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The role of reactive oxygen species and anti-oxidants during pre-cooling stages of axis cryopreservation in recalcitrant *Trichilia dregeana* Sond.

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Running title: Cryopreservation and oxidative stress
Abstract

Cryopreservation is currently the only feasible method for long-term conservation of non-orthodox germplasm. Previous attempts to cryopreserve embryonic axes of *Trichilia dregeana*, indicated that an inability for shoot production, suggestedly associated with a wound-induced burst of reactive oxygen species (ROS) accompanied by declining anti-oxidant potential, consistently accompanied procedures necessary for cryopreservation. The present study involved provision of cathodic protection, both in the aqueous phase (electrolysis of a solution containing dilute electrolytes) and dry state (rapid drying of axes on a grid on which a static field is generated via the cathode of a power-pack) with the addition of other anti-oxidants i.e. ascorbic acid and DMSO to counteract uncontrolled ROS activity. Superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH) production in conjunction with the corresponding total aqueous anti-oxidant activity and viability was quantitatively assessed after each stage of the cryo-procedure, including and excluding cathodic protection and/or anti-oxidants. No shoot production occurred in treatments without cathodic protection and/or anti-oxidants. In contrast, 80, 40 and 40% of axes produced seedlings after excision, cryoprotection and flash (rapid) drying, respectively, when the treatments included a form of cathodic protection.

Keywords: anti-oxidants, cathodic protection, cryopreservation, reactive oxygen species, recalcitrant seeds, *Trichilia dregeana*. 
Introduction

Cryopreservation is presently considered to be the only means of long-term storage of the germplasm of desiccation-sensitive recalcitrant-seeded species, as the seeds themselves cannot be stored under the low moisture and low temperature conditions used for orthodox seeds (Engelmann, 2011; FAO, 2013). Successful cryopreservation of the embryonic axes of some recalcitrant-seeded species, e.g., *Cocos nucifera* (Assy-Bah and Engelmann, 1992), *Citrus sinensis* (Santos and Stushnoff, 2003), *Poncirus trifoliata* (Wesley-Smith *et al*., 2004), various amaryllids (Sershen *et al*., 2007) and *Ilex brasiliensis* (Mroginski *et al*., 2008) have been reported. However, more often than not, cryopreservation of embryonic axes of a range of tropical/sub-tropical woody species has not been successful as shoot production was either suppressed (Poulsen, 1992; Beardmore and Whittle, 2005; Ballesteros *et al*., 2014) or totally absent following cryogen exposure (Wesley-Smith *et al*., 1992; Kioko *et al*., 1998; Perán *et al*., 2006; Goveia, 2007; Normah *et al*., 2011). Critical parameters compromising seedling establishment after cryopreservation include the stage of development of the axes: if under-developed, germination competence could be diminished as indicated by Goveia *et al*. (2004), or if already in the early stages of germination, metabolic rate is enhanced with a concomitant increase in desiccation sensitivity, as demonstrated for several amaryllid species (Sershen *et al*., 2007). In the case of axes which dry slowly, failure of seedling establishment may be because their survival is compromised by the prolonged period of dehydration necessary to reduce their WC to a level appropriate for cryogenic cooling (Sershen *et al*., 2012a; Ballesteros *et al*., 2014).

Successful cryopreservation of axes of *Trichilia dregeana* Sond., the subject of the present study, has been elusive, with the lack of shoot production being the
major problem. In an earlier work the suggestion was made that necrosis of the shoot apical meristem could be associated with an excision-related burst of reactive oxygen species (ROS) when the cotyledonary attachments, which are situated close to the shoot apex, are severed upon axis excision (Goveia et al., 2004). This has since been established to be the case (Whitaker et al., 2010; Pammenter et al., 2011). Additionally, the embryonic axes of these seeds have been reported to respond badly to cryoprotection (Berjak et al., 1999), and many previous attempts to cryopreserve them have been unsuccessful (Kioko et al., 1998; Goveia et al., 2004; Whitaker et al., 2010).

Cryopreservation of embryonic axes from recalcitrant seeds requires that they are rapidly dehydrated to relatively low WC s which should minimise or (ideally) preclude intracellular ice crystallisation when they are cooled to cryogenic temperatures (updated reviews by Berjak and Pammenter, 2014; Pammenter and Berjak, 2014). However, after these procedures, establishment of seedlings naturally producing both a root and a shoot by axes excised from recalcitrant seeds of tropical/sub-tropical provenance has seldom been achieved (Engelmann, 2011; Normah et al., 2011). In a break-through with T. dregeana axes, Naidoo et al. (2011) reported that shoot production was inhibited by the very first stage of the cryo-procedure, viz. excision. Those authors found that implementation of a pre-culture step exposing axes to dimethyl sulphoxide (DMSO) prior to complete removal of the cotyledons, and post-excision soaking in DMSO or 1% (w/v) ascorbic acid (AsA) promoted the production of shoots by most of the excised axes. That study, together with several others on T. dregeana axes (Goveia et al., 2004; Song et al., 2004; Whitaker et al., 2010; Pammenter et al., 2011; Varghese et al., 2011) have led to the suggestion that failure to produce shoots after excision (and hence cryopreservation)
may be a consequence of physical injury to shoot meristematic cells situated in close proximity to the point of excision (Ballesteros et al., 2014) combined with unregulated activity of ROS (Goveia et al., 2004).

