Is mitochondrial function the key to improving cryopreservation of threatened Australian flora?

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Summary

Cryopreservation is a method of ex situ conservation for threatened plant species. Although this method allows long-term storage, it also causes damage to plant tissues, which must be repaired for survival to occur. This repair process is fuelled by mitochondria, however little is known about how mitochondrial function is affected by cryopreservation in plants. While metabolic rates and mitochondrial function have been tested in animal cryopreservation, this is the first study to investigate the relationship between metabolic rate and cryopreservation in plants.

The project aimed to increase our understanding of the stresses experienced by Australian plants during cryopreservation. This project will used new non-invasive techniques to assess mitochondrial function during the cryopreservation process in threatened Australian species. This includes the ASTEC Global Technology Q2 Oxygen Sensing Technology to assess metabolic function before and after cryopreservation; mitochondria-specific fluorescent probes to visualise mitochondrial damage within cells during cryopreservation; and the Seahorse XF Flux analysis, to gain specific insight into the toxic nature of CPAs effect on the various components of mitochondrial function.

This work on the importance of mitochondrial function is ongoing, with two PhD students and an honours student in 2021 continuing experimental work on the vital role mitochondria play during cryopreservation. This report summarises the results collect so far, with a focus on the Q2 Oxygen Sensing Technology that has proven to be particularly applicable to analysing mitochondrial function pre- and post-cryopreservation. The overall findings of this study showed, as expected, that mitochondrial function is severely impacted by the stresses of cryopreservation; however, alterations to the cryopreservation protocol can help alleviate some of this stress and reduce the damage done to mitochondria. Funding from the Australian Flora Foundation went largely towards consumables, hiring a casual research assistant, and for attending the Buck Institute Bioenergetics Master Classes on flux analysis.

Introduction

This project aims to advance the fundamental science of metabolic function that impacts the successful cryopreservation of threatened Australian plant species. Australia is host to an incredibly diverse and endemic range of species, many of which require conservation. The energy produced by the mitochondria is vital in almost all aspects of cell metabolism, and it is of particular importance for cryopreservation due to its role in providing energy for repairing damaged DNA, the production of new proteins and lipids, and the energy to resume normal cell division and growth after storage. The project will increase our understanding of the stresses experienced by Australian plants during cryopreservation. Specifically, the characterisation of mitochondrial function and integrity in plant tissues will be pioneered as a novel approach to the development of species-specific cryopreservation protocols. This knowledge will enable the development of effective cryopreservation protocols for problematic Australian species in future, an essential step for the successful conservation of Australian plant diversity and indeed the management of rare and endangered species.

Cryopreservation is the safest and most effective long-term conservation method of storing valuable species and genotypes, involving storage of living biological material in liquid nitrogen (LN), whereby the retrieval of this material is in a viable state to be used to produce new individuals (Kaczmarczyk *et al.* 2012). This requires specific protocols to be developed that protect the biological material (e.g. seeds, seed axes or shoot tips) from freezing damage and allow it to be safely cryo-stored (Funnekotter *et al.* 2017b). The integrity of mitochondrial function within cells is essential for the successful recovery of cryopreserved material but there has been very limited investigation in plants. The energy stored in ATP produced by the mitochondria is vital in almost all aspects of cell metabolism, and it is of particular importance for cryopreservation due to its role in providing energy for repairing damaged DNA, the production of new proteins and lipids, and the energy to resume normal cell division and growth after storage. Reduced mitochondrial activity has been correlated to poor cryogenic survival in spermatozoa of fish, mammals and humans (De Baulny *et al.* 1997; He and Woods 2004; Henry *et al.* 1993).

Utilising a variety of different methods is crucial to gain insight into the various stresses cryopreservation imposes on mitochondria, from desiccation stress, toxic damage from CPAs and the large fluctuations in temperature. This project aimed to assess the following technologies:

Q2 Oxygen Sensing Technology: Use of non-invasive techniques will enable the identification of a direct correlation between mitochondrial function and post-cryogenic survival rates. Oxygen consumption in cryopreserved shoot tips will be measured for the first time during the recovery period using Q2 Oxygen Sensing Technology on a recently purchased, state-of-the-art instrument (ASTEC

Global Technology). This technique allows minute changes in oxygen concentration within a sealed container to be measured over time, with greater oxygen consumption correlating with increased mitochondrial activity. This will be tested to determine if metabolic activity in recovering shoot tips is linked to plant post-cryopreservation regrowth.

Mitochondrial Fluorescent Probes: While the Q2 provides information about metabolic activity of whole sample, knowledge of where and when damage occurs during cryopreservation within the sample is essential for the optimisation of cryopreservation protocols. Mitochondria-specific fluorescent probes will give insight into mitochondrial damage in real time when shoot tips are exposed to the desiccation media and the toxic effects of the CPAs, as well as on how mitochondria recover after cryopreservation.

