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Effects of cryopreservation of recalcitrant *Amaryllis belladonna* zygotic embryos on vigor of recovered seedlings: a case of stress 'hangover'?

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Cryopreservation is the most promising long-term storage option for recalcitrant (i.e. desiccation-sensitive) seed germplasm; however, its effects on the vigor of recovered seedlings are unclear. This study looked at the vigor of seedlings recovered from partially dried (D) and cryopreserved (C) recalcitrant zygotic embryos (ZEs) of *Amaryllis belladonna*. Seedlings recovered from fresh (F), D- and C-embryos were regenerated in vitro, hardened-off ex vitro and then exposed to 12 days of watering (W) or 8 days of water deficit (S), followed by 3 days of re-watering. Seedling vigor was assessed in terms of physiological and growth responses to the imposed water stress. Compared with F-embryos, partial dehydration and cryopreservation reduced the number of embryos that produced seedlings, as well as the subsequent in vitro biomass of these seedlings. DW- and CW-seedlings (i.e. seedlings recovered from dried and cryopreserved ZEs that were watered for 12 days) exhibited lower CO_2-assimilation rates and abnormal root growth. Stomatal density was also lower in C-seedlings. DS- and CS-seedlings were exposed to persistent low leaf water and pressure potentials and unlike FS-seedlings, displayed signs of having incurred damage to their photosynthetic machinery. CS-seedlings were less efficient at adjusting leaf water potential to meet transpirational demands and more susceptible to persistent turgor loss than DS- and FS-seedlings. DS-seedlings performed slightly better than CS-seedlings but drought-induced seedling mortality in both these treatments was higher than FS-seedlings. These results suggest that seedlings recovered from partially dried and cryopreserved embryos were less vigorous and more susceptible to hydraulic failure than those from fresh ZEs.

Introduction

Cryopreservation (i.e. storage at ultra-low temperatures, usually −196°C) is the most promising long-term storage option for recalcitrant (i.e. desiccation-sensitive; Roberts 1973) seed germplasm, which otherwise cannot be stored for any useful period of time (Berjak and Pammenter 2004). Unlike the success achieved with somatic (e.g. Mycock et al. 1995) and nucellar embryos (e.g. Kartha 1985), and even zygotic embryos (ZEs) from orthodox seeds (e.g. Gagliardi et al. 2002), recovery after cryopreservation of recalcitrant ZEs/embryonic axes (EAs) very seldom results in the production of callus-free plants, rid of morphological abnormalities (Mycock 1999). In fact, a number of studies (e.g. Dumet et al. 1997, Sershen et al. 2007, Steinmacher

Abbreviations – ANOVA, analysis of variance; Chl, chlorophyll; CP, cryoprotection; EAs, embryonic axes; FW, fresh weight; Gly, glycerol; md, midday; LN, liquid nitrogen; PSI, photosystem I; PSII, photosystem II; pd, predawn; RGR, relative growth rate; SEM, scanning electron microscope; ZEs, zygotic embryos.

et al. 2007, Wesley-Smith et al. 2001) have observed abnormal phenotype in seedlings recovered from recalcitrant ZEs/EAs exposed to partial dehydration or the combination of partial dehydration and cooling. Furthermore, the cryopreservation tissue-culture regeneration process may also expose recovered seedlings to the effects of somaclonal variation, resulting in changes to their genotypic and/or phenotypic profiles (reviewed by Harding 2004).

It has been known for some time now that exposure to different types of stress can alter subsequent plant responses (Bruce et al. 2007), but there is, at present, little to no understanding of how stresses imposed at the embryonic stage are translated or manifested during subsequent in and ex vitro seedling growth. A few reports suggest that there exists within developing embryos (zygotic and somatic) some 'memory'-based mechanism that senses environmental signals such as duration of imbibition (Forsyth and van Staden 1983) and temperature during embryogenesis (Kvaalen and Johnsen 2007), which in turn influence adaptive traits in the seedlings they give rise to. If such a mechanism were to exist within recalcitrant ZEs/EAs, the dehydration and freezing stresses imposed on these explants during cryopreservation, together with the morphological abnormalities that often characterise recovered seedlings (Mycock 1999), may compromise the aim of plant cryopreservation – to regenerate true-to-type seedlings.

There have been reports of no observed differences in morphological characters between plants recovered from control or cryopreserved: shoot apices (e.g. Helliot et al. 2002); meristems (e.g. Caswell and Kartha 2009); somatic embryos (Aronen et al. 1999); embryogenic cell suspensions (e.g. Côte et al. 2000); polyembryonic cultures (Konan et al. 2007); shoot tips (e.g. Benson et al. 1996); non-orthodox whole seeds (e.g. Popov et al. 2004); and even ZEs of recalcitrant seeds (Assy-Bah and Engelmann 1992). Also, there is an increasing number of reports indicating no phenotypical, biochemical, chromosomal or molecular modifications of thawed material attributed to cryopreservation (reviewed by Harding 2004; Engelmann 2004). Many of those observations have been made very soon after cryopreservation and on a small number of individuals, often using material still cultured in vitro or after a short period of growth ex vitro. However, there are cases (e.g. Engelmann 1991, Benson et al. 1996, Côte et al. 2000, Martínez-Montero et al. 2002, Konan et al. 2007, Caswell and Kartha 2009) where many plants (often hundreds) derived from cryopreserved germplasm have been grown and assessed in the field for many months, to years. Those studies have shown recovered plantlets of a number of (mainly crop) species (including potato, banana, sugarcane and apple and oil palm) to exhibit normal phenotype and even flower, fruit and produce seed. These observations were based on a comparison of a wide range of developmental and morphological growth characteristics of plants recovered from frozen and unfrozen samples; however, except for one report on the photochemical activities of two photosystems in frozen–thawed *Bratonia* orchid protocorms during in vitro recovery (Bukhov et al. 2006), there are, at present, no published reports on the in or ex vitro physiological performance and/or stress tolerance of plants recovered from cryopreserved samples. Ex vitro physiological performance and stress tolerance of seedlings recovered from cryopreserved germplasm are of special interest to programs concerned with the cryopreservation of the germplasm of endangered wild species, which ultimately aim to re-introduce plants or seedlings recovered from cryopreserved samples back into the wild. As the successful re-introduction of recovered seedlings will depend on their ability to tolerate abiotic stresses such as drought, most especially during the early seedling establishment phase, the current contribution looked at the in and ex vitro vigor of seedlings recovered from partially dried and cryopreserved, recalcitrant ZEs. The studies were undertaken on the recalcitrant ZEs of the wild geophyte *Amaryllis belladonna* (Amaryllidaceae) and seedling vigor was assessed in terms of physiological and growth responses to an ex vitro water stress.

Materials and methods

Plant material

Mature *A. belladonna* fruits were harvested directly from parent plants and transported in plastic bags to the laboratory with minimum delay (1–2 days) or water loss. Upon arrival, the seeds were dusted with a benomyl-based fungicide, Benlate [active ingredient: benomyl (benzimidazole), Dupont, Wilmington, DE] and stored 'moist' (i.e. seeds placed in a monolayer on a grid suspended approximately 200 mm above sterile, moistened paper towel that lined the base of individual buckets which was closed with lids), at 6°C.

