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Factors affecting stress tolerance in recalcitrant embryonic axes from seeds of four *Quercus* (Fagaceae) species native to the USA or China

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• **Background and Aims** *Quercus* species are often considered 'foundation' components of several temperate and/or subtropical forest ecosystems. However, the populations of some species are declining and there is considerable urgency to develop *ex situ* conservation strategies. In this study, the storage physiology of seeds within *Quercus* was explored in order to determine factors that affect survival during cryopreservation and to provide a quantitative assessment of seed recalcitrance to support future studies of this complex trait.
• **Methods** Water relations and survival of excised axes in response to water loss and cryo-exposure were compared for four *Quercus* species from subtropical China (*Q. franchetii*, *Q. schottkyana*) and temperate USA (*Q. gambelii*, *Q. rubra*).
• **Key Results** Seed tissues initially had high water contents and water potentials. Desiccation tolerance of the embryonic axis was not correlated with the post-shedding rainfall patterns where the samples originated. Instead, higher desiccation tolerance was observed in samples growing in areas with colder winters. Survival following cryo-exposure correlated with desiccation tolerance. Among species, plumule tissues were more sensitive than radicles to excision, desiccation and cryo-exposure, and this led to a higher proportion of abnormally developing embryos during recovery following stress.
• **Conclusions** *Quercus* species adapted to arid and semi-humid climates still produce recalcitrant seeds. The ability to avoid freezing rather than drought may be a more important selection factor to increase desiccation tolerance. Cryopreservation of recalcitrant germplasm from temperate species is currently feasible, whilst additional protective treatments are needed for *ex situ* conservation of *Quercus* from tropical and subtropical areas.

Key words: Seed desiccation tolerance, recalcitrance, embryonic axis, critical moisture content, cryopreservation, oak, *Cyclobalanopsis*, subtropical, plumule, *Quercus gambelii*, *Quercus rubra*, *Quercus franchetii*, *Quercus schottkyana*.

INTRODUCTION

Seeds are broadly classified as 'recalcitrant' and 'orthodox' based on their tolerance of desiccation. Desiccation tolerance has often been considered a qualitative feature of the species, with seeds either perishing or surviving after some level of drying (for example to ambient relative humidity). However, we know that recalcitrant seeds exhibit a range of tolerances to desiccation (Walters and Koster, 2007; Berjak and Pammenter, 2008) and that the extent of tolerated water loss depends on the intensity and duration of drying, the method and tissues used to assess survival, seed source, handling procedures and a host of other factors. The complexity of the incidence and measurement of seed recalcitrance impedes our abilities to probe it and to ultimately understand the mechanisms of damage or protection that contribute to variation in post-harvest physiology among all seeds. Comparative studies of the genetic regulation and ecological significance of the complex trait of seed recalcitrance will require reliable, quantitative assessments of the seed recalcitrance phenotype. Future studies will be expedited by demonstrated and repeatable phenotypic variation among related species or populations distributed across a range of climates.

Habitat or climate characteristics appear to contribute to variation in seed desiccation tolerance among populations. For example, *Coffea* species adapted to longer dry seasons tended to survive to lower water contents (Dussert *et al.*, 2000; Eira *et al.*, 2006). Desiccation tolerance in seeds of *Quercus petraea*, *Aesculus hippocastanum*, *Acer pseudoplatanus*, *Zizania palustris* and *Camellia sinensis* was related to duration of the growing season, which likely influences embryo maturity (Berjak *et al.*, 1993; Vertucci *et al.*, 1994*a*; Daws *et al.*, 2004, 2006; Daws and Jensen, 2011). Seeds of tropical rainforest species are reputed to be highly recalcitrant, despite the long growing season, because adaptations for seed desiccation tolerance were lost as species evolved in climates without seasonal drought or freezing temperatures (Farnsworth, 2000). Collectively, past research presents an apparent paradox about the evolution of seed recalcitrance in that recalcitrance may be favoured in areas without winter freezing temperatures as well as areas with short growing seasons bracketed by freezing winter temperatures.

The genus *Quercus* (Fagaceae) may be an ideal study system to explore the seed recalcitrance trait and to provide a set of carefully described, closely related species with known variation in seed physiology in anticipation of future genomic and ecological studies. Over 450 species of *Quercus* trees or shrubs are broadly distributed across the Northern Hemisphere in habitats ranging from temperate and tropical forests to semi-deserts and arid environments (Nixon, 1993, 1997; Huang *et al.*, 1999). *Quercus* species

are often considered a 'foundation' component of the ecosystems they inhabit (Ellison *et al.*, 2005), yet population sizes are declining as a result of disease, habitat loss or climate change. Hence, there is considerable urgency to develop *ex situ* conservation strategies to preserve the genetic diversity extant in wild *Quercus* populations (Kueppers *et al.*, 2005; Tyler *et al.*, 2006; Li and Pritchard, 2009).

The known recalcitrant physiology of seeds from several *Quercus* species complicates *ex situ* conservation efforts (Pritchard, 1991; Pence, 1992; González-Benito and Martín, 2002; González-Benito *et al.*, 2002; Fernandes *et al.*, 2008; Chmielarz *et al.*, 2011; Ganatsas and Tsakaldimi, 2013; Walters *et al.*, 2013). Because of their relatively high water content, recalcitrant seeds are susceptible to lethal damage during conventional freezer storage (Li and Pritchard, 2009; Walters *et al.*, 2013). Many efforts to cryopreserve recalcitrant seeds focus on rapid drying of excised embryonic axes followed by rapid cooling (Wesley-Smith *et al.*, 1992, 2001, 2004; Walters *et al.*, 2008). A target water content of ~0·25 g H_2O^{-1} (dry mass – lipid mass) is sufficiently dry to reduce the probability of lethal ice formation in cells that are cooled to liquid nitrogen temperatures at rates faster than ~10 °C min^{-1} (0·167 °C s^{-1}) (Vertucci, 1989; Pence, 1992; Wesley-Smith *et al.*, 1992, 2001, 2004, 2014). For this reason, a water content of 0·25 g H_2O g^{-1} is often considered a benchmark for cryopreservability and water at lower water contents is often described as unfreezable (Wolfe *et al.*, 2002).

Cryopreservation protocols using these principles are available for *Q. robur*; however, these methods generally result in high survival and normal root growth, but low recovery of the plumule (Chmielarz *et al.*, 2011). High sensitivity of plumule compared with radicle tissues has been noted in several species (Pritchard *et al.*, 1995; Wesley-Smith *et al.*, 2014) and is marked by deranged intracellular organization in shoot tips upon thawing (Berjak *et al.*, 1999). Differences in radicle and plumule responses to drying and cryoexposure may arise from differences in drying rates (and so different water contents during cryoexposure) or differences in sensitivity to desiccation or desiccation–low temperature interactions.

Here, we used *Quercus* as a study system to investigate factors presumed to influence the storage physiology of recalcitrant seeds and survival during cryoexposure. We tested the hypothesis that species originating from environments with low rainfall produced seeds with relatively greater tolerance to desiccation. We also compared water relations and survival of radicle and plumule tissues to test whether poor growth of shoots after cryoexposure could be attributed to greater sensitivity to desiccation or to greater susceptibility to lethal ice formation. Desiccation tolerance was quantified by the water content at which survival was negatively impacted (i.e. damaging water content) or by the drying time at which decreased viability was observed. This quantitative treatment allowed us also to test the hypothesis that desiccation tolerance correlates with survival following cryoexposure.

MATERIALS AND METHODS

Plant materials and viability assays

Our study considered four species of *Quercus* that have been classified in different subgenera. Species native to US temperate areas are members of subgenus *Quercus: Q. gambelii* (white oaks, section *Quercus*) and *Q. rubra* (black oaks, section *Lobatae*). Species native to China are members of subgenera *Quercus* [*Q. franchetii* (section *Cerris*)] or *Cyclobalanopsis* (*Q. schottkyana*, which is grouped with the Asian evergreen broad-leaf oaks) (Table 1). The US oaks are from desert (*Q. gambelii*) and mesic (*Q. rubra*) environments and the Chinese oaks are from subtropical semi-humid areas. Two populations *of Q. gambelii*, one from Wyoming (WY) and another from Nevada (NV), were studied to provide a comparison between groups with and without subfreezing winter temperatures (Table 1). Mature fruits were collected and stored at 5 °C in plastic bags, punctured to provide ventilation.

