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What can cell cycle and ultrastructure tell us about desiccation tolerance in *Leucaena leucocephala* germinating seeds?

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Abstract

Desiccation tolerance (DT) is the ability to tolerate dehydration to levels below 0.1 g(H_2O) g^{-1}(dry mass) and subsequent rehydration without lethal damage. Here, it is proposed that *Leucaena leucocephala*, a tree species, has potential to be model tolerant species in seed research. Using flow cytometry and transmission electron microscopy, cytological changes related to loss of DT in *Leucaena* primary roots were followed during germination. *Leucaena* seeds lost their DT at the end of germination and this coincided with an increase in cellular 4C DNA content. A negative correlation between the 8C DNA content and the capacity of germinating *Leucaena* seeds to tolerate desiccation was also observed. Apparently, the seeds of *Leucaena* underwent extra cycles of endoreduplication and accumulated a high content of DNA – an event not previously linked to DT. The ultrastructural damage imposed by drying overcame *Leucaena* primary root cell resilience and their ability to resume normal growth. Nuclear DNA content may be used as indicator of progress of germination and loss of DT in *Leucaena*.

Additional key words: DNA content, endoreduplication, flow cytometry, transmission electron microscopy.

Introduction

Desiccation tolerant organisms are able to dehydrate to water content below 0.1 g(H_2O) g^{-1}(d.m.), rehydrate, and resume normal growth (Oliver *et al*. 2000, Phillips *et al*. 2002). Ability to tolerate desiccation is common feature of angiosperm seeds (orthodox seeds; Potts 1994, Dinakar and Bartels 2013). Such seeds acquire desiccation tolerance (DT) during seed development and maturation (Bewley *et al*. 2013). After dispersal or harvest, DT remains during storage or in the soil seed bank. Once water is available, orthodox seeds imbibe and germinate if non-dormant. With progress of germination, such seeds gradually lose DT becoming completely desiccation sensitive around the point of radicle protrusion (Bewley *et al*. 2013). Desiccation tolerance in seeds has been studied over the past few decades with a particular emphasis on its acquisition during seed maturation (Blackman *et al*. 1992, Black *et al*. 1999, Sreedhar *et al*. 2002, Illing *et al*. 2005, Verdier *et al*. 2013). Association of DT with accumulation of sugars, late embryogenesis abundant proteins, and activity of antioxidant scavenging systems have been extensively reported (Blackman *et al*. 1992, Bailly 2004, Buitink and Leprince 2004, Kranner and Birtic 2005, Tunnacliffe and Wise 2007, Hundertmark *et al*. 2011, Dinakar and Bartels 2013). A less explored aspect of DT is its loss during germination in seeds of tropical tree species and cellular adjustments related to it.

Ultrastructural modifications and the cell cycle (DNA content) have been associated with desiccation sensitivity in seeds (Berjak and Pammenter 2000, Berjak and Pammenter 2008). For example, ultrastructural stabilizing processes, such as plasmalemma displacement and intracellular space vacuolization, are common features observed in DT tissues under dehydration stress (Farrant 2000, Berjak and Pammenter 2008, Moore *et al*. 2008). Progression of the cell cycle is considered a good marker for DT (Faria *et al*. 2005) and it is hypothesized that cells in the G1 phase of the cell cycle (2C nuclei) are more resistant to stress and have a greater longevity than cells in the G2 phase (4C nuclei) (Saracco *et al*. 1995, Faria *et al*. 2005). Endoreduplication, resulting in ploidy levels equal or higher than 4C, have been correlated to a larger cell size and connected with reserve deposition in seeds (Lemontey *et al*. 2000, Atif *et al*. 2013) and salt and

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Abbreviations: DT - desiccation tolerance; TEM - transmission electron microscopy; WC - water content.
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osmotic stress tolerance (Elmaghrabi *et al*. 2013, Skirycz *et al*. 2011). However, to our knowledge, endoreduplication resulting in ploidy levels equal or higher than 8C has not been linked to DT or sensitivity in seeds.

Here, the seeds from a pantropical tree species *Leucaena leucocephala* (Lam.) De Wit were used as system to investigate DT and sensitivity in plants. *Leucaena* annually produces large amounts of orthodox seeds, which have an easily breakable seed coat and normally reach high germination percentages. Such characteristics make *Leucaena* a potential model species in tree seed research. Using techniques such as flow cytometry and transmission electron microscopy, cytological changes related to loss of DT in *Leucaena* primary roots during and after germination were explored.