It is necessary, however, that embryonic axes have to be excised, rapidly (flash) dried and rapidly cooled to cryogenic temperatures. All these steps, as well as axis retrieval from, and thawing and rehydration after, cryogenic storage do or have the potential to – lead to the generation of ROS (Benson and Bremner, 2004; Roach et al., 2008; Whitaker et al., 2010; Berjak et al., 2011). The ROS having attracted the most attention in terms of oxidative damage are $\cdot \text{O}_2^-$, $\cdot \text{OH}$, and $\text{H}_2\text{O}_2$. Although ROS are implicated in normal metabolism under conditions where they must be strictly controlled by endogenous anti-oxidants (Mittler et al., 2002), any or all of the cryo-procedures may be accompanied by the production of a ROS burst with the potential to overwhelm the endogenous anti-oxidant capacity of small explants such as embryonic axes (Berjak et al., 2011; Varghese et al., 2011). Under such conditions, severe oxidative damage and death of all or part of the explant tissues are the likely consequences conjectured to underlie poor cryo-survival (Touchell and Walters, 2000; Goveia et al., 2004; Roach et al., 2008, 2010; Whitaker et al., 2010; Pammenter et al., 2011).

It has also been postulated that axes separated from the rest of the tissues of recalcitrant seeds by excision may not have fully functional endogenous anti-oxidant systems (Varghese et al., 2011; Sershen et al., 2012b) and hence not be capable of quenching stress-related ROS bursts induced by procedures involved in cryopreservation. Such imbalance could lead to a state of oxidative stress which may be an underlying cause of axis tissue necrosis – especially of shoot meristems – during cryo-procedures.
The present study examined the effectiveness of known chemical anti-oxidants (Naidoo et al., 2011) and novel means of cathodic protection (Pammenter et al., 1974; Berjak et al., 2011) to ameliorate the destructive consequences of unregulated ROS generation accompanying the various steps involved in *T. dregeana* axis cryopreservation. The premise of cathodic protection is that the free radicals/ROS generated as a consequence of the various steps of cryopreservation will be quenched, thereby obviating the cascade of events leading to oxidation of cellular macromolecules, particularly lipids, proteins and nucleic acids (Hanaoka, 2001; Hiraoka et al., 2004; Berjak et al., 2011).

Production of ROS and the corresponding anti-oxidant activity were chosen as indicators of oxidative damage (Halliwell and Gutteridge, 2007). These parameters were assessed in conjunction with *in vitro* germination of axes and particularly the capacity for shoot development after each stage of cryopreservation to establish whether poor seedling development could be attributed to disrupted cellular homeostasis (Kranmer et al., 2006; Varghese et al., 2011). The successful use of exogenously applied anti-oxidants in facilitating shoot development by axes of *T. dregeana* after excision (Naidoo et al., 2011) and recovery after cryopreservation of shoot tips (Reed et al., 2012) prompted the further investigation of changes in oxidative metabolism and the corresponding capacity for onwards development of axes in the context of the provision of anti-oxidant treatments. These parameters were evaluated in all cases on material that had been exposed to the conventional treatments (no anti-oxidants) and on material that was exposed to the treatments inclusive of an anti-oxidant or cathodic protection.

**Materials and Methods**

The sequence of procedures and parameters investigated are summarised in Figure 1.
Seed collection, surface decontamination and hydrated seed storage: Seeds were collected, decontaminated and stored as described by Naidoo et al. (2011).

Excision of primary and secondary explants: Primary explants consisted of the embryonic axis with a portion (2 mm x 2 mm x 0.5 mm; 2 mm³) of the basal segment of each cotyledon remaining attached to the axis (for the purpose of preventing injury to the shoot meristem before priming with an anti-oxidant treatment). Primary explants were excised using a sterile 11 pt blade and immediately subjected to anti-oxidant pre-conditioning (pre-culture). Subsequently, the secondary explant was isolated by removing the remaining attached cotyledonary segments using two hypodermic needles (Naidoo et al., 2011).

Anti-oxidant pre-conditioning (pre-culture) of primary explants: All procedural steps including pre-conditioning were conducted in a darkened laminar air-flow cabinet. Primary explants were incubated on a pre-culture medium containing the anti-oxidant, DMSO, for 6 h in the dark (n=20), the medium consisting of full strength (4.4 g L⁻¹) MS (Murashige and Skoog, 1962), 30 g L⁻¹ sucrose and 10 g L⁻¹ agar, pH 5.6 – 5.8. Filter-sterilised DMSO (1 ml L⁻¹, Sigma, ≥ 99.5% [GC grade]) was added to the medium after autoclaving.

