e.g. excision, osmotic treatments, dehydration, ultra-low temperatures) that frequently impose physiological stresses which could cause genetic instability (Harding, 2004; Panis and Lambardi, 2006). Furthermore, in some cases the *in vitro* culture procedures required in a cryopreservation protocol have been considered the main origin of the detected variations (Harding, 1997, 2004; Channuntapipat et al., 2003). Recent studies have reported no differences (Turner et al., 2001;

Nair and Reghunath, 2009; Atmakuri et al., 2009; Srivastava et al., 2009; Castillo et al., 2010; Salaj et al., 2011; Ai et al., 2012; Matsumoto et al., 2013) or small differences (Kaity et al., 2008, 2009; Zeliang et al., 2010; Martín et al., 2011, 2015) between cryopreserved or treated explants compared to the starting plant material.

Several stresses imposed on cells/tissues during cryopreservation are related to oxidative damage due to the over-production of Reactive Oxygen Species (ROS). For example, the osmotic pretreatment with sorbitol applied to cryopreserved olive somatic embryos increased their oxidative stress (Lynch et al., 2011). ROS are highly reactive and can alter the normal cellular activity affecting membrane lipids, proteins and nucleic acids (Imlay, 2003; Bhattacharjee, 2011). DNA damage produced directly by ROS has been detected in some plant cultures (Arora et al., 2002; Gill and Tuteja, 2010). Different antioxidants have been tested to reduce the ROS effect on cryopreserved explants with differing results. The use of ascorbic acid and tocopherol improved

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regrowth after cryopreservation in different plant species (Uchendu et al., 2010a, b). In a previous work, our group studied the effect of antioxidants applied in one step of a cryopreservation protocol by encapsulation-dehydration in two mint genotypes. Our hypothesis was that antioxidants could play a role, not only improving the recovery after cryopreservation, but also reducing genetic instability. However, results showed that the antioxidants used did not have a significant effect on recovery, although Vitamin E improved stability in the less stable genotype (González-Benito et al., 2016).

Stress conditions in cryopreservation procedures may not only affect the genetic stability or ROS production, but also the epigenetic stability of the cryopreserved material. It is well known that epigenetic changes are involved in plant response to stress situations (Kaepler and Phillips, 1993; Finnegan, 2001). Furthermore, epigenetic changes during *in vitro* culture and cryopreservation have been attributed to the stressful conditions of these techniques (Adu-Gyamfi et al., 2016). DNA methylation is probably the best studied epigenetic phenomenon in Eukaryotes, particularly the addition of a methyl group to cytosine residues. Cytosine methylation is related to gene transcription repression by acting on chromatin structure or DNA-binding regions (Inamdar et al., 1991; Davey et al., 1997).

Although many studies have been published on genetic stability after cryopreservation, the effect that these processes have on epigenetic stability has been more scarcely approached. Among the first works are those of Hao and collaborators on *Citrus* callus (Hao et al., 2002a, b) and strawberry plants (Hao et al., 2002a, b). Later, methylation was studied on *Humulus lupulus* (Peredo et al., 2008), *Ribes* (Johnston et al., 2009), papaya (Kaity et al., 2013), and cocoa (Adu-Gyamfi et al., 2016). All these authors reported changes in the DNA methylation status of the treated plants.

Methylation Sensitive Amplified Polymorphism (MSAP) is one of the techniques most widely used for epigenetic studies. It is a modification of the AFLP (Amplified Fragment Length Polymorphism) method and relies on the differential sensitivity of restriction nucleases to cytosine methylation in order to analyse the methylation status of the recognition sequences (Reyna-Lopez et al., 1997). The MSAP markers are a reliable method for the identification of genome regions with putative changes in DNA cytosine methylation in response to environmental and developmental stimuli (Fulneček and Kovařík, 2014). Furthermore, MSAP is an appropriate technique in non-model species, and it is very useful, not only to detect changes in the cytosine methylation pattern, but also to characterize the type of changes (demethylation or *de novo* methylation). However, MSAP analysis is complex and time consuming when a high number of samples are studied.

Approaches developed so far to easily carry out the analysis of MSAP data have been made in the field of ecology, to study population diversity or plasticity (Pérez-Figueroa, 2013). Variability is expected when working with natural populations, and its analysis and classification is part of the objective of the use of MSAP markers. On the contrary, in germplasm conservation studies, stability is required, and therefore the comparison to original plant material is necessary.

For plant germplasm conservation, it is important to guarantee the highest quality and safety of the maintained material for its future use. In this context, it is necessary to assure the phenotypic, genetic and epigenetic stability of the plant material. To achieve this objective, the use of fast and easy tools will be required. These analyses will facilitate the work of curators in order to guarantee the collection quality. Therefore, detailed knowledge of the mechanisms behind the conservation protocols and of their influence on the collections is required.

The aim of this study was to assess the genetic and epigenetic stability of mint apices throughout a cryopreservation (by encapsulation-dehydration) protocol, using a sequential analysis, and the effect of ascorbic acid on the process. In addition, a statistical approach, based on R programming, is implemented to analyse MSAP data. Based on this approach, a new application has been developed to facilitate the interpretation of DNA methylation studies in conservation experiments

(Methylation Analysis Inference or MAI application).

