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Production of reactive oxygen species in excised, desiccated and cryopreserved explants of *Trichilia dregeana* Sond

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Abstract

The tropical tree *Trichilia dregeana* Sond. has recalcitrant seeds that cannot be stored by conventional seed banking methods that include drying and storage at low temperatures, or by using cryopreservation protocols that have been successfully applied to recalcitrant-seeded species such as *Castanea sativa*. We recently showed that in *C. sativa* both excision of the embryonic axes and subsequent dehydration cause transitory (5 min) bursts of extracellular superoxide ($O_2^{\cdot-}$) production. Here we show that both excision and partial dehydration of the embryonic axes from seeds of *T. dregeana* cause large, prolonged extracellular bursts of $O_2^{\cdot-}$. Furthermore, during rehydration after cryopreservation, another burst of $O_2^{\cdot-}$ occurs with slightly different kinetics. Compared with *C. sativa*, rates of $O_2^{\cdot-}$ production in *T. dregeana* are approximately twice as great and decline much more slowly, suggesting that excessive radical formation may be responsible for poor survival of the axes following cryopreservation. Fractionating the cell wall proteins of embryonic axes and cotyledons in conjunction with electrophoretic analyses of the fractions showed that most $O_2^{\cdot-}$ was produced by two peroxidases with molecular masses of c. 50 and 80 kD that were loosely bound to the cell walls of the embryonic axes. Future successful cryopreservation of *T. dregeana* would appear to depend on manipulations of $O_2^{\cdot-}$ production, and the discovery of peroxidases as the enzymes responsible described here may help in the development of more effective protocols.

Keywords: Cryopreservation; Desiccation; Excision; Peroxidase; Reactive oxygen species; Recalcitrant seeds; *Trichilia dregeana*

1. Introduction

Orthodox seeds (Roberts, 1973; Dickie and Pritchard, 2002) progress through a series of developmental stages that end with maturation drying and quiescence while the seed remains attached to the parent plant (Black, 2000). These seeds dehydrate to a water content that is in equilibrium with the relative humidity of the atmosphere (Sutherland et al., 2002) and, depending on storage conditions, are capable of surviving in this state of dehydration for many years, renewing metabolic activity and growth when rehydrated and dormancy mechanisms are broken (Black, 2000). Good quality, dry orthodox seeds may be maintained relatively easily in cold storage or sub-zero temperatures for periods that can be predicted based on initial seed properties and storage parameters (Roberts, 1973; Ellis and Roberts, 1980). By contrast, recalcitrant seeds do not undergo cellular de-differentiation and metabolic shutdown during development (Berjak and Pammenter, 2004) and are shed from the parent plant in a state of "germinative metabolism" (Berjak et al., 1984, 1989). If such metabolically active seeds cannot retain water, metabolic damage (Berjak et al., 1984; Farrant et al., 1986, 1989; Pammenter et al., 1994), and the uncontrolled production of reactive oxygen species (ROS) can result (Pammenter and Berjak, 1999; Varghese and Naithani, 2002; Pukacka and Ratajczak, 2006; Cheng and Song, 2008). ROS can damage a variety of macromolecules, including those of the membrane (i.e. proteins and lipids) as well as nucleic acids (Hendry, 1993; Bailly, 2004; Bailly et al., 2008; Kranner and

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Birtic, 2005). This damage can become greater as a result of dehydration-induced impairment of the natural antioxidant systems of seeds (Hendry et al., 1992; Côme and Corbineau, 1996; Berjak and Pammenter, 2004; Kranner and Birtic, 2005; Cheng and Song, 2008; Varghese and Naithani, 2008). Therefore, the storage of recalcitrant seeds presents many challenges to seed banking. Cryopreservation protocols exist that enable successful long-term storage for some recalcitrant-seeded species, but there are still major problems regarding efficiency and applicability to a wider range of taxa (Engelmann, 2004).

Cryopreservation of recalcitrant seeds can be effective if partially dried embryonic axes are used (Berjak et al., 1989). While axes can be rapidly dried, whole large seeds dry so slowly that deterioration and pathogen infection inevitably occur before suitable water contents are reached. For example, the cryopreservation of the axes of Neem (*Azadarachta indica*) seeds is facilitated by partial dehydration from 1.69 g/g to 0.15 g/g, although further dehydration reduces survival after freezing (Varghese and Naithani, 2008). Similar success had been reported for excised embryos of *Aesculus hippocastanum*, *Castanea sativa* and *Quercus* spp., although recovery after cryopreservation tends to be rather variable (Pence, 1992). Recently we showed that excision and desiccation of embryonic axes can stimulate potentially harmful extracellular ROS production (Roach et al., 2008), which may limit the application of this approach. However, it is important to note that ROS also play important roles in cell signalling in seeds (Bailly et al., 2008; Oracz et al., 2009).

