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Recalcitrant and orthodox *Spartina* seeds or isolated embryos exhibit similar leachate patterns immediately following desiccation

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Abstract

Solute leakage is used as an indicator of membrane damage during desiccation of recalcitrant seeds. We re-examined this phenomenon and its utility by comparing recalcitrant *Spartina alterniflora* and orthodox *Spartina pectinata* seeds, as well as drying isolated embryos or embryos isolated after whole-seed drying. During drying, intact seed leakage (electrical conductivity and absorbance at 280 nm) was independent of seed moisture content, dormant/non-dormant state or desiccation tolerance. Embryos from both *Spartina* species, isolated after drying within the intact seed, exhibited increased leakage, especially below 40% (dry weight basis, DWB) seed moisture, the critical water content for viability loss in *Spartina alterniflora*. When isolated embryos were dried, the pattern and extent of increased solute leakage were similar in both *Spartina* species, even though *S. pectinata* embryos were >95% viable and *S. alterniflora* embryos were <20% viable. We conclude that increased solute leakage is an artefact of embryo excision and not an accurate indicator of desiccation damage to recalcitrant *Spartina alterniflora* seeds.

Keywords: desiccation tolerance, electrical conductivity, membrane damage, recalcitrant seeds, solute leakage, *Spartina*, viability

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Introduction

One of the proposed causes of desiccation-sensitive, recalcitrant seed death is membrane damage during drying. Ultrastructural, biochemical and physiological evidence to support this idea is widespread (Berjak and Pammenter, 2000). Since the initial report by Becwar *et al.* (1982), detection of increased organic and inorganic solute leakage during drying of intact recalcitrant seeds (e.g. Nautiyal and Purohit, 1985a; Oliveira and Valio, 1992; Pammenter *et al.*, 1998; Sunilkumar and Sudhakara, 1998; Chaudhury and Malik, 2004; Lan *et al.*, 2007), isolated embryos (Salmen Espindola *et al.*, 1994; Pukacka and Ratajczak, 2006), isolated axes (Berjak *et al.*, 1992; Poulsen and Eriksen, 1992; Wesley-Smith *et al.*, 2001; Shao *et al.*, 2009; Ntuli *et al.*, 2011) or axes assayed after whole-seed drying (e.g. Panza *et al.*, 2007) has been interpreted as evidence for membrane damage as a contributing cause of recalcitrant seed death (Côme and Corbineau, 1996; Pammenter *et al.*, 2002; Song *et al.*, 2003). For some recalcitrant species, increased solute leakage occurs throughout the drying regime (e.g. Fu *et al.*, 1990; Chaitanya and Naithani, 1994; Pammenter *et al.*, 1998; Xin *et al.*, 2010), while in other species leakage increases most extensively with drying below the critical moisture content for seed death (e.g. Pukacka, 1989; Pammenter *et al.*, 1991; Finch-Savage *et al.*, 1996; Li and Sun, 1999). Intact recalcitrant seeds may or may not exhibit solute leakage, depending upon the maturity and integrity of the propagule, while isolated axes tend to readily release solutes as they are dried (e.g. Becwar *et al.*, 1982 versus Pukacka, 1989 for *Acer saccharinum* L.). However, it is not clear if increased solute leakage during rehydration after drying is strictly associated with recalcitrant seed death (e.g. Kundu and Kachari, 2000), a post-mortem event (our interpretations of Nautiyal and Purohit, 1985b; Bilia *et al.*, 1999) or simply reflects rehydration of dried plant material (orthodox and recalcitrant seeds).

In several studies, increased leachate conductivity was not a consistent indicator of recalcitrant seed death (Chandel *et al.*, 1995; Bonner, 1996; Raja and Palanisamy, 2009). To investigate this issue, we compared the solute release from recalcitrant *Spartina alterniflora* and orthodox *Spartina pectinata* using intact dormant and non-dormant caryopses, as well as embryos isolated from the two species before or after desiccation. By comparing two species with differing desiccation tolerance from the same genus, we hoped to clarify the nature of the relationship between solute leakage and seed response to drying. Based upon our results, we conclude that solute leakage from the two *Spartina* species is not related to recalcitrant seed death.