2. Materials and method

2.1. Plant material

In vitro propagated shoots of *Mentha x piperita* L. genotype 'MEN 198' from the Leibniz Institute of Plant Genetics and Crop Plant Research genebank were used as plant material. They were monthly subcultured on MS medium (Murashige and Skoog, 1962) supplemented with 3% sucrose and 0.7% agar. Incubation took place at 22–25 °C with a photoperiod of 16 h light, 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD) from fluorescent cool white light.

2.2. Steps of the cryopreservation protocol by encapsulation-dehydration

Step N. From *in vitro* shoots cultures, nodal segments were extracted, which were grown on MS medium and incubated for 3 weeks at alternating temperatures for cold acclimation $-1 / 25$ °C, 8h dark/16h light at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPFD irradiance. For the control sample (step A), nodal segments were cultured on MS and incubated at 25 °C (with the same photoperiod and PPFD) for one week to obtain a similar level of development of the axillary buds to the incubation for 3 weeks at $-1 / 25$ °C.

Step P. After cold acclimation, apices that developed from axillary buds (1–2 mm) of the nodal segments were excised. Approximately fifteen apices were cultured on 2 mL MS liquid medium with 0.3 M sucrose (on top of 2 filter paper disks in 6 cm diameter Petri dishes). Apices were incubated for 1 day at 25 °C, with a PPFD of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and 16 h photoperiod.

Step S. Pre-treated apices were immersed in alginate solution (liquid MS + 0.35 M sucrose + 3% alginate). They were dispensed in drops into the polymerization solution (MS liquid medium + 100 mM CaCl_2 + 0.35 M sucrose) and maintained for 30 min in that solution. Subsequently, the already formed beads were placed in MS liquid medium + 0.75 M sucrose, and shaken at 120 rpm for 18–20 h.

Step D. After this period, the beads were removed from the medium, blotted on sterile filter paper and placed in an open Petri dish, into the flow chamber for drying during 5 h at $22 \pm 1\%$ water content fresh weight basis (Teixeira et al., 2014).

Step LN. Once dried, five beads were placed in a cryovial and immersed for one day in liquid nitrogen (LN). Subsequently the cryovials were reheated in a hot sterile water bath at 40 °C for 2 min.

2.3. Use of antioxidants

Ascorbic acid at a concentration of 0.43 mM was added to the MS liquid medium with 0.75 M sucrose, in Step S (named Step Sas). Successive steps carried out with apices treated with ascorbic acid were named followed by “-as”.

2.4. Culture of apices and beads

After the different steps of the protocol, five apices (steps N and P) or beads including apices (steps S, D and LN) were cultured in 6 cm diameter Petri dishes with recovery medium (MS + 0.5 mg L^{-1} 2-IP + 0.1 mg L^{-1} NAA + 3% sucrose + 0.7% agar) and kept in a growth chamber at 22–25 °C in the dark for 24 h. Later, cultures were incubated with a photoperiod of 16 h light and PPFD of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for four weeks. Subsequently, shoots were transferred to culture jars with MS medium and incubated at 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; after more four weeks, recovery (shoot development) after each step was recorded. Six replicates (of five apices each) were used per step or treatment for

regrowth assessment.

2.5. Genetic study

2.5.1. DNA isolation for RAPD and AFLP analysis

After the 8-week culture period, leaves were collected from recovered shoots and stored at -80°C until DNA isolation. Genomic DNA was extracted from frozen tissue, according to the Gaweł and Jarret (1991) procedure. Four samples (shoots) were studied in each step and treatment.

2.5.2. RAPD analysis

Eight primers for RAPD's analysis (Operon Technologies Inc., Alameda/CA, USA) were used: 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' CAGCACTGAC 3'), OPO-10 (5' TCAGAGCGCC 3') and OPO-20 (5' ACACACGCTG 3'). The PCR reaction mixture contained approximately 10 ng total DNA, 10 x PCR commercial buffer, 1.0 μL of 50 mM MgCl_2 , 0.4 μL of 50 mM dNTPs, 1U of BioTaq DNA polymerase, 1.0 μL of 20 mM primer, in a final volume of 25 μL . The PCR amplifications were performed in a DNA Thermal Cycler (Eppendorf). For initial denaturation the following procedure was used: 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 . The PCR products were prepared by adding 2.5 μL of bromophenol blue and then were resolved in 1.5% (w/v) agarose gel, using horizontal electrophoresis and 1 x TBE running buffer (Sambrook et al., 1989). The RAPD's markers were visualized by staining with ethidium bromide 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.

