DEVELOPMENT OF CRYOPRESERVATION FOR *Loxocarya cinerea* - AN ENDEMIC AUSTRALIAN PLANT SPECIES IMPORTANT FOR POST-MINING RESTORATION

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Abstract

We report the development of a cryopreservation protocol for the endemic Western Australian plant species *Loxocarya cinerea* (Restionaceae). Shoot tips from two genotypes, SXH404 and SXH804, were cryopreserved using the droplet-vitrification technique. Control explants, which were cryoprotected, but not cooled, showed regeneration for both genotypes (SXH404, 22.1 ± 5.9%; SXH804, 67.7 ± 9.6%). Extension of incubation in PVS2 from 30 to 60 min did not lead to survival after cryopreservation. Thermal analysis using differential scanning calorimetry confirmed the beneficial effect of a loading phase but also revealed no or very little ice formation after cryoprotection of shoot tips in other treatments. Regeneration following cryopreservation was obtained for genotype SXH804 (4.3 ± 2.1%) but not for SXH404. Regenerated explants of *L. cinerea* SXH804 were morphologically identical to tissue-cultured plants. As an alternative to shoot tips, callus tissues of clone SXH404 were successfully cryopreserved (>66.7% post LN survival) using the same protocol.

Keywords: cryopreservation, differential scanning calorimetry, droplet vitrification, *Loxocarya cinerea*, tissue culture

INTRODUCTION

Loxocarya cinerea (Restionaceae) is an endemic Gondwanan taxon unique to the Jarrah (*Eucalyptus marginata* Sm.) forest in southwest Western Australia. It is a low spreading dioecious species that forms large underground rhizome agglomerations that are capable of rapid re-sprouting after fire, which is a semi-regular occurrence in these forests. Both are useful traits that render it invaluable as a soil-stabilising species, a feature especially useful for post-mining restoration that often leaves bare ground to be rehabilitated, for example following bauxite mining. *Loxocarya cinerea* is also an important food source for native fauna (particularly marsupial species such as kangaroos and wallabies). Around 550 ha of native vegetation are removed annually in a region of high biodiversity with 300-400 plant

species found in the upland areas impacted by mining, many of which are found nowhere else in the world (8, 13, 14, 27). *Loxocarya cinerea* produces very few seeds and available seed is of indeterminate quality due in part to unresolved seed dormancy characteristics and cannot be effectively propagated by cuttings/division or direct transplanting; therefore it is one of a number of native species that must necessarily be returned to site as tube stock produced *via* tissue culture (14, 28).

Micropropagation of *L. cinerea* is achieved through extracting zygotic embryos from the few viable seeds that are available and culturing these under aseptic conditions to produce various seedling clonal lines of shoot cultures (28). To enhance genetic diversity of *L. cinerea*, new cultures are initiated from viable seeds from multiple provenance sites as opportunity arises. However seed production is unreliable, so this may only occur once every several years. Standard propagation involves transferring micropropagated plants of a suitable size to soil under greenhouse conditions (deflasking) until they are acclimatised, and later transplanting them into restoration sites, which occurs during the winter wet season (28). Tissue culture lines of *L. cinerea* are therefore highly valuable and cannot be readily replaced if losses occur due to equipment failure, microbial contamination or disaster (e.g. fire). Off-site duplicate culture collections (including cool storage) mitigate risks to some extent but still do not completely resolve accidental losses (as outlined above), nor somaclonal variation or culture decline over long periods of continuous culture maintenance. Hence other methods of safe long-term storage need to be considered.

Cryopreservation, *i.e.* storage of biological material at ultra low temperature (-196°C) in liquid nitrogen (LN) while maintaining cell viability and the capacity to regenerate and grow following rewarming (1, 2, 9, 10), is cost effective and space efficient, requiring only small portions of plant material to be stored such as shoot tips (or sometimes callus tissue). Once material is cryostored, no diseases are transmitted (1) and material cryopreserved to date in a wide variety of species has so far been proven to be genetically stable (6, 11). As all biochemical reactions and physical processes are basically halted at LN temperature, the material is storable for very long periods of time (2).

One of the key issues determining success in cryopreservation is the avoidance of intracellular ice formation, which can destroy cell membranes leading to cell death. Hence one of the aims of cryopreservation is to circumvent these deleterious processes through the addition of various cryoprotectants, which have a tendency to favour the vitrification (formation of glass) of water at low temperatures so that upon fast cooling the formation of intracellular ice can be altogether avoided (17, 18, 23). The physical mechanisms of these processes are reviewed elsewhere (15, 16, 29, 30). In practice, a vitrification cryopreservation protocol is applied where a small plant sample (e.g. shoot tip, 1-2 mm long) is dehydrated, cryoprotected and then vitrified during cooling in LN (22). Sample survival is also critically dependent on the warming rate, as the sample has to be quickly rewarmed from LN to prevent the formation of ice during this critical phase (recrystallization). The significance and extent of ice formation and vitrification of cellular contents can be measured and quantified using a thermophysical analysis method, such as differential scanning calorimetry (DSC). DSC measures the heat flow compared to a reference during cooling and warming, allowing the detection of any phase changes and knowledge of the prevalent state of water [liquid, ice or glass (7)]. DSC is an elegant tool to aid in the understanding of physical mechanisms of damage in cryobiology (3, 17). It has recently been used to assist with developing successful cryopreservation for the endemic native Australian plant species *Lomandra sonderi* (19).