Materials and methods

Plant materials

S. alterniflora seeds (i.e. intact dispersal units: caryopses and attached glumes) were obtained at the natural time of dispersal (late November–early December) from wild plants in the marshes at Port Fourchon, Louisiana (2005, 2006, 2011). Seeds were harvested by tapping spikes on the side of a bucket and collecting only seeds that dehisced. Seeds were then placed in sealed plastic bags for transport to the laboratory. Transport times from Port Fourchon was *c.* 3 h. Harvest seed moisture content prior to submerged storage averaged 45 ± 3% (DWB, dry weight basis) and ranged from 42 to 49%. In the lab, seed aliquots (10 g per container) were immediately placed in GA-7 culture vessels (Magenta vessels, Sigma Aldrich, St. Louis, Missouri, USA) that contained 250 ml of deionized water, and were stored submerged at 2°C; in 2011, a portion of freshly harvested seeds was freeze-clamped in liquid nitrogen, transferred to 50-ml plastic tubes (Corning Co., Lowell, Massachusetts, USA), sealed and stored at −80°C.

S. pectinata seeds were purchased from Western Native Seeds (Coaldale, Colorado, USA) (WNS-03A, Lot # 6042, 2003 harvest; Lot #10 950, 2007 harvest). *S. pectinata* seeds had average moisture contents of 8.5 ± 1% (DWB, 2003) and 9.7 ± 1% (2011). When received, the seeds were either placed in sealed Ball™ jars (Ball Corporation, Muncie, Indiana, USA) and stored at 23°C or submerged in water and stored at 2°C, as above. After several months of storage at 23°C, related studies indicated that *S. pectinata* seeds at low moisture contents could be safely stored at −20°C; to minimize ageing, unimbibed seeds in the Ball jar were transferred to −20°C.

Prior to each experiment, seeds were screened with backlighting to ensure that only 'full' seeds were assayed. If previously hydrated, full seeds were accumulated on moist towelling during screening, and were then lightly patted with tissue to remove excess external moisture prior to desiccation.

Seeds of both *Spartina* species are physiologically dormant at dispersal. Submerged stratification of viable seeds for an average of 2 months at 2°C elicits 50% germination after 14 d at 27°C (Chappell, 2008; Chappell and Cohn, in preparation). For each harvest vintage, seeds were classified as dormant if they had been stored submerged for <2 months. Seeds were classified as non-dormant and used in these experiments after the stratification requirement had been fulfilled and seeds subsequently germinated 100% at 27°C.

Desiccation and viability

A drying chamber, as described in Wesley-Smith *et al.* (2001), was used to dry the seeds rapidly. The dryer consisted of a 500-ml Nalgene jar (Thermo Fisher Scientific, Rochester, New York, USA) with a layer of fresh 8-mesh $CaSO_4$ desiccant (W.A. Hammond Drierite Company, Xenia, Ohio, USA) on the bottom (*c.* 75 g), a 12 V computer fan (a 12 V 1amp adapter was needed to run the fan from the wall current), and a Ball-jar rim lined with mesh that was used as a stage to hold the seeds. The computer fan, supported by four 200-µl pipette tips, was placed *c.* 5 cm above the desiccant, and was oriented so it pulled dry air from the desiccant up, over the seeds. To obtain consistent dry-down rates from run to run, it was imperative to

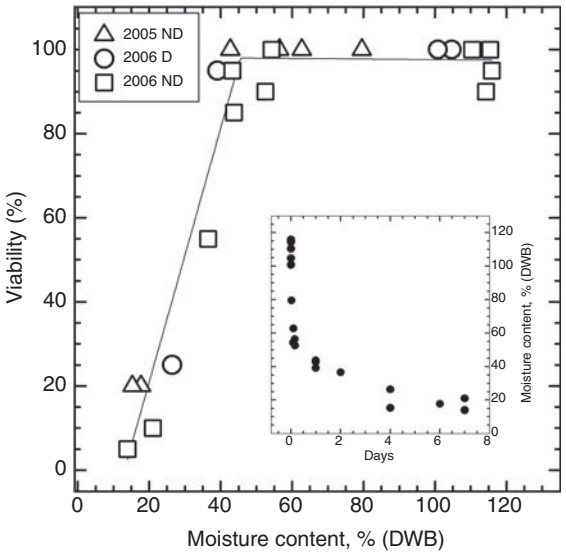

Fig. 1. Viability of *Spartina alterniflora* seed lots evaluated for leachates (Δ, 2005 harvest non-dormant; ○, 2006 harvest dormant; □, 2006 harvest non-dormant) after rapid drying (inset, ●).