Most studies used excised embryonic axes. The excision procedure may induce a wounding response that complicates interpretation of water-stress-induced responses. Freshly excised axes were kept moist on blotter paper containing citric and ascorbic acid (0·2 g L^{-1}) solution until sufficient numbers were accumulated for study (~2 h). Viability of excised axes was assessed by germination *in vitro*. Axes were surface-sterilized in 1 % sodium hypochlorite for 10 min, rinsed twice in sterile water, transferred to Woody Plant Medium with 0·3 % charcoal and kept in darkness under room conditions for 48 h. After this initial period, plates were transferred to 20 °C (*Q. franchetii*, *Q. gambelii* and *Q. rubra*) or 25 °C (*Q. schottkyana*) with a light/dark photoperiod of 16/8 h. After 2 and 4 weeks we noted whether axes had expanded, greened, formed callus or showed normal development of roots and shoots, considered as doubling

TABLE 1. *Collection and climate information for* Quercus *seed lots studied. Mean minimum and maximum temperature and total rainfall are reported for October to February, months between shedding and germination for these species. Climate data for USA locations (1961–90) and Chinese locations (1971–2000) were obtained from the World Wide Information Service (http://www.worldweather.org/093/c00781.htm) and Climatic Data Centre (http://cdc.cma.gov.cn/), respectively*

Species	Collection location	Location altitude (m)	Latitude and longitude	Collection date	Minimum temperature (°C)	Maximum temperature (°C)	Rainfall (mm)
Q. franchetii	Kunming, China	1900	25°01′ N, 102°41′ E	10 October 2012	5·64	17·14	164·4
Q. schottkyana	Kunming, China	1900	25°01′ N, 102°41′ E	10 October 2012	5·64	17·14	164·4
Q. gambelii (NV)	Kyle Canyon, Spring Mountains, NV, USA	2075	36°15′ N, 115°36′ W	3 October 2012	4·8	18·6	50·3
Q. gambelii (WY)	Crook County, WY, USA	1646–1890	44°59′ N, 104°56′ W	15 September 2012	−7·06	6·3	77·1
Q. rubra	Delta and Schoolcraft Counties, MI, USA	183–268	45°44′ N, 87°3′ W and 42°6 N′, 85°38′ W	8 October 2012	−8·52	0·52	348·5

of radicle length or greening of shoots. A normal seedling exhibited both root and shoot development. Drying and cryoexposure treatments used aliquots of ten axes each and treatments were repeated on two or three separate days; proportion data were pooled to give sample sizes of 20–30 axes according to Crawley (2007).

Dehydration treatments and pressure–volume relationships

Embryos were dried rapidly over a stream of nitrogen gas (i.e. 'flash drying') for different durations (0–1400 min) to adjust water content. Fresh and dry masses were measured on three to five individual embryos at each drying interval. In some treatments, the axis was bisected at the cotyledonary node so that the water contents of the plumule and radicle could be determined separately. Sample dry mass was assessed after heating tissues at 90 °C for 4 d. Water content (wc) was expressed on a dry mass basis. Drying time course curves were calculated from the relationship of time versus ln(Δwc), where Δwc was wc at time t – final wc, which was determined to be ~0·05 g water g^{-1} dry mass under ambient room conditions (30 % RH) in Fort Collins. The r^2 of these regressions was usually >0·85.

The relationship between water content and water potential was measured for embryonic axes, radicles, plumules and cotyledons using pressure–volume relationships. Plant materials were soaked in polyethylene glycol (PEG, MW 8000) solutions of concentrations between 0·1 and 1·15 g PEG g^{-1} water at room temperature for 2 d. Water contents were measured from fresh and dry mass assessments as described in the previous paragraph. Water potentials of PEG solutions were calculated according to Michel (1983) and verified using a thermocouple psychrometer (Decagon, Pullman, WA, USA). The accuracy of water content measurements for axes soaked in the most concentrated PEG solutions was verified with comparable water potential treatments delivered using saturated salt solutions to control RH. The pressure–volume curves were modelled from linear regressions of water content and ln(water potential) calculated separately for water potentials above and below −6 MPa. All relationships gave $r^2 > 0{\cdot}94$.

Tolerance of desiccation and cryoexposure

Desiccation tolerance of embryonic axes was assessed by electrolyte leakage from reimbibing axes and survival and growth following a drying challenge. Axes were flash-dried for different durations and then slowly rehydrated on damp blotter paper for 1 h. These prehydrated embryos were soaked in 2 ml of distilled water and electrical conductivity was measured every 5 min for 1 h using an ASAC 1000 (Applied Intelligence Systems, Neogen, Lansing, MI, USA) conductivity meter. The slope of the imbibition time versus conductivity relationship was used to compare treatments and species. Measurements represented the average of five replicates, each replicate consisting of two axes.

After the 1 h soak, embryos were surface-sterilized and placed on medium (described above) to assess survival and growth.

Embryos dried to non-lethal water contents were also challenged by exposure to liquid nitrogen using five cooling methods: (1) bare axes injected individually into N_2 slush (~−210° C, formed by pulling a vacuum over liquid nitrogen) using a spring-loaded desoldering pump (Elecon, Vallabh Vidyanagar, India) with axes affixed to pins with a drop of glycerol; (2) axes enclosed in 27 mg lightweight aluminium foil packets (2 cm × 3 cm) (two axes per packet) and forcibly plunged into N_2 slush with pre-cooled forceps; (3) axes enclosed in 1·2 ml screw-cap polypropylene cryovials (ten axes per cryovial) and submerged into liquid nitrogen; (4) the same cryovial treatment placed in vapour above liquid nitrogen (−150 °C); and (5) the same treatment as (4) except cryovials were placed in a Styrofoam box packed with vermiculite for insulation and the box was placed in vapour above liquid nitrogen. Temperature within the core of the axis was measured in a subset of axes by impaling them with fine-gauge (0·075 mm × 0·12 mm) bare

TABLE 2. *Dry mass and water status of embryonic tissues of* Quercus *acorns prior to experimentation. Values are mean ± s.d. (n > 5). Water potentials were calculated from the pressure–volume curves in Fig. 1. Pericarp dry mass was ~40 % of cotyledon dry mass among all species and pericarp water content was ~0·11 and 0·41 g g^{-1} for species from China and the USA, respectively. Arrows under the cotyledon water potential represent the direction of water flow from cotyledon to axis (→) or from axis to cotyledon (←)*

	Dry mass (mg)			Water content (g H_2O g^{-1} dry weight)			Water potential (MPa)		
Species	Cotyledons	Axis	Radicle and plumule	Cotyledons	Axis	Radicle and plumule	Cotyledons	Axis	Radicle and plumule
Q. franchetii	580 ± 50	2·47 ± 0·51	0·99 ± 0·24	0·80 ± 0·15	0·95 ± 0·02	1·06 ± 0·01	−2·3	−2·9[a]	−3·5[b]
			1·48 ± 0·51			0·88 ± 0·03	→	−3·4[b]	−3·4[b]
Q. schottkyana	530 ± 100	1·42 ± 0·31	0·22 ± 0·07	0·66 ± 0·04	1·06 ± 0·04	1·17 ± 0·03	−2·1	−2·8	−2·6
			1·20 ± 0·31			1·03 ± 0·04	→		−2·7
Q. gambelii	1130 ± 230	2·34 ± 1·32	nd	0·77 ± 0·05	1·20 ± 0·13	nd	−2·7	−3·1	nd
(NV)			nd			nd	→		nd
Q. gambelii	710 ± 370	2·38 ± 0·91	0·51 ± 0·23	0·85 ± 0·05	1·36 ± 0·14	1·34 ± 0·12	−3·2	−2·9	−3·0
(WY)			1·87 ± 0·81			1·36 ± 0·15	←		−3·0
Q. rubra	1890 ± 540	4·13 ± 1·01	1·82 ± 0·52	0·69 ± 0·04	1·18 ± 0·12	1·23 ± 0·08	−3·2	−2·7	−2·6
			2·52 ± 0·98			1·14 ± 0·18	←		−2·6

[a]Measurement taken in November 2012.
[b]Measurement taken in March 2013.
nd, not determined.

wire, type T thermocouples (Omega Engineering, Stamford, CT, USA). Temperature was recorded at millisecond intervals for fully hydrated axes of all species. Additional experiments were conducted using *Q. rubra* (the largest embryonic axis) to measure the effect of water content on cooling rate. Axes were flash-dried for 0, 45 and 120 min to obtain a range of water contents. Cooling rates were calculated from the linear regression of temperature and time for the temperature ranges between 5 and −10, −40 and −150 °C. Cooling rates for the narrower (i.e. higher) temperature ranges were slower, probably as a result of higher heat capacity. Only cooling rates between 5 and −40 °C are reported in this study.