The aim of this study centred on developing successful cryopreservation for *L. cinerea*. DSC analysis was used to detect the formation of potentially deleterious ice in shoot material at critical stages in the cryopreservation cycle, as this is a fundamental cause of lethal cryo-injury. Shoot tips from two clonal lines of *L. cinerea* (SXH404 and SXH804) were used to compare possible clonal differences, and regenerative callus tissue was also used with one

clone (SXH404) to determine whether different tissue types exhibited different responses to cryopreservation.

MATERIALS AND METHODS

Plant material

Loxocarya cinerea plants (grown *in vitro*), genotypes SXH404 and SXH804, were provided by Alcoa of Australia's Marrinup Nursery in Western Australia. Basal Medium (BM) consisted of half-strength Murashige and Skoog (MS) medium (20) with macro- and micronutrients (modified to include a total of 100 µM NaFeEDTA), 1 µM thiamine hydrochloride, 2.5 µM pyridoxine, 4 µM nicotinic acid, 500 µM myo-inositol and 500 µM 4-morpholineethanesulfonic acid (MES). BM was used for all experiments with some variations as specified. Shoots of *L. cinerea* were maintained on a multiplication medium (consisting of BM with 20 g L^{-1} sucrose, 8 g L^{-1} agar, 0.5 µM benzylaminopurine (BAP), with pH set at 6.0 prior to autoclaving at 121°C for 20 min) and cultured at 23±1°C with a 12 h light/12 h dark photoperiod (36 W fluorescent tubes, PPFD approximately 30 µmol m^{-2} s^{-1} at culture level) for 3 weeks, before isolation of shoot tips and thermal analysis or cryopreservation. Shoot cultures (five shoot clumps/jar with each clump comprising approximately 10-20 shoots) of *L. cinerea* were grown in 250 mL glass jars containing 50 mL medium with vented lids (~ 10 mm diameter vent with 0.2 µm micropore membrane). Callus induction and growth medium for *L. cinerea* consisted of BM as per multiplication medium above, except that BAP was replaced with 0.1 µM thidiazuron (TDZ) that was filter-sterilized and added to the medium after autoclaving. Callus tissues for cryopreservation experiments were grown in 55 mm plates containing 10 mL of medium (10 callus pieces per plate) and sealed with a thermoplastic film. Recovery medium (RM) consisted of BM as per the multiplication medium above but with choline chloride at 2 mM, BAP replaced with filter-sterilized zeatin (1 µM) and gibberellic acid (3 µM) and dispensed in 55 mm diameter sterile plates (~10 mL medium per plate) and sealed as described above. Sugars (e.g. raffinose), nutrient media, cryopreservation chemicals (e.g. DMSO, glycerol, etc.) and plant growth regulators (e.g. BAP, etc.) were obtained from Sigma-Aldrich, Australia.

Cryopreservation solutions

Preculture medium (PM) consisted of BM with 0.2 M raffinose. Loading solution (LS) consisted of 2 M glycerol with 0.4 M sucrose in BM minerals and organic supplements as above (but without standard sugar concentration and agar). Plant Vitrification Solution 2 (PVS2), consisted of BM with 0.4 M sucrose, 30% w/v glycerol and 15% w/v ethylene glycol and DMSO at 15% w/v (21). Loading solution and PM were autoclaved and PVS2 (minus DMSO) was autoclaved, then DMSO was filter-sterilized (0.45 µm Acrodisc® nylon membrane syringe filter, Sigma-Aldrich Australia) and added just prior to use.

Cryopreservation protocol

A modified droplet-vitrification protocol was applied (5). *L. cinerea* shoot tips (approximately 2 mm long and 0.5 mm thick) were isolated and placed on PM for either 24 or 48 h in darkness at 25±1°C. Shoot tips were then incubated in LS for 20 min. The LS step was only followed in the first experiment and later omitted. Shoot tips were then transferred into PVS2 or modified PVS2 with an altered DMSO concentration (10% w/v or 5% w/v) at 0°C for 30 or 60 min. Shoot tips were placed in 1 µL droplets of PVS2 on aluminium foils and then transferred into empty chilled cryovials, which were closed and then cooled rapidly from 0°C by immersion into LN.

After 1 h in LN, cryovials were rewarmed using a water-bath at 40°C for 10 s. The shoot tips were then immersed in half-strength MS medium containing 1 M sucrose for 20 min at room temperature. For regeneration, shoot tips were placed on RM as described for 2 weeks at

25±1°C with no light, and then into an incubator at 23±1°C with a 12 h light/12 h dark photoperiod (PPFD as above). Survival (green tissue, leaf development) and regeneration (shoot development) were determined 6 weeks after rewarming.

Callus tissue was induced from shoot tip explants incubated at 25±1°C with a 12 h light/12 h dark photoperiod (PPFD as above) for 6 weeks on TDZ medium (in 55 mm Petri plates, 10 shoot tips/plate and sealed as above). For cryopreservation experiments, callus tissue was trimmed to a defined size (~2 mm x 2 mm sections) and placed on PM for 48 h in darkness at 25±1°C, and then placed in PVS2 at 0°C for 30 min before being cryopreserved and rewarmed as described above. Rewarmed callus tissue was placed on BM with: (a) 0.1 µM thidiazuron (TDZ); (b) 0.1 µM TDZ with 2 mM choline chloride (CC), 1 µM zeatin (Z) and 3 µM gibberellic acid (GA); (c) 0.1 µM TDZ with 1% w/v activated charcoal (AC); and (d) 0.1 µM TDZ, 2 mM CC, 1 µM Z, 3 µM GA and 1% w/v AC. Callus tissue was checked for survival (green tissue, leaf development) and regeneration (shoot development, if present), after rewarming for up to 9 weeks.