Preparation of cathodic water: Cathodic water, which is a strong reductant (Hanaoka, 2001) was prepared by the electrolysis of an autoclaved solution of 1 µM CaCl₂ and 1 mM MgCl₂ (CaMg). The electrodes were platinum foil, the cathode and anode were in separate chambers connected via a salt bridge (saturated KCl in 3% agar) and electrolysis was conducted at 60 V for 1 h (Berjak et al., 2011). The non-electrolysed solution of Ca⁺ and Mg⁺ (CaMg solution; Mycock, 1999) in distilled water, served as the control.
Post-excision soaking: Pre-cultured primary explants were immersed in a 1% DMSO solution (v/v) in distilled water for cotyledon excision, leaving the axes only as the secondary explants. Axes (n=20) were immersed in one of the following post-excision soaking treatments for 30 min: i. distilled water; ii. cathodic water; iii. 1% ascorbic acid in distilled water (w/v); iv. 1% ascorbic acid prepared in cathodic water (w/v); or v. 1% DMSO in distilled water (v/v).

Cryoprotection: Subsequent to post-excision soaking in ascorbic acid prepared in cathodic water (treatment showing the highest viability after post-excision soaking), axes (n=20) were treated with the penetrating cryoprotectants, DMSO and glycerol dissolved in either cathodic or distilled water. The cryoprotectants were applied individually and in combination as 5 and 10% (v/v) solutions. Axes were immersed in the 5% cryoprotectant solutions for 60 min, followed by exposure to the 10% solutions for a further 60 min.

Desiccation: Axes were rapidly desiccated in a flash dryer (Berjak et al., 1990; Pammenter et al., 2002) with or without ‘dry’ cathodic protection, the former connecting the cathode of the power source to the stainless steel wire grid on which the axes were supported during flash drying (Pammenter et al., 1974). A potential difference of 300 V was applied throughout the duration of drying. Three sets of conditions were tested: i. excision of axes, pre-culture, post-excision soak and flash-drying (90 min); ii. excision of axes, pre-culture, post-excision soak, cryoprotection and flash-drying (120 min); iii. excision of axes and flash drying (75 min). In each case, the duration of flash drying was the time necessary to reduce WC to a range of 0.35-0.4 g g⁻¹ (i.e. the range avoiding desiccation damage sensu stricto, but which should facilitate cryogenic cooling with no, or minimal, ice crystallisation).
Rehydration after desiccation: To ascertain the impact of flash drying in each case, axes were rehydrated (n=20) in solutions of CaMg (control) or cathodic water for 30 min in the dark.

Cooling, thawing and rehydration: Naked axes (n=20) after respective treatments were plunged directly into nitrogen slush (-210°C) in a polystyrene cup (250 ml) where they were tumble-mixed with swirling for 5 min, during which the slush reverted to the liquid form (-196°C). Axes were stored in LN overnight. Upon retrieval they were transferred from cryovials directly into Petri dishes and thawed by direct immersion in cathodic water or the CaMg solution at 40°C for 2 min and thereafter transferred to the same solution at 25°C (room temperature) for 30 min (Berjak et al., 1999) for rehydration.

Surface decontamination and germination of axes: Axes were decontaminated by serial immersion in 2% (v/v) Hibitane® for 2 min, 70% ethanol for 2 min and 1% (v/v) sodium hypochlorite (NaOCl) for 5 min. Axes were subsequently rinsed three times with sterile distilled water before being immersed again for 5 min in 1% (w/v) NaOCl, followed by three rinses with a sterile CaMg solution (Kioko, 2003). After each stage of the protocol, axes were germinated on full strength (4.4 g L⁻¹) MS basal medium (Murashige and Skoog, 1962) containing sucrose (30 g L⁻¹) and agar (10 g L⁻¹) and supplemented with the growth hormone, 6-benzylaminopurine (BAP; 0.1 mg L⁻¹) at pH 5.6-5.8 (Perán et al., 2006).

Superoxide assessment: Extracellular production of \( \cdot \text{O}_2^- \) was assessed by the oxidation of epinephrine to adrenochrome, measured spectrophotometrically at 490 nm (Misra and Fridovich, 1972), using a UV-Vis spectrophotometer (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA). A 1 mM epinephrine solution (pH 7.0) in 1 M HCl was prepared, from which 0.5 ml was dispensed into 1.5 ml distilled water.
water to make up the incubation medium. Immediately after treatments, axes were divided into five replicate batches of four axes per treatment and placed in incubation medium on an orbital shaker (Labcon, Instrulab CC, Maraisburg, South Africa) at 70 rpm, at room temperature, for 15 min in dark (after Roach et al., 2008). Absorbance of each replicate was subsequently read. The extinction co-efficient of adrenochrome at 490 nm, 4.47 mM\(^{-1}\) cm\(^{-1}\), was used to calculate \(\cdot O_2^-\) production which was expressed in µmol min\(^{-1}\) g\(^{-1}\) on a dry mass basis. The specificity of this assay was confirmed via the 50% inhibition of \(\cdot O_2^-\) by 250 units ml\(^{-1}\) superoxide dismutase (SOD; data not shown).