Seahorse XF Flux analysis: Extracellular flux analysis enables real-time measurements of cellular oxygen consumption rate and extracellular acidification rate, providing the most detailed assessment of cellular bioenergetics. This project will be the first to assess the effects individual CPAs have on extracellular flux analysis as well as their combined effects, with the aim to develop new vitrification solutions that have reduced metabolic toxicity.

Methods

In vitro cultures

In vitro shoots from all species were obtained from Kings Park and Botanic Garden, Western Australia. All media used was based on basal medium (BM) containing half- strength Murashige and Skoog macro- and micro- elements, sucrose and agar at pH 6.0 prepared as previously described by Menon *et al.* (2012) All tissue culture propagation used BM with the addition of 0.5 µM benzyladenine (BA), or 1.25 µM kinetin (K) and 0.125 µM BA. 40 mL of the medium was dispensed into 250 mL jars capped with vented lids and autoclaved at 121°C for 20 min. *In vitro* shoots were micropropagated into fresh media every 3-5 weeks depending on the species.

Cell cultures were produced from the *in vitro* shoot cultures, inducing callus formation with the addition of auxins to the BM. Once callus had formed, the callus was transferred to liquid BM and maintained on a rotary shaker at 100 rpm to form friable callus. Individual cells were then sieved out of the liquid BM.

Protoplasts were extracted from the cells cultures or directly from the shoot cultures using the protocols described by Jeong *et al.* (2021). Digestion enzymes (cellulose, hemicellulose, pectolyase) were obtained from Desert Biologicals.

Cryopreservation protocol

Shoot tips were placed onto desiccation medium (BM containing 0.4 M sucrose) for 48 hours prior to cryopreservation. Shoot tips were cryopreserved using the cryo-mesh method, as described in Funnekotter *et al.* (2017a), using a 20 minute loading solution (LS, 2 M glycerol with 0.4 M sucrose), 30 minute PVS2 incubation (PVS2, 30% w/v glycerol, 15% w/v ethylene glycol, 15% w dimethyl sulfoxide, 0.4 M sucrose), one hour in liquid nitrogen, and 20 minutes in washing solution (1.0 M sucrose). After thawing and washing, the shoot tips were placed onto RM.

Q2 Oxygen Sensing Technology

Oxygen consumption as an indicator of metabolic activity was measured on the Astec Global Q2 instrument (Amsterdam, The Netherlands). Q2 vials only were autoclave sterilised while the vial caps were sterilised by soaking in a 10% v/v biocide solution (PPM®, Plant Preservation Mixture) solution for 30 min. The Q2 was turned on prior to use and the temperature set to 25°C, and the barometric pressure of the room was recorded using a portable weather station (TechBrands XC0394 Electus Distributions, Australia). Oxygen levels were measured every 30 min. Metabolic rate of each shoot tip was determined using calculations developed by Vleck (1987), with modifications described by Tomlinson *et al.* (2018) The starting oxygen concentrations were normalised to 1 (atmospheric oxygen concentration) to account for different fluorescence levels in each vial cap (see Figure 1A for the variation in O_2, a value of 1 represents atmospheric O_2). Respiratory chamber volume at standard temperature and pressure was calculated using chamber volume, mass of the shoot tip, a standard density of 0.3 g/cm^3, temperature, barometric pressure and saturation water vapour pressure.

Optimisation of headspace

Prior to analysis, shoot tips were placed into Q2 vials with varying quantities of medium added, to alter the headspace within the vials. Shoot tips were isolated and tested on a range of headspace values from 0.3 mL to 0.9 mL.

Assessing mitochondrial damage during cryopreservation

Shoot tips were isolated, weighed, and run on the Q2 using desiccation medium, for 48 h. These shoot tips were weighed again before being cryopreserved using the optimum PVS2 incubation time.

Once thawed, shoot tips were run on the Q2 using RM for a number of days, until oxygen levels in the vials began to drop to less than 80%. Vials with dramatically decreased oxygen levels were identified as likely to be contaminated and were set aside. Positive controls in the form of freshly isolated shoot tips were also run through the Q2. The shoot tips were weighed once more after

removal from the Q2 to determine any change in mass, to make measurement of metabolic rate more accurate, and were transferred onto petri dishes of RM to further recover in light conditions at 24^{o}C. Survival and regeneration were recorded after 3-4 weeks. Shoot tips were classed as surviving when they showed spots of green or any form of general tissue growth. Shoot tips were classed as regenerating if a new shoot was clearly visible growing from the shoot tip. Dead shoot tips showed none of the signs of survival or regeneration.

Utilising the Q2 to optimise the cryopreservation protocol

Using the optimised protocol developed above, the Q2 was used to test if improved mitochondrial function correlates to increased post-cryogenic survival. Shoot tips were placed onto a range of desiccation medium (0.2 M to 1.0 M sucrose) in Q2 vials prior to cryopreservation for 48 h where they were assessed as above. The shoot tips were then cryopreserved and placed back onto the Q2 on RM for another 48 h.