Embryo pre-treatment, in vitro seedling regeneration and ex vitro hardening-off

All experiments were carried out on seeds stored for between 7 and 10 days, never longer. For these experiments, ZEs were excised and collected within closed Petri dishes on filter paper moistened with sterile calcium-magnesium solution [CaMg solution: 1:1 solution of 1 μM $CaCl_2$ and 1 mM $MgCl_2$ (after Mycock

1999)] before being subjected to one of the following treatment combinations: (1) no cryoprotection (CP), no dehydration and no cooling (i.e. freshly excised ZEs possessing water content of *c.* 4.65 g g^{-1}); (2) CP with glycerol [Gly: a combination of 5 and 10% (v/v)], rapid dehydration to *c.* 0.31 g g^{-1} [using a flash-drier (originally used by Berjak et al. 1989)] and rapid rehydration in CaMg solution; and (3) CP with Gly, rapid dehydration to *c.* 0.31 g g^{-1}, rapid cooling (hundreds of °C s^{-1}) of naked ZEs in nitrogen slush [liquid nitrogen (LN) sub-cooled to −210°C], transfer to cryovials under LN and storage in LN for 24 h, followed by thawing in CaMg solution held at 40°C for 2 min and rehydration in CaMg solution at 25°C for 30 min. For CP, ZEs were immersed in 5% (v/v) aqueous Gly and thereafter transferred to a 10% (v/v) aqueous solution of the cryoprotectant, for 1 h at each concentration. Embryos subjected to each of these three treatment (300 embryos for each) combinations (1–3) were then decontaminated with 1% (w/v) aqueous calcium hypochlorite for 3 min, washed with sterile CaMg solution (three times) and then set to germinate with five embryos per Petri dish on full-strength Murashige and Skoog medium (Murashige and Skoog 1962), containing 3% (w/v) sucrose. All Petri dishes were initially placed in the dark, and transferred to a growth room with an 18-h photoperiod at approximately 23°C upon signs of root and shoot development (which usually occurred after 6–10 days of in vitro growth).

Embryos were grown in vitro for 150 days with two sub-cultures (at 50 and 100 days) and those that subsequently produced seedlings with callus-free roots and shoots were transplanted independently into plastic inserts (5-cm wide; 15-cm deep), filled with a mixture of one part pine bark and one part coarse river sand, and placed in a misthouse for 14 days to harden-off.

Ex vitro experimental design

From here on, seedlings generated from fresh ZEs will be labeled 'F,' dried 'D' and cryopreserved 'C.' Where any of these labels is followed by the letter 'S', it refers to seedlings exposed to a water deficit, imposed by with-holding water, whereas the letter 'W' indicates the absence of this stress (i.e. seedlings that were watered daily).

After hardening-off, plants were transferred to natural conditions of illumination in a polycarbonate-clad greenhouse. Plants were fertilised upon transfer to the greenhouse and after 7 days, D-, F- and C-seedling groups, now composed of 174 (randomly selected) seedlings each, were further sub-divided into two batches of 87. Within each group, one seedling batch was subjected to a water stress by with-holding water for 8 days, followed by 3 days of re-watering, whereas the second batch was watered daily for 12 days. Instantaneous leaf-based CO_2-assimilation rate, potential photochemical efficiency as well as leaf water, osmotic and pressure potential, were measured across all embryo pre-treatment × watering regimes (referred to as 'all treatments' from here on) on days 0, 8 and 12, whereas growth responses (seedling biomass and biomass partitioning) and leaf chlorophyll (Chl) content were assessed after the in and ex vitro growth period. Root morphology and stomatal density were assessed across all treatments at the end of the ex vitro growth period.

Photosynthetic capacity

Chlorophyll content

Chl content was measured on experimental day 0 (ex vitro) and at the final harvest (day 12) using one leaf from each of four seedlings (only living leaf material sampled), across all treatments. After determining their fresh weight (FW), individual leaves were ground in a pestle and mortar with LN and Chl immediately extracted in 5 ml of 80% acetone, in the dark (after Arnon 1949). After 6 h, the leachate was filtered and its absorbance read at 663 nm for Chl *a* and 645 nm for Chl *b* (after Arnon 1949). Chl *a*, *b* and total Chl content were thereafter expressed on a FW basis. The Chl assay employed here may seem outdated in light of UPLC- and HPLC-based methods (e.g. Rodrigues-Amaya and Kimura 2004) presently used for qualitative and quantitative measurement of caretenoids in plant tissue. However, rather than aiming to identify and quantify individual caretenoids present, the current study simply aimed to quantify changes in leaf Chl content in response to drought stress. In such cases, spectrophotometric pigment assays such as the one used here (after Arnon 1949) are sufficient (Bulda et al. 2008), but stability of the pigment extract should be checked because extraction with acetone solutions can lead to Chl degradation to phaeophytin by co-extracted acids (Rodrigues-Amaya and Kimura 2004). This was unlikely to have affected the results obtained here because the absorbance of acetone Chl extracts of *A. belladonna* leaves remained stable (to the third decimal) for as long as 6 h after filtration (when kept in the dark).

Steady-state gas exchange

All gas exchange measurements were carried out using the Li-Cor 6400 portable photosynthesis measuring system, fitted with an *Arabidopsis* chamber and configured as an open system (Li-Cor, Lincoln, NE).

On experimental days 0, 8 and 12, instantaneous (spot) measures of leaf-based CO_2-assimilation rates (A) were carried out at C_a: 400 μmol mol^{-1} and then at above ambient C_a: 600 μmol mol^{-1}, across all treatments. Measurements were carried out around midday, on non-senescing leaves and under natural illumination. For each treatment, three consecutive measurements were taken for one leaf from each of seven seedlings, when the total percentage coefficient of variance (% ΔH_2O; ΔCO_2; Δ flow rate) was <1%. The mean of these three measurements yielded the final reading for that leaf. Only values measured at a photosynthetic photon flux density greater than 800 μmol m^{-2} s^{-1} (i.e. light saturated) were used for subsequent analyses.

The ratio between A at C_a: 600 and 400 μmol mol^{-1}, referred to as 'A@600:A@400' from here on, was also calculated. This ratio has been used to assess the effects of water stress on CO_2-assimilation in other studies and, like stomatal conductance, the response of A to changing C_a is believed to be a reliable indication of stomatal limitation of A (Osmond et al. 1980); a higher ratio is indicative of greater stomatal limitation.

Potential photochemical efficiency

On experimental days 0, 8 and 12, a Plant Efficiency Analyzer (Hansatech Instruments Ltd., Kings Lynn, UK) was used to measure Chl fluorescence transients on one fully expanded, non-senescing, mature leaf from each of six seedlings, across all treatments. After samples were dark adapted for 20 min, transients were induced by red light of 1500 μmol m^{-2} s^{-1} generated by six light-emitting diodes (peak 650 nm). These diodes covered the exposed area of the leaf (4 mm in diameter) in homogenous illumination and fluorescence signals were recorded within a time span of 10 μs to 1 s with a data acquisition rate of 10 μs for the first 2 ms and 1 ms thereafter. F_v/F_m, the ratio of variable (F_v) to maximum fluorescence (F_m), is a measure of potential photochemical efficiency of photosystem II (PSII) and was calculated from the fluorescence data using Biolyzer 3.0 software (developed by Maldonado-Rodriguez 2002).

Leaf water status

At predawn (pd) and 5–6 h into the light period (midday, md), on experimental days 0, 8 and 12, leaf water potential (Ψ_w) measurements were carried out using thermocouple psychrometers (Model C-52; Wescor, Logan, UT) in combination with a microvoltmeter. For this, leaf discs were excised from the middle of one fully expanded, non-senescing, mature leaf from each of four seedlings, across all treatments. The microvoltmeter was calibrated against NaCl standards, at 25°C, and a cooling period of 20 s was used to measure the dew point. The difference between pd Ψ_w and md Ψ_w, referred to as 'pd–md' from here on, was also calculated. pd–md Ψ_w reflects the extent to which md Ψ_w is lowered from that at pd, to generate the Ψ_w gradient between soil and leaf and to drive the water flow through the plant to meet the transpirational demand during the light period.