Axes were held at liquid nitrogen temperatures for 18 h prior to viability assessments. Axes were warmed rapidly by immersing them in a solution heated to 42 °C (Wesley-Smith *et al.*, 2014); we used a 0·5 M sucrose solution to thaw axes.

Statistics

Statistical analyses were performed using R statistical software (R Development Core Team, 2014). Water content data were pooled within species for each drying time and differences in drying rate among species or tissue types were assessed by comparison of slopes calculated for water contents >0·15 g g^{-1}. Cooling rates for at least five axes per species and moisture level were calculated from linear regressions of time versus temperature between 5 and −40 °C and fresh mass effects on cooling rate were assessed by analysis of variance.

Survival and root and shoot development were treated as proportion data and binomial distributions were used to calculate error (Crawley, 2007). Desiccation tolerance was inferred by damaging water contents that marked the points of deviation from damaged to non-damaged physiology (Pammenter *et al.*, 1991; Farrant and Walters, 1998). We calculated the damaging water content from the intersection of two lines: one line was the lower limit of the confidence interval for unaffected viability (a horizontal line drawn at mean survival −1·5 × the standard deviation of the mean) and the other line was the regressed relationship between water content and survival at water contents giving survival below the confidence interval for undamaged physiology. The 90 % uncertainty range for damaging water content was calculated from the intersection of lines drawn from calculated slopes ± the standard error of the slopes (i.e. the 90 % confidence intervals of regression lines). We also tested the feasibility of using the R library function for dose–response to calculate the water content or drying time giving a 25 % reduction in viability or growth (LD75).

RESULTS

Size, water status and viability of axes prior to drying and cooling challenges

Acorns from different sites and species varied in size (Table 2), species from the USA tending to be larger or more variable than species from China. Embryonic axes constituted between 0·12 and 0·33 % of total dry mass of the cotyledons. Upon receipt at the National Center for Genetic Resources Preservation (NCGRP), the water content of the cotyledons and embryonic axes ranged from 0·66 to 0·85 g g^{-1} and from 1·36 to 0·95 g g^{-1}, respectively, with *Q. gambelii* from Wyoming having the highest water content and species collected from China tending to be drier than US species.

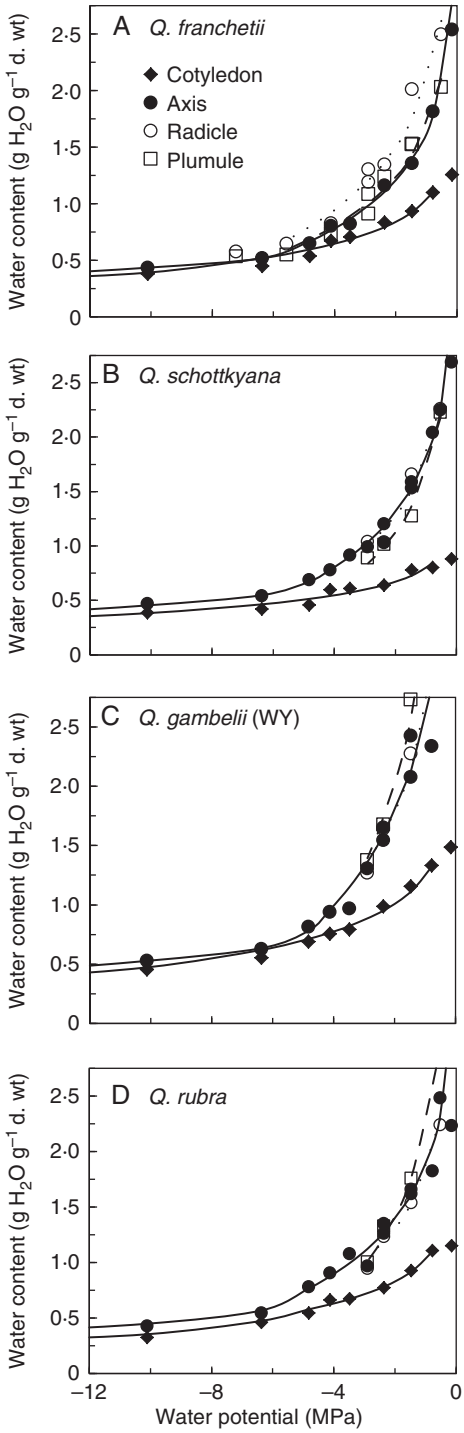

FIG. 1. Pressure–volume relationships of samples of *Quercus* embryonic axes (solid circles), axis parts (open squares, plumules; open circles, radicles) and cotyledon pieces (solid diamonds) osmotically dried using polyethylene glycol (PEG, MW 8000) solutions of differing concentrations. Curves were calculated from separate regressions of water content and ln(water potential) for water potentials above and below −6 MPa, all relationships having $r^2 > 0.94$. Pressure–volume relationships quantified in these curves were used to convert measured water content into water potential.

TABLE 3. *Initial viability and growth of embryonic axes, radicles and plumules of* Quercus *species and coefficients of drying rates modelled in Fig. 2. Percentage viability of freshly excised axes was assessed by general appearance and growth of the root, shoot or both (i.e. a normal seedling), and the standard deviation, calculated from binomial error distribution, is also indicated. Incidence of root growth compared with shoot growth was tested by a Tukey multiple comparisons of means test, with superscripted letters indicating significance at the* $P < 0.01$ *level. The drying rate model was calculated from the linear relationship between drying time* t *and ln(water content at* t *– 0·05 g* H_2O g^{-1} *dry weight). The intercept of the model was constrained by the average maximum water content for the tissue (given below); this value corresponds to the water content of excised axes that have rested on damp blotter paper for 2–3 h prior to drying treatments. The slope of the model is the rate coefficient and the standard error of the coefficient is provided after the* ± *symbol. Letter superscripts indicate significant differences among species for shoot growth or axis drying rate (*$P < 0.05$*) and asterisks represent significant differences between plumule and radicle as indicated*

Species	Initial viability ((%)				Maximum water content (g H_2O g^{-1} dry weight)			Drying rate coefficient (min^{-1})		
	Expansion and greening	Root or shoot growth	Shoot growth	Normal seedling	Axis	Radicle	Plumule	Axis	Radicle	Plumule
Q. franchetii	86·3 ± 5·5	81·5 ± 7·4	33·3 ± 3·3^{b}	33·3 ± 3·3	2·204 ± 0·560	2·112 ± 0·078	1·765 ± 0·155**	0·0196 ± 0·0013^{a}	0·0208 ± 0·0021	0·0265 ± 0·0022**
Q. schottkyana	95·5 ± 5·9	94·1 ± 6·8	88·8 ± 9·1^{a}	79·4 ± 21·3	1·588 ± 0·476	1·528 ± 0·136	1·341 ± 0·227	0·0177 ± 0·0011^{a}	0·0179 ± 0·0014	0·0253 ± 0·0025***
Q. gambelii (NV)	97·4 ± 4·4	95·7 ± 7·4	80·9 ± 11·7^{a}	80·9 ± 11·7	1·834 ± 0·472	nd	nd	0·0078 ± 0·0006^{b}	nd	nd
Q. gambelii (WY)	81·7 ± 2·4	72·5 ± 3·5	2·4 ± 4·8^{b}	2·4 ± 4·8	2·443 ± 1·045	2·366 ± 0·998	2·654 ± 1·151	0·0078 ± 0·0007^{b}	0·0063 ± 0·0003	0·0101 ± 0·0012**
Q. rubra	98·7 ± 2·6	98·7 ± 2·6	41·7 ± 19·8^{b}	41·7 ± 19·8	1·426 ± 0·585	3·006 ± 0·380	2·382 ± 0·419	0·0274 ± 0·0023^{a}	0·0297 ± 0·0042	0·0323 ± 0·0036^{ns}

Significance of differences between radicle and plumule drying rates: ns, not significant; $^{**}P < 0.05$; $^{***}P < 0.01$.
nd, not determined.