**Hydroxyl radical assessment:** After each treatment, five replicates of four axes each were incubated in 1.5 ml of potassium phosphate buffer (prepared using 20 mM K\(_2\)HPO\(_4\) and 20 mM KH\(_2\)PO\(_4\), pH 6.0), containing 20 mM 2-deoxy-d-ribose, on the orbital shaker at 70 rpm, at room temperature, for 45 min (as described by Schopfer et al., 2001). Debris originating from the axes was cleared by centrifugation at 9 000 rpm for 2 min. After centrifugation, a mixture of 0.5 ml of incubation medium (from each replicate), 0.5 ml of 2-thiobarbituric acid (TBA; 10 g L\(^{-1}\) in 50 mM NaOH) and 0.5 ml tricholoroacetic acid [TCA; 28 g L\(^{-1}\) in distilled water]) was used to estimate the formation of the breakdown product malondialdehyde. The mixture was heated in a water bath at 95°C for 10 min, and then cooled on ice for 5 min. Débris was once again cleared by gentle spinning as described above. The reaction product was measured by dispensing 300 µl of solution into each well of black Elisa\(^{\oplus}\) plates, and subsequently reading the fluorescence using a fluorescence spectrophotometer (FLx 800, Bio-Tek Instruments Inc.; excitation: 530 nm and emission: 590 nm).

**Hydrogen peroxide assessment:** Hydrogen peroxide levels were measured using the xylenol orange assay (Gay and Gebicki, 2000; Bailly & Kranner, 2011),
with a few minor modifications. Five replicates of four axes per treatment were incubated in 1.0 ml distilled water on the orbital shaker and rotated at 70 rpm, at room temperature, for 30 min. Thereafter, 300 µl incubation medium was added to 1.5 ml working reagent, prepared by mixing 0.1 ml of reagent A (containing 25 mM FeSO₄, 25 mM (NH₄)₂SO₄, 2.5 M H₂SO₄) and 10 ml of reagent B (125 mM xylenol orange (Sigma-Aldrich) and 100 mM sorbitol). The reaction mixture was incubated for 20 min after which the absorbance of samples were read at 560 nm (Cary 50 Conc UV Vis spectrophotometer, Varian, Palo Alto, CA). The extinction coefficient of Fe³⁺ xylenol orange complex at 560 nm is 267 mM⁻¹ cm⁻¹, which was used in calculations to estimate H₂O₂ content in axes on a dry mass basis. The specificity of this assay was confirmed by the inhibition of > 80% H₂O₂ by 500 units ml⁻¹ catalase (CAT; data not shown).

All procedures subsequent to primary excision of the explant were conducted under dark conditions to limit ROS generation initiated and perpetuated by photoxidation (Touchell and Walters, 2000).

Total aqueous anti-oxidant activity (TAA): Total aqueous anti-oxidants (enzymatic and non-enzymatic) was measured in treated and control axes (n=5) using the 2,2’-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid; ABTS, Sigma-Aldrich) decolorisation assay, which is applicable to lipophilic and hydrophilic anti-oxidants, including flavonoids, hydroxycinnamates and carotenoids (Re et al., 1999). Four replicated batches of five axes were separated for each treatment and extraction was effected immediately in 2 ml chilled extraction buffer (50 mM KH₂PO₄ buffer; pH 7.0) containing 1mM CaCl₂, 1mM KCl and 1mM EDTA. Samples were centrifuged for 15 min at 14 000 rpm, 9 m s⁻² at 4°C. Supernatant was extracted and
further centrifuged for 5 min after which the final supernatant was used for the total antioxidant assay.

The assay was performed twice on 100 µl of extract from each of the four replicates per treatment, in the dark (after Johnston et al., 2006). A standard curve was generated using a 5 mM Trolox® (6-hydroxy-2,5,7,8-tetramethylchromeane-2-carboxylic acid, 97%) solution prepared in the anti-oxidant extraction buffer. Changes in absorbance were estimated spectrophotometrically and expressed on a fresh mass basis as Trolox equivalents using the standard curve.

Oxidative parameters and viability assessments were conducted after each of the treatments (with and without anti-oxidants) subsequent to secondary excision as outlined in Figure 1.

Statistical analysis: Production of ROS (O$_2^-$, OH and H$_2$O$_2$) and TAA were tested for significant inter-treatment differences between cathodic and non-cathodic treatments using an independent samples t-test for all stages of cryopreservation, except for post-excision soaking treatments where differences were tested by analysis of variance (ANOVA). For the soaking treatments, the Tukey HSD post-hoc test was analyzed to determine where differences were significant between treatments. A Pearson Chi-Square test was done to assess significant inter-treatment differences in viability between cathodic and non-cathodic treatments. A Mann-Whitney-U test was performed in instances where the assumptions of normality or equal variance were not met. All statistical analyses were performed at the 0.05 level of significance using SPSS statistical package (Version 19; SPSS Inc. Chicago, Illinois, USA).