2.5.3. AFLP analysis

AFLP analysis was performed according to Vos et al. (1995). Genomic DNA was digested with two restriction enzymes Eco RI and Mse I. The digestion was carried out in a final volume of 35 μL , using 12.5 U of Eco RI, 6 U of MseI (New England Biolabs), 500 ng of genomic DNA in 1 x buffer RL [10 mM Tris-HAc (pH 7.5), 50 mM KAc, 10 mM MgAc, 5 mM DTT, 50 ng/ μL] and maintained for 3 h at 37°C . Two different adaptors that had been designed to avoid the reconstruction of these restriction sites, one for the Eco RI sticky ends and one for the Mse I sticky ends, were ligated to the DNA fragments by adding to the digestion 5 μL of a mix containing 5 pmol Eco RI adaptor, 50 pmol Mse I adaptor, 10 mM ATP, and 1.2 U T4 DNA ligase (Boehringer) in 1 x buffer RL. The ligation was incubated for 3 h at 37°C and subsequently overnight at 4°C . Digested and ligated DNA fragments were diluted fivefold to be used as templates for the first amplification reaction. 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 x 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. Pre-amplified fragments were diluted tenfold to be used as starting material for the selective amplification. In this amplification, only the Eco RI primer was labelled with FAM fluorochrome; both, the Eco RI and the Mse I primers, contain the same sequences as those used in the pre-amplification but with three selective nucleotides at the 3' end. For the selective amplification the primers used were Eco RI ACG/Mse CCA, ACC/CCA, ACC/CAG, and ACA/CCA. The PCR reaction was performed in a volume of 20 μL containing 6 ng of labelled Eco RI primer, 30 ng of Mse I primer, 0.1 mM of each dNTP, 1.5 mM MgCl_2 , 0.4 U Taq DNA polymerase and 5 μL of diluted pre-amplified DNA in the 1 x PCR buffer provided by the manufacturer (Ecogen). The 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 decreased 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 analyzed in an automated ABI3730 sequencer by the company SECUGEN S.L. (Madrid, Spain). The resulted electropherograms were analyzed using GeneMarker® v1.90 software (SoftGenetics, LLC).

2.6. Epigenetic study: MSAP analysis

The evaluation of the epigenetic stability, through MSAP analysis, was carried out on apices sampled after the different cryopreservation steps. Three replicates (of one apex each) were used per step and antioxidant treatment. Control apices (step A) were excised from nodal segments cultured on MS and incubated at 25°C for one week (see section 2.2).

DNA was isolated from apices immediately after the corresponding treatment (without regeneration period), with the exception of some samples from the LN step which were recovered for 24 h (named LN_r). Apices from steps D, Das, LN and LN_s were processed within the alginate bead and their DNA was purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (G&E).

The MSAP analysis was performed following the protocol of Cervera et al. (2002). For each sample, MSAP analyses were performed using both EcoRI/MspI and EcoRI/HpaII digests. The pre-selective and selective amplifications were carried out with the same parameters used previously for AFLP analysis. The primers used for the pre-selective amplification were EcoRI + A and HpaII/MspI + A, while for the selective amplification the combination of primers used were Eco AC with HpaII/MspIAAT; AC/ATC; and AC/ACT. At the end of the selective PCR, amplification products were analyzed in an automated ABI3730 sequencer by the company SECUGEN S.L. (Madrid, Spain). The resulted electropherograms were analyzed using GeneMarker® v1.90 software (SoftGenetics, LLC).

2.7. Data analysis

2.7.1. Recovery data

Recovery data were analyzed by logistic regression for events data and pairwise comparisons were carried out using, for the adjustment for multiple comparisons, the least significant difference (LSD) method with a 5% significance level (Agresti, 2002).

2.7.2. Analysis of AFLP and RAPD data

Amplified fragments from the RAPD and AFLP analyses were scored as present (1) or absent (0). Genetic similarities were calculated using the Jaccard similarity coefficient (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 . The processed 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-PC version 1.80 (Rohlf, 1992).

The effects of the protocol steps and the use of the antioxidant on the recovery and genetic stability percentages were analyzed by logistic regression models. Due to the small size of the sample for the genetic stability analysis and the fact that the events analyzed were not frequent, the statistical analyses were performed using logistic regression models with reduction of bias (Heinze and Schemper, 2002); analyses were carried out with the brglm2 package in R (Kosmidis, 2018).

2.7.3. Analysis of MSAP data

With MSAP technique, there are two sets of amplification data (one from each restriction enzyme) for each sample. MSAP profiles were recorded as 1/0 binary matrix where 1 indicated the presence and 0 the absence of a given fragment.

In order to establish the methylation changes, all samples were compared with the control sample (step A). The resultant code, expressed as four binary digits, describes the presence/absence of each fragment in the HpaII and MspI digest of the control sample and the treated sample. The changes produced in each sample with respect to the control sample were classified by considering three events at each fragment: no change observed in the pattern between the two samples (stability, S), demethylation (D) and *de novo* methylation (M), according to patterns established by Bardini et al. (2003) and Fulneček and Kovařík (2014).

The interpretation of MSAP is based on the known restriction enzyme activities at recognition sequences modified by methylation, these data and the corresponding literature can be found at the REBASE website (The Restriction Enzyme Database) <http://rebase.neb.com>.

The effect of the -step and use of antioxidant on the changes in methylation status was analyzed using a multinomial logistic regression model (Agresti, 1996). We used the multinom function from the nnet-package in R (Venables and Ripley, 2002). The response variable was the methylation change (S, D and M) in each sample and the explicative variables were the factors step and ascorbic acid. Percentages of observed changes in DNA methylation state, for every combination of the factors “Step” and “Ascorbic acid”, were compared with the Tukey test using the contrast function from the emmeans package in R (Lenth, 2019).

The statistical application ‘MAI’ was developed to analyse MSAP data. MAI is an interactive web app built with Shiny package in R (see Supplementary Material) especially developed to analyse stability for germplasm conservation purposes.