After Ψ_w was determined, leaf samples were wrapped in at least four layers of Parafilm™ (American National Can, Chicago, IL), covered with aluminium foil and plunged repeatedly into LN for 30 s intervals, over a period of 5 min. After thawing, samples were unwrapped, placed immediately into C52 sample chambers and Ψ_w was recorded after a pre-determined equilibration period. If apoplastic water is negligible, Ψ_w after cooling and thawing of tissue in this way is generally regarded as the 'osmotic' potential (Ψ_s), or water activity. Pressure potential (Ψ_p) was calculated as the difference between osmotic and water potential.

Growth and biomass

After the in vitro growth period (150 days) six seedlings were separated into bulb, leaves and roots, on a per individual basis, and oven-dried for 72 h at 80°C to a constant weight, for dry mass estimates. Dry mass of these organs was similarly determined for six seedlings at the end of the ex vitro growth period and used to calculate relative growth rate (RGR) via Eqn 1:

$$\frac{\text{Individual seedling dry mass after 33 days ex vitro growth (g)} - \text{Mean seedling dry mass after 150 days in vitro growth (g)}}{\text{Mean seedling dry mass after 150 days in vitro growth (g)}} \Big/ 33 \text{ days} \quad (1)$$

Stomatal density

Only fully expanded leaves were used to investigate the effect of embryo pre-treatment and subsequent ex vitro growth (with and without an imposed water stress) on stomatal density. In *A. belladonna* leaves, the region of highest density and most regular stomatal distribution was found to lie in a 1-cm wide band across the middle portion of the adaxial surface, so this portion of the leaf was excised and used for all subsequent estimates. One leaf from each of 15 seedlings across all treatments was sampled early in the morning, on day 12 and fixed in gluteraldehyde immediately after excision. After fixation, samples were dehydrated using an ethanol series, critical

point dried and directly sputter-coated with gold in a Polaron E5100 sputter coater. Surface structure was viewed using a Leo 1450 scanning electron microscope (SEM; Zeiss, Germany).

Stomatal density on the left- and right-hand side of the selected leaf portion was captured at a constant magnification and field of view. The left- and right-hand sides of each leaf sample were further sub-divided into a top and bottom half, such that each sample was represented by four sectors: left-top and left-bottom and right-top and right-bottom. The number of stomata in each sector was counted, keeping the sample area constant. When the counts for the four sectors of each leaf were homogenous [tested for by one-way analysis of variance (ANOVA), $P < 0.05$], they were averaged to yield a mean stomatal density for each leaf. Only leaves with homogenous distribution of stomata were selected for subsequent analyses.

Root morphology

Roots from each of 10 seedlings across all treatments were excised after the ex vitro growth period. Roots of individual seedlings were divided into tips, middle (portion between the bulb base and root tip) and bulb base (portion immediately beneath the bulblet), prior to fixation in gluteraldehyde. After fixation, samples were dehydrated using an ethanol series, critical point dried, directly sputter-coated with gold and viewed using a Leo 1450 SEM.

Statistical analysis

In and ex vitro biomass, RGR, stomatal density and Chl content data were compared across treatments by a two-way ANOVA. CO_2-assimilation (A at C_a: 400 µmol mol^{-1}) and the ratio between A at 400 and 600 µmol mol^{-1}, photochemical efficiency (F_v/F_m) and water, osmotic and pressure potential data were tested for differences across treatments, within time intervals, by a two-way ANOVA.

All two-way ANOVAs were factorial in design, testing for the main-effects of embryo pre-treatment (referred to as 'embryo') and water stress (referred to as 'stress') and the interaction between these (referred to as 'embryo × stress'); performed using STATISTICA Version 8 (StatSoft Inc., Tulsa, OK). Where the original data was expressed as a proportion (%), these values were transformed (arcsin) to conform data to ANOVA assumptions. Multiple comparisons were then made with data that were significantly different ($P < 0.05$) using a Scheffe's mean separation test. All statistical tests were performed at the 0.05 level of significance.

At the end of the in and ex vitro growth period embryo/seedling viability (%) was compared across all treatments using null-model chi-squared analyses at the 0.05 level of significance [EcoSim Version 7.72 (developed by Gotelli and Entsminger 2009)].

Results

In vitro growth

Although 98% of freshly excised ZEs developed into seedlings, significantly ($P < 0.05$) fewer embryos produced seedlings after partial dehydration (83%) and the combination of partial dehydration and cooling (72%) during in vitro regeneration (data not shown).

After the in vitro growth period leaf, bulb, root and total dry mass were highest in F-seedlings and lowest in C-seedlings ($P < 0.05$; Fig. 1A). Although biomass partitioning to bulbs and roots in C-seedlings was significantly lower than F- and D-seedlings, biomass partitioning to leaves was significantly higher (Fig. 1B). Biomass partitioning to roots in D-seedlings was also significantly lower than F-seedlings.

Ex vitro growth

RGR in CW-seedlings was significantly lower than DW- and FW-seedlings (Table 1). In DS- and FS-seedlings,

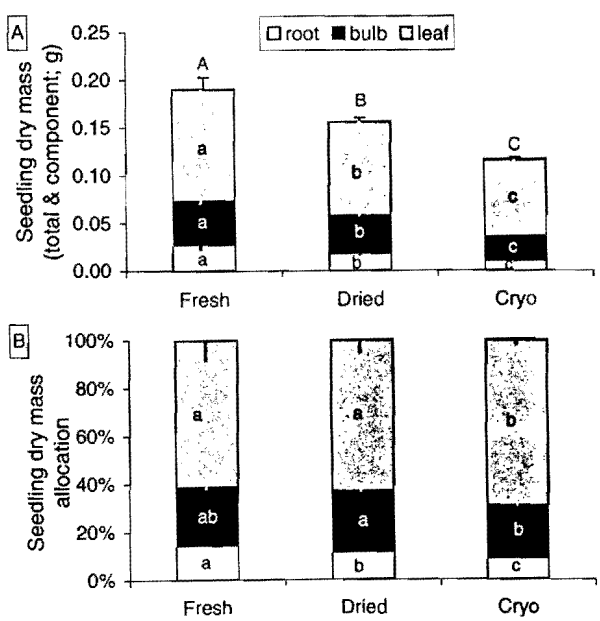

Fig. 1. (A) Total and organ-based dry mass and (B) biomass partitioning of recovered seedlings after 150 days in vitro growth. Blocks labeled with different lower case letters are significantly different across treatments when compared within organs, whereas upper case letters indicate differences in total dry mass ($P < 0.05$, ANOVA, $n = 6$). Bars represent SD.

Table 1. Ex vitro seedling relative growth rate (RGR; g g^{-1} DM day^{-1}). RGR = relative growth rate [(individual seedling dry mass after 33 days ex vitro growth (g) − mean seedling dry mass after 150 days in vitro growth (g)/mean seedling dry mass after 150 days in vitro growth (g))/33 days] of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Values represent mean ±SD and are significantly different when followed by different letters ($P < 0.01$ for embryo, stress and embryo × stress, ANOVA, n = 6).

FW	0.049 ± 0.008^{a}	FS	0.017 ± 0.002^{b}
DW	0.041 ± 0.009^{a}	DS	0.019 ± 0.001^{b}
CW	0.023 ± 0.007^{b}	CS	0.023 ± 0.001^{b}

RGR was significantly reduced relative to their respective unstressed controls but stress did not further affect the already low RGR of C-seedlings significantly.