Results
Results presented here show selected treatments from each stage of cryopreservation, the non-cathodic treatment in each case represents the control.

*Post-excision soaking:* Figures 2 and 3 show viability and biochemical data respectively for freshly excised (non-treated) axes. Post-excision soaking in a 1% solution of ascorbic acid prepared in cathodic water promoted shoot development by the greatest number of excised axes of *T. dregeana* (*p* < 0.05; Fig. 2). Significant differences occurred in O$_2^-$ and H$_2$O$_2$ production between the anti-oxidant soak and distilled water treatments, and each of these treatments compared with freshly excised axes (*p* < 0.05; Fig. 3). Each of these ROS was lower in axes treated with ascorbic acid used in combination with cathodic water.

*Cryoprotection:* Results pertaining to cryoprotectant treatment showed significant root and shoot production (*p* < 0.05) by 50 and 40% of axes, respectively, compared with neither root nor shoot development when the solvent was distilled water (Fig. 4). Viability results were observed to correspond with a significant difference in H$_2$O$_2$ production such that levels were lower in axes treated with cryoprotectants prepared in cathodic water (Fig. 5).

*Desiccation:* The influence of dry cathodic protection during flash drying was investigated at three stages of the protocol and the most effective cathodic and non-cathodic regime was selected for comparative purposes (Figs. 6 and 7). Dry cathodic protection was observed consistently and significantly to reduce O$_2^-$ and H$_2$O$_2$ levels across the regimens (*p* < 0.05; Fig. 7). However, no root or shoot production occurred unless the full spectrum of pre-culture, post-excision soaking, cryoprotection and dry cathodic protection during dehydration was applied, under which circumstances only 10% of the axes each produced a root (Fig. 6).
Rehydration: Further positive results were attained after rehydration of axes. Following the drying regime that resulted in 10% root production and subsequently rehydrating axes in cathodic water for 30 min facilitated significantly enhanced onwards development of the excised axes (p < 0.05; Fig. 8), exemplified by root and shoot production by 50 and 40% of the axes, respectively. There was significant reduction (p < 0.05) of both \( \cdot O_2^- \) and \( H_2O_2 \) in axes rehydrated in cathodic water (Fig. 9).

Cooling: Processing of axes with cathodic water after rapid cooling viz. rewarming (thawing) and rehydration, resulted in significantly lowered levels (p < 0.05) of the three ROS measured compared with the situation when CaMg was used (Fig. 10). Nevertheless, despite the seemingly efficient reduction of oxidative indicators, neither roots nor shoots were produced by axes after exposure to cryogenic temperatures (-210 followed by -196°C; data not shown). In this regard, \( \cdot O_2^- \) and \( \cdot OH \) production was considerably higher after cooling (Fig. 10) compared with levels in axes that had been only rapidly dried and rehydrated (Fig. 7).

No significant differences occurred in anti-oxidant activity at any stage of cryopreservation or in \( \cdot OH \) production except after retrieval from cooling.

Discussion

An oxidative burst has been defined as the rapid production of ROS as a defence mechanism in response to an external stimulus (Ross et al., 2006). It has been well documented that excision of the cotyledons from the embryonic axis of many recalcitrant seeds (Roach et al., 2008; Berjak et al., 2011; Pammenter et al., 2011) including \( T. dregeana \) seeds (Goveia et al., 2004; Whitaker et al., 2010; Varghese et al., 2011) elicits an oxidative burst in response to wounding thus
precluding shoot development (Goveia et al., 2004) by possibly causing injury to the shoot meristatic cells (Ballesteros et al., 2014). The high levels of ROS observed in freshly excised axes were significantly reduced by post-excision soaking in the ascorbic acid and cathodic water solution (Fig. 3) and the amelioration of detrimental ROS production seems to have facilitated the highest percentage of shoot development by excised axes (Fig. 2). Levels of ROS were reduced even by the distilled water post-excision soak (Fig. 3) but this result was obtained in conjunction with the use of a pre-culture treatment containing DMSO prior to excision of cotyledons (see Fig. 1). Dimethyl sulphoxide has been documented to have powerful radical scavenging properties and is particularly potent as a OH scavenger (Rosenblum and El-Sabban, 1982; Yu and Quinn, 1994), and the use of DMSO in the concentrations applied in this study is unlikely toxic, compared to the concentrations used in Plant Vitrification Solutions (PVS) where toxicity to plant cells is possible. The observed results for germination (Fig. 2) and oxidative indicators (Fig. 3) after treatment of axes using the anti-oxidant treatment supports the action of cathodic water as an enhancer of proton donors, suggesting that O\textsuperscript{2-} and H\textsubscript{2}O\textsubscript{2} are efficiently scavenged by this treatment as a result of the increased dissociation activity of AsA when dissolved in cathodic water, in accordance with Hanaoka (2001).