3. Results

3.1. Genetic stability

After an 8-week culture period, 100% recovery was obtained in steps A to P. Recovery significantly decreased (Wald's $\chi^2 = 13.224$, $df = 6$, Sig. = 0.04) as the protocol advanced to 83% in Step S and further significant decrease was found in steps D and LN (52–62%). The addition of ascorbic acid in Step S did not significantly improve recovery.

The eight RAPD primers used produced 89 bands for the 35 samples studied, with a size ranging from 2500 to 300 bp (Fig. 1). All the analyzed markers were monomorphic and no differences were detected among samples.

The used AFLP primer combinations produced 113 scorable fragments. Only three of them were polymorphic (2.65%). These variations affected only two out of the 35 analyzed samples (5.7%), and with a low impact since the similarity coefficient of these samples with the control was 0.95 (Fig. 2). The affected samples were a sample from Step P (P1, two variations), and another one from step Sas (Sas1, one difference).

The statistical analysis confirmed the high genetic stability of the

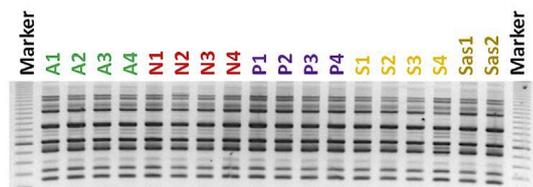


Fig. 1. RAPD profiles of DNA samples (after 8 week-recovery) from mint ('MEN 198') apices after different steps [control (A), cold acclimation (N), preculture in sucrose (P), alginate beads in sucrose (S) and alginate beads in sucrose treated with ascorbic acid (Sas)] of the encapsulation-dehydration cryopreservation protocol. Amplification products were generated by primer OPO-7. Marker: 100 Base-Pair Ladder Marker.

screened DNA analyzed by RAPD and AFLP markers. No significant statistical differences related to the cryopreservation process (among steps) were observed. Likewise, the addition of ascorbic acid on Step S did not entail any variation in the genetic stability of the portion of DNA studied.

3.2. Epigenetic stability (MSAP)

The epigenetic stability throughout the encapsulation-dehydration cryopreservation protocol, with or without the addition of ascorbic acid in Step S, was evaluated with MSAP markers from 33 samples. A total of 126 fragments ranging from 40 bp to 300 bp were generated by the three primer combinations. Of these, 97 epiloci were polymorphic (74.6%). A representation of electropherograms from samples of the different steps of the cryopreservation protocol is shown in Fig. 3.

Statistical analysis of the methylation status changes with the multinomial model showed a significant effect of the protocol step ($p < 0.001$); however, no significant differences were found due to the addition of ascorbic acid ($p = 0.051$), nor in the interaction between the step and the use of the antioxidant ($p = 0.157$; Table 1).

A clear variation of the methylation pattern was observed from the first Step of the protocol: only 65% of the markers remained unchanged at Step N (Table 2). Differences at this step could be attributed at the same proportion due to *de novo* methylations and demethylations. From this step to recovery after immersion in liquid nitrogen (LN step), variations in the cytosine methylation pattern increased throughout the process. In all the steps, the highest proportion of changes was due to *de novo* methylation. The lowest values of stability were reached at the immersion in liquid nitrogen step (47% for explants without ascorbic acid, and 50% when ascorbic acid had been added at Step S). However, a significant increase in stable patterns, compared to the control samples, was observed when apices were cultured for 24 h on recovery medium (LNr). After one day recovery, stability percentages increased to values close to the first steps of the protocol: 60% for samples without and 64% for samples with ascorbic acid treatment (Fig. 4).

The ‘MAI’ application was developed during this study to easily analyse MSAP data. This application not only allows the statistical analysis of the methylation or demethylation events, but also the graphic presentation of the observed or estimated results.

4. Discussion

Quality of the conserved plant material (i.e. its epi-genetic stability, maintenance of the phenotype and capacity of regeneration and multiplication) is one of the main objectives for germplasm conservation. Consequently, it is necessary to optimize the protocols currently used and to deepen in the knowledge of the mechanisms involved. In the present study, the genetic and epigenetic stability after each step of an encapsulation-dehydration protocol for cryopreservation of mint shoot tips was assessed. This allows the detection of the effect of each of the procedures on the quality of plant material. In addition, an antioxidant (ascorbic acid) has been added in the preculture medium with 0.75 M sucrose (step Sas) to test its possible beneficial effect. Genetic analyses revealed the stability of the cryopreserved mint apices; moreover, the supplement of ascorbic acid did not show any significant effect on recovery or genetic stability. The same genotype, ‘MEN 198’, was previously studied in an encapsulation-dehydration protocol in which three antioxidants (glutathione, vitamin E and ascorbic acid) were included in a preceding step of the protocol (Step P (González-Benito et al., 2016)). High genetic stability was also observed, and there was no significant effect associated with the use of the antioxidants. However, in the same study, genetic stability was improved in a different, more unstable, mint genotype. Recovery improvement due to the presence of antioxidants has been observed mainly in vitrification protocols (Uchendu et al., 2010a, 2010b; Mathew et al., 2018). The particular protection mechanisms of the different cryopreservation protocols