The main aim of the present study was to investigate wound- and desiccation-induced $O_2^{\cdot-}$ production in excised axes of the tropical tree species *Trichilia dregeana*. *T. dregeana* is a recalcitrant-seeded tree occurring in the evergreen forests of southern Africa, and can reach heights of 30 m (Choinski, 1990). The oily seeds are surrounded by a waxy scarlet aril, and are produced from January to May (Coates Palgrave, 1977). Tissue culture of axes excised from newly shed seeds of this species results in the production of roots but not shoots, but shoot proliferation can occur if portions of cotyledon are left attached to the axes (Goveia et al., 2004). However, all attempts to develop reliable protocols to cryopreserve *T. dregeana* have been unsuccessful. We investigated the patterns and mechanisms of $O_2^{\cdot-}$ production during the excision of embryonic axes and their subsequent desiccation and cryopreservation. Our ultimate aim was to gain more insights into the consequences of the stresses that accompany cryopreservation, namely wounding and desiccation, to enable the future development of protocols for successful long-term storage of this species.

2. Materials and methods

2.1. Seed material

Seeds were collected from *T. dregeana* Sond. trees on the Life Sciences Campus of the University of KwaZulu-Natal, Pietermaritzburg, South Africa.

2.2. Flash drying, moisture content (MC) determination and tissue culture conditions

As recommended by Goveia et al. (2004), axes were always excised with a few mm of cotyledonary material attached. After excision, axes were flash dried (Berjak et al., 1990; Wesley-Smith et al., 2001) for up to 5 h and then MCs determined by oven drying at 103 °C for 17 h. Axes were cultured on full-strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 3% sucrose, and viability assessed after 8 weeks.

2.3. Cryopreservation

Embryonic axes were placed in plastic cryovials and plunged into liquid nitrogen. Vials were removed after 24 h and the axes thawed at room temperature.

2.4. Extracellular superoxide assay

Unless indicated otherwise, all reagents were obtained from Sigma (St Louis, MO, USA). Extracellular $O_2^{\cdot-}$ production in *T. dregeana* axes and segments of cotyledonary tissue was measured by the oxidation of epinephrine to adrenochrome, which was detected spectrophotometrically (A_{490}; $\varepsilon_{adrenochrome} = 4.02\ mM^{-1}\ cm^{-1}$) (Misra and Fridovich, 1972; Takeshige and Minakami, 1979). Specificity of the assay was confirmed by a c. 50% inhibition of $O_2^{\cdot-}$ production by $250\ \mu l\ ml^{-1}$ superoxide dismutase (SOD) (Table 1). The effects of SOD and enzyme inhibitors (KCN and NaN_3 for peroxidases and diphenylene iodonium (DPI) for NAD(P)H oxidases) on extracellular $O_2^{\cdot-}$ production were tested by adding the effectors at the same time as the epinephrine, and measuring adrenochrome formation after 5 min.

2.5. Fractionation of enzymes from selected tissues

The axes and cotyledons excised from *T. dregeana* seeds were immediately frozen in liquid nitrogen, freeze dried, and then fractionated after Rast et al. (2003). Freeze dried tissues were ground in ice cold 0.25 M Tris–HCl buffer pH 8 and 2% solid polyvinylpyrrolidone (PVP), then centrifuged at 4 °C for 15 min at 4000 ×*g*. The supernatant represented the cytosolic

Table 1
Effects of SOD and the enzyme inhibitors KCN, NaN_3 and DPI on the extracellular $O_2^{\cdot-}$ production of freshly excised *T. dregeana* axes and segments of cotyledonary tissue.

Effectors	$O_2^{\cdot-}$ production (nmol $s^{-1}\ g^{-1}$ DW)
Control	7.0±0.5 (100%)
SOD ($250\ \mu l\ ml^{-1}$)	3.6±0.4 (52%)
KCN (1 mM)	3.7±0.3 (53%)
NaN_3 (1 mM)	6.9±0.5 (98%)
DPI (10 μM)	7.1±0.2 (102%)

SOD and the inhibitors were added concurrently with the epinephrine and $O_2^{\cdot-}$ production was detected as formation of adrenochrome after 5 min of incubation. Values are given±standard error, n=5–7.

enzyme fraction ("C"). The pellet was suspended in 20 ml 50 mM phosphate buffer pH 7, and re-centrifuged as above. This procedure was repeated three times; the sum of the supernatants contained enzymes loosely bound to the cell wall (e.g. by hydrogen bonds, "B1"). The pellet was re-suspended in 10 ml phosphate buffer and solid digitonin added to give a final concentration of 0.3%. The solution was stirred for 3 h at 10 °C, centrifuged as above, and the step repeated. The combination of the two supernatants contained enzymes bound to the cell wall by hydrophobic interactions ("B2"). The pellet was re-suspended in 15 ml phosphate buffer and NaCl added to give a final concentration of 2 M. The solution was stirred for 3 h at 10 °C, centrifuged as above, and the step repeated. The combination of the two supernatants contained enzymes bound to the cell wall by ionic bonds ("B3"). The final pellet was re-suspended in 10 ml phosphate buffer, and contained enzymes bound by covalent linkages ("B4").