Materials and methods

Leucaena seeds were collected in the vicinity of Lavras (Minas Gerais, Brazil) from at least 20 trees. All seeds were pooled, air-dried, and stored in plastic bags at 10 °C and a 40 % relative humidity. Previous assays revealed that *Leucaena* seeds exhibit physical, coat-imposed dormancy. To overcome this dormancy, mechanical scarification was performed prior to germination assays using sand paper, followed by immersion in distilled H_2O at 25 °C for 16 h. Germination tests were performed at 25 °C and a constant irradiance of 26 μmol m^{-2} s^{-1} and determined from four independent replicates of 25 seeds using radicle protrusion as criterion for germination. Germination was carried out in standing paper rolls moistened with distilled water. Loss of DT and its dynamics after germination were determined by drying *Leucaena* seeds with a primary root of 1, 3, or 5 mm long. Four replicates of 20 seedlings for each stage were dried for 3 d over an activated silica gel in a closed box with a forced air flow at 20 °C until a water content of 1.0, 0.67, 0.43, 0.25 and 0.08 g(H_2O) g^{-1}(d.m.) was reached. Water content was expressed on a dry mass basis (Cromarty *et al*. (1985). After dehydration, the germinated seeds were immediatelly pre-humidified at a 100 % relative humidity and at 25 °C for 24 h in the dark to avoid imbibitional damage (Leopold and Vertucci 1986) and rehydrated as previouslly described. Germinated seeds that continued their development and transformed into viable seedlings were considered desiccation-tolerant. Primary root survival and lateral root development were also evaluated.

Water content (WC) of dried seeds and germinated seeds with increasing protruded primary root length (1, 3, and 5 mm) was assessed in four replicates of 30 seeds by oven drying at 103 ± 2 °C for 17 h. The water content was expressed as g(H_2O) g^{-1}(d.m.). Imbibition pattern was assessed in 3 replicates of 10 seeds which were mechanically scarified, soaked in distilled water, and germinated as described above. After imbibition/ germination, water content was estimated. To evaluate seedling formation, *Leucaena* germinated seeds with a 3 mm primary root length were dehydrated up to 1.0, 0.67, 0.43, and 0.25 g(H_2O) g^{-1}(d.m.), pre-humidified, and rehydrated as previously described. Seedling fresh mass was evaluated after 7 d.

Relative DNA content was assessed by flow cytometry of intact nuclei prepared from radicles excised from seeds after 16, 24, 40, and 44 h of imbibition. Samples were prepared according to Carvalho *et al*. (2008) and analyzed on a *Partec PAS* flow cytometer (*Partec*®, Munster, Germany) equipped with a laser source and a series of filters (*TK 420*, *TK 560*, and *RG 610*). Each replicate was processed with no less than 5 000 nuclei. Samples with variation coefficients greater than 5 % were discarded and re-evaluated.

For transmission electron microscopy (TEM) analysis, samples of 3 mm long primary roots, fresh with 2.03 g(H_2O) g^{-1}(d.m.) and dried with 0.43 and 0.09 g(H_2O) g^{-1}(d.m.), and radicles excised directly from control dry seeds with 0.09 g(H_2O) g^{-1}(d.m.), were cut transversally and immersed in a modified Karnovsky fixative solution containing 2.5 % (m/v) glutaraldehyde, 2 % (m/v) formaldehyde, a 0.05 M sodium cacodylate buffer, and 0.001 M $CaCl_2$ (pH 7.2) and stored in a cold chamber (4 °C) until analysis. The samples were washed in a 0.05 M cacodylate buffer three times for 10 min, post-fixed in a 1 % (m/v) aqueous osmium tetroxide solution for 1 h, washed twice for 15 min in distilled water, transferred to a 0.5 % (m/v) uranyl acetate solution (4 °C) for 12 h, and finally washed once more in distilled water and dehydrated in a series of acetone solutions [25, 50, 75, 90 and 100 % (v/v)] three times. The dehydrated tissue was gradually infiltrated with Spurr epoxy resin [Spurr/acetone, 30 % for 8 h, 70 % for 12 h and 100 % (m/v) twice for 24 h each]. The specimens obtained were set in molds and polymerized at 70 °C for 48 h forming blocks. The blocks were trimmed using a diamond knife in a *Reichart-Jung* ultramicrotome. Ultra-thin sections (< 100 nm) were selected using a gold ring, placed on glass microscope slides, stained with toluidine blue (1 g of toluidine blue, 1 g of $Na_2B_4O_7 \cdot 10\ H_2O$ and 100 cm^3 of H_2O, filtered with a *Millipore* filter (0.2 mm) and mounted in *Permalt*. The ultra-thin sections were collected on gold slot grids and dried on aluminium racks coated with *Formvar*. The contrast of the sections was improved by staining in uranyl acetate followed by lead citrate for 3 min and then examined with a transmission electron microscope (TEM) (*Zeiss EM-109*) operating at 80 kV.