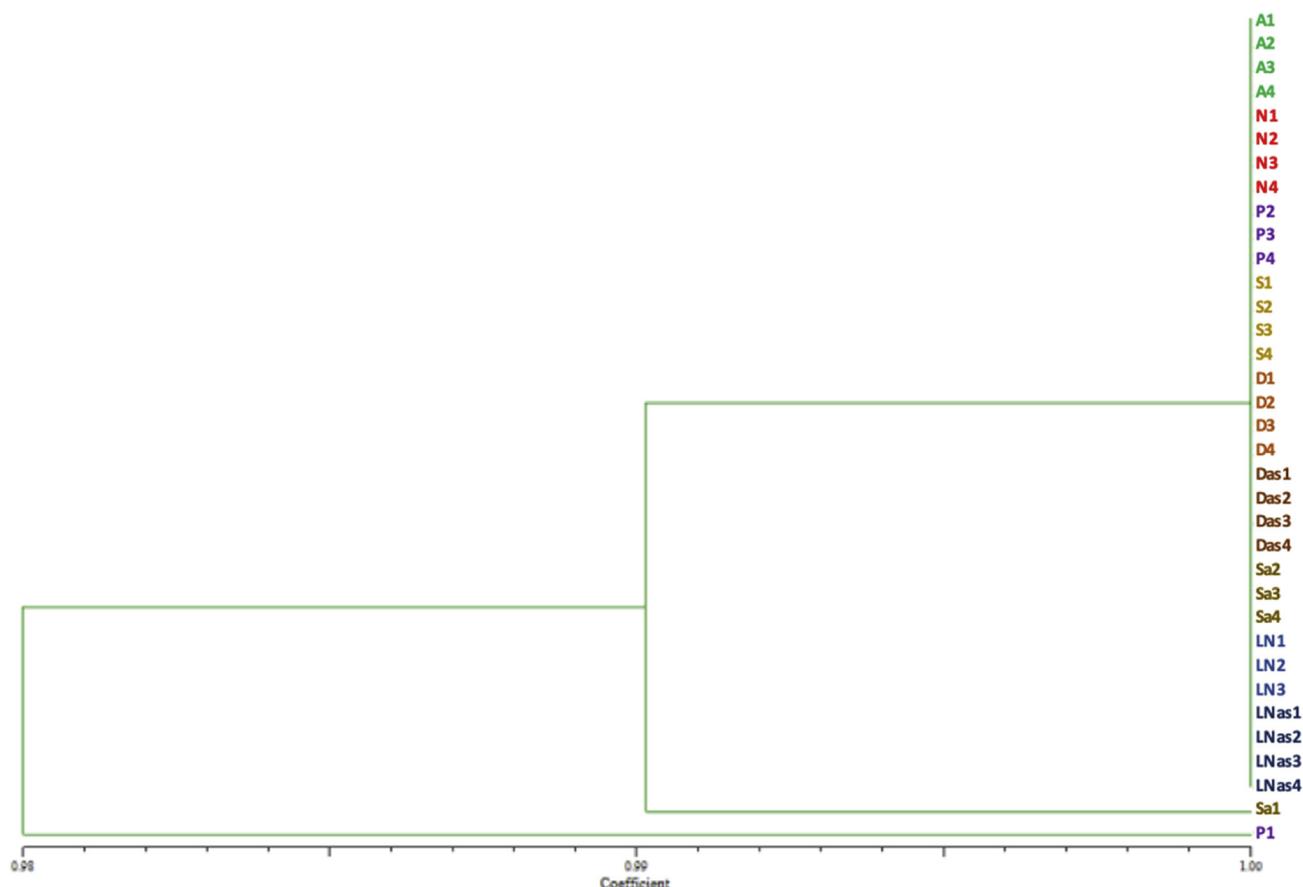


Fig. 2. Dendrogram generated by the UPGMA method using Jaccard's similarity coefficient, based on AFLP data of samples from different steps of a cryopreservation protocol by encapsulation-dehydration [control (A), cold acclimation (N), preculture in sucrose (P), alginate beads in sucrose (S), dehydration (D), immersion in liquid nitrogen (LN), and the same steps after addition of ascorbic acid in Step S: Sas, Das and LNas].

(encapsulation-dehydration vs. vitrification) could be related to their capacity to interact with antioxidant substances in order to improve the cell protection.

RAPD and AFLP markers did not show genetic changes in the DNA screened throughout the encapsulation protocol after an 8 week-recovery period. However, MSAP markers from apices revealed significant differences related to the step of the protocol although, as it was also observed in the genetic analysis, no significant effect of the ascorbic acid addition was detected.

Epigenetic changes have been associated to genetic variations, or as precursors of somaclonal variation (Kaeppler et al., 2000). However, our results from cryopreserved mint apices did not confirm this theory, since molecular markers indicated a high genetic stability while significant epigenetic changes associated to the protocol step were observed. There are previous works in which DNA methylation changes were detected after cryopreservation, without a clear correlation to genetic instability. Hao et al., 2002a, Hao et al., 2002b found similar results with *Citrus* callus and strawberry plants. Likewise, Peredo et al. (2008), with RAPD and AFLP markers, did not find genetic instability in cold-stored or cryopreserved hop plants, even though with MSAP markers epigenetic variations were detected.