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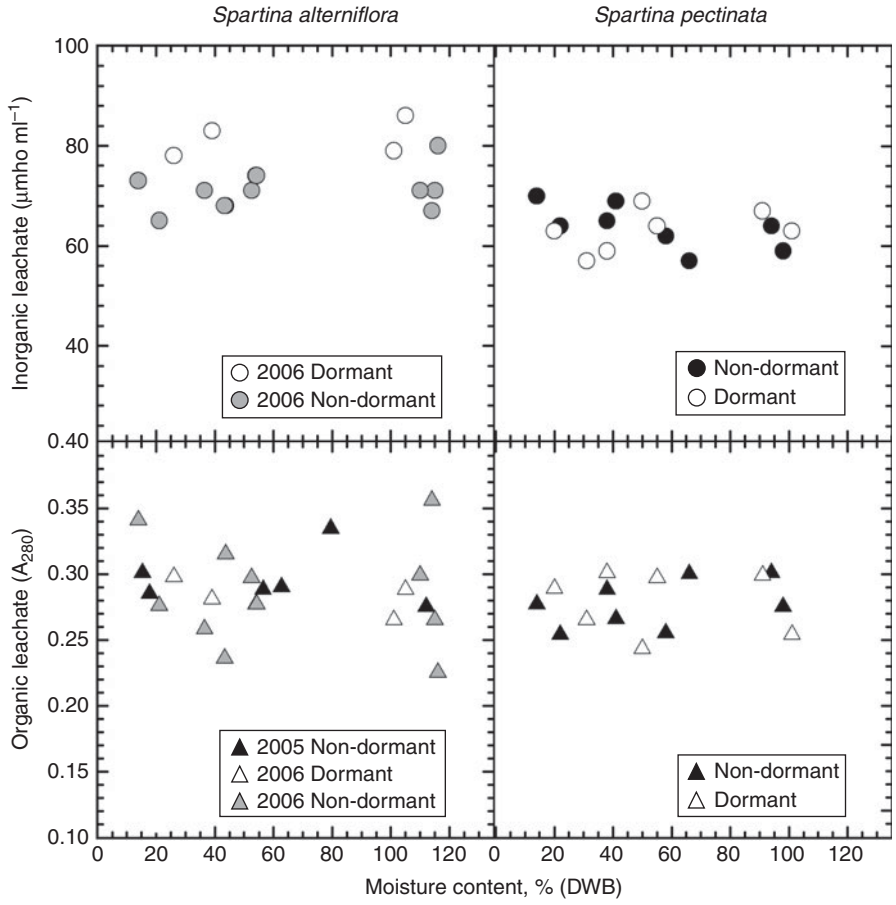

Fig. 2. Whole-seed leachates obtained immediately after rapid drying of *Spartina alterniflora* (left) and *Spartina pectinata* (right).

use fresh desiccant (heat-regenerated desiccant yielded inconsistent dry-down patterns). After drying, the seeds were assayed immediately for viability, seed moisture content and leakage. Seeds designated for solute leakage, moisture content and viability assays were present in each dry down, so there were matching moisture content and viability data points for each leachate assay. Percentage seed water content was calculated on a dry weight basis, and dry weights were obtained by placing aliquots of 20 seeds at 105°C for 7 d to attain constant weight, followed by cooling over desiccant to prevent moisture accumulation.

Viability of *Spartina* seeds after each dry-down run was determined by a cut test. The apical one-third of a seed was removed with a scalpel, clipping the coleoptile. Twenty seeds, at most, were placed in 9 × 9 × 1.5 cm plastic Petri dishes, containing two pieces of brown germination paper (Anchor Paper, St. Paul, Minnesota, USA) and 8 ml distilled water. A folded Kim-Wipe™ tissue was placed over the seeds. The covered Petri dishes were positioned at an angle of *c*. 45° in humidity boxes lined with moist paper towels and incubated at 27°C in the light. Radicle and shoot emergence were recorded after 14 d. This incubation protocol was also used to assess isolated embryo viability, scored positively when shoot elongation was observed.

Freezing and thawing

Ten grams of *S. pectinata* seeds (stored at −20°C) (2007 harvest) were hydrated in 250 ml of deionized water for 1 week at 2°C. Fifty fully hydrated *S. pectinata* seeds were placed in a mortar and freeze-clamped in liquid nitrogen. Frozen seeds were warmed to room temperature for 2 h and frozen again in liquid nitrogen. *S. alterniflora* seeds (2011) were freeze-clamped in liquid nitrogen at harvest and stored at −80°C for 8 months. After seeds were taken out of the freezer and warmed at room temperature for 2 h, they were frozen in liquid nitrogen again. Subsequently, both species were thawed for 2 h, and intact seeds subjected immediately to leachate analysis.