Mitochondrial Fluorescent Probes

MitoTracker™ Green FM (ThermoFisher Scientific #M7514) was used to label active mitochondria in shoot tips and cells. A working concentration of 500 nM MitoTracker was used to stain the samples in liquid BM. Samples were observed under a Nikon Eclipse 80*i* microscope with a Nikon C-SHG1 Super High Pressure 100 W Mercury Lamp. Nikon B-2A (Excitation 470/40) filter cube was used, and images were captured using a Nikon DS-Qi2 camera.

Seahorse XF Flux analysis

Cell cultures were sieved for a uniform size, centrifuged and resuspended in an unbuffered liquid BM before being transferred to the Seahorse cell culture plates. Basal oxygen consumption rate (OCR) was determined for each well before the addition of respiratory inhibitors; Oligomycin, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), Antimycin A and Rotenone to identify ATP-linked respiration, maximal respiration and the spare capacity available above basal metabolic rate.

Results and Discussion

The results presented below are the major findings from the Q2 trials, and the initial findings from the fluorescent probes and flux analysis. The results from the Q2 trials are in preparation for publication (2021); with a more detailed analysis into the findings discussed there. Experiments with the mitochondrial fluorescent probes and the Seahorse XF flux analysis are ongoing, with the aim to publish these results 2021/22 if the current limitations can be overcome (see below). The works presented here are the collaborative results from the Conservation Biotechnology team at Kings Park Science and Curtin University.

Q2 Oxygen Sensing

Q2 optimisation

Prior to any analysis, the Q2 headspace required optimisation for each species. Without proper optimisation, species will either consume too little oxygen and be below the detectible limits of the Q2, or consume all the oxygen and expose the samples to extreme hypoxic conditions. As seen in Fig. 1A, an excessively large headspace showed no decline in vial O_2, Fig. 1B with an optimal headspace showed a linear decline over time in O_2, and, Figure 1C with an insufficient headspace where the medium interfered with the function of the Q2 vials, producing incorrect O_2 readings. Many species had an optimum headspace between 0.3 mL and 0.6 mL analysed over a 48 h period. Overall the Q2 instrument shows decent sensitivity when analysing samples over multiple hours (>6 h); however, shorter time scales may not show a sufficient decrease in oxygen to accurately determine metabolic rate from variations in background signal (as seen in Fig. 1A).

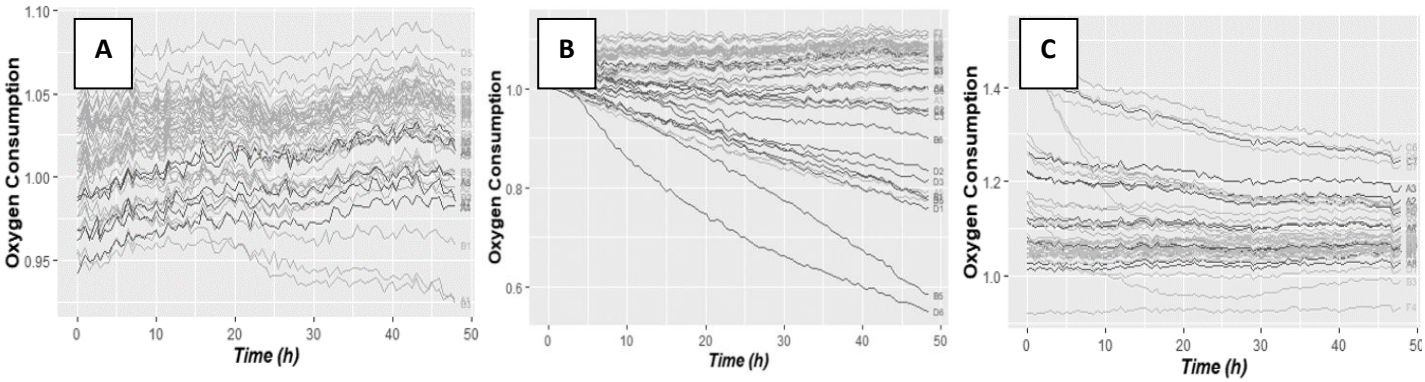

Figure 1. Examples of raw data showing O_2 consumption over time for fresh shoot tips on recovery medium comparing different headspaces over 48-h (using 0.9 mL Q2 vials). (A) Excessive 0.65 mL headspace. (B) Optimal 0.4 mL headspace. (C) Insufficient 0.2 mL headspace. Each line indicates the O_2 consumption of a single shoot tip.

Basal metabolic rates in Australian species

The basal metabolic rate of five Australian species was assessed to determine, if as hypothesised, that different species have different metabolic rates. Significant differences in average metabolic rates of freshly isolated shoot tips were observed between all species except *Grevillea scapigera* and *Lomandra sonderi* (Fig. 2). *Anigozanthos viridis* had the highest average fresh MR (0.99 mL $O_2.h^{-1}.g^{-1}$), while *Lasiopetalum moullean* (0.69 mL $O_2.h^{-1}.g^{-1}$) had the lowest (Fig. 2). More analysis will need to be done to determine if basal metabolic rate has any links to cryo-capability of species; however, it is more likely that that the ability of the species to maintain their metabolic rates during the stresses of cryopreservation is of greater importance.