Some of the methylation changes observed in our study could be considered transient, since samples analyzed 24 h after recovery showed a reduction of the epigenetic variation, with similar patterns to those observed after the first steps of the protocol (steps N and P). These results agree with the theory that non-permanent changes are caused by transient and reversible modifications of gene regulation which can allow plants to acclimate to the stressful conditions of *in vitro* and cryopreservation protocols (Baranek et al., 2016; Chatterjee et al.,

2017). Similarly, in an encapsulation-dehydration protocol, Johnston et al. (2009) observed changes in the DNA methylation status of cryopreserved *Ribes* genotypes which regressed to control values after successive subcultures. Authors concluded that these DNA methylation changes were reversible epigenetic changes related to the modulation of gene activity during the altered conditions. However, it is not possible to completely reject that part of the detected variations could be permanent and, therefore, meiotically heritable (Kou et al., 2011; Johannes and Schmitz, 2019).

Several studies have found phenotypic variability attributable to epigenetic diversity rather than to genetic changes (Joyce and Cassells, 2002; Adu-Gyamfi et al., 2016). The detected changes in DNA methylation along the cryopreservation protocol in mint apices were not associated to any phenotypic variation that could be noticed at the developmental stage that was studied during *in vitro* growth.

The analysis of DNA methylation changes detected by MSAP after each step of the cryopreservation protocol allowed discriminating the type of variation, when compared to control (*in vitro*) samples. Although *de novo* methylation and demethylation events could be found simultaneously in a particular sample, most of the changes detected in this work corresponded to *de novo* methylation events.

Some previous studies on epigenetic stability after plant cryopreservation described, however, demethylation as the most frequent change. In this regard, Hao et al., 2002a, Hao et al., 2002b, reported hypomethylation after cryopreservation in *Citrus* callus and strawberry apices, by vitrification and encapsulation-dehydration, respectively, although the number of changes observed was very low (4 and 1 markers out of approximately 300) to be representative. On the other hand, Peredo et al. (2008) found a high number of changes, mainly

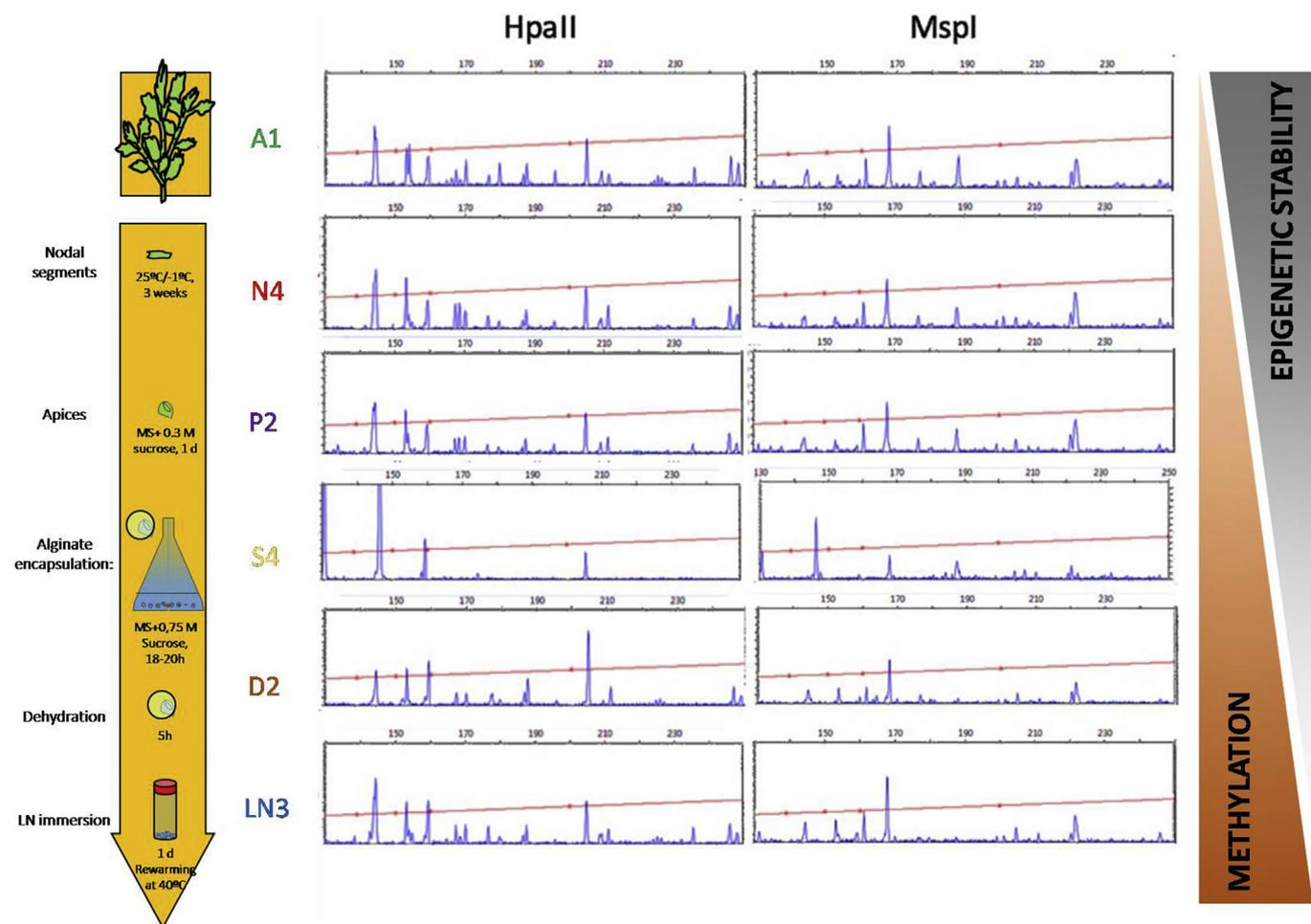


Fig. 3. MSAP electropherograms of mint samples from the different steps of the encapsulation-dehydration cryopreservation protocol, amplified with the primers Eco RI + AC/HpaII/MspI + AAT. The x-axis represents the size (bp) of the DNA fragments and the y-axis indicates the amount of the amplified products (see Fig. 2 legend for explanation of the sample codes).

