SIMPLE STRATEGY FOR THE IN VITRO CONSERVATION OF *Alnus glutinosa* (L.) Gaertn. GERMPLASM

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Abstract The aim of this study was to develop a simple method for the medium-term storage of *Alnus glutinosa* (L.) Gaertn. explants obtained from trees aged 20-30 years. Several parameters were evaluated, including type of explant (shoot apex or nodal segments), pre-storage treatment (0 or 10 days after the last subculture) and duration of cold storage (3-24 months) at 2-4°C. Explants were maintained at this temperature under dim lighting on Woody Plant Medium supplemented with 0.1 mg l⁻¹ 6-benzyladenine and 0.5 mg l⁻¹ indole-3-acetic acid. Under these conditions, a high percentage (75-87%) of cultures remained viable after 18 months in cold cabinets. The stored material was successfully recovered and multiplied normally in the same medium, showing good growth and developing into normal shoots that were morphologically similar to those of non-stored controls. At the histological level, the main change observed was the accumulation of starch granules in cells of the shoot apex, as well as in cells located close to the vascular bundles, after 3 months of cold storage. As the duration of cold storage increased, the number and size of the starch granules decreased but cell plasmolysis and the content of lipid droplets increased. Cold damage was generalized after 24 months at 4°C. This study provides new insights into the changes occurring in *A. glutinosa* during cold storage.

Key words alder, *Alnus glutinosa*, cold storage, histology, micropropagation, medium-term storage

Author Contribution Statement MC San José and E Corredoira designed the research, analyzed the data and wrote the paper. LV Janeiro conducted the research. All authors have read and approved the final manuscript.

Key Message This study provides a simple protocol for storage of alder clones. The technique described is useful for in vitro germplasm collections, decreasing the risk of genetic changes and associated costs.

Conflict of Interest The authors declare that they have no conflict of interest.

Introduction

European alder (*Alnus glutinosa* (L.) Gaertn.), also called black alder or European black alder, is a medium-sized tree that is widely distributed throughout Europe. It occupies riversides, valley bottoms, flooded areas and wet hillsides, at altitudes ranging from sea level to 1700 m. Black alder populations have declined drastically throughout Europe in the past few decades, partly as a result of deforestation and the disappearance of riparian habitats, but mainly because of alder blight disease, caused by *Phytophthora alni* (Brasier et al. 2004). Heavy loss of alders may have serious effects on forest and soil composition, wildlife food and habitats, and soil erosion (Cree 2006). In order to protect these important genetic resources, existing conservation methods must be improved and new tools must be developed.

The approach traditionally used for conservation of wild species is in situ conservation in natural habitats. Although efficient, collections are exposed to natural disasters and attacks by pests and pathogens. Moreover, distribution and exchange from field gene banks is difficult because of the vegetative nature of the material and the greater risk of disease transfer (Engelmann 1997). It is now recognized that ex situ techniques may effectively complement in situ methods, and may represent the only option for conserving certain highly endangered and rare species (Sarasan et al. 2006; Engelmann 2011). The importance of ex situ conservation is internationally accepted and widely used by numerous biodiversity conservation organizations (UNEP 2002; Capuana and Di Lonardo 2013). Since the early 1990s, attention has turned to biotechnological methods, specifically in vitro or tissue culture methods. Use of in vitro tools can yield additional backup collections and provide alternative means of propagation and conservation of species (Reed et al. 2011). However, in vitro techniques require periodic transfer of cultures to fresh medium and the inclusion of plant growth regulators as well as organic and inorganic components in the culture media. These requirements increase the cost of the conservation techniques, and more importantly, they increase the risk of somaclonal variation, and therefore the genetic fidelity of the stored germplasm is not ensured (Ozden-Tokatli et al. 2010). These risks can be decreased by using other unconventional conservation techniques such as slow growth storage and cryopreservation (Ashmore 1997; Reed et al. 2011).