Responses of cells to cope with oxidative stress may not always involve increasing levels of endogenous anti-oxidants (Halliwell, 2006), external provision of protection can act in inhibiting ROS-producing systems and enhancing other mechanisms such as chaperones for transport of anti-oxidants (Halliwell, 2006). The details of the mechanism by which AsA (in cathodic water) best promoted shoot development (Fig. 2) and reduced O\textsuperscript{2-} and H\textsubscript{2}O\textsubscript{2} levels after excision (Fig. 3), remain
to be resolved. Nevertheless, this combination for a post-excision soaking solution was selected as the treatment of choice in view of the results obtained in this study and in consideration of the synergistic function of AsA with an array of other anti-oxidants in ROS-scavenging, its role in maintaining membrane and lipoprotein integrity and in promoting cell growth and development (Halliwell, 1994; Potters et al., 2002; Shao et al., 2008).

While the application of anti-oxidants pre- and post-excision was adequate to reduce ROS and facilitate significant establishment of seedlings (i.e. showing both root and shoot development) by excised axes (Fig. 2), successive steps of cryopreservation viz., cryoprotection, partial dehydration, cooling and rewarming are likely to induce stresses far more difficult to counteract. Excised axes of *T. dregeana* had not previously been reported to produce shoots or to show substantial root development after cryoprotection (Kioko, 2003; Goveia, 2007) where those authors described callus production or swelling to be the most common indications of survival. In the present study it was believed that preparation of the cryoprotectants, DMSO and glycerol, as solutions in cathodic water may have had a positive effect on survival with 55% root and 40% axes exhibiting shoot production (Fig. 4).

Considering both cryoprotective treatments exposed axes to the same concentration of the cryoprotectants for the same duration, the inability of axes to survive conventional cryoprotection (when the solvent was distilled water) was not a consequence of cryoprotectant toxicity. Dimethyl sulfoxide and glycerol are penetrating cryoprotectants that act by increasing membrane permeability to aid in water removal from cells and facilitate protective dehydration during freezing (Fuller, 2004). Increased membrane permeability could facilitate an influx of ROS, specifically $\text{H}_2\text{O}_2$ that travels freely across membranes (Halliwell, 2006), making the
uncontrolled generation of this species all the more harmful during cryoprotection. The unregulated generation of $H_2O_2$ during conventional cryoprotection, as shown in this study (Fig. 5), possibly had markedly cytotoxic effects, and the scavenging of this ROS alone by cathodic water (Hanaoka, 2001) is suggested to have facilitated survival during cryoprotection (Fig. 4).

In terms of cryopreservation, these results are significant as it is beneficial to have $T. dregeana$ axes survive the step of cryoprotection where both DMSO and cathodic water are present: i.e. both may provide the means to regulate oxidative metabolism. Cryoprotection is a stress inducing stage during cryopreservation of a variety of organs (Best, 2015) and the preparation of cryoprotectants in a highly reducing solution can be applied to mediate ROS production and improve viability.

During desiccation, water from the cytoplasm is removed and WC is lowered. These circumstances in recalcitrant axes induces ROS generation and hinders anti-oxidant defences (Varghese et al., 2011), where even the slightest removal of water causes a large decrease in the ascorbate pool (Smirnoff, 1993). This initiates metabolic derangement (Pukacka and Ratajczak, 2006) and the situation is exacerbated in the shoot pole by the extended duration needed to dehydrate the whole axis to an appropriate, minimally-injurious WC range (Ballesteros et al., 2014). Additionally, effects of possible mechanical damage accompanying drying could induce secondary oxidative events such as lipid peroxidation (not investigated in this study), while concomitantly reducing anti-oxidant capacity (Bailly, 2004; Varghese et al., 2011), thus severely affecting first the shoot meristematic region and subsequently, the root pole.

The results observed after desiccation of $T. dregeana$ (Fig. 6) suggest that the long periods of drying axes to reduce WC to that amenable for cooling imposes so
severe a stress to the shoot meristem that none of the potentially ameliorative measures involving cathodic protection was adequate to counteract oxidative events. It has been reported that survival after desiccation and subsequent cooling, is significantly higher in species where axes rapidly lose water (Ballesteros et al., 2014) and are therefore exposed to desiccation for a much shorter period, as exemplified by Strychnos gerardii (Berjak et al., 2011) and certain monocot Amaryllid species (Ballesteros et al., 2014). Extensive periods of drying hold axes at intermediate WCs for a longer duration, during which time ROS production is exacerbated (Walters et al., 2001; Varghese et al., 2011). This potentially inhibited root production (10% only) by the majority of axes and precluded shoot development by all (Fig. 6). In species such as T. dregeana, where axes are inherently resistant to water loss, long periods of desiccation cannot be avoided. A secondary complication is the differential drying rates of the root and shoot meristems (Ballesteros et al., 2014). Kioko (2003) also showed that the axis shoot tip dries considerably more rapidly than does the root pole: hence the shoot apical meristem may be far more adversely affected as a result of the duration of the applied stress (shoot meristem may be dried to a WC far lower than that which would permit survival). These results highlight that cathodic protection, while successful in regulating oxidative stress during stages prior to desiccation of axes of T. dregeana, was not adequate to mitigate ROS-induced (or other) damage, presumably particularly of the shoot meristematic region.