Table 1
Likelihood-ratio test (G2) results of methylation status changes determined by MSAP markers.

Factors	d.f.	G2	P-value
Step	14	79.1	< 0.001
Ascorbic acid	8	15.4	0.051
Step x Ascorbic acid	6	9.31	0.157

Table 2
Percentages of observed changes in DNA methylation state for the combination of the factors “Step” and “Ascorbic acid”, versus the control sample.

Step/Ascorbic acid	% Stability	% <i>de novo</i> methylation	% Demethylation
N	64.9 ^c	17.1 ^a	18.1 ^{bc}
P	62.0 ^{bc}	21.4 ^{ab}	16.5 ^{abc}
S	65.9 ^c	25.6 ^{ab}	8.5 ^a
Sas	63.3 ^c	21.7 ^{ab}	15.0 ^{abc}
D	50.1 ^{ab}	32.0 ^b	17.8 ^{bc}
Das	56.6 ^{abc}	27.9 ^b	15.5 ^{abc}
LN	47.0 ^a	31.3 ^b	21.7 ^{bc}
LNas	50.4 ^{ab}	26.9 ^{ab}	22.7 ^c
LNr	59.2 ^{abc}	26.9 ^{ab}	14.0 ^{abc}
LNras	64.1 ^c	23.5 ^{ab}	12.4 ^{ab}

Means with the same letter within a column are not significantly different according to Tukey test, $\alpha = 0.05$.

attributable to demethylation events, in cryopreserved hop apices. One of the differences with our study lies in the developmental stage of the cryopreserved apices and the control sample used. These authors analysed the methylation status of the cryopreserved apices after four months of *in vitro* culture following recovery, and this was compared to a control plant grown under field conditions. In our work, DNA samples were isolated without regeneration, just after each step of the protocol (except for the last one, where also a 24 h recovery was used), and our control sample consisted of apices from *in vitro* cultured shoots.

Increased methylation has been reported in some tissue culture studies (Cecchini et al., 1992; Smulders and Klerk, 2011) associated with a response to stress conditions. Furthermore, different abiotic stresses induce an increase in the methylation levels of several species in field experiments (Labra et al., 2002; Marfil et al., 2019). Hypermethylation could be considered, therefore, a common genome response to abiotic stresses (Marfil et al., 2019). This hypothesis would agree with our results, as samples might have suffered a high degree of stress caused by the different pretreatments of the protocol, and the analysis was carried out just after those treatments, previous to recovery.

Although MSAP, or other similar methylation markers, give information on the type of change produced (demethylation, *de novo* methylation), the complexity of their analysis makes it difficult to extract all the potential information. Indeed, in many studies, there is not usually an exhaustive evaluation of the statistical significance of the changes observed. Kaity et al. (2009) analyzed the genetic integrity of cryopreserved papaya clones using RAF-PCR markers (with HpaII