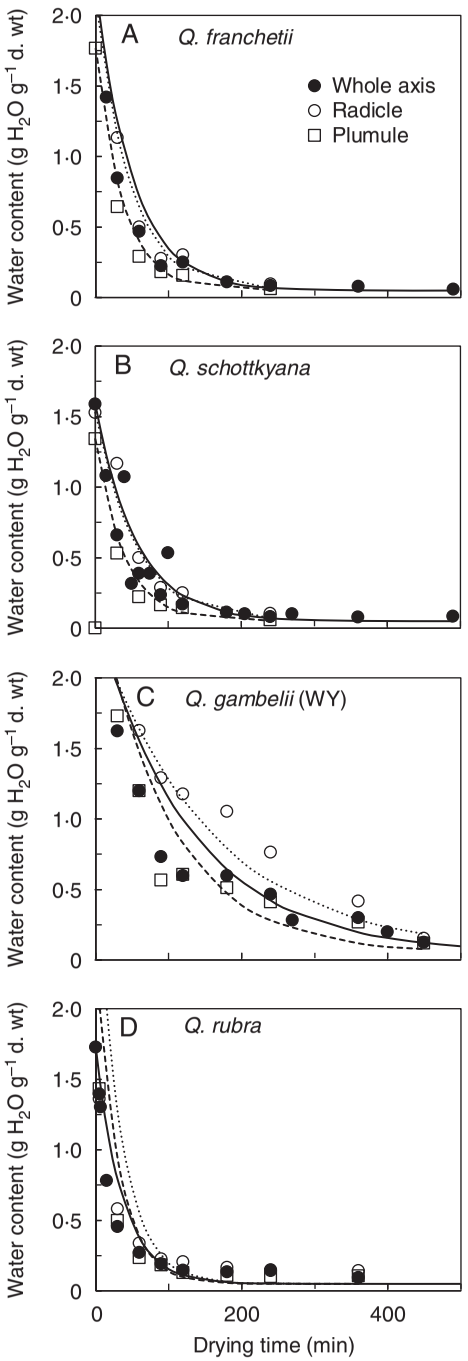

FIG. 2. Drying time courses for samples of *Quercus* embryonic axes (solid circles) and axis parts (open squares, plumules; open circles, radicles). Excised axes were dried over a stream of nitrogen and divided above and below the cotyledonary node to assess drying rate at different terminals. Curves were calculated from the linear relationship between ln(water content – 0·05) and drying time with the intercept constrained to ln(maximum water content) as given in Table 3 ($r^2 > 0.9$).

Water potential (Ψ_w) corresponding to the initial water content of cotyledons and axes was interpolated from pressure–volume relationships (Fig. 1), and ranged from −2·1 to −3·2 MPa and from −2·7 to −3·1 MPa, respectively (Table 2). Water content in pressure–volume curves appeared typical of embryonic tissues from other recalcitrant seeds (Farrant and Walters, 1998), decreasing sharply with very small changes in water potential

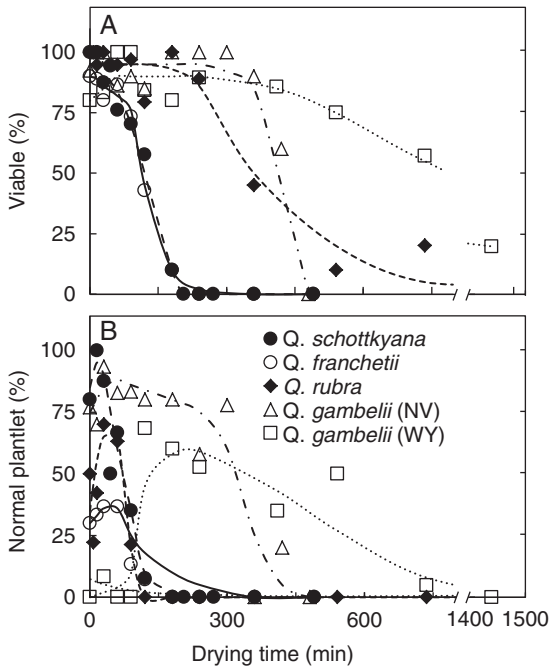

FIG. 3. Effect of drying time on viability (A) and normal plant growth (B) of *Quercus* embryonic axes. Symbols indicate different species as indicated. Curves were drawn as an aid to the eye.

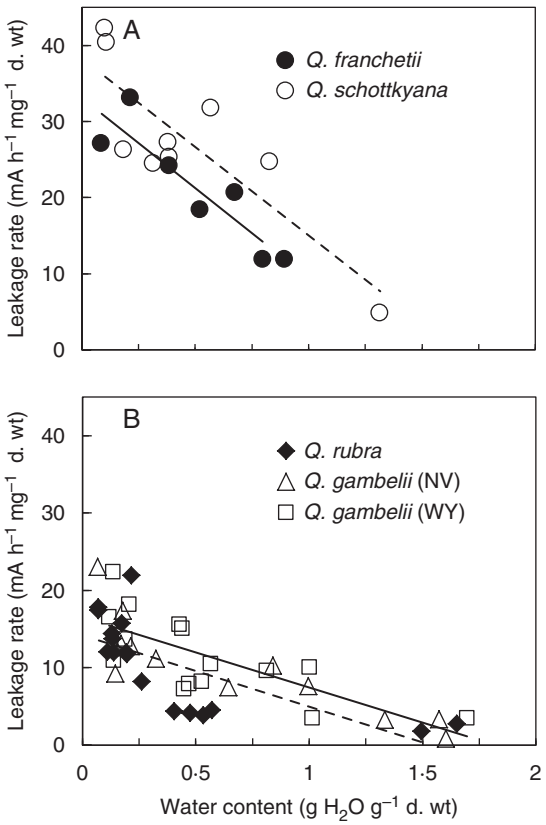

FIG. 4. Electrolyte leakage from embryonic axes of *Quercus* that were flash-dried to different water contents. Panel (A) shows the Chinese species, *Q. franchetii* (solid circles) and *Q. schottkyana* (open circles); panel (B) shows the US species, *Q. gambelii* (NV) (open triangles), *Q. gambelii* (WY) (open squares) and *Q. rubra* (solid diamonds). Leakage rates were positively correlated with the water content to which embryonic axes were dried ($P < 0{\cdot}01$). The slopes of the regressions are summarized in Table 4.

at $\Psi_w > -6$ MPa, and decreasing rather obliquely at $\Psi_w < -6$ MPa. For $\Psi_w > -6$ MPa, water contents of embryonic axes were higher than those of cotyledons. Despite having lower water content, cotyledons of *Q. franchetii*, *Q. schottkyana* and *Q. gambelii* (NV) had initial water potentials that were higher than the respective axes and also higher than the initial water potentials of *Q. rubra* and *Q. gambelii* (WY) cotyledons (Table 2). No obvious differences in initial water potential were noted among embryonic axes of different species and the water relations of plumule and radicle portions were similar to each other and to those of whole embryonic axes, except in *Q. franchetii* (Table 2). The discrepancy for this species is attributed to a 4-month separation (November to March) between measurements of whole axes and axis parts; during that storage period embryo germination may have progressed. As water potentials approached 0 MPa, plumules tended to have higher water contents than radicles in the species from the USA; however, the reverse pattern appeared in the species from China (Fig. 1).

Freshly excised embryonic axes (i.e. no drying treatment) showed high viability in culture, with at least 80 % of all axes expanding and greening after 1 month (Table 3). Most axes readily developed roots in culture; however, shoot development did not occur as regularly ($P < 0{\cdot}001$ in paired *t*-test comparison), shoot growth being particularly rare in *Q. gambelii* (WY). In other words, viability assessed by axis expansion and greening was usually higher than viability assessed by normal seedlings having both roots and shoots, even in control treatments that received no water stress.

TABLE 4. *Effects of drying on electrolyte leakage of* Quercus *embryonic axes. Slope ± standard error of slope,* r^2 *and probability range of the correlation between rate of conductivity increase in the leachate (data given in Fig. 4) and water content achieved during drying are given. Water contents are interpolated from the drying model coefficients provided in Table 3*

Species	Conductivity in leachate (mA h^{-1} mg^{-1} H_2O)	
	Slope	r^2
Q. franchetii	23·7 ± 4·9	0·82**
Q. schottkyana	23·3 ± 5·6	0·71**
Q. gambelii (NV)	8·9 ± 1·7	0·72***
Q. gambelii (WY)	9·1 ± 2·3	0·53**
Q. rubra	9·3 ± 2·3	0·52**

**$P < 0{\cdot}01$; ***$P < 0{\cdot}001$.

Drying treatment and response to water stress

Embryonic axes were dried over a stream of N_2 gas to nearly ambient conditions (i.e. 30 % RH) within ~3–8 h depending on species (Fig. 2). Axes from both *Q. gambelii* populations dried more slowly than those of the other species ($P < 0{\cdot}002$) [the drying time course for *Q. gambelii* (NV) is not given, but was similar to data in Fig. 1C for *Q. gambelii* (WY)]. Plumule tissue