Results and discussion

Understanding dynamics of germination and simultaneous or subsequent loss of DT in orthodox seeds creates opportunities for examining how organisms tolerate desiccation stress (Bruggink and Van der Toorn 1995, Buitink *et al*. 2003, 2006, Maia *et al*. 2011, 2014). By observing changes in nuclear DNA content and ultrastructural modifications, this study provides insights into mechanisms underlying sensitivity to drought and desiccation in *Leucaena* seeds.

Like most species of the *Leguminosae* family, *Leucaena* produces seeds which have dormancy imposed by an impervious tegument (Rolston 1978, Baskin and Baskin 1998). If properly treated for dormancy release, the seeds of *Leucaena* could achieve high germination indexes and synchronization when compared with non-treated seeds (Fig. 1*A*). The *Leucaena* seeds were dispersed with a low water content of approximately 0.14 g(H_2O) g^{-1}(d.m.) and tolerated drying prior to storage down to 0.07 g(H_2O) g^{-1}(d.m.) without loss of viability. The fully imbibed *Leucaena* seeds with primary root lengths of 1, 3, and 5 mm reached water content of 1.93, 1.98, and 2.12 g(H_2O) g^{-1}(d.m.), respectively (Table 1). The imbibition pattern found for the *Leucaena* seeds (Fig. 1*B*) matched a pattern described by Bewley and Black (1994). This pattern is characterized by a rapid initial uptake of water (phase I), followed by a stationary phase (phase II) which culminates in a marked increase in fresh mass due to radicle protrusion and elongation. The point corresponding to the radicle protrusion occurred 40 h after the beginning of imbibition (Fig. 1*B*). The germinated seeds at different developmental stages exhibited similar rates of water loss and achieved similar water content by the end of drying (Fig. 2).

To relate developmental stages after germination with DT in a more accurate way, the germinated seeds at precise developmental stages with primary root lengths of 1, 3, and 5 mm were sampled and analyzed. These seeds

Table 1. Water content [g(H_2O) g^{-1}(d.m)] of *Leucaena* seeds at different developmental stages and conditions. Means ± SEs, $n = 4$.

Samples	Water content
Dry seeds from the seed batch	0.13 ± 0.002
Dry seeds further dried above silica gel for 3 d	0.08 ± 0.004
Germinated seeds (1 mm primary root lengh)	1.93 ± 0.012
Germinated seeds (3 mm primary root lengh)	1.97 ± 0.006
Germinated seeds (5 mm primary root lengh)	2.12 ± 0.013

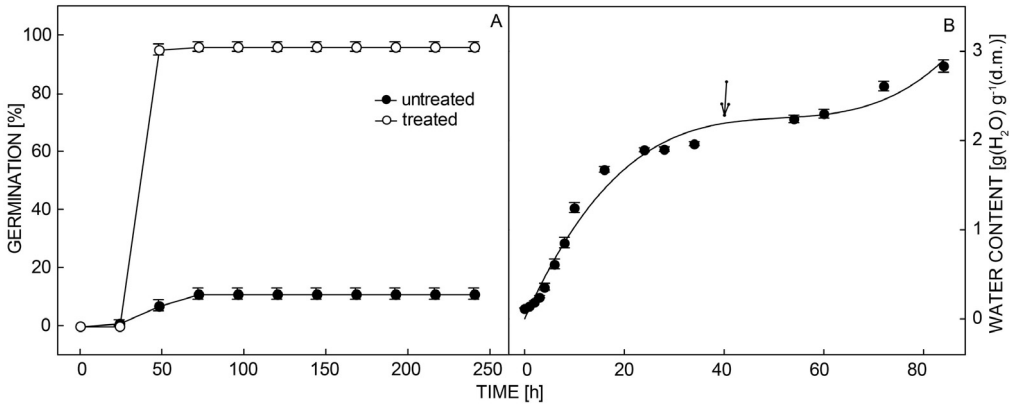

Fig. 1. Time course of germination and water content during imbibition of *Leucaena* seeds at 25 °C. Seeds treated with scarification (*open circles*) or untreated (*closed circles*). Means ± SEs, $n = 4$ (*A*) or 10 (*B*). The *arrow* indicates the point of radical protrusion.