In the absence of a stress, total seedling dry mass in F-seedlings was significantly higher than D- and C-seedlings whereas water stress significantly reduced dry mass accumulation across all embryo pre-treatments (Fig. 2A). Within the stressed treatments, dry mass accumulation was significantly higher in FS- and lower in CS-seedlings. Irrespective of whether the seedlings were stressed or not, bulb and root dry mass was significantly higher in F-seedlings and lower in C-seedlings. Water stress decreased bulb and root dry mass across all embryo pre-treatments; these differences were significant for F- and D-seedlings for bulb and for F- and C-seedlings for root. Although leaf dry mass in DW- and CW-seedlings was significantly lower than FW-seedlings, water stress significantly reduced leaf dry mass across all embryo pre-treatments.

In stressed and unstressed material, although biomass partitioning to bulbs and roots in C-seedlings was significantly lower than F- and D-seedlings, biomass partitioning to leaves was significantly higher (Fig. 2B). With a stress, biomass partitioning to roots in D- and F-seedlings increased significantly, relative to their respective unstressed controls, whereas biomass partitioning to leaves decreased significantly. In CS-seedlings, biomass partitioning to bulbs increased significantly relative to CW-seedlings, whereas partitioning to leaves decreased.

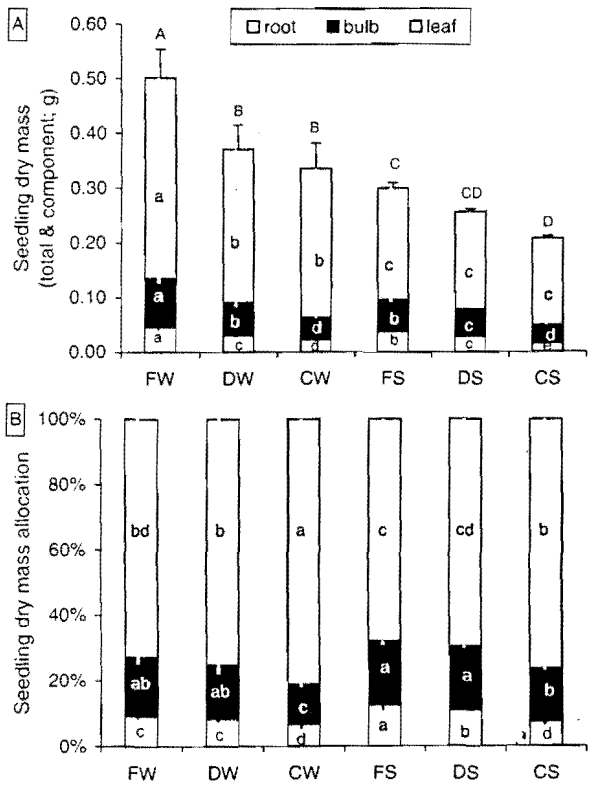

Fig. 2. (A) Total and organ-based dry mass and (B) biomass partitioning of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Blocks labeled with different lower case letters are significantly different across treatments when compared within organs, whereas upper case letters indicate differences in total dry mass (for (A): $P < 0.01$ for embryo and stress and $P = 0.01$ for embryo × stress; for (B): $P < 0.01$ for embryo; roots: $P < 0.01$ for stress and embryo × stress; leaves: $P < 0.01$ for stress, ANOVA, $n = 6$). Bars represent SD.

Leaf stomatal density

As there were no significant differences in stomatal density between stressed and unstressed leaves, stomatal density data were pooled based on embryo pre-treatment for all subsequent analyses, which showed stomatal density of C-seedlings to be significantly lower than F- and D-seedlings (Table 2).

Root morphology

This was assessed at the end of the ex vitro growth period across all treatments; however, within embryo pre-treatments there were no obvious differences between stressed and unstressed seedlings so root morphology are presented only for non-stressed F-, D- and C-seedlings. Results of these studies were not quantitative and in light of their descriptive nature only features that occurred

Table 2. Adaxial stomatal density. Estimated from SEM images of individual leaves of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs after 33 days ex vitro growth. Values represent mean ±SD (data for stressed and unstressed treatments were pooled within embryo pre-treatments) and are significantly different when followed by different letters ($P < 0.01$, ANOVA, n = 20).

Treatment	Stomatal density (no. of stomata mm^{-2})
Fresh	36.4 ± 5.2^{a}
Dried	35.8 ± 8.3^{a}
Cryopreserved	15.6 ± 5.1^{b}

in more than 5 of the 10 replicates for each embryo pre-treatment are shown.

Scanning electron micrographs of roots produced by unstressed F-, D- and C-seedlings revealed root hair density to be the greatest (qualitative assessment) in F-seedlings (Fig. 3A, D, G). Root hairs in F-seedlings occurred across all three sections of the root (i.e. tip, middle and bulb base) but were most abundant in the middle portion (Fig. 3D). In D-seedlings, root hair density was also greatest in the middle portion of the root but unlike F-seedlings, root hairs were sporadically distributed across the three portions and clearly absent in some areas (Fig. 3B, E, H). Root tips in D-seedlings were characterised by root hairs that were relatively shorter (qualitative assessment) than those associated with the root tips of F-seedlings (Fig. 3G–H). In C-seedlings, large areas across all three root portions were devoid of root hairs (Fig. 3C, F, I) whereas root hair density in the middle portion, the site of the greatest root hair density in other treatments, was also far lower (qualitative assessment) than F- and D-seedlings (Fig. 3D–F).

The roots of D- and F-seedlings tapered toward the tip, whereas the root tips of C-seedlings were almost nodular and often exhibited a tuft of relatively long root hairs (Fig. 3G–I). In C-seedlings, the section of the root immediately beneath the bulb was almost always abnormally thick, when compared with F- and D-seedlings (Fig. 3A–C).

Plant water relations

On day 0, Ψ_w, Ψ_s and Ψ_p in CW-seedlings were significantly lower than FW- and DW-seedlings at pd, but not at md (Figs 4A, 5A, B and 6A, B). Although pd–md Ψ_w was close to zero in CW-seedlings on day 0, this parameter was significantly higher in DW- and FW-seedlings (Fig. 4B), suggesting that DW- and FW-seedlings were more efficient at adjusting Ψ_w to meet transpirational demands than CW-seedlings. Within the unstressed treatments, pd Ψ_w and pd–md Ψ_w in C-seedlings remained significantly (on days 8 and 12) lower than D- and F-seedlings throughout the experimental period (Fig. 4A, B) but pd–md Ψ_w values did become more positive (relative to day 0) in CW-seedlings as the experimental period progressed.