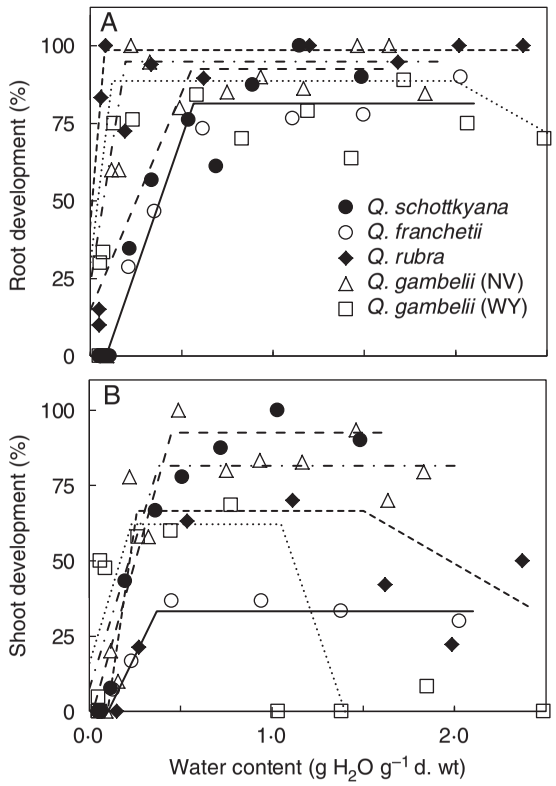

FIG. 5. Effect of drying to different water contents on normal root (A) and shoot (B) growth of *Quercus* embryonic axes. Horizontal lines represent the proportion of normal growth in axes before stress symptoms. Slanted lines on the left represent the regression of water content versus growth at lower water contents. The intersection of the two lines was calculated as the damaging water content, the point at which abnormal growth or organ mortality was observed with further drying. Slanted lines on the right represent lower initial growth.

tended to dry faster than the respective radicle tissue when drying coefficients were compared among species ($P < 0{\cdot}02$) (Table 3).

Drying had variable effects on survival and organ development depending on the species and duration of treatment (Fig. 3). Slight drying had no effect on signs of viability (expansion and greening of axes) (Fig. 3A) but appeared to stimulate shoot growth in species exhibiting initially low plumule development (Fig. 3B). As drying time increased, normal seedling development declined and eventually signs of viability were also lost. The drying time at which changes to the physiology of axes were observed differed among species. Water contents corresponding to drying times were calculated from drying time course models (Table 3; Fig. 2) and are used henceforth to describe embryonic axis responses to drying.

Leakage of electrolytes from rehydrating axes was used to assess damage of embryonic axes following desiccation. Conductivity of leachate increased as embryos were flash-dried to progressively lower water contents ($P < 0{\cdot}01$ in regression analyses for all samples) (Fig. 4; Table 4). Drying had a greater effect on electrolyte leakage in the species from China, as indicated by greater slopes of regression models: 23 mA h^{-1} mg^{-1} H_2O for *Q. franchetii* and *Q. schottkyana* versus ~9 mA h^{-1} mg^{-1} H_2O for *Q. gambelii* (NV), *Q. gambelii* (WY) and *Q. rubra*.

TABLE 5. *Water contents limiting survival and growth of* Quercus *embryonic axes calculated using various approaches. Damaging water contents were calculated from the intersection of horizontal and sloped lines as shown in Fig. 5; numbers in parentheses represent intersection of the horizontal line with the 90 % confidence interval for the sloped lines. The water content and drying time giving a 25 % reduction in viability or growth (LD75) was calculated using the dose function from the R library; standard error of the 'dose' giving 75 % of the maximum survival is also provided. The range of water contents corresponding to LD75 ± standard error was calculated from the drying time course function (Table 3) and is provided in parentheses beneath the LD75 drying time*

Species	Damaging water content (g H_2O g^{-1} dw) (90 % confidence interval)				LD75 – water content (g H_2O g^{-1} dw) ± standard error				LD75 – dry time (min) ± standard error (water content (g H_2O g^{-1} dw))			
	Expansion and greening	Root growth	Shoot growth	Normal seedling	Expansion and greening	Root growth	Shoot growth	Normal seedling	Expansion and greening	Root growth	Shoot growth	Normal seedling
Q. franchetii	0·40*,††† (0·30–0·55)	0·56***,††† (0·53–0·61)	0·37^{ns,††} (0·35–0·38)	0·57^{ns,ns} (0·52–0·63)	0·72 ± 0·08‡‡	0·92 ± 0·16‡‡‡	1·86 ± 0·36^{ns}	1·98 ± 0·32^{ns}	69 ± 9 (0·66–0·48)	43 ± 9 (1·03–0·73)	75 ± 14 (0·44–0·24)	34 ± 18 (1·50–0·76)
Q. schottkyana	0·36 (0·28–0·48)	0·41 (0·38–0·44)	0·45 (0·42–0·49)	0·43 (0·40–0·47)	0·44 ± 0·04	0·64 ± 0·07	0·49 ± 0·05	0·8 ± 0·07	83 ± 7 (0·42–0·34)	54 ± 8 (0·68–0·51)	52 ± 7 (0·50–0·38)	36 ± 8 (0·93–0·70)
Q. gambelii (NV)	0·19 (0·13–0·32)	0·19 (0·14–0·29)	0·38 (0·30–0·52)	0·44 (0·32–0·70)	0·09 ± 0·24	0·39 ± 0·18	0·95 ± 0·12	1·04 ± 0·12	319 ± 36 (0·25–0·16)	256 ± 32 (0·36–0·24)	205 ± 22 (0·48–0·36)	121 ± 21 (0·87–0·64)
Q. gambelii (WY)	0·13 (0·09–0·22)	0·12 (0·09–0·16)	0·23 (0·18–0·34)	0·34 (0·22–0·72)	0·05 ± 0·12	0·38 ± 0·10	0·51 ± 0·09	0·70 ± 0·10	759 ± 84 (0·06–0·05)	484 ± 65 (0·22–0·13)	360 ± 52 (0·16–0·09)	287 ± 39 (0·41–0·24)
Q. rubra	0·06 (0·05–0·11)	0·08 (0·06–0·11)	0·26 (0·22–0·32)	0·23 (0·19–0·28)	0·19 ± 0·04	0·30 ± 0·05	2·24 ± 0·31	2·23 ± 0·32	263 ± 27 (0·05–0·05)	263 ± 27 (0·06–0·05)	94 ± 17 (0·24–0·11)	72 ± 17 (0·57–0·25)

Correlations between damaging water content and LD75 calculated using water content among samples: ns, not significant; $^{*}P < 0{\cdot}1$; $^{***}P < 0{\cdot}01$.
Correlations between damaging water content and LD75 calculated using water content among samples: ns, not significant; $^{\dagger\dagger}P < 0{\cdot}05$; $^{\dagger\dagger\dagger}P < 0{\cdot}01$.
Correlations between damaging water content and LD75 calculated using water content among samples: ns, not significant; $^{\ddagger\ddagger}P < 0{\cdot}05$; $^{\ddagger\ddagger\ddagger}P < 0{\cdot}01$.