2.6. Peroxidase activity

Peroxidase activity in various cellular fractions of the axes and cotyledons of *T. dregeana* seeds was investigated by the oxidation of 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Fluka, Buchs, Switzerland) to the stable cation radical ($ABTS^{+}$) in the presence of H_2O_2 (Childs and Bardsley, 1975). The rate of the reaction was determined spectrophotometrically ($\lambda = 420$ nm, $\varepsilon_{ABTS^{+\bullet}} = 36\ mM^{-1}\ cm^{-1}$). Each assay (1 ml) contained 10–200 µl extract, 1 mM ABTS and 50 mM phosphate buffer pH 6. The reaction was started by the addition of H_2O_2 to give a final concentration of 10 mM in the assay solution. The reaction mixture was incubated for 10–15 min at 25 °C. For the "B4" fraction, reagents were added to the suspended pellets and, the incubation tubes shaken. After a suitable time the reaction was stopped by centrifuging the tube, and the absorption of the supernatant measured.

2.7. Polyacrylamide gel electrophoresis (PAGE)

Fractions were concentrated by dialysis against a 20% solution of polyethylene glycol 20000 (Fluka), and then loaded onto 12% (w/v) polyacrylamide gels (Laemmli, 1970). Running buffer and gels contained 0.1% sodium dodecyl sulfate (SDS), but samples were not heated and mercaptoethanol and SDS were omitted from the loading buffer. After electrophoresis, peroxidase bands were visualized by incubating gels in 20 mM guaiacol (Fluka) and 20 mM H_2O_2 in a 10% glycerol, 0.25 M sodium acetate buffer pH 5. After a few minutes incubation, orange bands appeared at the position of the peroxidases. Experience from our studies on other plants (including estimating molecular masses by size exclusion chromatography) indicated that the molecular masses of cell wall peroxidases derived from polyacrylamide gels are accurate (Minibayeva et al., 2009). Superoxide production was visualized by pre-equilibrating the gels in 50 mM phosphate buffer pH 7.4 containing 10% glycerol, 0.1 mM $MgCl_2$ and 1 mM $CaCl_2$ for 30 min followed by incubation in the same buffer containing 0.5 mM nitroblue tetrazolium (NBT) and 0.4 mM NADH at room temperature in the dark for 1 h. The appearance of blue formazan bands was observed at the position of $O_2^{\cdot -}$ production (Serrano et al., 1994; López-Heurtas et al., 1999). Gels were run with molecular mass markers (Broad range, Bio-Rad, Hercules, CA, USA) which were stained with Coomassie Brilliant Blue G250 (Sigma).

3. Results

3.1. Moisture content and germination

During flash drying of the excised axes, the MC declined from c. 65–70% at excision to 21% after 5 h (Fig. 1a). Reducing the MC to 21% reduced viability, assessed by growth in tissue culture, from

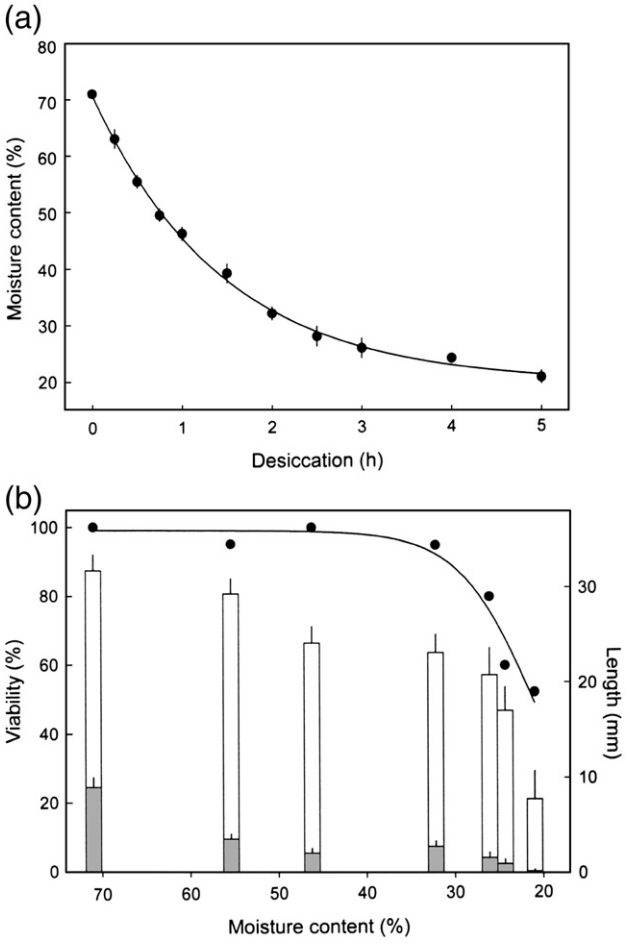

Fig. 1. Effect of desiccation on *T. dregeana* axes with attached cotyledonary segments. (a) Moisture content (MC) of isolated embryonic axes. Axes with attached cotyledonary sections (c. 1 mm) were excised from intact seeds and desiccated immediately by flash drying. To determine the MC of the axes alone, the cotyledonary tissue was removed after flash drying. Data points represent mean ± SE, $n = 10–20$. (b) Correlation between MC, viability and vigour of isolated axes with cotyledonary tissue. Viability is defined by the proportion of axes displaying growth after 8 weeks in tissue culture (left *y*-axis; circles denote means, $n = 20$). Vigour is assessed by root and shoot length (white and grey bars, respectively) following 8 weeks of culture (right *y*-axis; bars represent mean ± SE, $n = 20$).