By day 8, water stress had depressed pd Ψ_w across all embryo pre-treatments and even with re-watering (i.e. day 12) pd Ψ_w across all stressed treatments never

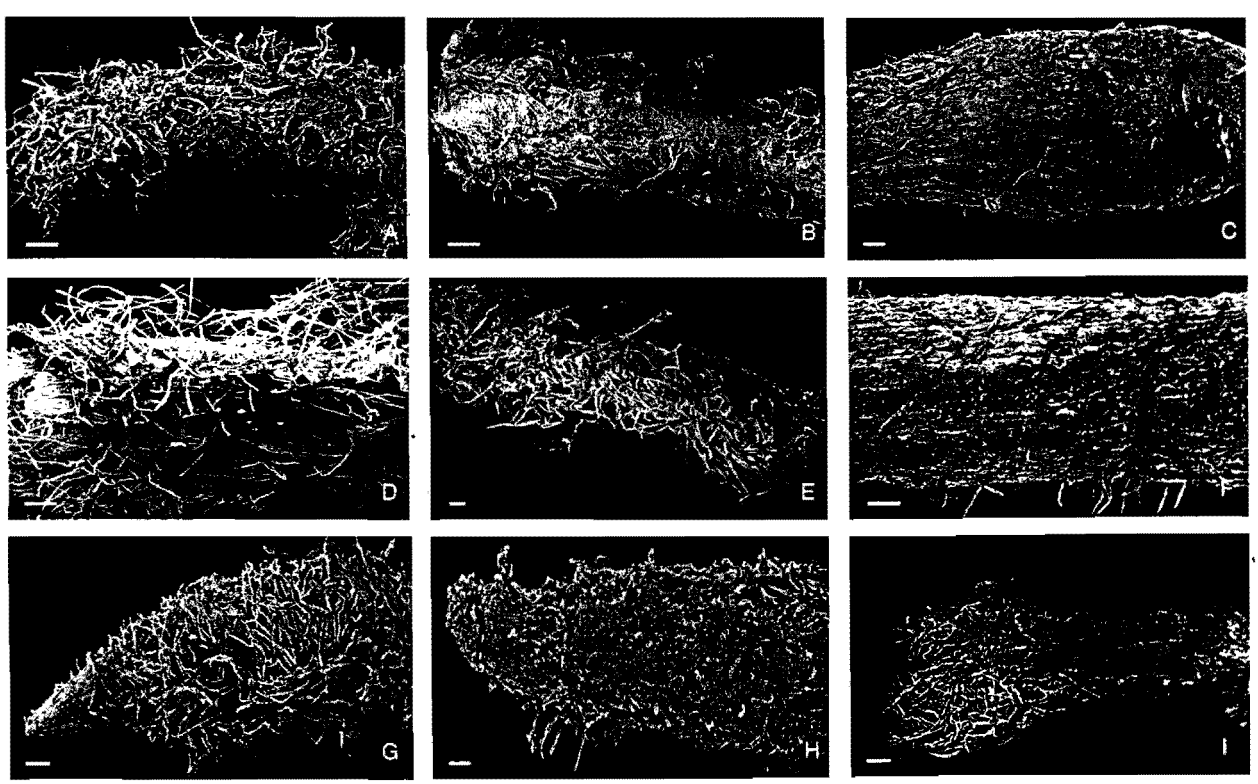

Fig. 3. Scanning electron micrographs of roots of seedlings derived from (A, D, G) fresh, (B, E, H) partially dried and (C, F, I) cryopreserved ZEs after 33 days ex vitro growth. (A–C) root portion immediately beneath bulb; (D–F) middle portion of root; (G–I) root tip. Bar represents 200 µm.

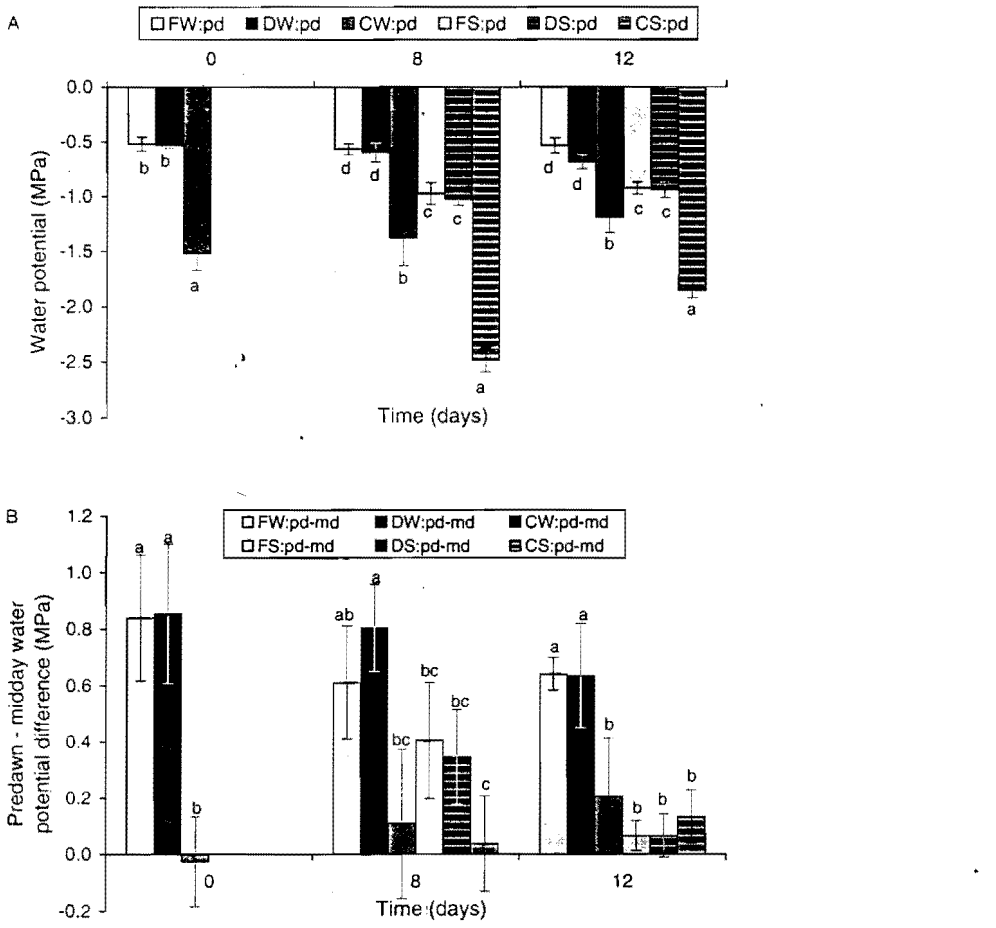

Fig. 4. (A) Predawn (pd) leaf water potential and (B) Predawn–midday water potential difference (pd–md) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Columns labeled with different letters are significantly different when compared within experimental days ($P < 0.01$ for embryo, stress and embryo × stress, ANOVA, $n = 4$). Bars represent ±SD.

recovered to levels comparable with their respective unstressed controls (Fig. 4A, B). On day 8, pd–md Ψ_w across all stressed treatments was relatively lower than their respective unstressed controls (significant for D-seedlings only), whereas within the stressed treatments pd–md Ψ_w was significantly lower in CS-seedlings (Fig. 4B). When stressed plants were re-watered, pd–md Ψ_w in these treatments remained significantly lower than their respective unstressed controls.

Irrespective of whether they were stressed or not, Ψ_s in C-seedlings was significantly lower than D- and F-seedlings at pd and md on days 8 and 12 (Fig. 5A, B). On days 8 and 12, Ψ_s in FS- and DS-seedlings was significantly lower than their respective unstressed controls at pd, and at pd and md for CS-seedlings.

On day 8, water stress depressed Ψ_p across all embryo pre-treatments but this trend was significant at md only (Fig. 6A, B). Even though Ψ_p in FS-seedlings was slightly higher than DS- and CS-seedlings at pd and md on day 8, these differences were not significant. With re-watering Ψ_p in DS- and FS-seedlings recovered to levels comparable with their respective unstressed controls on day 12 (being more pronounced at pd); however, Ψ_p in CS-seedlings remained relatively lower than in CW-seedlings (significant at md only) and DS- and FS-seedlings.