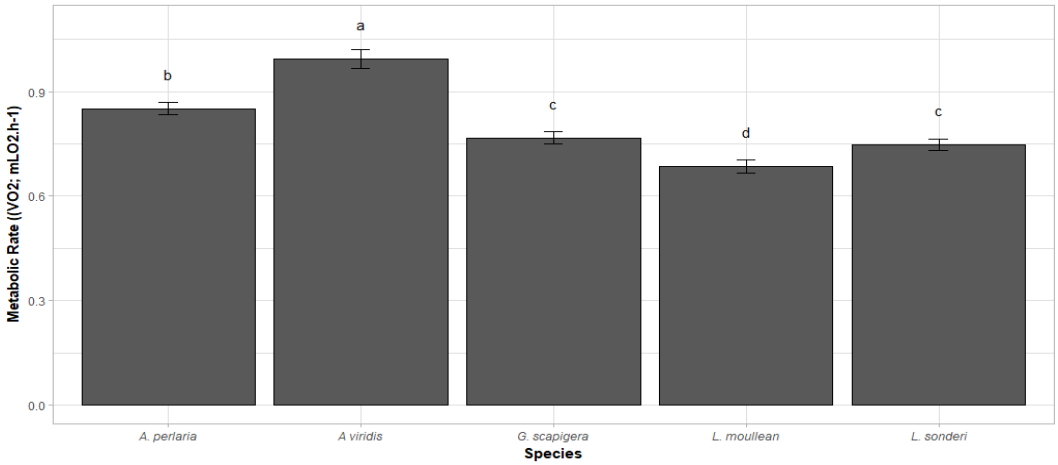

Figure 2. Average metabolic rate of freshly isolated shoot tips of Androcalva perlaria, Anigozanthos viridis, Grevillea scapigera, Lasiopetalum moullean, and Lomandra sonderi.

Effect of cryopreservation on metabolic rate

Anigozanthos viridis and *A. perlaria* were further tested on the Q2, comparing pre- and post-cryopreservation metabolic rates. A significant decrease in average metabolic rate was observed between pre- and post-cryopreservation periods for both *A. viridis* and *A. perlaria* (Fig. 3). Average metabolic rate dropped from 1.42 mL $O_2.h^{-1}.g^{-1}$ to 0.78 mL $O_2.h^{-1}.g^{-1}$ between pre- and post-cryopreservation for *A. viridis* and 1.43 mL $O_2.h^{-1}.g^{-1}$ to 0.2 mL $O_2.h^{-1}.g^{-1}$ for *A. perlaria*. It is well know that cryopreservation is a stressful process and a drop in metabolic rate was expected, however, metabolic rates did not recover back anywhere near the initial levels. Even *A. viridis*, which is a cryo-tolerant species, showed a 50% reduced metabolic rate post-cryopreservation compared to the initial basal metabolic rate. Developing cryopreservation protocols that can limit this damage done to mitochondria will likely improve the health and vigour of the cryopreserved samples once they are rewarmed.

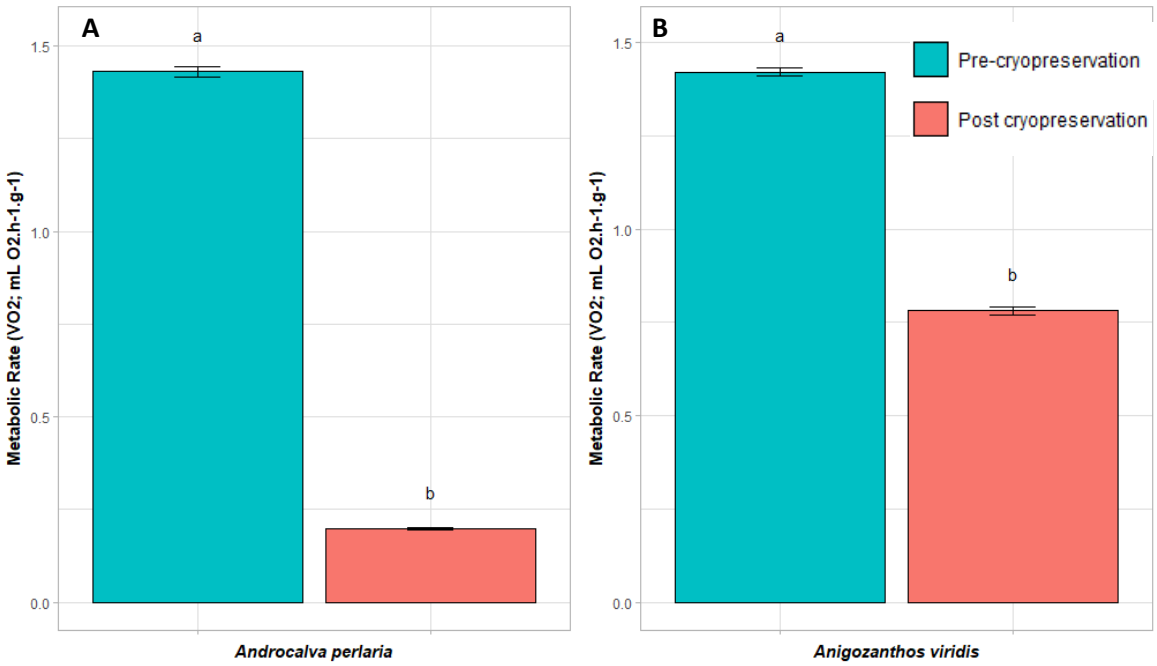

Figure 3. Pre and post cryopreservation metabolic rates for Androcalva perlaria (A) and Anigozanthos viridis (B). Different letters indicate statistically significant differences between treatments for metabolic rate.