In vitro slow growth techniques are routinely used for medium-term conservation of numerous species, of both temperate and tropical origin, including crop plants, e.g. potato, *Musa*, yam, cassava

(Ashmore 1997; Engelmann 1999, 2011). The goal is to modify the physical environment of the culture, the medium composition, or both, in order to slow down plant growth and thus increase the time between consecutive subcultures. The most widely applied strategy is to maintain cultures at low temperature either in complete darkness or under low irradiance (Marco-Medina and Casas 2012). These techniques are clearly useful for their flexibility, simplicity and practicality, and they are the most direct way of restricting the growth and development of explants in vitro (Engelmann 1997; Turner 2001). However, as with most in vitro techniques, the successful implementation of minimal growth technology requires the establishment of specific protocols for each type of explant and species under consideration (Watt et al. 2000).

Although in vitro conservation techniques have been developed for numerous plant species, until now only two reports on the cryopreservation of alder have been published (Chmielarz 2010; San José et al. 2014). In order to stem the loss of biodiversity, an attempt to conserve *Alnus glutinosa* (L.) Gaertn. by in vitro methods was made in this study. Shoot explants from different mature trees were successfully cultured in vitro (San José et al. 2013), and the micropropagated shoots were maintained without loss of the multiplication capacity for over five years. However, shoot cultures required regular 3-week subculture at 25°C and 16 h photoperiod, and therefore the development of reliable methods of medium-term storage is crucial. It has been estimated that almost half of the production costs of micropropagation are attributable to labour costs (Standardi and Piccioni 1998). Therefore, the objective of the present study was to assess the in vitro storage of black alder under slow growth conditions, in order to develop a simple and efficient protocol for conservation of the genetic diversity of the species. Information about the histological events occurring within those explants submitted to these culture conditions is very scarce. To help fill this gap, we focused on the histological changes in explants during minimal growth storage to gain insights into the anatomical changes occurring in explants.

Material and methods

Plant material and culture conditions

Stock shoot cultures (Clones G1, R1 and R4) were established in vitro from mature *A. glutinosa* trees in 2009, as previously described (San José et al. 2013). Clonal shoot multiplication was maintained by

subculturing shoots in 500 ml glass jars containing 70 ml of Woody Plant Medium (WPM, Lloyd and McCown 1980), supplemented with 0.1 mg l⁻¹ 6-benzylaminopurine, 0.5 mg l⁻¹ indole-3-acetic acid, 20 g l⁻¹ glucose and 7 g l⁻¹ Difco agar (proliferation medium). The multiplication cycle consisted of a 9-week period with transfers every 3 weeks to fresh medium. Cultures were kept in a growth chamber with a 16-h photoperiod (50-60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) provided by cool-white fluorescent lamps, and 25°C day/20°C night temperatures (standard conditions). All media were adjusted to pH 5.6-5.7 before being autoclaved at 115°C for 20 min.

Slow growth treatments

Shoot apex and nodal segments (10-15 mm in length) excised from 9-week-old in vitro-grown shoots were defoliated and used as explants for the in vitro conservation experiments. Two pre-storage treatments were considered: 1) Day 0, in which the explants were placed in the cold immediately after subculture, and 2) Day 10, in which the explants were placed in the cold 10 days after subculture. During these 10 days, the cultures were kept under standard growth conditions.

Eight explants were placed in each 500 ml glass jar. Each experiment was carried out with a minimum of 16 explants and was repeated at least twice. The glass jars were kept in 340 L Sanyo Medicoool Cabinets at 2-4°C under dim lighting (8-10 $\mu\text{mol m}^{-2} \text{s}^{-1}$) provided by exterior cool-white fluorescent lamps. After 3, 6, 9, 12, 18 or 24 months of cold storage, the jars were removed from the cabinets. All explants were immediately transferred to fresh proliferation medium and were kept in a growth chamber under standard growth conditions. Controls (0 months in cold) were maintained under these standard conditions during the experiment.

Data recording and statistical analysis

After 9 weeks in the proliferation medium, the following parameters were recorded: recovery, as the percentage of cultures able to proliferate; number of new shoots per explant; number of shoots ≥ 0.5 cm per explant; and length of the longest shoot per explant. For each of the dependent variables (type of explant, pre-storage and time of cold storage), analysis of variance was conducted to evaluate the effect of the main experimental factors and their possible interaction. Percentage data were subjected to arcsine transformation prior to analysis, and non-transformed data are shown in the tables. The three clones (G1,

R1 and R4) were analyzed separately. All statistical analyses were conducted using SPSS for Windows (version 19.0, Chicago, USA).