Seed and embryo leakage

To measure leachates, 90 *Spartina* intact seeds or isolated embryos were dried rapidly. In addition, intact seeds were dried rapidly and embryos were

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Table 1. Average inorganic and organic leachate values of *Spartina alterniflora* and *S. pectinata* seeds during the drying time course. Means ± standard deviation (SD)

Harvest year	Seed condition	Inorganic leachate (μmho ml^{-1})	Organic leachage (A_{280})
S. alterniflora			
2005	ND	–	0.296 ± 0.019
2006	ND	71 ± 4	0.286 ± 0.039
2006	D	82 ± 3	0.283 ± 0.012
2004	Dry, stored 2°C, 10 months	525	1.36
S. pectinata			
2003	ND	63 ± 4	0.281 ± 0.020
2003	D	63 ± 4	0.276 ± 0.021

D, dormant; ND, non-dormant.

excised after drying. After reaching the desired moisture content, 40 seeds/embryos were used for viability and moisture content calculations, and the remaining 50 were submerged in 10 ml of distilled water at room temperature (*c*. 23°C) for 24 h, at which time leakage had plateaued. An aliquot of water was removed from the test tubes for leachate measurement. Inorganic leachates were measured using a conductivity meter (model number, RC 16B2, Industrial Instruments, Cedar Grove, New Jersey, USA) and recorded as μmho ml^{-1}. Organic leachates were measured spectrophotometrically at 280 nm.

To determine whether leachate amounts differed between freshly dried versus dried and aged seeds, *S. alterniflora* seeds were dried to 8% moisture content and stored in sealed zip-lock bags for 6 to 16 months at 2°C.

Results

The critical moisture content (40% DWB) for *S. alterniflora* was attained by drying for *c*. 24 h, and seed viability decreased to <10% with 6 d of further desiccation (Fig. 1). Throughout the drying time-course, inorganic and organic leachates from whole seeds did not increase, even as viability declined dramatically. The quantity and pattern of organic leachate of non-dormant seeds from the 2005 and 2006 harvests were similar. Leachate patterns from orthodox *S. pectinata* seeds were comparable to those obtained from recalcitrant *S. alterniflora*. For both species, dormant and non-dormant seeds leaked to a similar extent (Fig. 2, Table 1).

In contrast to intact *Spartina* dispersal units, embryos leaked substantial quantities of inorganic and organic substances, particularly just prior to, or coincident with, reaching the critical moisture content for *S. alterniflora*. Such increased leakage was observed for embryos excised after intact seed drying (Fig. 3) or from isolated hydrated embryos that were dried (Fig. 4). Furthermore, orthodox *S. pectinata* embryo leachate was comparable in trend and extent to that observed for recalcitrant *S. alterniflora* embryos (Figs. 3 and 4),

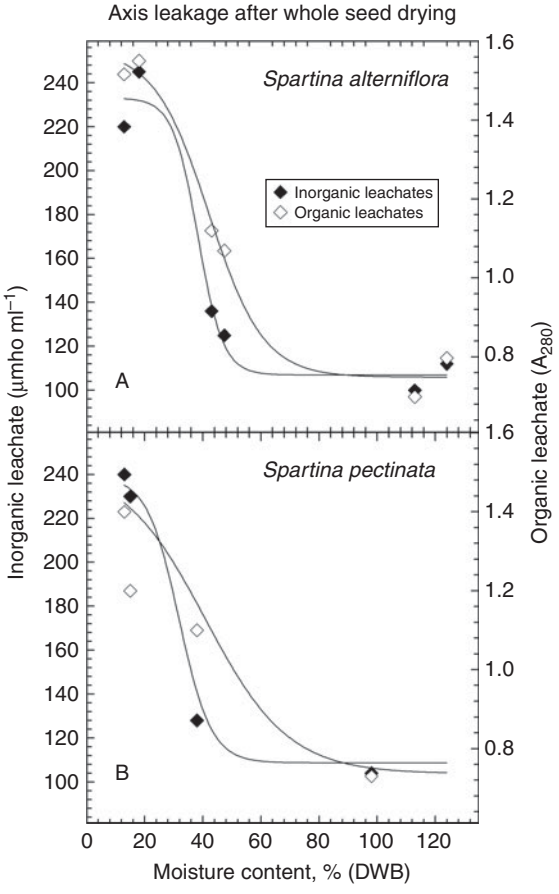

Fig. 3. After whole-seed drying, isolated embryo leachate amounts from (A) non-dormant, recalcitrant *S. alterniflora* (Port Fourchon, 2006) and (B) orthodox *S. pectinata*: inorganic (◆) and organic (◇) leachates. Each value represents an independent sample. Curves obtained by best fit regression for *P* values <0.01 for each curve.