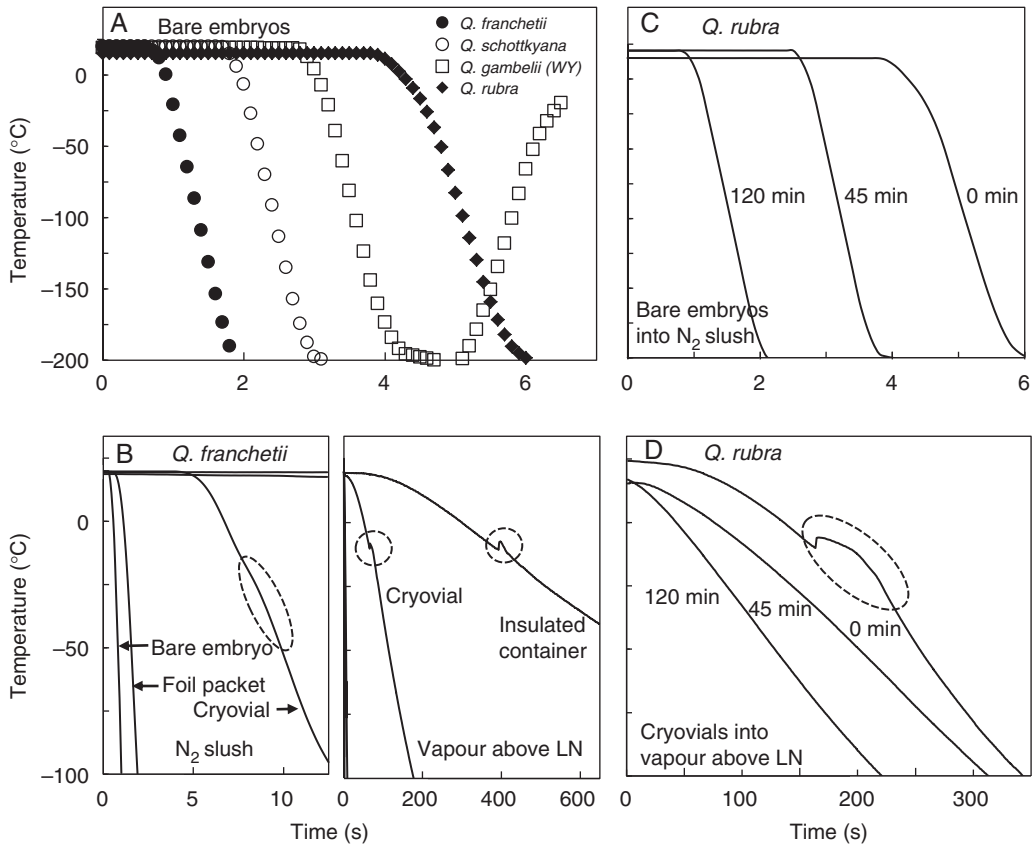

FIG. 6. Cooling time courses of fully hydrated (A, B) and dried (C, D) embryonic axes of *Quercus* embryos. In (A), bare embryos of *Q. franchetii*, *Q. schottkyana*, *Q. gambelii* (WY) and *Q. rubra* (see key) were plunged into N_2 slush and warmed in 40 °C water [only *Q. gambelii* (WY) is shown]. Panel (B) compares cooling time courses of embryos of *Q. franchetii* cooled using various methods as indicated. Panels (C) and (D) give representative time courses for *Q. rubra* embryos dried for 0, 45 and 120 min and then cooled in N_2 slush or in cryovials placed in vapour above liquid nitrogen-LN. The encircled discontinuities indicate ice formation. Sample sizes and statistics are summarized in Table 6.

TABLE 6. *Cooling rates obtained by various methods of exposing* Quercus *embryonic axes to liquid nitrogen-LN. Cooling rates were calculated from linear regressions of cooling time courses between +5 and −40 °C. Values represent average ± s.d.* (n= *5). Cooling rates of* Q. gambelii *(NV) were not measured because of the small size of the seed lot. Average fresh mass and water content of axes are indicated. Cooling rates using different methods are significantly different at* $P < 0{\cdot}001$ *except the two vapour cooling treatments; however, these treatments were significantly different when time to reach −40 °C (i.e. the reciprocal of rate) was compared*

Species	Water content (g H_2O g^{-1} dry weight)	Fresh mass (mg)	Cooling rate between +5 and −40 °C (°C s^{-1}): Bare axis plunged into N_2 slush (***,†††)	Foil packet immersed in liquid nitrogen-LN (***,†††)	Cryovial submerged in liquid nitrogen-LN (***,†††)	Cryovial placed in vapour above liquid nitrogen-LN (ns,††)	Insulated box placed in vapour above liquid nitrogen-LN (ns,nd)
Q. franchetii	1·90 ± 0·11	5·66 ± 0·73	164 ± 18	55 ± 12	7·8 ± 0·6	0·36 ± 0·03	0·06 ± 0·01
Q. schottkyana	1·51 ± 0·34	4·00 ± 0·37	170 ± 16	80 ± 9	8·4 ± 0·4	0·40 ± 0·03	0·06 ± 0·01
Q. gambelii (NV)	1·83 ± 0·47	7·35 ± 4·68	nd	nd	nd	nd	nd
Q. gambelii (WY)	2·02 ± 0·63	5·87 ± 2·24	161 ± 28	56 ± 11	4·5 ± 1·8	0·56 ± 0·09	0·06 ± 0·01
Q. rubra	1·73 ± 0·58	10·87 ± 2·27	98 ± 20	31 ± 11	2·8 ± 0·7	0·36 ± 0·08	0·05 ± 0·01
	0·45 ± 0·13	6·17 ± 1·38	154 ± 18	61 ± 5	6·2 ± 0·8	0·40 ± 0·02	
	0·15 ± 0·02	5·05 ± 1·43	181 ± 5	70 ± 11	8·0 ± 0·7	0·51 ± 0·10	

nd, measurement not done.
Correlations between cooling rate and fresh mass of fully hydrated axes: ns, not significant; $^{***}P < 0{\cdot}001$.
Correlations between cooling rate and fresh mass of *Q. rubra* at different water contents: $^{††}P < 0{\cdot}01$; $^{†††}P < 0{\cdot}001$.

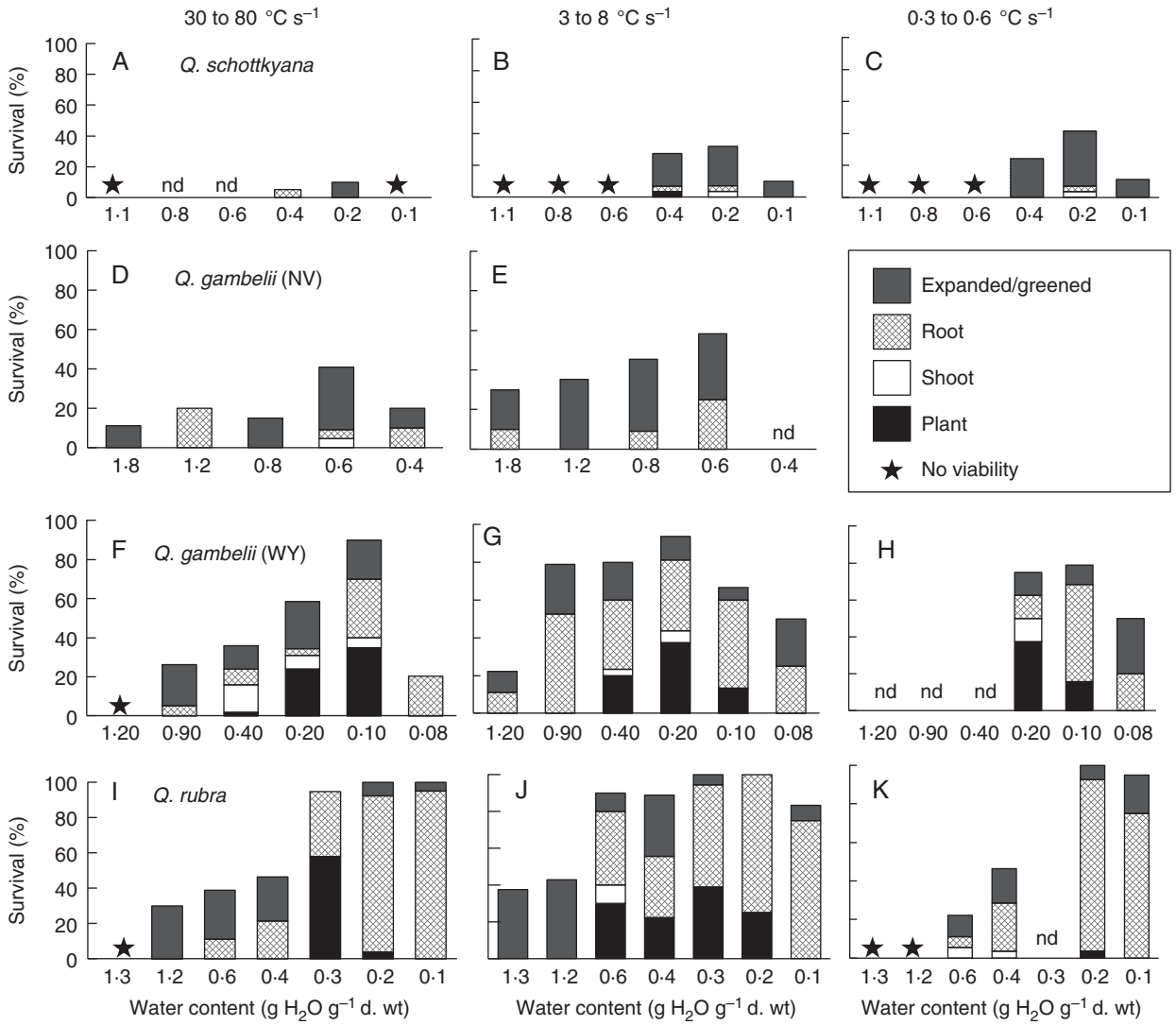

FIG. 7. Survival of embryonic axes of *Quercus* species at different water contents after exposure to liquid nitrogen. Samples were cooled using different methods to achieve the range of cooling rates indicated above each column. Each row of graphs represents a different species or population. The bars show the percentages of the recovery growth for survival (expansion and greening, dotted bars), normal development of roots (hatched bars), shoots (white bars) and regrowth of whole plants (black bars). Stars represent treatments obtaining no survival, and 'nd' stands for treatments that were not studied. There was no survival in any treatment of *Q. franchetii* (data not shown).