Histological analysis

Shoot apex and nodal samples (0.5 cm in length) of control and 3, 6, 9, 12, 18 and 24 months cold-storage explants (Clone R4) were placed in FAA (formalin, glacial acetic acid, and 50% ethanol [1:1:80 (v/v/v)]). The samples were dehydrated in graded solutions of n-butanol (Jensen 1962). Fully infiltrated tissues were embedded in paraffin wax, and a series of longitudinal sections, 10 µm thick, was obtained using a Reichert-Jung 1130-Biocut rotatory microtome. The sections were double stained with periodic acid-Schiff (PAS)-naphthol blue-black (O'Brien and McCully 1981). This stain is commonly used to reveal total insoluble polysaccharides and total protein content of the cells. PAS-Sudan black was used to determine the lipid content (O'Brien and McCully 1981). Histological preparations were examined under a standard NIKON-FXA microscope, and images were captured and digitized using an OLYMPUS DP71 camera (Japan), and the Microimage Analysis Software program (Olympus, Hamburg, Germany).

Results

In terms of morphology, cold storage moderately affected the appearance of the cultures in all clones tested. During storage at 2-4°C, some shoots developed in both types of explant. These shoots were a yellowish-white colour as a result of the low intensity of light ($8-10 \mu\text{mol m}^{-2} \text{s}^{-1}$) to which they were subjected during storage. After some time, shoot tip necrosis was observed and the leaves appeared to be necrotic, and some were finally shed. This effect became more evident as the duration of cold storage increased. Necrosis usually starts in the upper part of the shoot and spreads to the lower part over time. This effect was most evident in the shoots developed from the shoot apex explants. No differences between the explants under the different pre-storage regimes were observed (Day 0 and Day 10). Callus formation at the base of the explants was almost negligible. Once transferred to standard conditions, the stored explants successfully regenerated into new shoots that were morphologically similar to those of non-stored controls (Fig 1 A and B).

In all three clones, the duration of cold storage significantly affected the percentage of shoot recovery ($p \leq 0.001$; Tables 1 to 3), which was highest (100%) during the first nine months of cold storage.

The percentage of shoot recovery decreased significantly after 12 months, although it remained higher than 75% even after 18 months of cold storage, which represents a relatively high survival rate. After 24 months, the explant recovery percentage did not surpass 50%. No significant differences were observed in relation to pre-storage time (0 or 10 days after the last subculture) or type of explant (apical or nodal), and the interaction between explant type, pre-storage and time of cold storage was also not significant.

In all clones evaluated, the type of explant and the duration of cold storage had significant effects ($p \leq 0.001$) on the number of shoots formed (Tables 1 to 3). The interaction between both variables was also significant. In all three genotypes, the nodal explants yielded a significantly higher number of shoots than the apical explants, and the number increased as the duration of cold storage increased up to 18 months. The best results were obtained with nodal segments stored for 9 months at 4°C. As with the percentage of survival, the number of shoots decreased significantly when the duration of cold storage was extended to 24 months. The number of shoots that developed in the treatment with no pre-storage was significantly higher ($p \leq 0.001$) for nodal explants than for apical explants. Maintenance of explants for 10 days under standard conditions (pre-storage 10 d) prior to cold storage led to a significant increase ($p \leq 0.001$) in the number of shoots arising from the shoot apex explants of clones R1 and R4, but not clone G1. The interaction between pre-storage treatment and explant type was significant for clones R1 and R4 (Tables 2 and 3). The interaction was significant for these clones because the number of shoots that developed from nodal segments was higher without pre-storage, while the number of shoots that developed from shoot apices was higher with pre-storage. In all three genotypes, the interaction between the three factors did not significantly affect the number of shoots formed.