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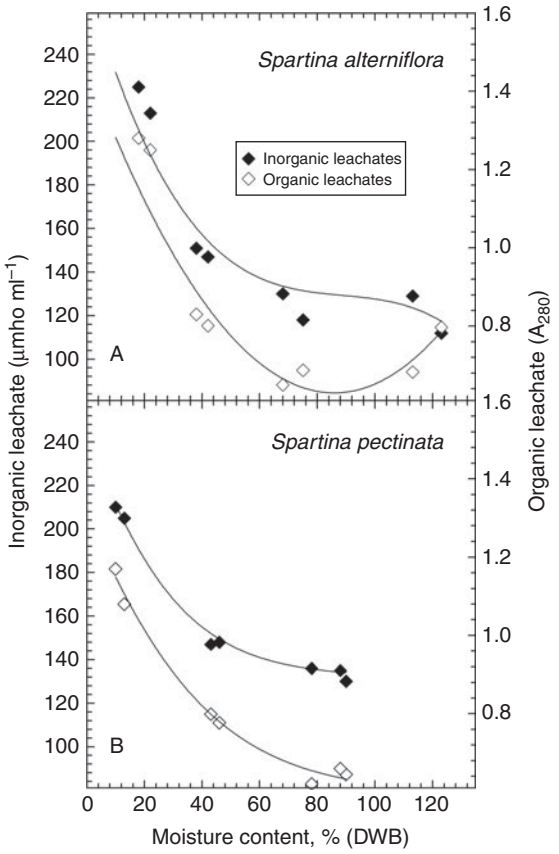

Fig. 4. Leachate amounts from (A) non-dormant *S. alterniflora* (Port Fourchon, 2006) and (B) *S. pectinata* embryos isolated before drying: inorganic (◆) and organic (◇) leachates. Each data point represents an independent sample. Curves obtained by best fit regression for P values <0.001 for each curve.

in spite of the difference in desiccation tolerance of isolated embryos between the two *Spartina* species (Fig. 5).

In a subsequent experiment with different seed lots, the total available leachate was estimated after freezing and thawing (FT). Intact *S. pectinata* seeds released more A_{280}-absorbing substances than intact *S. alterniflora* seeds, both before and after FT, when expressed on a leachate concentration, seed weight or per seed basis. Ratios of leachate between unfrozen controls and FT seeds were similar for both species (Table 2).

Discussion

Solute leakage into the incubation medium is a common symptom of imbibitional stress or injury of orthodox seeds and pollen (Simon, 1974; Hoekstra *et al.*, 1999; Osborne *et al.*, 2002). It has been used as an indicator of seed viability for decades (e.g. Hibbard and Miller, 1928) and is commonly observed during hydration of dried recalcitrant seeds. To ascertain whether increased seed leakage was associated with recalcitrant seed mortality during desiccation or was merely a phenomenon associated with drying but not necessarily death, the leachate patterns of recalcitrant *S. alterniflora* and orthodox *S. pectinata* seeds were compared during drying. For these *Spartina* species, the results clearly indicated that whole-seed leakage does not increase during desiccation. More importantly, we demonstrated that isolated embryo leakage increases dramatically as either *S. alterniflora* or *S. pectinata* are dried, irrespective of whether isolated embryos are dried or embryos are isolated after whole-seed drying. Axes isolated before drying leaked as extensively as axes that had been isolated from drying seeds, analogous to observations with *Landolphia kirkii* (Pammenter *et al.*, 1991; Berjak *et al.*, 1992). Furthermore, the embryo leakage commences between *c.* 40-60% seed moisture content in both species; just before reaching the critical water content (40%) associated with initiation of recalcitrant *S. alterniflora* seed death.