Optimising cryopreservation protocols for metabolic function

Desiccation prior to cryopreservation is vital to remove as much freezable water as possible. However, excessive desiccation can be just as detrimental as ice formation to the samples. A range of desiccation media (0.2 M, 0.4 M, 0.75 M and 1.0 M sucrose) were compared against the control recovery medium (RM) where no desiccations stress is applied. This was applied to two species, *Arabidopsis thaliana* and *Grevillea dryandroides,* which show differing cryo-tolerance, with a maxiumum post-cryogenic recovery of 41% on 0.2 M sucrose (Fig. 4) and 8% on 0.4 M sucrose (Fig. 5) respectively. It is of interest to note that the more cryo-tolerant *A. thaliana* showed much higher metabolic rates, and was far less sensitive to the desiccation media and toxic cryoprotective agents (CPAs) (Fig. 4 D & -LN respectively). However, even *A. thaliana* showed significantly lower metabolic rates once cryopreserved in all treatments (Fig. 4 +LN). *Grevillea dryandroides* in comparison, showed far more sensitivity to both desiccation, where increased osmotic concentrations reduced metabolic rate (Fig. 5 D), and was extremely sensitive to the CPAs, showing significant declines in metabolic rates for most treatments (Fig. 5 -LN). Thus, by the time the shoot tips were submerged into LN to cryopreserve them, they were in poor health. Obtaining good post-cryogenic survival for this species will require further optimisations, potentially with less toxic CPAs.

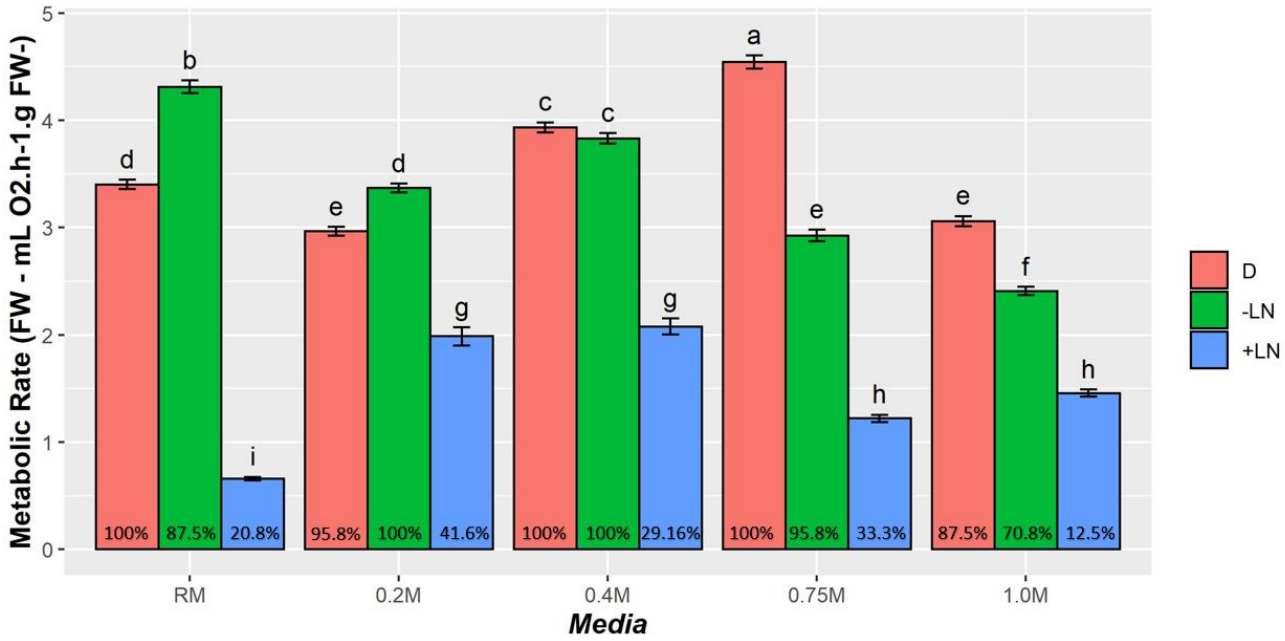

Figure 4. Post-cryopreservation metabolic rates for Arabidopsis thaliana. Cryopreservation treatments are separated as Desiccation only (red), -LN (green) and +LN (blue). Comparison between shoot tip MR during desiccation period on 0.2 M, 0.4 M, 0.75 M, and 1.0 M sucrose DM and RM, and post-cryopreservation MR. Percentage of regenerating shoot tips post-cryopreservation per treatment is indicated in each bar (%).