100% to c. 50%, with the shoots being more severely affected than the roots (Fig. 1b).

3.2. Kinetics of $O_2^{\cdot-}$ production by undesiccated axes

In freshly isolated axes, extracellular $O_2^{\cdot-}$ production was inhibited by c. 50% by KCN, but unaffected by DPI or NaN_3 (Table 1). In the first five min after excision axes produced $O_2^{\cdot-}$ at c. 7 nmol s^{-1} g^{-1} dry mass, declining after 50 min to c. 3.5 nmol s^{-1} g^{-1} dry mass (Fig. 2). The rates of extracellular $O_2^{\cdot-}$ production in desiccated axes were c. 4 and 5.5 nmol s^{-1} g^{-1} dry mass for axes desiccated to 39 and 21% respectively, and were almost constant for 30 min following rehydration.

3.3. Effect of cryopreservation of desiccated explants on $O_2^{\cdot-}$ production and survival

During rehydration following cryopreservation, extracellular $O_2^{\cdot-}$ production was initially c. 2 nmol s^{-1} g^{-1} dry mass in axes dried to both 39 and 21% (Fig. 3). Rates of production increased to a maximum of c. 5 nmol s^{-1} g^{-1} dry mass after 30 min, and then declined slightly, remaining a little higher in explants desiccated to 39%. After cryopreservation, axes were transferred into tissue culture to test viability. No explants survived cryopreservation after desiccation to a MC of 39%. After 8 weeks, c. 7% of the explants dried to 21% formed callus, but root or shoot formation was never observed.

3.4. Localisation and characterisation of peroxidases and $O_2^{\cdot-}$ producing enzymes in cell wall fractions of axes

Peroxidase activity was present in all isolated cellular fractions, and was higher in the axes than in the cotyledons by up to two orders of magnitude (Table 2). In the axes, the intracellular (C) and electrostatically bound (B3) fractions had highest activities

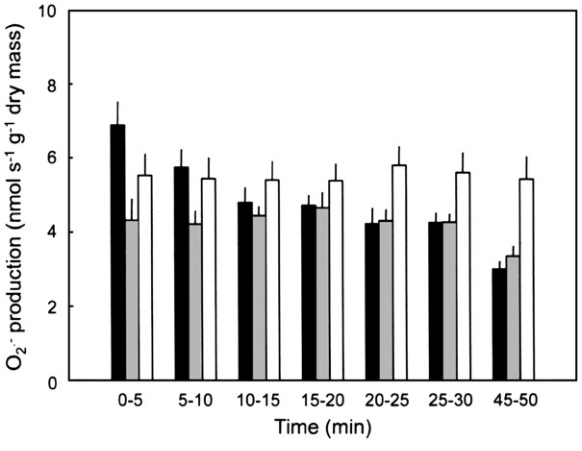

Fig. 2. Effect of desiccation to different moisture contents following excision of *T. dregeana* axes with attached sections of cotyledonary tissue on the rate of extracellular $O_2^{\cdot-}$ production. Black, grey and white bars represent axes with MCs of 63±2% (undesiccated), 39±2% and 21±1%, respectively. The same plant material was used throughout and the incubation solution was changed every 5 min over which $O_2^{\cdot-}$ production was estimated (mean±SE, n=5 replicates of four axes).

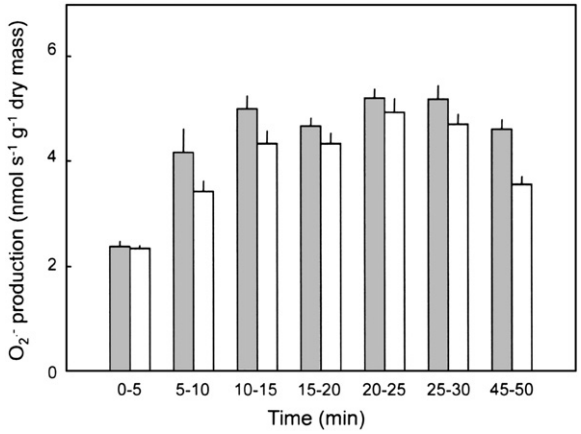

Fig. 3. Rate of extracellular $O_2^{\cdot-}$ production following cryopreservation of excised axes with cotyledonary sections of *T. dregeana*, desiccated to MCs of 39±2% (grey bars) or 21±1% (white bars). The same plant material was used in all measurements and the incubation solution was changed at the start of each 5 min interval over which production was estimated (mean±SE, n=7 replicates of four axes).