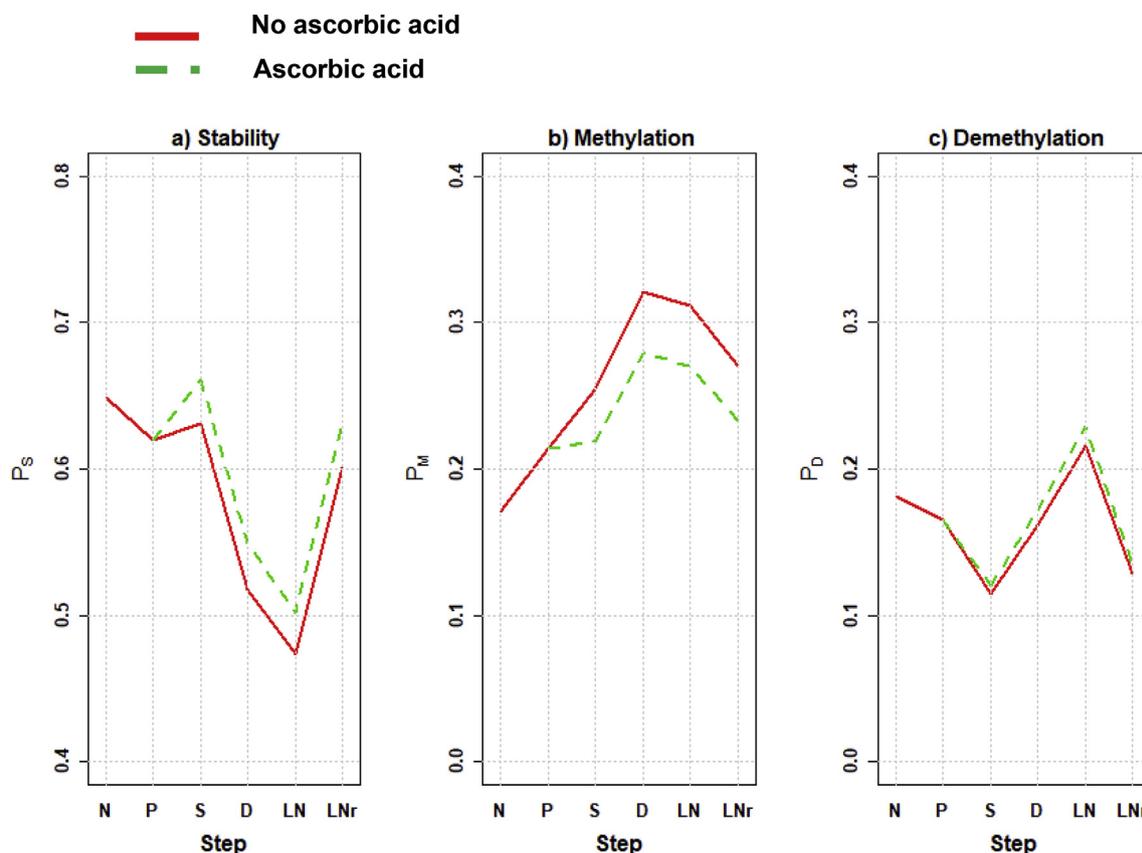


Fig. 4. Estimated proportions obtained with MAI of epigenetic stability, demethylation or *de novo* methylation throughout the different steps of the encapsulation-dehydration cryopreservation protocol without or with addition of ascorbic acid in Step S.

digested genomic DNA as template) to study methylation modifications. These authors detected methylation changes in cryopreserved samples or treated with PVS2, however, the type of change (methylation or demethylation) was not stated. Likewise, using MSAP markers most authors do not statistically analyse the methylation changes detected. Maki et al. (2015) studied cryopreserved wasabi plants and only reported differences in the band pattern without the analysis of the type of change. Other authors stated the kind of differences found in their studies, using a 4-digit code, similar to the one used in this work to compare samples with a control. Yet, no statistical analysis of their significance was carried out making the interpretation of the results difficult: e.g. Hao et al., 2002a, Hao et al., 2002b in *Citrus* callus and strawberry, and Kaczmarczyk et al. (2010) in potato. Peredo et al. (2008) working with hop, also were able to establish the changes in methylation (*de novo* methylation/demethylation), without a statistical analysis, although they carried out a cluster analysis of the similarity of the band patterns obtained. Likewise, Adu-Gyamfi et al. (2016) analyzed the MSAP bands obtained in cryopreserved cocoa plant material by cluster analysis, principal coordinates analysis and AMOVA.

Peredo et al. (2008) and Adu-Gyamfi et al. (2016) used an analysis similar to that proposed by Pérez-Figueroa (2013) for diversity studies. In these cases, the main objective is to reveal the degree of diversity of a group of samples, detecting the different methylation patterns that can be present in the sample population. Using similar tools, cluster analysis and AMOVA, Pérez-Figueroa (2013) developed the program 'msap' to make this kind of analysis easier. However, this approach does not allow analysis of the type of modification (demethylation or *de novo* methylation) produced along a treatment or time, or even groups of plants, when the comparison with a control sample is required to understand the changes produced.

Although we have focused on cryopreservation works, MSAP analyses in other stability studies as *in vitro* culture procedures (Peredo

et al., 2006; Ghosh et al., 2017) or slow-growth conservation (Hao and Deng, 2005) are similar to those mentioned before, without a statistical analysis of the type of methylation changes observed.

Our statistical approach allows a rapid and easy analysis of the MSAP profile data. MAI application not only detects stability, *de novo* methylation and demethylation, but also the statistical signification of the factors affecting the process. Likewise, the developed interface allows its use without any previous knowledge of R program, making the stability analysis of methylation changes for conservation approaches easier.

In conclusion, our MSAP results reveal an important variation in the DNA methylation pattern of the plant samples subjected to an encapsulation-dehydration cryopreservation protocol, even when the molecular markers (RAPD and AFLP) showed an almost complete genetic stability. The sequential analysis also demonstrated that ultra-low temperature and stressful pretreatment conditions contributed to DNA methylation changes. These results support the idea that conservation procedures should be optimized to avoid any type of change that may affect the quality of the conserved material. A better understanding of all the processes involved in each of the steps of the protocols used will allow the development of increasingly safe conservation procedures for the maintenance of high value plant material. Likewise, the development of useful analysis tools that allow a better and easier evaluation, such as the MAI application for the study of DNA methylation changes through MSAP markers, will facilitate the development of more appropriate conservation methodologies.

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Author contribution

Miguel Angel Ibáñez- Conceptualization, Software, Formal Analysis, Writing – Original Draft. **Ana Álvarez-Mari**- Software, Formal Analysis. **Héctor Rodríguez**- Formal Analysis, Writing – Original Draft. **Carolina Kremer**- Investigation. **María Elena González-Benito**- Conceptualization, Investigation, Writing – Original Draft. **Carmen Martín**- Conceptualization, Investigation, Writing – Original Draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.08.026>.

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