Of the three variables evaluated, only the duration of the cold storage affected shoot length in all clones ($p \leq 0.001$; Tables 1 to 3). In clone G1, cold storage led to a significant increase ($p \leq 0.001$) in the length of the shoots in both types of explants, and the highest values for all treatments were reached after 6-9 months at 4°C and decreased significantly after 24 months (Table 1). Without pre-storage, the nodal explants responded better than the apical explants and the interaction between the type of explant and the duration of cold storage was significant ($p \leq 0.001$). Maintenance of the explants for 10 days under standard conditions (pre-storage 10d) yielded a significant increase ($p \leq 0.001$) in the length of the shoots produced in both types of explant. The increase was greater for a cold storage period of 9 months, and therefore the interaction between these variables (pre-storage time and duration of cold storage) was significant ($p \leq 0.001$).

In clone R1, the length of the shoots was not significantly affected either by the type of explant or by the pre-storage regime (Table 2). However, the interaction between the three factors significantly affected the shoot length ($p \leq 0.001$). The longest shoots were produced by apical explants that had been precultured for 10 d under standard conditions and kept in cold storage for 18 months. The duration of cold storage significantly affected the length of the shoots in both types of explants ($p \leq 0.001$) and in both pre-storage regimes (0 or 10 d) up to 18 months. The interaction between both factors was also significant ($p \leq 0.05$).

In clone R4, the length of the shoots was significantly affected by three factors, although there was no significant interaction between these ($p \leq 0.001$; Table 3). The shoots yielded from nodal explants were significantly longer than those obtained from the apical explants in both pre-storage regimes (0 or 10d) and all cold storage times. Cold storage of nodal and apical explants for 24 months yielded the shortest shoots in both pre-storage regimes. The interaction between the duration of cold storage and time of pre-storage was also significant ($p \leq 0.01$) and the longest shoots were obtained from both apical and nodal explants stored for 6 and 9 months at 4°C.

Histological analysis

The histological study of clone R4 revealed the presence of the main shoot apex and several primordial leaves at various stages of development, and axillary buds were present (Fig. 2A). The ground meristem, from which the cortex and stem pith originate, was localized in the sub-apical zone. Procambial strands that would later differentiate into vascular tissues were also observed. Cells in the meristematic zone of shoot tips displayed typical features of actively dividing cells, i.e. small size, isodiametric shape and low vacuolization. They also had a high nucleocytoplasmic ratio, a voluminous nucleus, and clearly evident nucleolus, often situated in a central position. In the vascular cells, differentiation of tracheal elements was observed with secondary wall deposition in a helicoidal pattern. Starch granules were not observed in the apical explants, and only small granules were visible in the cortical cells close to the vascular bundles (Fig. 2B).

After 3 months of cold storage, the most notable change was the presence of a large number of starch granules, which were visible in cells at the base of the meristematic cortex, in leaf primordial and in cells adjacent to procambial strands (Fig. 2C). More starch granules were also observed in the parenchymatic cells of the cortex and of the pith in the basal explants (Fig. 2D). Some cells in the

meristematic region were slightly plasmolyzed. Lipid droplets appeared in the outermost cells of the primordial leaves (Fig. 2E). The meristematic cells of the apical and axillary shoots stained intensely with naphthol blue.

As the duration of cold storage increased, the number and intensity of the plasmolyzed cells increased and some dead cells were observed in the apical meristem and primordial leaf zones (Fig. 2F). After 24 months of cold storage, the damage to the explants was much more intense, especially in the cells in the subapical region (Fig. 2G). The number and size of the starch granules decreased (Fig. 2H) and lipid droplets became more evident in the apical and nodal explants (Fig. 2 I, J). After 18 months of cold storage, a secondary protective tissue (phelloderm) developed in the nodal explants and took on the primary function of the epidermis (Fig. 2K).