(Table 2). Fig. 4 illustrates electrophoretic separation of proteins from fractions B1, B2 and B3 treated to visualize peroxidase activity and $O_2^{\cdot-}$ production. Gels in which no $O_2^{\cdot-}$ production was detected are omitted from the figure. Peroxidases were present as a diversity of isoforms, which varied between fractions and between the axes and the cotyledons. Fraction B3 contained peroxidases which did not migrate into the gels. Most $O_2^{\cdot-}$ was produced by peroxidases with molecular masses of c. 50 and 80 kD in the B1 fraction of the axes, although some, apparently cationic, peroxidases from the cotyledons in B3 also appeared to produce $O_2^{\cdot-}$. Small amounts of $O_2^{\cdot-}$ were produced by unindentified high molecular mass proteins in B2.

4. Discussion

Ex situ storage of recalcitrant seeds is possible only in the short term, while long-term storage of germplasm requires cryopreservation (Berjak and Pammenter, 2004). As discussed in the Introduction, studies suggest that the most suitable materials for cryopreservation are either the embryo or the embryonic axis, which need to be excised and partially dried prior to cryogenic cooling. However, in many cases, particularly for seeds such as

Table 2
Peroxidase activity in various cellular fractions of axes and cotyledons of *Trichilia dregeana* seeds.

	Peroxidase activity (nkat g^{-1} dry mass)	
Fraction	Axes	Cotyledons
C	1770±80	60±2
B1	410±10	33±2
B2	140±10	15±0
B3	710±30	56±3
B4	60±0	44±3

Peroxidase activity was measured by the oxidation of ABTS to the stable cation radical $ABTS^+$ in the presence of H_2O_2. Figures are given±standard error, n=5.

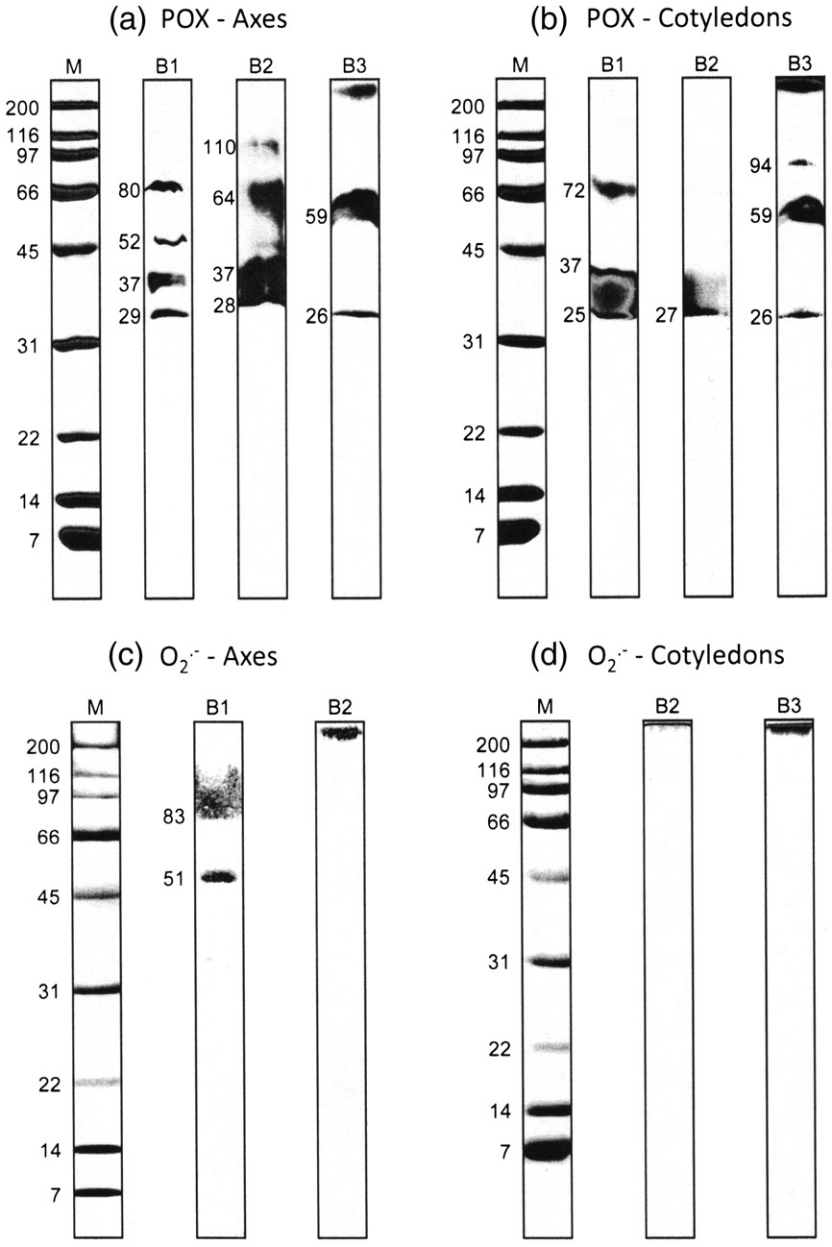

Fig. 4. Characterisation of peroxidases and other $O_2^{\cdot-}$-producing enzymes in cell wall fractions of axes and cotyledons of *T. dregeana* seeds. (a) Peroxidase staining with guaiacol in axes and (b) cotyledonary fractions. (c) Superoxide production visualized using NBT staining in axes and (d) cotyledons fractions.