Thermal analysis

Thermal analysis of the shoot tips of *L. cinerea* genotypes SXH404 and SXH804 and of different cryopreservation solutions was undertaken using two differential scanning calorimeters. Firstly, a Perkin Elmer DSC1 with intercooler (Perkin Elmer, Waltham, Massachusetts, USA) was used. Calibration was achieved using Indium and Zinc, $[T_m(In)$ 156.6°C, $T_m(Zn)$ 419.1 °C, $\Delta H(In)$ 28.7 J/g, $\Delta H(Zn)$ 107.5 J/g]. Samples of single shoot tips per measurement were weighed on a Mettler-Toledo microbalance (accuracy ± 1 µg, Columbus, Ohio, USA) and placed in aluminium pans, which were hermetically sealed. Data was evaluated using Perkin Elmer software, Pyris and TA Universal Analysis 2000 Version 4.3A. The following programme was used for all samples. Samples were cooled from 25°C at a rate of 100°C/min to -65°C. At -65°C, samples were held isothermally for 5 min before ramping up the temperature to 30°C at a rate of 10°C/min. The runs were repeated three times, each time with new plant material.

The Perkin Elmer instrument was unable to reach sufficiently low temperatures to observe glass transitions in water. Therefore a second DSC instrument (Linkam Scientific Instruments DSC600, Guildford, Surrey, England) was used, which is capable of cooling to -196°C, allowing the measurement of cryoprotective glass formation. The data was evaluated using Linksys32 V2.2.3 software. Samples were cooled from 25°C at a rate of 100°C/min to -130°C. At -130°C, samples were held isothermally for 5 min before ramping up the temperature to 25°C at a rate of 50°C/min. The warming rate used here is higher than in the previous experiments, in order to maximise the resolution of possible glass transitions, which have much lower enthalpy than freezing transitions, and are therefore much harder to detect.

Statistics

Each experiment for thermal analysis and cryopreservation was repeated at least three times. Cryopreservation experiments involved 10 to 15 cryopreserved and 10 to 15 control shoot tips (which underwent the entire cryopreservation protocol, except for cooling and rewarming). Statistical differences were calculated with One-Way-ANOVA with the software Sigmaplot (Version 12.0, 2011, Systat Software, Inc.). The post hoc analysis used was the Holm-Sidak method.

RESULTS

Preliminary cryopreservation experiments comprised a basic vitrification method (without a loading phase), whereby shoot tips of *L. cinerea* (SXH404) were precultured on BM with 0.2 M raffinose for 1 or 2 days and then treated with PVS2 at 0°C for 30 or 60 min. Preculture on 0.2 M raffinose had no apparent deleterious effect on shoot tips with up to 100% survival (minus LN) in contrast to much poorer performance with higher concentrations of raffinose and other sugars examined (data not presented). Measurements of shoot tip regeneration including PVS2 treatment established that the upper limit of PVS2 exposure (minus LN) was most likely 30 min – particularly when combined with a 48 h desiccation treatment using 0.2 M raffinose (Fig. 1A and B). Exposure to LN was lethal to shoot tips in all treatments indicating that the basic vitrification protocol was inadequate regardless of PVS2 exposure time or time of desiccation treatment.
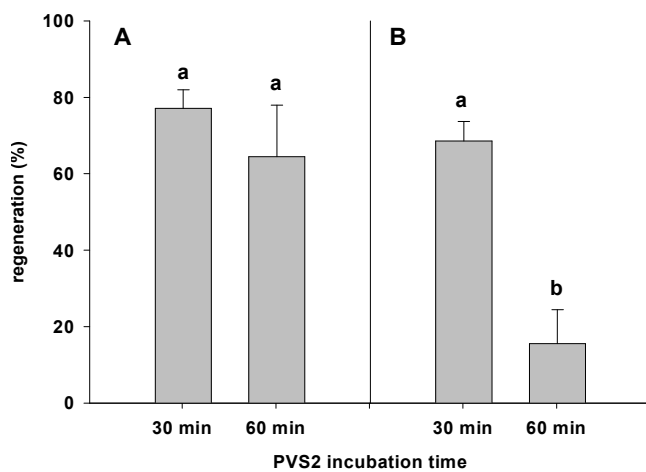

Figure 1. Regeneration (%) of control shoot tips of *Loxocarya cinerea* genotype SXH404 (without the loading phase) using 30 and 60 min incubation times in PVS2 on ice after a (A) 24 h and (B) 48 h preculture on 0.2 M raffinose medium. Explants exposed to LN did not survive in any treatments. Different lowercase letters (a, b) denote significant differences ($P < 0.05$) between treatments (One-Way-ANOVA). Bars indicate standard errors.

Further examination of the effect of varying the DMSO fraction in PVS2 showed that DMSO at the standard 15% v/v appears to be toxic to *L. cinerea* based on survival and regeneration of SXH404 shoot tips (Fig. 2). The addition of a loading phase prior to PVS2 treatment (but without exposure to LN) reduced shoot tip survival significantly at the standard 15 % v/v DMSO concentration but not at 5% or 10% DMSO concentrations (Fig. 2). Shoot tips of SXH404 failed to survive exposure to LN in any treatment in this experiment.