Superoxide is known to inactivate a spectrum of enzymes that are fundamental in energy and amino acid metabolism (Halliwell, 2006), hence elevated levels of this ROS could result in inhibition of metabolic pathways crucial to growth and repair, and ultimately could result in death. It is possible that the levels of the major scavengers of O$_2^-$ and H$_2$O$_2$ i.e. superoxide dismutase (SOD) and catalase (CAT), respectively
(Halliwell, 2006), might decline or become dysfunctional under water stressed conditions in recalcitrant tissues (Chaitanya and Naithani, 1994; Varghese et al., 2011). The results of cryo procedures thus far, indicate that external provision of anti-oxidants, even when dry cathodic protection is provided, is inadequate to overcome the oxidatively-stressed state as shown by the levels of $O_2^-$ and $H_2O_2$ immediately after desiccation (Fig. 7), which bring about localised necrosis in, or even death of, axes. Regardless of the minimal capacity for ongoing development (10% of axes producing roots; Fig. 6) obtained using the selected drying treatment which incorporated wet (post-excision soaking and cryoprotection) and dry cathodic protection, this was the procedure selected for assessment of rehydration and cooling on each parameter.

Rehydration of axes in cathodic water facilitated 50 and 40% root and shoot production respectively (Fig. 8): such success after desiccation and rehydration has never before been achieved with *T. dregeana* (Kioko, 2003; Goveia, 2007). The rehydration step could impose a stress disrupting the oxidative balance (Smirnoff, 1993) in desiccated recalcitrant axes such that survival is uncertain. Nevertheless, the use of cathodic water at this stage seemed to curb excessive production of ROS adequately, and is suggested to alleviate oxidative stress (Fig. 9) to facilitate survival (Fig. 8). However, it should be noted that it may not be the use of cathodic water alone at the rehydration stage that acts as a key promoter of survival. The axes had, up to this point in the procedure, been exposed to protection in the form of AsA, DMSO and dry cathodic protection. It is the cumulative effect of protective mechanisms at each procedural stage that fine-tuned oxidative metabolism such that survival of axes after rapid dehydration was attained.
Inspite of 50% and 40% of axes producing roots and shoots respectively after the cryo-procedure preceding cryogen immersion (Fig. 8), none of the axes exhibited root or shoot formation after retrieval from LN and thawing with CaMg or cathodic water. Two possible causes could underlie the lack of capacity for onward development by axes after cooling. Firstly, oxidative stress here caused axes to respond in a “plastic” manner, where irreversible damage as a result of failed repair mechanisms resulted in cell death (Kranner et al., 2010). This response was initiated by the level of ROS production (Fig. 10) completely overwhelming the anti-oxidant capacity within the cells (Benson and Bremner, 2004) even when reducing power was provided by the cathodic water. This is in contrast to pre-cooling stages where axes displayed an “elastic response” (Kranner et al., 2010) i.e. changes were reversible and could be repaired such that function and viability were somewhat maintained. Secondly, physical damage could have been incurred as a result of intracellular ice crystallisation (Benson, 1990; Pegg, 2001), particularly in terms of lethal numbers, dimensions and locations of the ice crystals (Wesley-Smith et al., 2013). It is probable that cell/tissue death resulted from a combination of both, but detailed quantitation of individual ROS and anti-oxidants will be necessary, as will be electron microscopical examination of freeze-substituted and freeze-fractured specimens. The latter investigation will determine the size range, frequency and distribution of intracellular ice crystals commensurate with survival or death, as demonstrated by Wesley-Smith et al. (2013).
Concluding comments

It is acknowledged that the diminished capacity for embryonic axes to survive various stages of the cryo-procedure could be attributed to causes other than the oxidative state; however, results presented here show significant differences in ROS production and shoot development between treatments including exogenously-provided anti-oxidants or not. Furthermore, there were significant differences in oxidative parameters and survival of embryos between stages of cryopreservation.

The higher production of ROS post-cooling (Sershen et al., 2012b) compared with any other procedural stage is suggested to be a function of cumulative stress. While provision of anti-oxidant protection was adequate to mitigate oxidative damage after each individual stage prior to cryogen exposure, the accumulated stress after cooling was too great to overcome.

The freezing process itself is highly injurious to recalcitrant germplasm and, in the WC range, 0.40 – 0.35 g g⁻¹, it is probable that residual intracellular solution (freezable) water was present. This possibility is to be resolved using sub-ambient differential scanning calorimetry.