T. dregeana with fleshy cotyledons, even these treatments only allow very poor survival after cryopreservation. In the present study, isolation of *T. dregeana* embryonic axes caused a burst of extracellular $O_2^{\cdot-}$ production, which declined slowly over the next 50 min (Fig. 2). These kinetics differed from those reported from *C. sativa* (sweet chestnut) seeds (Roach et al., 2008) which can be readily cryopreserved (Pence, 1992). In *C. sativa*, initial rates of $O_2^{\cdot-}$ production were only half those of *T. dregeana*, and the oxidative burst ended 5 min after excision. Production of $O_2^{\cdot-}$ from moderately desiccated *T. dregeana* axes (MC 39%) was similar to that of undesiccated controls (here 63%), while in axes with a MC of 21% $O_2^{\cdot-}$ production was higher, and did not vary significantly over the sampling period (Fig. 2). Axes dried to 39% had lost some viability (Fig. 1), while the vigour and viability of those desiccated to 21% was significantly reduced when compared with controls. By comparison, desiccating axes of *C. sativa* (Roach et al., 2008) to 32% (70% of axes still viable), increased extracellular $O_2^{\cdot-}$ production within the first 5 min. Further desiccation to a MC 25% reduced rates of O_2^{-}. production, and slightly prolonged the burst, coinciding with viability loss. These limited data suggest that a brief (5 min) oxidative burst in excised axes that is stimulated by mild (sublethal) desiccation but decreased by slow and lethal desiccation is characteristic of recalcitrant seeds that can be readily cryopreserved. By contrast, in recalcitrant seeds which are more difficult to cryopreserve, such as *T. dregeana*, the short

burst of extracellular $O_2^{\cdot-}$ production appears to be replaced by more prolonged $O_2^{\cdot-}$ production in response to excision and dehydration.

During rehydration following cryopreservation, desiccated explants of *T. dregeana* again displayed a burst of extracellular $O_2^{\cdot-}$, although maximum rates of production occurred 20–30 min after imbibition (Fig. 3). Rates of $O_2^{\cdot-}$ production were similar in material dried to 39 or 21% MC, although they tended to be higher in material dried to 39% MC. Survival after cryopreservation by the axes was, as predicted, very poor. No axes dried to 39% MC survived, and only 7% of those dried to 21% MC produced callus. It seems likely that the failure of the axes to recover following cryopreservation is the result of the cumulative harmful effects of prolonged or repeated bursts of extracellular $O_2^{\cdot-}$ production caused by the manipulations necessary to prepare the tissue for freezing, and the cryopreservation itself.

The identification of the ROS-producing enzymes will be important for future studies that attempt to manipulate redox status and improve the survival of the embryonic axis. In higher plants, depending on species, apoplastic $O_2^{\cdot-}$ production has often been attributed to either NAD(P)H oxidases or peroxidases (Bolwell et al., 2002). First experiments suggested that in *T. dregeana* peroxidases were responsible, because $O_2^{\cdot-}$ production was insensitive to the NAD(P)H oxidase inhibitor DPI (Segal and Abo, 1993), but highly sensitive to cyanide, a peroxidase inhibitor (Prohaska et al., 1977) (Table 1). Superoxide production was not sensitive to a frequently used inhibitor of peroxidases, NaN_3, but recent data show that plant peroxidases can be NaN_3-insensitive (Basile et al., 2006). All cell wall fractions of the embryonic axis and cotyledons of *T. dregeana* possessed peroxidase activities (Table 2). Separating proteins of these fractions by PAGE showed that peroxidases were present as a variety of isoforms (Fig. 4). Further evidence for the involvement of some peroxidases in $O_2^{\cdot-}$ production came from comparing bands with peroxidase activity with those that produce $O_2^{\cdot-}$ (Fig. 4). Results suggested that most $O_2^{\cdot-}$ was produced by two loosely bound ("B1" fraction) peroxidase isoforms with molecular masses of c. 50 and 80 kD in the embryonic axes. Some $O_2^{\cdot-}$ was also produced by an electrostatically bound ("B3" fraction) putatively cationic peroxidase in the cotyledons. Peroxidase-dependent apoplastic ROS production, often stimulated by stress, appears widespread in higher plants such as peas (Kukavica et al., 2009), maize (Vianello and Macri, 1991), French beans (Bolwell et al., 1995) and wheat (Minibayeva et al., 2009). The present study shows that peroxidase-mediated apoplastic ROS production also occurs in the seeds of *T. dregeana*.

In the vegetative tissues of higher plants, extracellularly produced ROS have been suggested to act as a defence against pathogens, to participate in wound healing and play roles in intracellular signal transduction (Bolwell, 1999; Mika et al., 2004; Gechev et al., 2006). It is reasonable to assume that extracellular ROS plays the same roles in seeds. Hence, a short, controlled burst of extracellular $O_2^{\cdot-}$ production as reported for chestnut seeds (Roach et al., 2008) may play important roles in wound responses, regeneration and growth. However, excessive ROS production and accumulation which occurs during the excision, desiccation and cryopreservation of the embryonic axes of seeds such as *T. dregeana*, can also have deleterious effects and be the reason for the limited success of cryopreservation. Future research needs to focus on ways of manipulating ROS production in these plant species, and the discovery of peroxidases as the enzymes responsible might greatly facilitate these studies. Targeting this group of enzymes to modulate ROS levels during axis preparation and cryopreservation may enable the development of enhanced cryopreservation protocols for recalcitrant seeds.