To identify and quantify the phase transitions of water, the cryoprotective solution PVS2 was cooled and warmed within the Perkin Elmer DSC. Similar to Volk and Walters (26), no ice formation or melting of ice was found during cooling and warming of the solution (Fig. 3). A glass transition could not be measured, as the lowest cooling temperatures were -60°C. The cryoprotective solution PVS2 was confirmed to form a glass, using the Linkam Scientific Instrument DSC600, where a temperature of -130°C was achieved, with PVS2 forming a glass at -115.4 ± 3.2°C (results not shown). Similar results were found by Volk and Walters for PVS2 with a glass transition temperature of -115°C (26).

Analysis of shoot tips of *L. cinerea* of both clones (SXH404 and SXH804), where a loading phase was included, showed that ice formation was excluded from the cooling phase (Fig. 3A) for clone SXH404, while a very small amount of ice appeared with clone SXH804. The warming phase (Fig. 3B) showed no melting of ice with either clone. Untreated shoot tips showed distinct ice formation and ice melting, while PVS2 alone showed that ice formation/melting was completely absent, indicating that the cryoprotectant properties of PVS2 were working effectively (Figs. 3A and B).
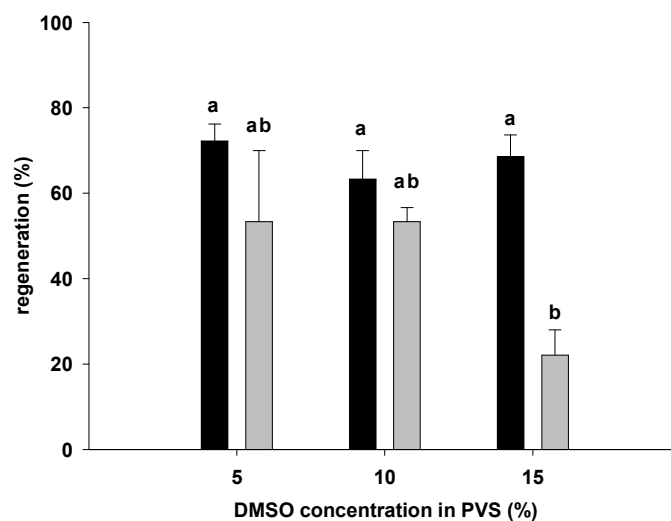
Figure 2. Plant regeneration (%) of shoot tips of *Loxocarya cinerea* genotype SXH404 after isolation and treatment with (or without) LS, PVS2 and with (or without) exposure to LN. Treatments included a 0.2 M raffinose preculture for 48 h either with the loading solution step (2.0 M glycerol with 0.4 M sucrose in 1/2MS medium) (grey bars) or without it (black bars), before incubation in the modified PVS2 with different DMSO concentrations for 30 min, followed by unloading with a 1.0 M sucrose solution and regeneration. Bars represent the standard error of the means. Different letters denote significant differences ($P < 0.05$, One-Way-ANOVA). No survival was observed for shoot tips exposed to LN hence data not shown.

As a control, freshly isolated shoot tips of *L. cinerea* genotypes SXH404 and SXH804 were thermoanalysed in the Perkin Elmer DSC1 instrument. Ice formation was detected during cooling while melting of ice was detected during warming of shoot tips (Fig. 3). DSC measurements were performed after preculture, after LS and after cryoprotection in PVS2 for 30 min. With each additional step the amount of freezing and melting of water within the plant tissue decreased (Fig. 4). Using the original protocol including the LS step, there was no or very little ice formation and melting of ice in both genotypes SXH404 and SXH804 (Figs. 3 and 4). Some ice formation was still found in the successfully cryoprotected genotype SXH804 after 30 min of exposure to PVS2. This suggests that despite some ice formation, successful cryopreservation could be achieved.

Application of a cryoprotective protocol including preculture, LS and PVS2 resulted in four out of 10 successful experiments (total recovery of five shoot tips into plants out of 131) where a few shoot tips of *L. cinerea* (clone SXH804) survived cryopreservation (Fig. 5) and were able to regenerate into plantlets that could be established in a potting medium and appeared morphologically identical to plants transferred from tissue culture onto soil and also to control plants (Fig. 6a-c). No survival of clone SXH404 shoot tips was achievable using the same protocol (Fig. 5).