TABLE 7. *Regression analyses using a general linear model between damaging water content for survival (Table 3) and maximum viability achieved after exposure to liquid nitrogen-LN (Fig. 7) observed for* Quercus *species cooled at different rates (Table 6)*

Exposure method and average cooling rate	*F*	d.f.	*P*	r^2
Foil packet (30–80 °C s^{-1})	55·2	4	0·005	0·948
Cryovial (3–8 °C s^{-1})	24·9	4	0·016	0·892
Vapour phase (0·3–0·6 °C s^{-1})	10·5	3[a]	0·083	0·840

[a]Vapour-phase treatments were not conducted for *Q. gambelii* (NV) because there were too few seeds.

After no or mild stress, normal root development was observed in 73 ± 4 % [*Q. gambelii* (WY)] to 99 ± 3 % (*Q. rubra*) of axes (Fig. 5A). Approximately 93 ± 7, 82 ± 8 and 33 ± 3 % of axes developed shoots in *Q. schottkyana*, *Q. gambelii*((NV) and *Q. franchetii*, respectively, following no or mild water stress. In contrast, mild stress appeared to increase shoot development in *Q. gambelii* (WY) and *Q. rubra*, from initial low levels near 2 and 42 %, respectively (Table 3), to maximum levels of 64 ± 14 and 67 ± 12 %. Shoot and root development decreased under severe water stress, and all signs of viability were lost in axes that were dried for a prolonged period.

Desiccation tolerance among species and tissues was quantified by the water content at which loss of viability or reduced

growth was apparent (calculations are described in Materials and methods). Damaging water content ranges were calculated from the intersection of horizontal lines representing non-lethal stress and sloped lines representing decreasing survival or growth with decreasing water content (Pammenter *et al.*, 1991; Farrant and Walters, 1998) (Table 5). Representative graphs for root and shoot growth are given in Fig. 5A, B and are similar in principle to analyses for viability and normal seedling development, respectively (data provided in Fig. 3A, B). Additional assessments of desiccation tolerance used the dose–response function from the R library to determine the water content or drying time corresponding to a 25 % reduction in viability or growth (LD75) (Table 5). The values for water contents varied among calculation approaches, but they were significantly correlated for expansion and greening, root growth and shoot growth (for analyses of damaging water content versus LD75 drying time but not for LD75 calculated from water content measurements). The low and variable incidence of shoot growth in unstressed axes made LD75 calculations for shoot growth or normal seedlings unreliable. In general, normal seedling growth (both root and shoot development) was compromised during the early stages of drying and preceded loss in the ability to develop either organ. Ability to expand and green persisted to lower water contents, but was eventually lost in all species. Plumules appeared to have similar or greater tolerance than radicles in *Q. franchetii* and *Q. schottkyana*; however, plumules were more sensitive to water stress compared with radicles in *Q. gambelii* and *Q. rubra*. Based on water content ranges that support viability and organ development, the species can be ranked for desiccation tolerance as *Q. rubra* > *Q. gambelii* (WY) > *Q. gambelii* (NV) > *Q. schottkyana* > *Q. franchetii*.

Liquid nitrogen treatment and response to low-temperature stress

Interactions between water status and viability below 0 °C are confounded by cooling rate, which affects the extent and cellular location of freezing transitions. Cooling rate, in turn, can be affected by specimen size, water content, containers, exposure methods and temperature range (Walters *et al.*, 2008). In our hands, bare embryos plunged into N_2 slush cooled from room temperature to −200 °C in 1–2 s (Fig. 6A), while axes cooled in an insulated box placed in vapour above liquid nitrogen (Fig. 6B) reached −150 °C after 1–1·5 h. Cooling rates increased as temperature decreased below −80 °C, likely because the heat capacity of the sample also decreased (data not shown). Discontinuities in cooling time courses, indicative of freezing events, were observable between −10 and −30 °C when fully hydrated samples (Table 3) placed in cryovials were cooled in either liquid nitrogen or vapour above liquid nitrogen (Fig. 6B). These discontinuities were not observed in more rapidly cooled samples (Fig. 6B, C) or in samples dried and cooled relatively slowly (Fig. 6D). Fresh mass, affected by either axis dry mass or water content, had significant effects on cooling rate in the faster cooling methods (i.e. plunging bare embryos, foil packets or cryovials into liquid nitrogen; $P < 0{\cdot}01$), but no effect was detected in embryos cooled relatively slowly in the vapour above liquid nitrogen (Table 6).

Survival of axes following liquid nitrogen exposure was assessed as a function of cooling method, axis water content and species. For all species and water contents, plunging bare axes into N_2 slush was mostly lethal; embryonic axes frequently shattered and only a small percentage (<10 %) showed signs of callus formation upon recovery (data not shown). A range of responses was observed among water contents and species when embryos were cooled in liquid nitrogen in foil packets (cooling rate 30–80 °C s^{-1}) or cryovials (3–8 °C s^{-1}) (Fig. 7). *Q. franchetii* embryos at all water contents tested (0·13–0·99 g g^{-1}) did not survive cryoexposure by any method (data not shown). For other species, survival and organ development tended to increase as the water content of cryoexposed axes decreased to levels close to the damaging water content marking desiccation damage (Table 5). Samples dried below damaging water contents had low survival, presumably in response to the desiccation treatment. Higher survival and more normal development for a wider range of water contents was noted in axes cooled at 3–8 °C s^{-1} (cryovials plunged into liquid nitrogen) compared with 30–80 °C s^{-1} (foil packets containing axes plunged into liquid nitrogen). Cooling at 0·3–0·6 °C s^{-1} by placing cryovials in the vapour above liquid nitrogen reduced survival and organ development in axes of *Q. rubra* (Fig. 7J, K), but gave survival results comparable to those found with faster cooling treatments for *Q. schottkyana* and *Q. gambelii* (WY) axes (Fig. 7B, C, G, H). We did not test survival of axes exposed to liquid nitrogen using the slowest treatment (~0·06 °C s^{-1}).

A higher proportion of root compared with shoot development was noted in recovering axes of all the species and cryoexposure treatments (paired *t*-test, $P < 0{\cdot}001$). Overall, embryos of *Q. rubra* and *Q. gambelii* were most amenable to cryopreservation treatments while axes of *Q. schottkyana* required a narrow range of water contents and cooling rates for survival. The highest four survival proportions for each species (Fig. 7) were averaged (0 % for *Q. franchetii*) and regressed with damaging water contents for expansion and greening given in Table 5 (Table 7). Significant relationships suggest that desiccation tolerance and tolerance to cryoexposure are related.

DISCUSSION

Survival following desiccation and cryoexposure was measured in embryonic axes of four *Quercus* species in order to compare stress tolerance among species and among radicle and plumule tissues within species. Our assessments of desiccation tolerance by water contents that limit normal seedling development (Table 5) are comparable to previous reports for *Quercus* (Pritchard, 1991; Finch-Savage 1992; Sun, 1999; Ganatsas and Tsakaldimi, 2013; P. Chmielarz, Institute of Dendrology, Kórnik, Poland and C. Walters, unpubl. res.) and other genera (Pammenter *et al.*, 1991; Berjak *et al.*, 1993; Farrant and Walters, 1998). The LD75 calculations presented here provide some statistical advantages over the more typical damaging water content analysis and are correlated with damaging water contents if survival and growth are initially high. We show here that pressure–volume relationships for embryonic axes of diverse oaks are similar (Fig. 1), confirming that water content measurements provide a reliable means to quantify and compare water stress in excised axes of different species. Our overall results are consistent with the general presumption that seeds from all *Quercus* species are recalcitrant (Hong *et al.*, 1998; Dickie and Pritchard, 2002; Xia *et al.*, 2012*a*) in that

lethal effects of drying were observed and would preclude freezer storage.

Increasingly, seed tolerance or sensitivity to desiccation is regarded as a quantitative trait rather than a category describing seeds that cannot be stored in the freezer. Ability to survive and grow after low water stress is influenced by complex interactions among phylogeny, growth conditions, maturity and post-shedding conditions (Tweddle *et al.*, 2003; Berjak and Pammenter, 2008; Daws and Jensen, 2011). Here we also show that these relationships are expressed differently in radicles and plumules, depending on species (Fig. 5, Table 5). The higher water contents of plumules compared with radicles at a given water potential (compare pressure–volume curves in Fig. 1) perhaps suggest that plumule cells were more vacuolated, which can predispose cells to greater sensitivity to water loss.