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References

Bailly, C., 2004. Active oxygen species and antioxidants in seed biology. Seed Science Research 14, 93–107.

Bailly, C., El-Maarouf-Bouteau, H., Corbineau, F., 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. Comptes Rendus Biologies 331, 806–814.

Basile, A., Senatore, F., Gargano, R., Sorbo, S., Del Pezzo, M., Lavitola, A., Ritieni, A., Bruno, M., Spatuzzi, D., Rigano, D., Vuotto, M.L., 2006. Antibacterial and antioxidant activities in *Sideritis italica* (Miller). Journal of Ethnopharmacology 107, 240–248.

Berjak, P., Pammenter, N.W., 2004. Recalcitrant seeds. In: Benech-Arnold, R.L., Sánchez, R.A. (Eds.), Seed Physiology: Applications to Agriculture. Haworth Press, New York, pp. 305–345.

Berjak, P., Dini, M., Pammenter, N.W., 1984. Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation-associated subcellular changes in propagules of *Avicennia marina*. Seed Science and Technology 12, 365–384.

Berjak, P., Farrant, J.M., Pammenter, N.W., 1989. The basis of recalcitrant seed behaviour. In: Taylorson, R.B. (Ed.), Recent Advances in the Development and Germination of Seeds. Plenum Press, New York, pp. 89–110.

Berjak, P., Farrant, J.M., Mycock, D.J., Pammenter, N.W., 1990. Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation-sensitivity. Seed Science and Technology 18, 297–310.

Black, M., 2000. Travels with seeds: a cartography of seed science. In: Black, M., Bradford, K.J., Vázquez-Ramos, J. (Eds.), Seed Biology: Advances and Applications. CABI Publishing, Oxford, pp. 3–10.

Bolwell, G.P., 1999. Role of active oxygen species and NO in plant defence responses. Current Opinion in Plant Biology 2, 287–294.

Bolwell, G.P., Buti, V.S., Davies, D.R., Zimmerlin, A., 1995. The origin of the oxidative burst in plants. Free Radical Research 23, 517–532.

Bolwell, G.P., Bindschedler, L.V., Blee, K.A., Butt, V.S., Davies, D.R., Gardner, S.L., Gerrish, C., Minibayeva, F.V., 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. Journal of Experimental Botany 53, 1367–1376.

Cheng, H.-Y., Song, S.-Q., 2008. Possible involvement of reactive oxygen species scavenging enzymes in desiccation sensitivity of *Antiaris toxicaria* seeds and axes. Journal of Integrative Plant Biology 50, 1549–1556.

Childs, R.E., Bardsley, W.G., 1975. The steady-state kinetics of peroxidase with 2, 2′-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochemical Journal 145, 93–103.

Choinski Jr, J.S., 1990. Aspects of viability and post-germinative growth in seeds of the tropical tree, *Trichilia dregeana* Sonder. Annals of Botany 66, 437–442.

Coates Palgrave, K., 1977. Trees of Southern Africa. C. Struik, Cape Town.

Côme, D., Corbineau, F., 1996. Metabolic damage related to desiccation sensitivity. In: Ouédraogo, A.S., Poulsen, K., Stubsgaard, F. (Eds.), Intermediate/Recalcitrant Tropical Forest Tree Seeds. IPGRI, Rome, pp. 83–97.

Dickie, J.B., Pritchard, H.W., 2002. Systematic and evolutionary aspects of desiccation tolerance in seeds. In: Black, M., Pritchard, H.W. (Eds.), Desiccation and Survival in Plants. CABI, Oxford, pp. 239–262.

Ellis, R.H., Roberts, E.H., 1980. Improved equations for the prediction of seed longevity. Annals of Botany 45, 13–30.

Engelmann, F., 2004. Plant cryopreservation: progress and prospects. In vitro Cellular & Developmental Biology. Plant 40, 427–433.

Farrant, J.M., Pammenter, N.W., Berjak, P., 1986. The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. Physiologia Plantarum 67, 291–298.

Farrant, J.M., Pammenter, N.W., Berjak, P., 1989. Germination-associated events and the desiccation sensitivity of recalcitrant seeds — a study on three unrelated species. Planta 178, 189–198.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I., Laloi, C., 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays 28, 1091–1101.

Goveia, M., Kioko, J.I., Berjak, P., 2004. Developmental status is a critical factor in the selection of excised recalcitrant axes as explants for cryopreservation: a study on *Trichilia dregeana* Sond. Seed Science Research 14, 241–248.

Hendry, G.A.F., 1993. Oxygen, free radical processes and seed longevity. Seed Science Research 3, 141–153.

Hendry, G.A.F., Finch-Savage, W.E., Thorpe, P.C., Atherton, N.M., Buckland, S.M., Nilsson, K.A., Seel, W.E., 1992. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. New Phytologist 122, 273–279.