Photosynthetic characteristics

Leaf chlorophyll content

After 150 days in vitro growth, Chl *a*, *b* and total leaf Chl content did not differ significantly with embryo pre-treatment (data not shown). With ex vitro growth, Chl *a* and total Chl content in F-leaves were significantly higher than D- and C-leaves, whereas Chl *b* content in F- and D-leaves was significantly higher than C-leaves

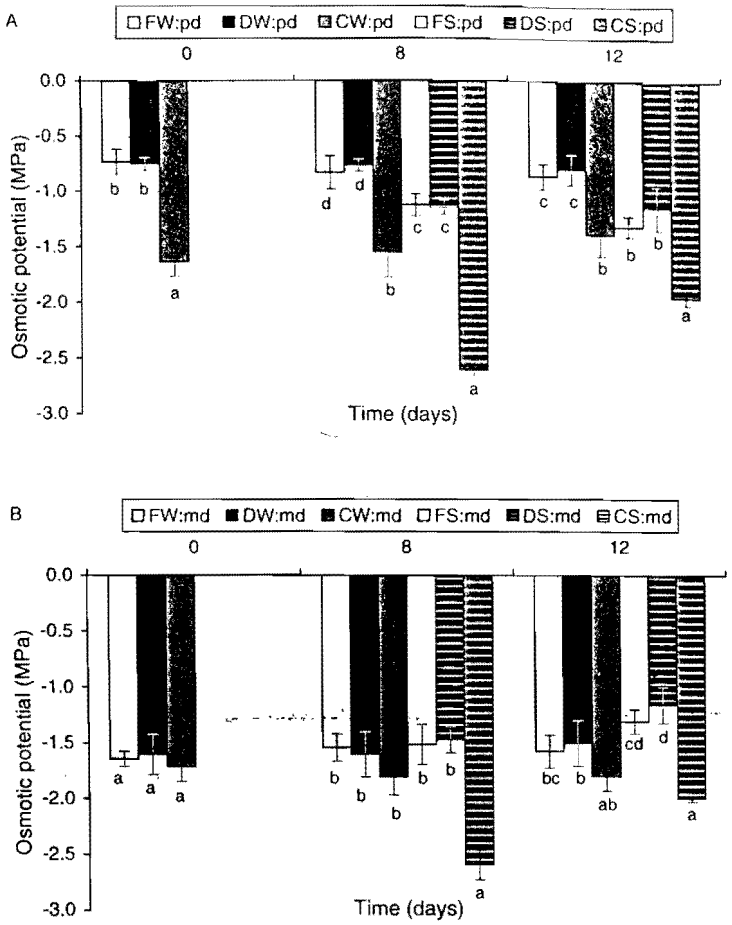

Fig. 5. (A) Predawn (pd) and (B) midday (md) leaf osmotic potential of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Columns labeled with different letters are significantly different when compared within experimental days ($P < 0.01$ for embryo and embryo × stress, ANOVA, $n = 4$). Bars represent ±SD.

(Fig. 7). Total Chl content in D-leaves was also relatively higher than C-leaves but this trend was significant for unstressed material only. DS- and CS-seedlings exhibited a decline in Chl*a*, *b* and total Chl content, relative to their respective unstressed controls (significant for total Chl in D-seedlings only) but this decline was not observed in FS-seedlings.

CO_2-assimilation rates

On day 0 and throughout the experimental period, leaf-based CO_2-assimilation rates (A) at C_a: 400 μmol mol^{-1} were significantly higher in FW-seedlings (Fig. 8). On days 8 and 12, A in DW-seedlings was relatively higher than CW-seedlings (significant on day 12 only). On day 8, water stress depressed A across all embryo pre-treatments (significant for F-seedlings only). Within the stressed treatments, A was highest in FS-seedlings and lowest in CS-seedlings (not significant). With re-watering (i.e. day 12), A in stressed treatments did not recover to levels comparable with their respective unstressed controls.

Within the unstressed treatments, the ratio of A at C_a: 600 μmol mol^{-1}:A at C_a: 400 μmol mol^{-1} (A@600:A@400), and hence the degree of stomatal limitation of A, did not differ significantly across embryo pre-treatments throughout the experimental period (Table 3). However, A@600:A@400 in FS-seedlings was significantly higher than DS- and CS-seedlings on days 8 and 12, respectively. Interestingly, A@600:A@400 in FS-seedlings was also significantly greater than FW-seedlings on days 8 and 12.

Chlorophyll fluorescence

On days 0 and 8, potential photochemical efficiency (F_v/F_m) did not differ significantly with embryo pre-treatment in unstressed material (Fig. 9). On day 8 and

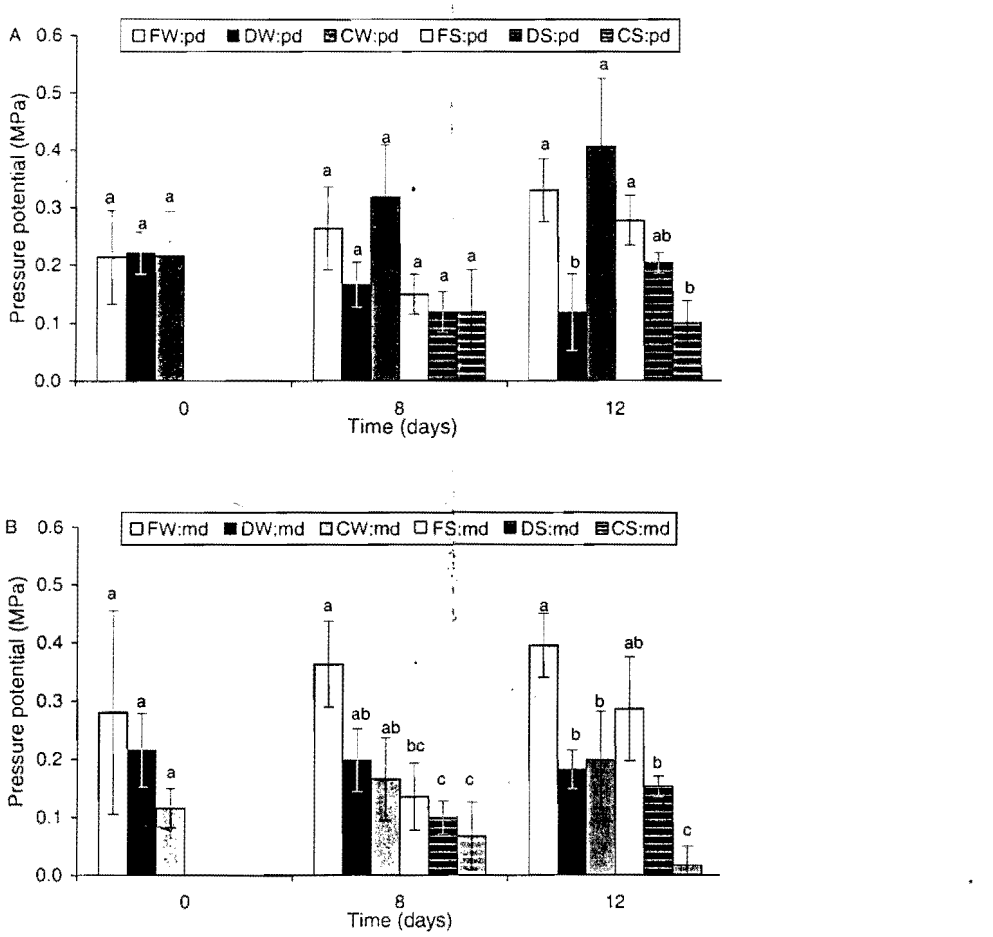

Fig. 6. (A) Predawn (pd) and (B) midday (md) leaf pressure potential of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Columns labeled with different letters are significantly different when compared within experimental days ($P < 0.01$ for embryo, stress and embryo × stress, ANOVA, $n = 4$). Bars represent ±SD.

after re-watering (i.e. day 12), F_v/F_m in CS-seedlings was significantly lower than CW-seedlings and DS- and FS-seedlings.