The present study has advanced progress in understanding oxidative stress associated with the entire cryo-procedure, and the positive influence of cathodic water on viability has significantly moved forward the goal of cryopreservation of axes of tropical/sub-tropical recalcitrant-seeded species as exemplified here by T. dregeana.

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References


International Symposium for *In Vitro* Conservation and Cryopreservation. Tepatitlan de Morolos, Jalisco, Mexico.


Sershen, Berjak, P., Pammenter, N.W., and Wesley-Smith, J. 2012a. Rate of dehydration, state of subcellular organisation and nature of cryoprotection are critical factors contributing to the variable success of cryopreservation: studies on recalcitrant zygotic embryos of *Haemanthus montanus*. Protoplasma **249**: 171-186.


Captions to Figures

Fig. 1: Flow diagram illustrating steps of the cryopreservation protocol after each of which selected redox parameters were assessed. CaMg, solution containing 0.5mM CaCl$_2$.2H$_2$O and 0.5µM MgCl$_2$.6H$_2$O; cathodic water, cathodic fraction of electrolysed solution of CaMg; DMSO, dimethyl sulphoxide; nitrogen slush (-210°C), produced by applying a vacuum to liquid nitrogen. See Materials and Methods for detailed description of cryoprotection.

Fig. 2: Axes of *T. dregeana* producing roots and shoots or roots only after exposure to pre-culture and AsA + cathodic water post-excision soaking (n=20). AsA, ascorbic acid.

Fig. 3: Reactive oxygen species production and total aqueous anti-oxidant (TAA) levels in excised axes of *T. dregeana* after pre-culture and post-excision soaking treatments (n=4 for ROS estimations and n=10 for TAA activity). AsA, ascorbic acid. Error bars represent mean ± standard deviation. Letters above bars represent significant differences between treatments.

Fig. 4: Axes of *T. dregeana* producing roots and shoots after exposure to pre-culture, post-excision soaking (AsA + cathodic water) and cryoprotection (n=20). AsA, ascorbic acid; PC, pre-culture.

Fig. 5: Reactive oxygen species production and TAA levels by excised axes of *T. dregeana* after exposure to pre-culture, AsA + cathodic water post-excision soaking and cryoprotection (n=4 for ROS estimations and n=10 for TAA activity). Error bars represent mean ± standard deviation. Letters above bars symbolise significant differences. AsA, ascorbic acid; PC, pre-culture.
Fig. 6: Axes of *T. dregeana* producing roots after exposure to dry cathodic and non-cathodic desiccation treatments (n=20). Axes in both treatment regimes rehydrated in CaMg. AsA, ascorbic acid; PC, pre-culture; FD, flash dried.

Fig. 7: Reactive oxygen species production and TAA levels by excised axes of *T. dregeana* after exposure to dry cathodic and non-cathodic desiccation treatments (n=4 for ROS estimations and n=10 for TAA activity). AsA, ascorbic acid; FD, flash dried; PC, pre-culture. Error bars represent mean ± standard deviation. Letters above bars symbolise significant differences.

Fig. 8: Axes of *T. dregeana* producing roots only or shoots and roots after exposure to a selected drying treatment and wet cathodic or non-cathodic rehydration (n=20). AsA, ascorbic acid; PC, pre-culture; FD, flash dried.

Fig. 9: Reactive oxygen species production and TAA levels by excised axes of *T. dregeana* after exposure to a selected drying treatment and wet cathodic or non-cathodic rehydration (n=4 for ROS estimations and n=10 for TAA activity). PC, pre-culture; FD, flash dried. Error bars represent mean ± standard deviation. Letters above bars symbolise significant differences.

Fig. 10: Reactive oxygen species production and TAA levels by excised axes of *T. dregeana* after exposure to cathodic water or CaMg thawing and rehydration after the selected drying and cooling treatment (n=4 for ROS estimations and n=10 for TAA activity). PC, pre-culture; FD, flash dried; LN, liquid nitrogen. Error bars represent mean ± standard deviation. Letters above bars symbolise significant differences.
Primary excision of explant from seed (axis with a cotyledonary block)

- DMSO pre-culture for 6 h

Final excision of explant

(Removal of axis from cotyledonary block under a 1% [v/v] solution of DMSO)

Post-excision soak for 30 min in selected anti-oxidant solutions

(One of DMSO, AsA, cathodic water or distilled water {control})

Cryoprotection of axes: 10% DMSO + 10% glycerol (made up in cathodic water or cryoprotectant prepared in distilled water {control})

Flash drying of axes to appropriate WCs (cathodic flash drying; conventional flash drying {control})

Cooling (nitrogen slush)

Thawing & Rehydration (cathodic water; CaMg {control})

Decontamination

Regeneration on appropriate medium
Fig. 2: Axes of *T. dregeana* producing roots and shoots or roots only after exposure to pre-culture and AsA + cathodic water post-excision soaking (n=20). AsA, ascorbic acid.

254x190mm (300 x 300 DPI)
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