Loxocarya cinerea SXH404 showed no ice formation in three runs (Fig. 3) or only very little in two runs, resulting in an average heat flow during warming of 2.96 ± 2.00 J g^{-1} (Fig. 4). This suggests that there are no differences between genotypes SXH404 and SXH804 regarding ice formation within their tissues, as measured by DSC. Since the amount of ice formed was minor and similar in both genotypes SXH404 and SXH804, it would appear that cryoprotection using PVS2 and the LS step were working successfully and ice formation was prevented in the tissue (the thermographs for PVS2, SXH404 and SXH804 in Fig. 3 are nearly identical). Therefore, ice formation can be reasonably excluded as the reason why no survival after cryopreservation was observed for genotype SXH404.
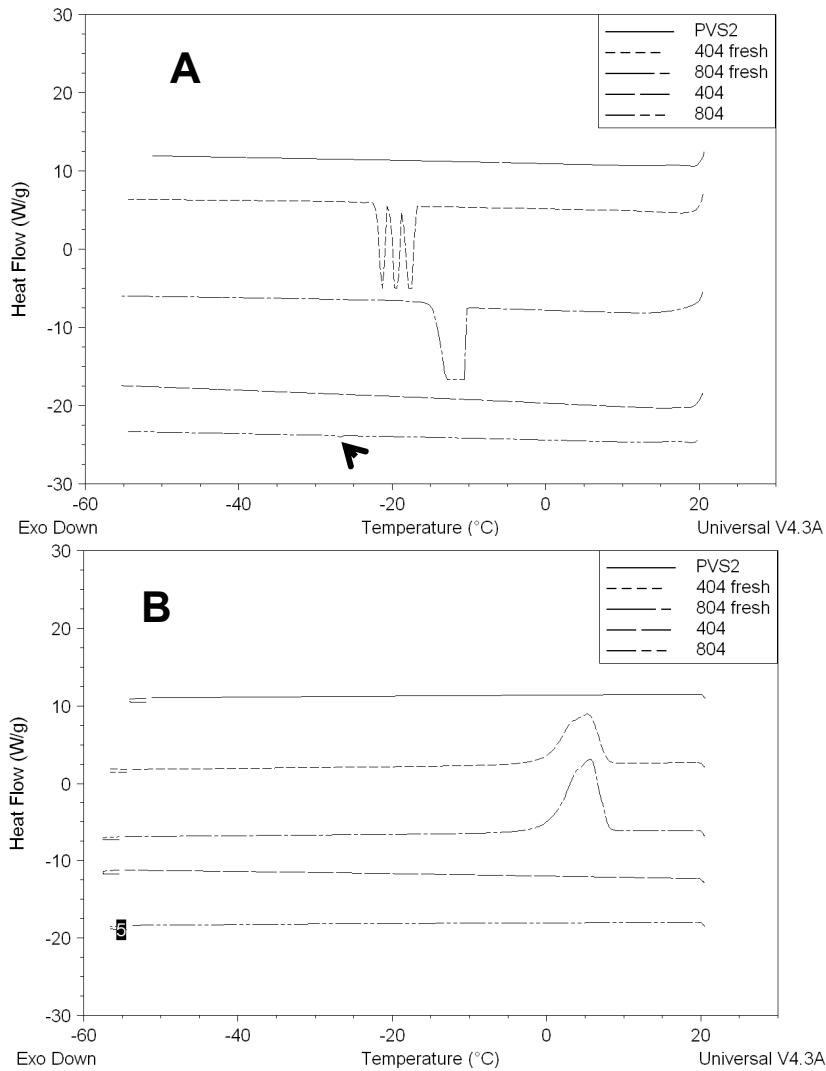
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Figure 3. Thermographs of cooling (A) and warming (B) cycles obtained with the Perkin Elmer DSC1. PVS2 graphs show no ice formation or melting. Freshly isolated shoot tips of *Loxocarya cinerea* genotypes SXH404 (404 fresh) and SXH804 (804 fresh) show ice crystallisation peaks during cooling (A) and melting peaks during warming (B). Shoot tips of genotypes SXH404 and SXH804 after isolation, preculture 48 h, loading solution 20 min and cryoprotection with PVS2 for 30 min show a small ice peak (black arrow) during cooling in SXH804 (A, 804) and no ice formation in SXH404 (A, 404), and no melting of ice during warming (B, 404, 804). Curves are vertically offset for better comparison.

Since the LS step decreased survival of control explants in genotype SXH404 (Fig. 2), shoot tips of this genotype were thermo-analysed without the LS step. Some ice formation was found during cooling and ice melting was found during warming (data not presented). The amount of ice formed and melted was larger (30.39 ± 12.36 J g^{-1}) in comparison to the original
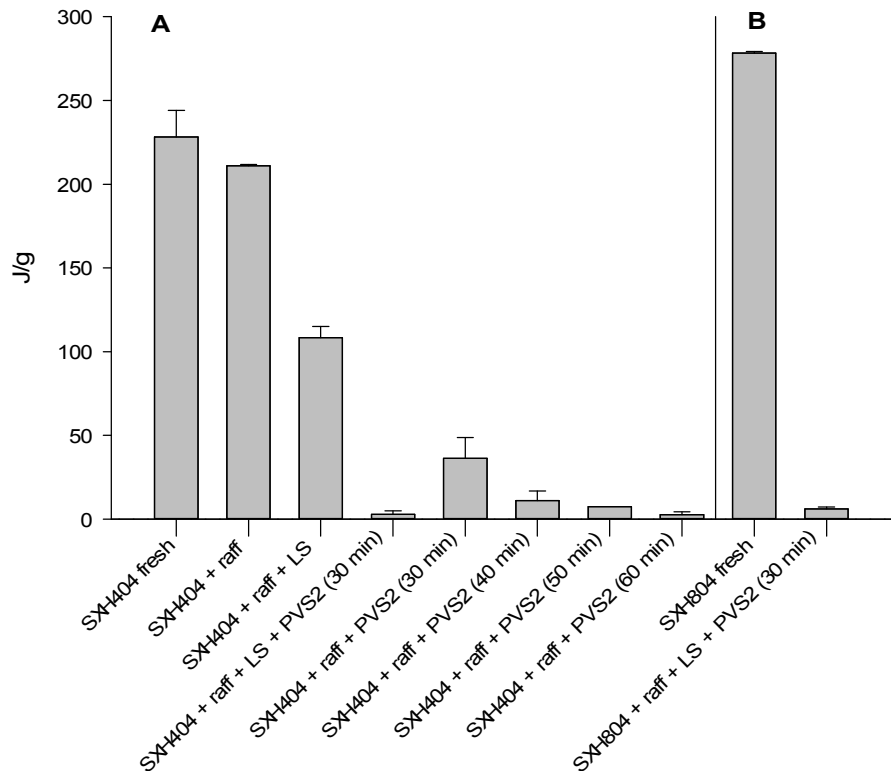
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Figure 4. Comparison of heat enthalpies of shoot tips during warming after different treatments for *Loxocarya cinerea* genotypes SXH404 (A) and SXH804 (B). Enthalpies are reduced with each step in the cryopreservation protocol. (fresh – freshly isolated shoot tips, raff – shoot tips after 0.2 M raffinose preculture on ½ MS with 2% sucrose and 8 g/L agar 48h, LS – loading solution (2.0 M glycerol and 0.4 M sucrose in ½ MS), PVS2 – plant vitrification solution 2 (½ MS medium, 0.4 M sucrose, 30% w/v glycerol, 15% w/v ethylene glycol, 15% w/v DMSO). Perkin Elmer DSC1 was used. Bars represent the standard error of the means.