Kranner, I., Birtic, S., 2005. A modulating role for antioxidants in desiccation tolerance. Integrative and Comparative Biology 45, 734–740.

Kukavica, B., Mojović, M., Vučinić, Ž., Maksimović, V., Takahama, U., Jovanović, S.V., 2009. Generation of hydroxyl radical in isolated pea root cell wall, and the role of cell wall-bound peroxidase, Mn-SOD and phenolics in their production. Plant and Cell Physiology 50, 304–317.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

López-Heurtas, E., Corpas, F.J., Sandalio, L.M., del Río, L.A., 1999. Characterisations of membrane polypeptides from pea leaf peroxisomes involved in superoxide radical generation. Biochemical Journal 337, 531–536.

Mika, A., Minibayeva, F.V., Beckett, R.P., Lüthje, S., 2004. Possible functions of extracellular peroxidases in stress-induced generation and detoxification of active oxygen species. Phytochemical Reviews 3, 173–193.

Minibayeva, F.V., Kolesnikov, O., Chasov, A., Beckett, R.P., Lüthje, S., Vylegzhanina, N., Buck, F., Böttger, M., 2009. Wound-induced apoplastic peroxidase activities: their roles in the production and detoxification of reactive oxygen species. Plant Cell and Environment 32, 497–508.

Misra, H.R., Fridovich, I., 1972. The univalent reduction of oxygen by reduced flavins and quinines. Journal of Biological Chemistry 247, 188–192.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15, 473–495.

Oracz, K., El Maarouf-Bouteau, H., Kranner, I., Bogatek, R., Corbineau, F., Bailly, C., 2009. The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. Plant Physiology 150, 494–505.

Pammenter, N.W., Berjak, P., 1999. A review of recalcitrant seed physiology in relation to desiccation tolerance mechanisms. Seed Science Research 9, 13–37.

Pammenter, N.W., Berjak, P., Farrant, J.M., Smith, M.T., Ross, G., 1994. Why do stored hydrated recalcitrant seeds die? Seed Science Research 4, 187–191.

Pence, V.C., 1992. Desiccation and the survival of *Aesculus*, *Castanea*, and *Quercus* embryo axes through cryopreservation. Cryobiology 29, 391–399.

Prohaska, J.R., Oh, S.H., Hoekstra, W.G., Ganther, H.E., 1977. Glutathione peroxidase: inhibition by cyanide and release of selenium. Biochemical and Biophysical Research Communications 74, 64–71.

Pukacka, S., Ratajczak, E., 2006. Antioxidative response of ascorbate–glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. Journal of Plant Physiology 163, 1259–1266.

Rast, D.M., Baumgartner, D., Mayer, C., Hollenstein, G.O., 2003. Cell wall associated enzymes in fungi. Phytochemistry 64, 339–366.

Roach, T., Ivanova, M., Beckett, R.P., Minibayeva, F.V., Green, I., Pritchard, H.W., Kranner, I., 2008. An oxidative burst of superoxide in embryonic axes of recalcitrant sweet chestnut seeds as induced by excision and desiccation. Physiologia Plantarum 133, 131–139.

Roberts, E.H., 1973. Predicting the storage life of seeds. Seed Science and Technology 1, 499–514.

Segal, A.W., Abo, A., 1993. The biochemical basis of the NADPH oxidase of phagocytes. Trends in Biochemical Sciences 18, 43–47.

Serrano, A., Córdoba, F., Conzález-Reyes, J.A., Navas, P., Villalba, J.M., 1994. Purification and characterisation of two distinct NAD(P)H dehydrogenases from onion (*Allium cepa* L.) root plasma membrane. Plant Physiology 106, 87–96.

Sutherland, J.R., Diekmann, M., Berjak, P., 2002. Forest Tree Seed Health for Germplasm Conservation. IPGRI Technical Bulletin, no. 6. IPGRI, Rome.

Takeshige, K., Minakami, S., 1979. NADH- and NADPH-dependent formation of superoxide anions by bovine heart submitochondrial particles and NADH-ubiquinone reductase preparation. Biochemical Journal 180, 129–135.

Varghese, B., Naithani, S.C., 2002. Desiccation-induced changes in lipid peroxidation, superoxide level and antioxidant enzymes activity in neem (*Azadirachta indica* A. Juss) seeds. Acta Physiologia Plantarum 24, 79–87.

Varghese, B., Naithani, S.C., 2008. Oxidative metabolism-related changes in cryogenically stored neem (*Azadirachta indica* A. Juss) seeds. Journal of Plant Physiology 165, 755–765.

Vianello, A., Macri, F., 1991. Generation of superoxide anion and hydrogen-peroxide at the surface of plant-cells. Journal of Bioenergetics and Biomembranes 23, 409–423.

Wesley-Smith, J., Pammenter, N.W., Berjak, P., Walters, C., 2001. The effects of two drying rates on the desiccation tolerance of embryonic axes of recalcitrant jackfruit (*Artocarpus heterophyllus* Lamk.) seeds. Annals of Botany 88, 653–664.

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