The purported link between increased leakage and recalcitrant seed death, routinely observed in similar published studies, could be explained by a number of alternative considerations. (1) Many studies consider the leakage patterns of isolated embryos of a single recalcitrant species in isolation. In such studies, there is little doubt that leakage was accurately reported; however, such studies do not have a closely related orthodox species with which to compare. (2) In the course of our work, several lots of dried and non-viable *S. alterniflora* seeds (8% moisture content) were stored at 5°C for periods of 6–16 months. These dried

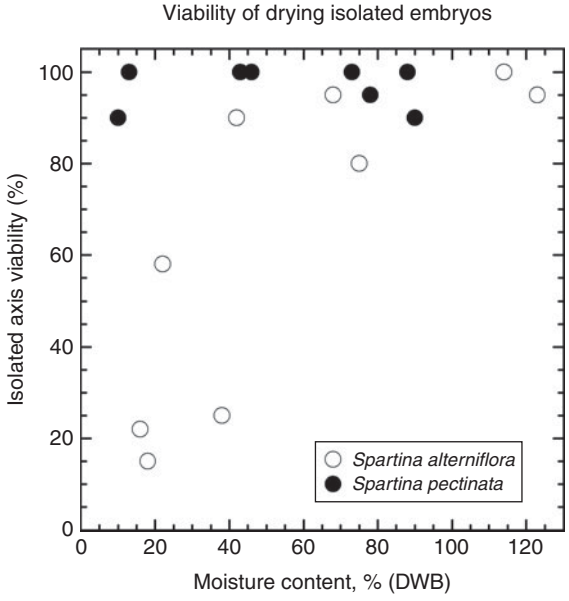

Fig. 5. Viability of isolated embryos of *Spartina alterniflora* and *Spartina pectinata* during desiccation.

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Table 2. Effect of freezing and thawing on organic leachate values of *Spartina alterniflora* (2011 harvest) and *S. pectinata* (2007 harvest) seeds. Values are means ± SD

		Organic leachate, A_{280} units		
Harvest year	Treatment	per ml leachate	per gram DW seeds	per seed
S. alterniflora				
2011	Control (C)	0.289 ± 0.033	23	0.058
	Freeze–thawed (FT)	0.567 ± 0.016	43	0.113
	C/FT ratio	0.51	0.53	0.51
S. pectinata				
2007	Control (C)	0.340 ± 0.017	25	0.068
	Freeze–thawed (FT)	0.814 ± 0.082	56	0.163
	C/FT ratio	0.42	0.45	0.42

C, Unfrozen control; DW, dry weight.

and stored intact *S. alterniflora* seeds leaked extensively (e.g. Table 1) when assayed by the method described here; values were at least five times greater than those obtained when freshly dried seeds were assayed (Table 1). Such a large increase suggests that whole-seed leakage represents a post-mortem event, rather than a cause of seed death. We do not know how quickly this change in whole-seed permeability occurs in storage, nor do we know if this is primarily a function of time or the low storage temperature. Nevertheless, this alteration of the dried intact seed leachate pattern indicates the need for prompt analysis of the samples versus accumulating them in storage for batch processing. (3) Our work and the related response syndrome reported by Roach *et al.* (2008) and Whittaker *et al.* (2010) suggest that embryo isolation is a potent trigger of seed physiological response: both increased leachate and free-radical production seem to be elicited by surgical removal of the embryo/embryonic axis from a dispersal unit. In *Trichilia dregeana* axes, leakage can be ameliorated, and viability enhanced, by ascorbate treatment prior to drying (Song *et al.*, 2004), suggesting suppression of an excision-induced oxidative burst as described by Whitaker *et al.* (2010) for this species. (4) The leachate response pattern may be a function of the extent of developmental arrest of the species under study: for seeds that are physiologically dormant (even though they are also recalcitrant), one might expect a minimal loss of solutes as a consequence of reduced metabolic activity associated with the dormant state. *S. alterniflora* would be an example. In contrast, seeds that begin germination processes during seed formation (e.g. *Avicennia marina*) would be expected to leak copiously due to the vigour of ongoing metabolism and accelerated cellular differentiation – a scenario formulated by Berjak *et al.* (1989) (see their fig. 2). (5) Rapid solute leakage from various non-dormant, recalcitrant seeds at elevated moisture contents may be more analogous to leaf responses to drought stress (e.g. Leopold *et al.*, 1981).

In summary, seed leakage patterns and amounts were similar in both the recalcitrant and orthodox *Spartina* species. Isolated embryo leakage seems to be a function of seed moisture status, rather than an indication of desiccation sensitivity in these species. Our results with intact seeds are consistent with the lack of mortality-associated TBARS (thiobarbituric acid reactive substances) lipid peroxidation products during the drying of *Spartina* species (Chappell and Cohn, 2011). Based upon our data, we would be hesitant to suggest the association of cell membrane damage with recalcitrant seed death of *S. alterniflora*.

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Conflicts of interest

None.

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