Ex vitro seedling mortality

In the stressed treatments, ex vitro seedling mortality was higher than in their respective unstressed controls (Table 4). Within the stressed treatments, mortality was highest in CS-seedlings and lowest in FS-seedlings. Mortality in DS-seedlings was only slightly lower than CS-seedlings, whereas within the unstressed treatments mortality in DW- and CW-seedlings was marginally higher than FW-seedlings.

Discussion

There are differences among species and explants in growth and morphological responses of recovered plantlets or seedlings following cryopreservation, with some studies showing no effects, whereas others have demonstrated morphological abnormalities and reduced development rates (see Introduction for details). Of the studies cited, there are a few in which plants (of mainly crop species) derived from cryopreserved germplasm have been grown in the field for a long period (e.g. Côte et al. 2000, Martínez-Montero et al. 2002, Konan et al. 2007). However, those studies were almost always based on phenotypic descriptions and there are presently few, if any published reports on the physiological performance of seedlings or plantlets recovered from cryopreserved material. The present contribution reports on the growth and physiological performance of seedlings derived from cryopreserved ZEs of the wild species *A. belladonna*. The ultimate aim of cryopreservation of the germplasm of endangered wild species is to re-introduce plantlets or seedlings back into the wild. Under such conditions, the

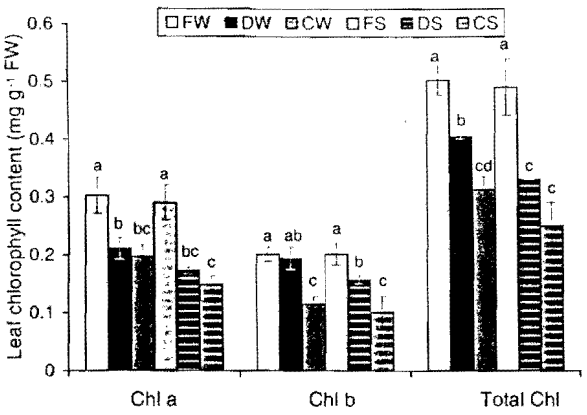

Fig. 7. Leaf chlorophyll content of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Chlorophyll content was measured only at the end of the ex vitro growth period. Columns labeled with different letters are significantly different when compared within categories ($P < 0.01$ for embryo and stress, ANOVA, $n = 4$). Bars represent ±SD.

seedlings are unlikely to be carefully tended after planting and will be subject to the vagaries of the weather, and so the vigor of such seedlings is important; this was assessed by observing the response of such seedlings to a water stress.

Growth characteristics

Phenotypic variation in in vitro recovery times, plant heights and modes of regeneration in plants recovered from cryopreserved germplasm have been previously reported (Harding and Benson 1994, Harding 1996). Although seedlings recovered from cryopreserved orthodox EAs have been reported to be similar to those generated from partially dehydrated and control axes, going on to produce flowers and fruit in vivo (Gagliardi et al., 2002), Steinmacher et al. (2007) showed partial dehydration and cryopreservation of recalcitrant peach palm ZEs to result in plantlets with significantly lower plant height and deficient haustorium development. In the case of excised ZEs of *A. belladonna*, both partial drying alone, and drying followed by exposure to cryogenic temperatures, reduced dry matter accumulation and partitioning to roots during the in vitro growth phase, relative to untreated embryos (Fig. 1). The final biomass achieved during the ex vitro growth period (Fig. 2A) could be a consequence of either or both ex vitro growth rates or the amount of material at the start of this growth phase. To assess this, RGRs (relative to the dry mass at the end of the in vitro growth period) were calculated. The adverse effect of embryo partial drying observed during the in vitro growth phase was reversible during subsequent ex vitro growth, but the adverse effect of exposure to cryogenic temperatures as well, was carried through to early ex vitro growth (Table 1). However, a water stress during this period dominated over the effects of embryo pre-treatment and RGRs within the stressed treatments did not differ significantly with embryo pre-treatment. This lack of response to the stress by C-seedlings, in terms of RGR, is probably because the combination of partial dehydration and cryopreservation impaired their ability to acquire resources to such an extent that a withdrawal of resources (i.e. water) had no effect on overall performance.

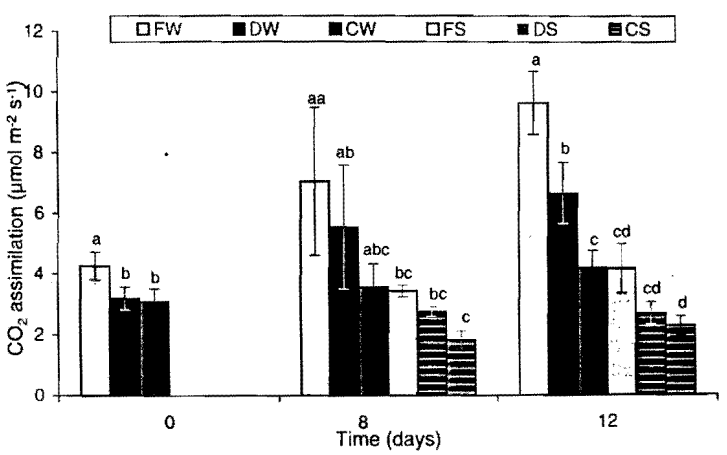

Fig. 8. Instantaneous leaf-based CO_2-assimilation rates (at C_a: 400 µmol mol^{-1}) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Columns labeled with different letters are significantly different when compared within experimental days ($P < 0.01$ for embryo on days 0 and 12 and $P = 0.01$ on day 8; $P = 0.04$ for stress and $P = 0.01$ for embryo × stress on day 8, ANOVA, $n = 7$). Bars represent ±SD.

Table 3. Ratio of CO_2-assimilation rate at C_a: 600 μmol mol^{-1}; CO_2-assimilation rate at C_a: 400 μmol mol^{-1}. Ratio was calculated for seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Values followed by different letters are significantly different when compared within a measurement day ($P < 0.01$ for embryo on days 8 and 12 and for stress and embryo × stress on day 12; $P < 0.05$ for stress and embryo × stress on day 8, ANOVA). Values represent mean ±SD (n = 7). NA, not applicable.

Day	FW	DW	CW	FS	DS	CS
0	2.29 ± 0.60^a	2.11 ± 0.55^a	1.85 ± 0.35^a	NA	NA	NA
8	1.68 ± 0.44^b	1.66 ± 0.70^b	1.81 ± 0.35^b	2.48 ± 0.23^a	1.57 ± 0.15^b	1.76 ± 0.47^b
12	1.68 ± 0.10^b	1.61 ± 0.17^b	1.51 ± 0.15^b	2.36 ± 0.53^a	1.66 ± 0.45^b	1.43 ± 0.20^b

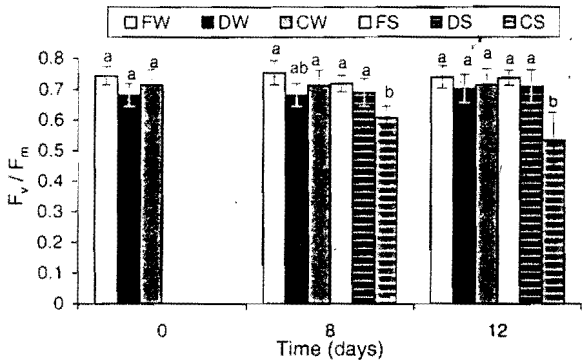

Fig. 9. Potential photochemical efficiency (F_v/F_m) of seedlings recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). Columns labeled with different letters are significantly different when compared within experimental days ($P < 0.05$ for stress and embryo × stress on days 8 and 12 and $P < 0.01$ for embryo on day 8, ANOVA, $n = 5$). Bars represent ±SD.