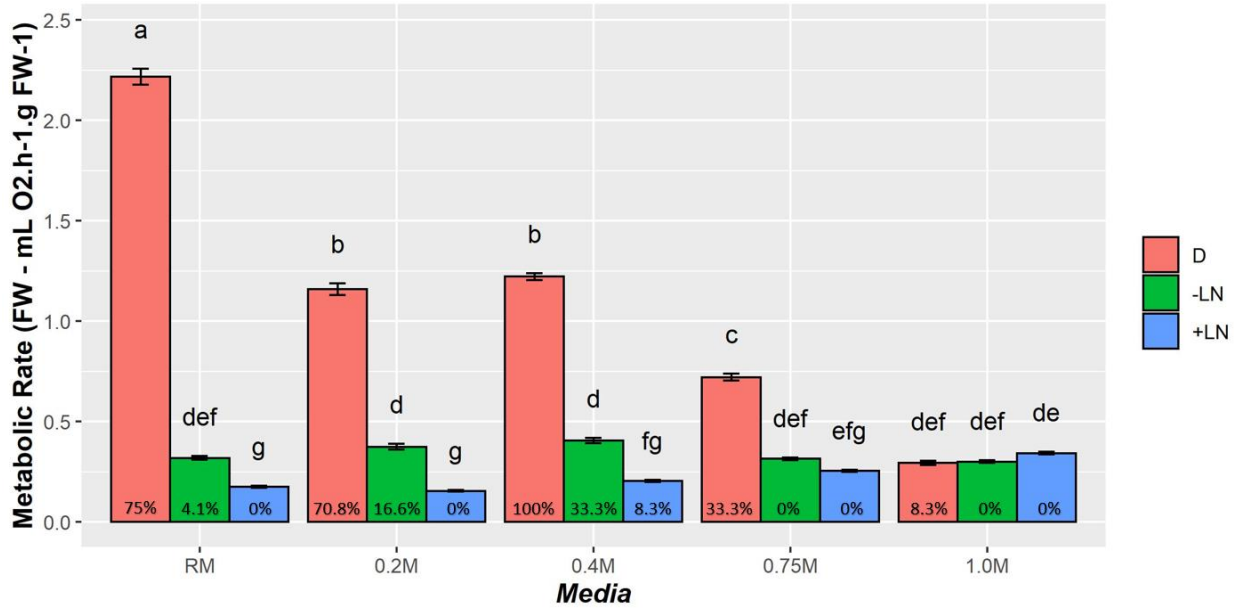

Figure 5. Post-cryopreservation metabolic rates for Grevillea dryandroides. Cryopreservation treatments are separated as Desiccation only (red), -LN (green) and +LN (blue). Comparison between shoot tip MR during desiccation period on 0.2 M, 0.4 M, 0.75 M, and 1.0 M sucrose DM and RM, and post-cryopreservation MR. Percentage of regenerating shoot tips post-cryopreservation per treatment is indicated in each bar (%).

Mitochondrial Fluorescent Probes

A range of tissues were assessed with the MitoTracker Green FM fluorescent probe, as we were unsure how well these probes would penetrate plant cell tissues. It is expected that the probe will passively penetrate into the cells and localise in functional mitochondria. This was observed with some of the cell cultures (Fig. 6B), showing bright green dots where the mitochondria are located in the cells with little background noise; however often only the peripheral mitochondria showed fluorescence (Fig. 6C), or no clear localisation in the mitochondria was seen (Fig. 6D). Penetration of the probe into plant cells seems to be inconsistent and requires optimisation, perhaps with the addition of compounds that increase membrane fluidity like dimethyl sulfoxide. Testing MitoTracker Green on shoot tips required very long incubations for the probe to penetrate (>2 h); however, the results indicate that the actively growing regions around the meristem seem to have increased fluorescence, indication a greater number of mitochondria present (Fig. 6A). It is unknown how well the probes have penetrated the woodier basal tissue, potentially just staining the outer cells, so care must be taken not to overstate the results seen in this trial. Future work using specialised confocal microscopy at the Centre for Microscopy, Characterisation and Analysis (CMCA) at UWA is planned, giving greater resolution and the ability to construct stacked images into 3D data sets, with the aim to better map the location of the mitochondria and compare shoot tips throughout the cryopreservation process. Additionally, protoplasts as an alternative to cell cultures can be tested to determine if the cell wall inhibits penetration of the fluorescent probe.