To further understand the complex interactions leading to differences in desiccation tolerance, we need reliable assays to quantify the phenotype, which will involve precise control of the duration and intensity of water stress and accurate measurement of response. We believe that excising embryonic axes provides needed experimental control for comparative studies among samples and tissues that vary in size (Table 2) and pressure–volume relationships (Fig. 1). We show here that water content assessments of the whole seed, containing diverse tissues, may provide a relatively crude assessment of water stress (Fig. 1) and response (Table 5) at the tissue level, and that drying rate varies among species and tissue types (Fig. 1, Table 3), such that drying time alone cannot be used to quantify water stress. For these reasons, it is difficult to compare results presented here with previous surveys of post-harvest physiology of oaks in which the entire seed was used to assess bulk short-term storage or recruitment of seedlings under natural conditions (Olson, 1992; Bonner, 1996; Xia *et al.*, 2012*a*; Joët *et al.*, 2013).

We hypothesized that embryos from *Quercus* species adapted to drier climates would exhibit greater tolerance to desiccation compared with congeners growing in mesic environments. Instead, we found that embryonic axes of the two Chinese subtropical species from a semi-humid habitat were least tolerant to desiccation, and a North American species adapted to cold, wet winters was most tolerant to desiccation (Table 5). Embryos of the desert-adapted North American species *Q. gambelii* displayed intermediate desiccation tolerance; moreover, the population originating from the temperate desert (Wyoming) was more tolerant than the population originating from the warm desert (Nevada).

Studies of the effect of growth environment on desiccation tolerance within species and among congeners consider the duration of both the maturation period (Vertucci *et al.*, 1995; Daws *et al.*, 2004, 2006) and the post-shedding, pre-germination period (Dussert *et al.*, 2000; Joët *et al.*, 2013). Longer periods of warmth during seed maturation allow seeds to accumulate more dry matter (Daws *et al.*, 2004, 2006; Daws and Jensen, 2011), which would also change pressure–volume relationships and tend to decrease mechanical stress when water is removed from cells (Walters and Koster, 2007). Therefore, one might hypothesize that seed dry mass positively correlates with desiccation tolerance (Daws *et al.*, 2004, 2006). However, there was no strong correlation between damaging water contents (Table 5) and axis or cotyledon dry mass (Table 2) in the samples studied here ($P > 0{\cdot}12$). Seed survival after shedding may require adaptive mechanisms to avoid desiccation stress over a long dry period. Traits such as water permeability or water gradients from cotyledon to axis may serve as protective mechanisms to prevent water removal rather than provide tolerance of desiccation (Farnsworth, 2000; Dussert *et al.*, 2004; Hill *et al.*, 2012; Xia *et al.*, 2012*b*; Joët *et al.*, 2013). Anatomical adaptations of the pericarp might help to resist water loss (Xia *et al.*, 2012*b*) and higher water potentials of the cotyledons compared with axes [as seen in *Q. franchetii*, *Q. schottkyana* and *Q. gambelii* (NV)] (Table 2) might provide a water reservoir for embryonic axes. As can be expected, protection against water loss has little advantage when water is actively removed, as in the current study. Instead, the more desiccation-tolerant axes originated from populations that must cope with freezing temperatures during winter (Table 1), and mechanisms that promote water movement away from the embryonic axis would effectively protect embryos from lethal freezing injury. For example, *Q. rubra* seeds (Xia *et al.*, 2012*a*) and axes (Fig. 2D) tended to dry rapidly despite their large size, indicating a lack of effective barriers to water loss. Moreover, initial water potentials of cotyledons from the more desiccation-tolerant axes [i.e. *Q. gambelii* (WY) and *Q. rubra*] were lower than those of the axes (Table 2), suggesting that the cotyledons did not serve as a water reservoir. Here, we present the case that recalcitrant *Quercus* seeds may exhibit desiccation avoidance or tolerance strategies depending on whether they are adapted to non-freezing or freezing winters, respectively. Intraspecific differences observed here further suggest that survival mechanisms may be relatively plastic and dependent on ecotype rather than species.

Survival following exposure to low temperature was tested further for the purposes of developing cryopreservation strategies for *Quercus* germplasm. In these studies, avoidance of lethal intracellular ice formation was attempted by balancing drying duration and cooling rate (Wesley-Smith *et al.*, 2004; Walters *et al.*, 2008). A preliminary assessment of water freezing transitions is provided by cooling time courses, such as those given in Fig. 6, showing discontinuities between −10 and −30 °C in slower-cooled, fully hydrated samples. More exact measurements of water freezing events are gleaned from differential scanning calorimetry, which did not detect transitions in axes having water contents less than ∼0·25 g H_2O g^{-1} dry weight (Vertucci, 1989). Samples that tolerate drying below this so-called unfreezable water content are also expected to tolerate cryoexposure. Consistently, axes of *Q. rubra* and *Q. gambelii* (WY), which had damaging water contents $\leq 0{\cdot}25$ g g^{-1} (Table 3), exhibited high survival (>75 %) for several moisture–cooling rate combinations (Fig. 6). *Quercus franchetii*, *Q. schottkyana* and *Q. gambelii* (NV) had damaging water contents near to or greater than typical unfreezable water contents (Table 5) and these samples had low survival (<50 %) following cryoexposure, likely because desiccation and freezing damage could not be avoided simultaneously. The lowest water content tested for axes of *Q. gambelii* (NV) was 0·4 g g^{-1} because of limited seed availability (Fig. 6); further drying may boost survival in this sample and will be tested in subsequent years. Cryoprotectants may be required to increase survival of *Q. franchetii* and *Q. schottkyana* embryos, which had relatively high damaging water contents.

Our results do not support the hypotheses that survival increases with faster cooling in axes containing freezable water. In fact, the highest survival was obtained in samples

cooled at 3–8 °C s^{-1} (Fig. 7). The shattering of bare embryos plunged directly into N_2 slush suggests to us that this rapid cooling method imparted mechanical damage.

The results reported here (Fig. 7) are also consistent with a growing number of reports in several species that embryonic axes recovering from cryoexposure often fail to develop normally (e.g. Berjak *et al*., 2011; Normah *et al*., 2011; Wesley-Smith *et al*., 2014). In oak, roots may grow but development of shoots is rare (Chmielarz, 1997; González-Benito *et al*., 2002; Chmielarz *et al*., 2011). Shoot growth from non-stressed axes was limited after 4–6 weeks (Fig. 5), suggesting that plumule tissue may have more fastidious growth requirements than radicles, and hence shoots may have poorer survival after extreme stress. Alternatively, the greater sensitivity to cryoexposure of plumule relative to radicle tissues may be attributed to differences in desiccation tolerance, freezing tolerance and wounding effects resulting from excision. The combination of greater desiccation sensitivity and faster drying of *Q. gambelii* (WY) and *Q. rubra* plumules compared with radicles (Figs 2 and 5; Tables 3 and 5) should result in a relatively narrow optimum axis water content and overall lower proportion of normal seedling development (Fig. 7). Plumules and radicles of *Q. franchetii* and *Q. schottkyana* were similarly sensitive to desiccation and more sensitive than respective tissues from US species (Fig. 5, Table 5), possibly explaining the consistently poor organ development in these species following cryoexposure.

Conclusions

Comparisons of damaging water contents for survival and growth in embryonic axes of four *Quercus* species suggested that dry-adapted *Quercus* species do not produce the most desiccation-tolerant seeds. Rather, strategies to survive freezing temperatures during winter may contribute to innate desiccation tolerance. In other words, desiccation tolerance is acquired during embryo development and may be influenced by environmental factors such as heat sum (*sensu* Daws), but additional adaptations may involve freeze avoidance mechanisms during winter. Embryonic tissues with greater tolerance to desiccation had higher survival following cryoexposure. These interrelationships may explain why cryopreservation of recalcitrant embryonic axes from temperate species is currently feasible, and more research is needed to preserve germplasm from tropical species (Walters *et al*., 2013). The results reported in this paper also demonstrate faster drying and greater sensitivity of plumules to desiccation compared with radicles, which may predispose plumules to greater damage during cryoexposure. Overall, these studies contribute to the understanding of the relationship between geographical origin and seed post-harvest physiology and demonstrate differential expression of desiccation sensitivity in mature embryo tissues.

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