protocol using the LS step (2.96 ± 2.00 J g^{-1}; Fig. 4). Therefore, shoot tips were incubated in PVS2 for longer time periods to determine the maximum time in PVS2 to prevent the formation of ice (40, 50 and 60 min); however, thermographs continued to show ice formation after 40 min of incubation in PVS2, before decreasing to minimal ice formation after 50 and 60 min of incubation in PVS2 (Fig. 4). The heat flow during warming after 60 min of incubation in PVS2 was slightly lower (2.67 ± 1.77 J/g) than when using the original protocol with the LS step and 30 min of incubation in PVS2 (2.96 ± 2.00 J/g, Fig. 4). It was also lower than the heat flow in genotype SXH804 with the original protocol (LS step and 30 min of incubation in PVS2: 6.16 ± 1.02 J g^{-1}, Fig. 4). Therefore, for the same incubation time in PVS2, the LS step led to reduced water crystallization. A shorter incubation time in PVS2 is usually an advantage, as prolonged incubation in PVS2, which contains DMSO, can be toxic to cells and damage the tissue (25).

Examination of heat enthalpies of shoot tips of *L. cinerea* (clone SXH404) during warming following various cryoprotective treatments indicated that successive steps in the cryopreservation protocol contribute to reducing endothermic enthalpies compared to fresh (untreated) material (Fig. 4). The largest reductions in enthalpy occurred with the loading solution (LS) and PVS2 steps compared with much less reduction with only the raffinose treatment. The most effective combination of steps to reduce ice formation appeared to be treatment with 0.2 M raffinose (2 days), then LS (0.4 M sucrose + 2 M glycerol) for 20 min followed by PVS2 treatment at 0°C for 30 min; or raffinose treatment followed by 60 min

exposure to PVS2 at 0°C. The protocol combining raffinose treatment, LS and PVS2 provided a similar outcome with *L. cinerea* clone SXH804. Despite the apparent beneficial effect of a cryoprotective protocol that included a LS step (as determined by DSC and heat enthalpy analysis), shoot tip survival following cryopreservation was not observed with genotype SXH404.
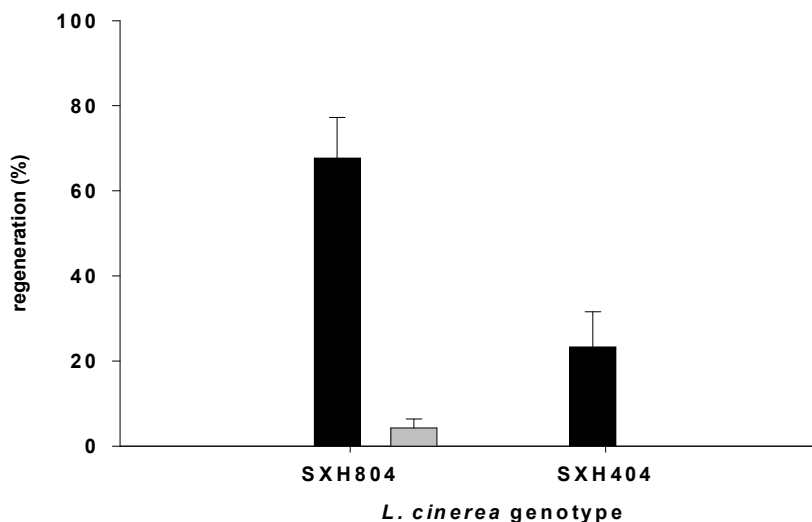