Not only did partial drying and cryopreservation affect partitioning of biomass to roots (Fig. 2B), but they also resulted in abnormal roots (Fig. 3). These phenotypic and morphological responses could be expected to reduce the abilities of plants derived from D- and C-embryos to acquire water, particularly under water-limited conditions. In addition, unlike FS- and DS-seedlings, CS-seedlings failed to significantly increase biomass partitioning to roots relative to their unstressed control.

Plant water relations

In unstressed seedlings, there were no significant differences in the measured water relations parameters between F- and D-seedlings (except for Ψ_p), indicating that the effects of partial drying of excised ZEs was reversible in the long term (Figs 4–6). C-seedlings had lower pd Ψ_w and did not develop lower md Ψ_w, indicating that little transpiration was occurring, in keeping with the reduced partitioning of biomass to roots and the observed root abnormalities. The application of a water stress gave rise to responses that would typically be expected (see Hsiao 1973). There were declines in both pd Ψ_w and pd–md Ψ_w (Fig. 4), and an indication of long-term osmotic adjustment, reflected in a decrease in Ψ_s in both F- and D-seedlings relative to well watered controls (Fig. 5). There also appeared to be some diurnal osmotic adjustment with md Ψ_s being lower than pd Ψ_s, maintaining positive (although reduced) Ψ_p (Fig. 6), permitting some transpiration and hence positive pd–md Ψ_w. C-seedlings demonstrated further reductions in Ψ_w and Ψ_s, but no diurnal osmotic adjustment. Permanent wilting was observed in CS-seedlings (Fig. 6): cell plasmolysis and membrane rupture are associated with permanent leaf wilting (Leopold et al. 1981, Galmés et al. 2007), and the decrease in Ψ_s observed in CS-seedlings on day 8 (Fig. 5) may have been a consequence of tissue dehydration, rather than osmotic adjustment. By 3 days after re-watering, there was no recovery in pd Ψ_w in any of the stressed treatments and no development of substantial differences between pd and md Ψ_w. Positive turgor is a requirement for cell expansion and the generally lower values of pd Ψ_p for stressed seedlings, although not significant using a factorial ANOVA design, are consistent with the lower RGRs measured. Similarly, the generally lower values of pd–md Ψ_w of stressed seedlings are also consistent with the reduced RGR (Table 1) observed for these seedlings.

Table 4. Total ex vitro seedling mortality (%) over the entire ex vitro growth period. Seedlings were recovered from fresh (F), partially dried (D) and cryopreserved (C) ZEs, subjected to watering (W) or water deficit (S). $P < 0.05$ when values were compared across treatments (null-model chi-squared test, n = 87).

Treatment	FW	DW	CW	FS	DS	CS
Mortality (%)	5	11	9	26	41	48

Photosynthetic characteristics

Although not always statistically significant (day 8), there was a consistent trend of D- and C-seedlings having lower A than F-seedlings (Fig. 8), possibly because of lower leaf Chl content compared with F-seedlings (Fig. 7). The significantly lower stomatal density of leaves of C-seedlings, compared with F- and D-seedlings (Table 2), may have also contributed to the relatively

lower A in C-seedlings, by reducing the influx of CO_2 for photosynthesis.

A stress-induced reduction in A, as observed across all embryo pre-treatments here (Fig. 8), can be a consequence of restriction of CO_2 supply, through reduction in stomatal conductance, or of the rate of mesophyll processes (these could be the result of either damage or a controlled down-regulation of the biochemical and/or photochemical components of CO_2 fixation) (see McDowell et al. 2008). Except for one report of photosynthetic electron transport being strongly inhibited in freeze-treated *Bratonia* protocorms, examined immediately after thawing (Bukhov et al. 2006), there is little by way of studies on the photosynthetic characteristics of plants recovered from cryopreserved germplasm. Bukhov et al. (2006) suggested that in *Bratonia* protocorms freeze–thawing caused partial disorders in linear electron transport between PSII and photosystem I (PSI), with the functional interactions among carriers in the electron-transport chain being disturbed between the plastiquinone pool and the PSI reaction center, resulting in a reduction in photosynthetic capacity during in vitro culture.

In the case of *A. belladonna* the ratio A@600:A@400, taken as a measure of stomatal limitation, did not differ among embryo pre-treatments in unstressed plants (Table 3), suggesting that differences in A were not a consequence of stomatal limitation (despite the reduced stomatal density of C-seedlings). When subjected to a water stress, stomatal limitation was greater in F-seedlings (A@600:A@400 was higher for FS-than FW-seedlings) than in D- and C-seedlings (A@600:A@400 was similar for DS- and DW-seedlings and for CS- and CW-seedlings). These data suggest that the reduction in A experienced by D- and C-seedlings was, to some degree, a consequence of damage to the photosynthetic machinery. Consistent with this, leaf Chl content was lower in D- and C-seedlings than F-seedlings, and F_v/F_m was significantly reduced in CS-seedlings.

As was the case with leaf water potential, within 3 days of re-watering none of F_v/F_m, leaf Chl or A recovered to levels similar to their respective unstressed control values, but recovery from a water stress can be slow, from days to weeks (Flexas et al. 2006).

Seedling mortality

Steinmacher et al. (2007) showed ex vitro mortality in seedlings generated from cryopreserved recalcitrant ZEs to be almost 50% higher than seedlings recovered from fresh ZEs. Similarly, in the present study ex vitro seedling mortality within the unstressed treatments was slightly higher in DW- and CW- than FW-seedlings (Table 4). Water stress led to higher mortality in all embryo pre-treatments (Table 3), being more severe in DS- and CS-seedlings (Table 4). Seedling mortality was highest in CS-seedlings, possibly because of the combination of insufficient adjustment of Ψ_w to meet transpirational demands (Fig. 4), a failure to adopt growth patterns that reduce transpirational water loss, exposure to significantly lower pd Ψ_w than DS- and FS-seedlings (Fig. 4), the production of abnormal roots (Fig. 3), and the onset of permanent leaf wilting (Fig. 6), all of which promote hydraulic failure (see McDowell et al. 2008) in juvenile plants (e.g. Hsiao 1973). The signs of hydraulic failure were not as pronounced in DS-seedlings but, D-seedlings, like C-seedlings exhibited abnormalities in root morphology (Fig. 3) and when stressed incurred damage to the photosynthetic machinery (Fig. 8; Table 3) and developed relatively lower Ψ_w and Ψ_p than FS-seedlings (Figs 4 and 6, respectively).

Concluding remarks

Partial dehydration and cryopreservation of recalcitrant *A. belladonna* ZEs can compromise vigor and drought tolerance of recovered seedlings. The effect of partial drying alone did appear to be reversible (in terms of RGR) within the time-frame of this study, and so an extended period of ex vitro acclimatisation before re-introduction of such seedlings into the wild may alleviate this 'stress hangover'; but this remains to be tested. Cryopreservation studies involving explants other than recalcitrant ZEs 'indicate a clear "consensus" for plants displaying morphological normality after cryopreservation' (reviewed by Harding 2004) but in seedlings recovered from cryopreserved recalcitrant ZEs/EAs, the retention of 'morphological normality' may not necessarily be equated to stress-related physiological responses. The results of this study highlight the need to investigate the potential impacts of cryoinjury on the genome, transcriptome, proteome and metabolome of recovered plants (see Harding and Benson 1994, Harding et al. 2009), which in disrupting established patterns of growth and reproduction, may impact on the re-introduction of such plants into natural environments.

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