Discussion

Exposure of plants to low temperatures induces changes in the morphology, histology, gene expression, cell biochemistry and ultrastructure, and sensitivity to this stress factor varies depending on species and genotype (Garbero et al. 2012). The results of this study demonstrated that alder explants can be stored for up to 18 months at 2-4°C, with survival percentages higher than 75%. Storage of in vitro shoots at low temperature (generally 2-5°C for temperate species) and in the dark or under low light intensity is by far the most widely used approach for medium-term conservation of in vitro cultures (Engelmann 1997; Lambardi and De Carlo 2003). In chestnut, survival percentages of up to 82% after 48 months at 8°C were achieved (Capuana and Di Lonardo 2013). Low temperature and light intensity induce modifications to the physiological state of stored explants, such as reduced respiration, water loss, wilting and ethylene production, thus allowing storage of cultures from between several months and several years (depending on the species) with only infrequent subculturing required (Rao 2004; Ozudogru et al. 2010).

Several types of plant material have been used for in vitro preservation of clonally propagated plants. However, meristem-derived explants such as shoot tips and buds are usually recommended for in vitro preservation due to high survival and regrowth percentage and high genetic stability of this material (Reed et al. 1998). In the present study, both types of explant used (shoot tips and nodal segments), responded well to storage. Cold storage led to an increase in the number of shoots that developed in each

explant, although the proliferation rates returned to normal levels in subsequent subcultures. Similar studies have documented more vigorous growth after low temperature storage (0°C for 8 months) in *Podophyllum peltatum* (Lata et al. 2010) and an increment in the number of new shoot/explant in apricot after chilling preconditioning at 4°C for 100-300h (Kouboris and Vasilakakis 2006).

The survival pattern is probably also influenced by the developmental stage of the plants under storage (Hansen and Kristiansen 1997). These authors suggested that when the cultures were placed in cold storage as early as 3 days after transfer of single shoots to new medium, the shoots may simply not have reached the optimal developmental stage for storage. Others authors also supported this idea, e.g., Kovalchuk et al. (2009) for apple, Lukoseviciute et al. (2012) for *Fragaria* and *Pyrus*, Ozudogru et al. (2010) for *Sequoia sempervirens*. The time between the last transfer and the moment when the cultures are placed in storage may be very importance (Engelmann 1991; Corredoira et al. 2011). Higher survival rates were obtained with shoot cultures of wild cherry, chestnut and oak kept for 10 days under standard conditions after the last subculture before transfer to the cold storage chamber (Janeiro et al. 1995). These authors indicated that this length of time is required for the explants to recover from the stress induced by the subculture process. This contrasts with the results obtained by Orlikowska (1992) in *Malus*, which had to be stored directly after transplantation to fresh medium, thus avoiding the occurrence of necrosis and production of phenolic compounds (Engelmann 1991). In alder, the pre-storage period (0 or 10 days after the last subculture) did not significantly affect explant survival. However, in clones R1 and R4 the number of shoots that developed from the shoot apex explants increased significantly when the explants were maintained under standard conditions for 10 days before cold storage.

We obtained very good results in this study, with explant survival percentages higher than 75% after 18 months of cold storage and using the usual multiplication method . One of the advantages of restricted-growth storage techniques is that they may only require the same basic in vitro facilities as required for micropropagation. Moreover, the storage regimes are usually based on modifications of the procedures that would in other circumstances be used for micropropagation. This may result in the cultures being able to be readily switched to rapid multiplication regimes when required (Blakesley et al. 1996).

The histological study of alder explants after cold storage under dim lighting showed that, after 3 months of cold storage, the main change was the accumulation of starch granules, which were initially

present in the subapical zone, but which eventually spread to all apical tissues, including the apical meristem and cells adjacent to procambial strands. The starch content of cells decreased as the duration of cold storage increased. In chilled *Arabidopsis* plants (2.5-4°C for 72h), Vella et al. (2012) observed larger and more abundant starch granules than in the control plants. This is consistent with the findings of other studies showing increased number of starch granules in chill-stressed rice plants (Mamum et al. 2006) and *Populus* (Zhang et al. 2011). However, other studies involving maize (Saropulus and Drennan 2007), *Malus* (Orlikowska et al. 2010) and *Digitaria* (Garbero et al. 2012) report smaller starch granules or the disappearance of starch granules under chilling stress. Low-temperature stress (5°C for 12h) has been shown to be associated with starch degradation due to β -amylase, a major enzyme of starch breakdown in *Arabidopsis* chloroplasts (Fulton et al. 2008) leading to release of cold-protective maltose (Kaplan and Guy 2004). These results supported a model in which cold acclimation involves accumulation of maltose as a compatible solute (Li et al. 2011). A study of *Arabidopsis* showed that, among other genes, a specific starch-debranching enzyme, AtISA3, known to be involved in starch degradation, is specifically induced during cold adaptation (Li et al. 2007). The different responses to chilling in regard to the size and number of starch granules may depend on the plant species and developmental stage.