Figure 5. Regeneration (%) of shoot tips of *Loxocarya cinerea* genotypes SXH804 and SXH404 control (-LN, black bars) and cryopreserved (+LN, grey bars). The droplet vitrification protocol was followed, including shoot tip isolation, a 0.2 M raffinose preculture for 48 h, a loading solution for 20 min, cryoprotection with PVS2 on ice for 30 min, rapid cooling in LN, rapid rewarming in a 40°C water bath for 10 s, and regeneration on recovery medium for 6 weeks. Bars represent the standard error of the means.
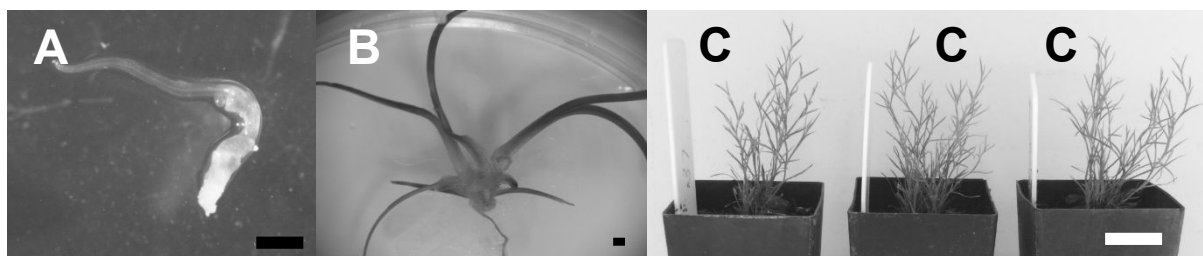

Figure 6. Regeneration of shoot tips of *Loxocarya cinerea* genotype SXH804. (A) 2 months after rewarming from cryopreservation; (B) 5 months after rewarming from cryopreservation; (C) approx. 2 yrs old after rewarming from cryopreservation, transplanted to soil (C1 – tissue culture (TC) plants, only transferred from TC to soil, C2 – plants derived from cryopreservation, C3 – control plants, derived from isolated shoot tips, subject to all cryopreservation steps except LN and warming phases). Black bars indicate 1 mm and the white bar indicates 20 mm.

Callus tissue of *L. cinerea* SXH404 was cryopreserved using the standard protocol as described above (without the LS step) for shoot tips. Survival of callus was lowest (66.7%) using standard callus induction medium (BM + 0.1μM TDZ) as the recovery medium (TDZ-O). The addition of activated charcoal to TDZ-O (TDZ-O +AC) or TDZ-O including 2 mM choline chloride, 1 μM zeatin and 3 μM gibberellic acid (TDZ-O +R) increased survival by around 20%, resulting in a very high proportion of callus tissue surviving cryopreservation (over 90%) (Fig. 7). Unusually, callus that was cryostored appeared to regenerate and recover slightly better than non-frozen callus (control treatments), which was a significant increase

using the TDZ-O + AC recovery medium, but not for the other recovery media tested (Fig. 7). About 40% of callus explants were able to regenerate into shoots after revival from cryostorage (Fig. 8A and B).
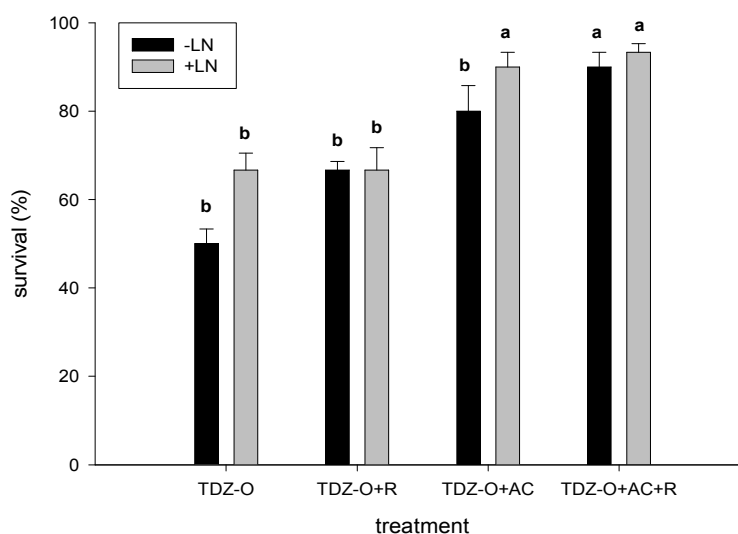

Figure 7. Survival (%) of callus tissue of *Loxocarya cinerea* genotype SXH404 control (-LN, black bars) and after cryopreservation (+LN, grey bars).**TDZ-O:** BM with 0.1 µM thidiazuron (TDZ); **TDZ-O + R**: BM with 2 mM choline chloride (CC), 1 µM zeatin (Z) and 3 µM gibberellic acid (GA) + 0.1 µM TDZ; **TDZ-O + AC:** BM with 0.1 µM TDZ and 1% w/v activated charcoal (AC); **TDZ-O + AC + R**: BM with 2 mM CC, 1 µM Z, 3 µM GA and 0.1 µM TDZ with 1% w/v AC. Different letters denote significant differences ($P < 0.05$, One-Way-ANOVA). Bars indicate standard errors.
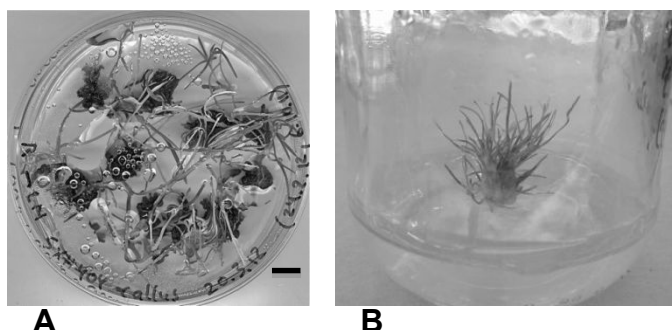

Figure 8. Regeneration of *Loxocarya cinerea* from callus after cryopreservation. (A) 22 weeks after rewarming from cryopreservation and (B) 50 weeks after rewarming from cryopreservation.
Bar (▬) = 1 cm.