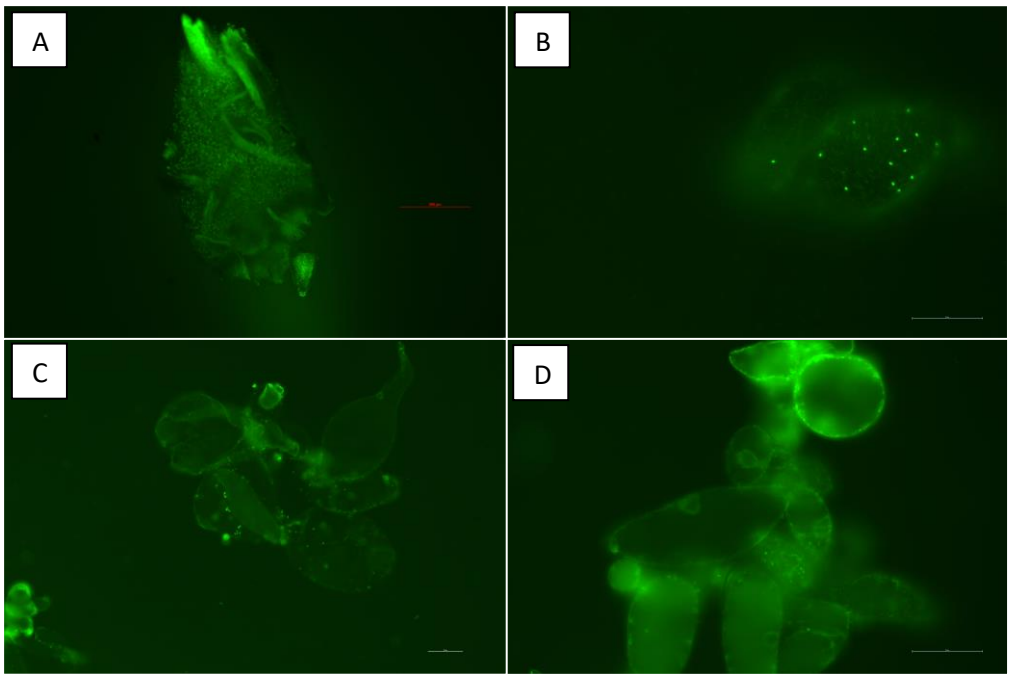

Figure 6. MitoTracker Green FM labelled shoot tip (A) and cell cultures (B-D)

Seahorse XF Flux analysis

Analysing the extracellular flux, both oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), can provide a more detailed assessment of mitochondrial function through the use of mitochondrial inhibitors and promotors. Obtaining reproducible results with the Seahorse XF Flux analyser has been a challenge, as seen in Fig. 7, even the initial basal OCR between samples varies considerably. This could be due to uneven cell loads into the wells, or obtaining good cell adhesion within these wells, as this equipment is optimised for animal cell cultures. The addition of the mitochondrial inhibitors and promotors is vital for obtaining information on ATP-linked respiration (Oligomycin), maximal respiration (FCCP) and the spare capacity available above basal metabolic rate (Antimycin A or Rotenone); however, while Oligomycin shows a good response, the other compounds showed very little change in OCR when added, despite using at higher than recommended amounts (Fig. 7). It is thought that these compounds are not entering either the cells, or the plants are resistant to their effects. Future work will focus on protoplasts, and alternative compounds, such as salicylhydroxamic acid, that will show a greater OCR response, with the aim to combine the findings with the MitoTracker fluorescent probe analysis.