Cells undergo plasmolysis in response to cold storage, and the amount of damage increased with the duration of cold storage. An increase in the affinity of the cells, particularly the apical cells, for PAS-naphthol blue stain was also observed. Increased concentrations of cellular protein can be considered one of the early physiological responses of stressed cells and may contribute to increasing the tolerance to cold exposure (Jutsuyama et al. 2002). Shrinkage and intense staining of nuclei observed in shoot apex after cold storage are due to chromatin contraction and increased protein concentration, respectively (Barraco et al. 2013). Chromatin condensation is a common cell reaction to stress conditions; in this state, chromatin is locked in an inaccessible configuration, which arrests transcription in a genomic region and thus stops cell growth (Eberharther and Becker 2002). Under stress conditions, growth arrest is thought to be advantageous for tissues as slower growth may allow plants to redirect resources to overcome the temporary stress (Jarillo et al. 2009).

In conclusion, this study provides an effective and simple protocol for storage of *A. glutinosa* under minimum growth conditions. The study findings show that cultures can be conserved in vitro for 18 months at 2-4°C under dim lighting. The recovered shoots multiplied normally, without any loss of their regeneration capacity and without any apparent morphological changes. The techniques described, i.e.

lengthening the time between subcultures to 18 months and consequently reducing maintenance requirements, may be useful for in vitro germplasm collections in which frequent subculture enhances the risk of genetic changes. They will also decrease the associated costs (for personnel, energy and material), which currently limit the number of genotypes that can be handled. In vitro culture for extended periods under these conditions may allow the cost-efficient storage of alder germplasm, thus contributing to improved conservation of alder genetic diversity.

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Figure Captions

Fig. 1 Medium-term storage of in vitro cultures of *Alnus glutinosa*. A. Shoot apex explants after 18 months storage on proliferation medium at 4°C under dim lighting. B. Nodal explants recovered after 18 months storage at 4°C and regrowth under standard conditions for 9 weeks (scale: glass jars are 90 mm in diameter)

Fig. 2 Anatomical modifications in *Alnus glutinosa* explants during cold storage. A. Histological section of an untreated shoot apex (bar = 100µm). B. Longitudinal section of an untreated nodal explant showing the presence of starch granules in cells adjacent to vascular bundles (arrows) (bar = 100µm). C. Shoot apex stored at 4°C for 3 months. Note the presence of a huge number of starch granules in the cells (bar = 100µm). D. Longitudinal section of a nodal explant stored at 4°C for 3 months, showing the presence of numerous starch granules (bar = 200µm). E. Lipid droplets (arrows) present in the primordial leaf cells in apical explants, after 3 months of cold storage (bar = 50µm). F. Alder shoot apex explant stored at 4°C for 9 months. Note the destruction of cells in the meristematic zone (arrows) (bar = 50µm). G. Shoot apex explant after 24 months of cold storage (at 4°C). Note the disorganized appearance of the whole explant and the presence of many dead areas (bar = 100µm). H. Nodal explant after 9 months of cold storage, showing a decrease in the number and size of starch granules (bar = 200µm). I. Shoot apex explant stored for 18 months at 4°C, showing an increase in the content of lipid droplets (arrows) (bar = 200µm). J. Nodal explant stored for 18 months at 4°C showing the presence of numerous lipid droplets (arrows) (bar = 100µm). K. Nodal explant stored for 18 months at 4°C, showing the formation of phellogen (ph) and numerous lipid droplets (arrows) (bar = 100µm)