DISCUSSION

Thermal analysis of shoot tips of *L. cinerea* revealed the formation of minimal amounts of ice after cryoprotection in PVS2 and cooling to -65°C. Genotype SXH404 showed little or no ice formation in DSC runs after 30 min cryoprotection, similar to SXH804. If ice formed during cooling, it is possible that it was either located outside of the cells, or if inside of the cells, it could have been located in those regions which are not necessary to regenerate the plant, for example, outside of the meristematic area. Ice formation could also take place in only some areas of the tissue, while other areas remain viable. Since the extent of ice formation was relatively small in this study, it could also be the case that this ice formation did not in fact take place during the actual cryopreservation experiment because cooling rates in the droplet-vitrification method used are much higher, with a cooling rate of approximately 7,800°C min^{-1}, compared to the cooling rate applied in the DSC instrument of 100°C min^{-1} (11). In any event, cryoprotection in PVS2 for up to 30 min would appear to be amply sufficient for successful cryopreservation, as DSC applies much lower cooling rates than in the actual cryopreservation protocol and, hence, the minimal amount of ice that is formed in

the DSC experiments is likely to be suppressed altogether during cryopreservation. The main conclusion that can be drawn from these thermal analyses is that ice formation is very unlikely to be the reason why *L. cinerea* genotype SXH404 is unable to regenerate following cryopreservation. Since ice formation during cooling and warming was successfully suppressed under the tested conditions, other mechanisms may be responsible for the failure of regeneration from shoot tips after cryopreservation.

Loxocarya cinerea proved very difficult to regenerate after cryopreservation using shoot tips grown under *in vitro* conditions. Thermophysical analysis using DSC indicated that a cryopreservation protocol comprising pre-culture on medium containing 0.2 M raffinose for 2 days, a loading phase (0.4 M sucrose + 2 M glycerol) for 20 min, treatment for 30 min in PVS2, transfer of shoot tips to foil strips in a drop of PVS2 (*i.e.* droplet-vitrification) and immersion in LN, failed to show evidence of significant ice formation in either the cooling or warming phases. In addition, heat enthalpy data indicated that preculture, LS and PVS2 phases alone and in combination all showed reductions in enthalpy consistent with results published for other species (12). This, however, contrasted sharply with overall poor survival of *L. cinerea* shoot tips after exposure to LN. A few shoot tips of one genotype were able to be regenerated into plants and grown in soil after cryostorage on four out of 10 repeats of the same protocol in this study. However individual survival and regeneration in each experiment was very low and not able to be significantly improved within the study's time frame. In comparison, callus tissue of a second genotype *L. cinerea,* was able to be successfully revived and re-grown following cryostorage whereas shoot tips from the same genotype were not. This survival of callus tissue was achieved without a LS step, which is reported to be essential, or at least highly beneficial, for survival of shoot tips of other species following cryostorage (12). Generally, shoot tips, as organised and genetically stable tissues, are the preferred material to use for cryopreservation; however, under some circumstances callus has also been found to be a highly effective way to conserve valuable plant germplasm and may provide several key advantages: callus is easier to prepare, requires significantly less processing time and may result in a larger number of regenerating plantlets and shoots from a similar amount of starting material (compared to a shoot tip) (4, 31). The cryopreservation of callus tissue could be one way to preserve *L. cinerea* in the interim (subject to further assessment, such as genetic stability and the capacity to reform viable shoots and plantlets following cryopreservation) until successful cryopreservation of apices becomes a viable alternative.

It is possible that *L. cinerea* shoot tips (or perhaps the shoot meristematic tissues) are sensitive to certain phases of the cryopreservation process, possibly a phytotoxic reaction to one or more cryoprotectants (glycerol and/or DMSO). A 2-week delay in growth of isolated and cryoprotected shoot tips was observed in contrast to only isolated non-treated shoot tips, which started growing after 2-3 days. It could also be useful in future to further examine the effects of a wider suite of phytohormones that can induce cellular growth after rewarming, extend the examination of the effects of antioxidants in the protocol and possibly a structural analysis of the cells to determine if there are other regions of the cells where damage may be occurring. The current study shows that the basic biophysical parameters of the cryopreservation process are effective at minimising or eliminating ice formation in tissues (also evident by data showing the reduction of the heat enthalpy of tissues in response to various cryopreservation stages); however, despite this, even a moderate rate of reproducible cryopreservation success with shoot tips of *L. cinerea* has been elusive. In contrast callus tissue appears to be far less sensitive to the cryopreservation process, as indicated by the fact that high post-cryostorage survival (>65%) of callus material was readily achievable. In addition, regrowth of cryostored callus appeared to be enhanced mostly by the addition of AC to the recovery medium, indicating that the well-known adsorption properties of AC (21) may be actively nullifying the detrimental effects of, for example phytotoxins, or modifying the

effects of other components of the culture medium in some way (24). This aspect is worthy of further investigation, as is the trial of LS variations in future experiments with shoot apices.

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