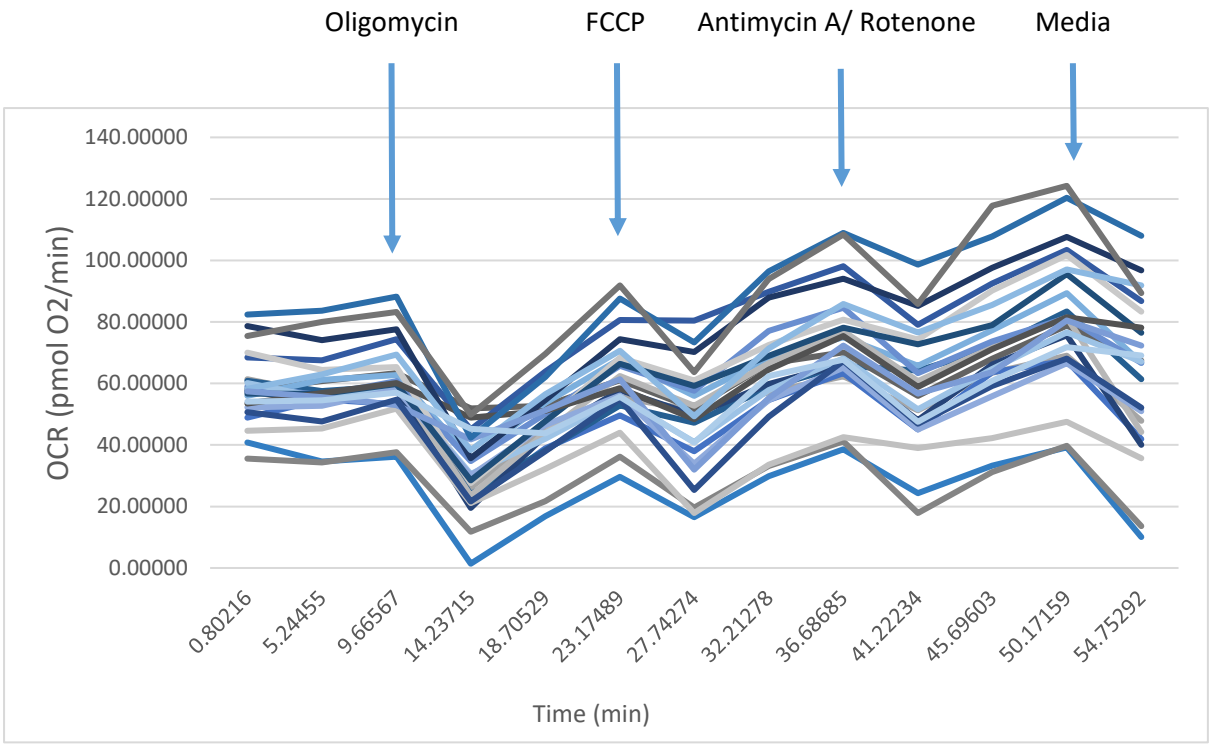

Figure 7. Data from initial trials using the Seahorse XF Flux Analyser. Oxygen consumption rates of carrot cells over time with addition of respiratory inhibitors at different concentrations. Timing of inhibitor addition indicated by arrows above.

Protoplasts

Both the fluorescent probe analysis and extracellular flux analysis have shown potential issues with penetration of the fluorescent probe, and mitochondrial promotors and inhibitors into the cells. Protoplasts are an ideal candidate to test whether the cell wall is inhibiting this penetration, as well as providing an simple model for single cell analysis. The initial protoplast protocol was tested on *A. thaliana*, showing overall good morphology in the isolated protoplasts (Fig. 8A); however, some modifications to the osmoticum was required to reduce swelling leading to the protoplasts bursting (Fig. 8B). The protoplast from *A. thaliana* also survived cryopreservation (Fig. 8C), and work is underway optimising this protocol to work on Australian native species.

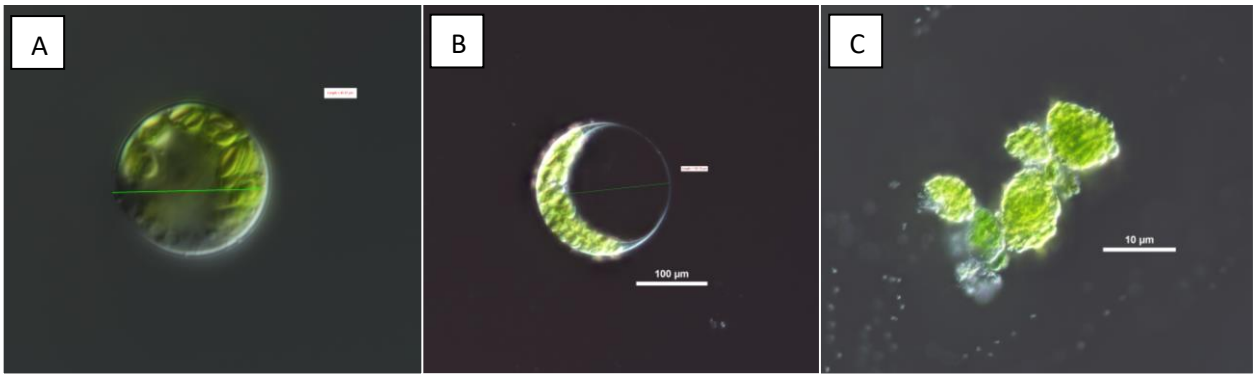

Figure 8. Protoplasts of Arabidopsis thaliana showing normal morphology (A), swelling (B) and 24 h after cryopreservation (C).

Key findings

This study aimed to answer the following question, is mitochondrial function the key to improving cryopreservation of threatened Australian flora? Using a range of new technologies, many of which have never been tested on plant shoot tips before, we identified that the stresses of cryopreservation severely affect mitochondrial function, particularly for cryo-sensitive species. Optimisations to the cryopreservation protocol did show improved mitochondrial function, and further work will aim to develop new less stressful cryopreservation protocols. The Q2 Oxygen Sensing Technology seems easily applicable to assessing mitochondrial function in shoot tips during the desiccation and recovery phases of cryopreservation, both of which are vital stages for achieving optimal post-cryogenic regrowth. The Seahorse XF Flux analyser, while having the potential to provide more detailed insights into mitochondrial health, requires further testing to develop a reliable protocol for plant cells before experimental work on the effect CPAs have on mitochondrial function can be done.

Acknowledgements

I would like to express my gratitude to the Australian Flora Foundation for their contribution to this study. This funding has allowed us to cover the consumable costs for three Honours students, two who have continued into a PhD position, hiring a casual research assistant, and for attending the Buck Institute Bioenergetics Master Classes on flux analysis, in this novel area of research. We thank Kings Park Science for access to *in vitro* cultures and the Q2 instrument, and we thank Curtin University for access to the Seahorse instrument and providing consumables and solutions for the preliminary trials.

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