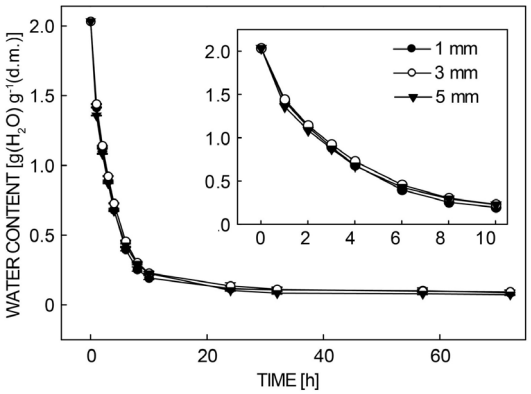

Fig. 2. Water content changes of *Leucaena* seeds upon dehydration above dry silica gel. Germinated seeds with a primary root length of 1 mm (*closed circles*), 3 mm (*open circles*), and 5 mm (*triangles*). The *inset* depicts the first hours of drying in more detail. Means ± SEs, $n = 4$.

were dehydrated to different water content ranging from 2.15 to 0.07 g (H_2O) g^{-1}(d.m.) and evaluated for their survival, *i.e.*, an ability to resume normal growth after being dehydrated and rehydrated as well as for survival of their primary roots and secondary root development (Fig. 3). Desiccation tolerance was completely lost around the point of radicle protrusion. The majority of the seeds in all developmental stages did not tolerate drying to a water content of 0.07 g (H_2O) g^{-1}(d.m.) (Fig. 3). Only 16 % of the seeds with a 1 mm root survived, and no survival was observed for those with a primary root length of 3 and 5 mm (Fig. 3*A*,*B*,*C*). In addition, the more germinated seed, the quicker loss of root viability in relation to drying (Fig. 3*A*,*B*,*C*). Together, these data suggest a developmental stage-dependent sensitivity to desiccation. Noticeably, more seedlings survived due to secondary root formation (Fig. 3*A*,*B*,*C*,*D* and Fig. 4*A*,*B*), a phenomenon also observed by other authors (Koster and Leopold 1988, Bruggink and Van der Toorn 1995, Vieira *et al*. 2010, Maia *et al*. 2011). Although all

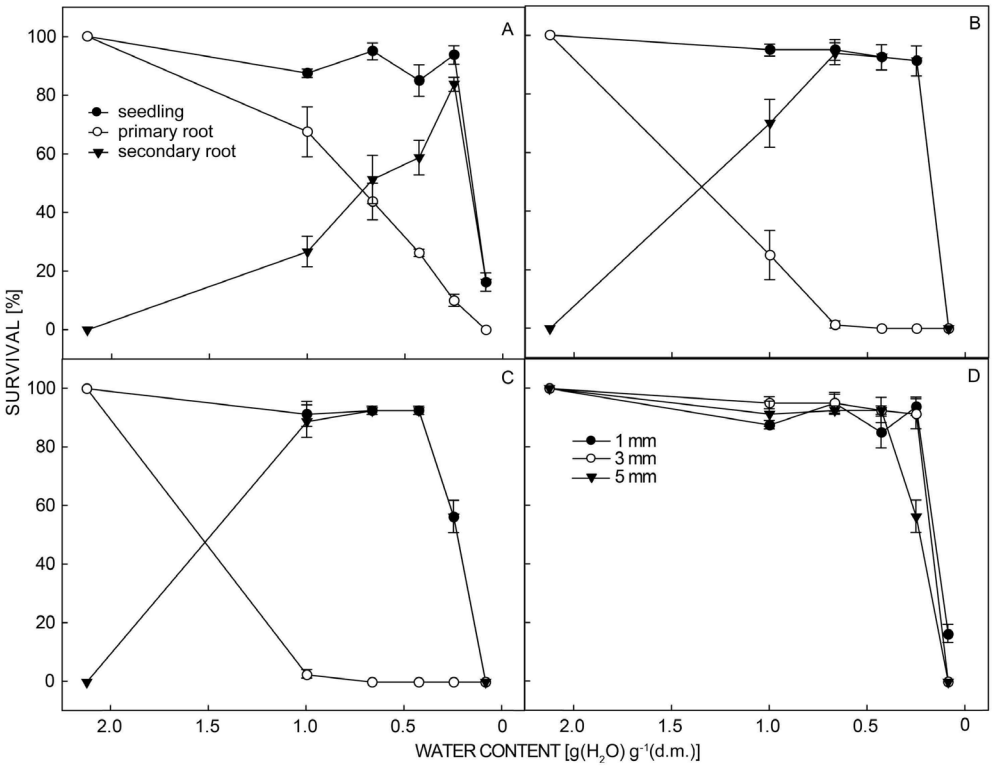

Fig. 3. Loss of desiccation tolerance in germinated *Leucaena* seeds dried to a different water content. Seeds with primary roots of 1 mm (*A*), 3 mm (*B*), and 5 mm (*C*), and seedling formation (*D*). Means ± SEs, $n = 4$.

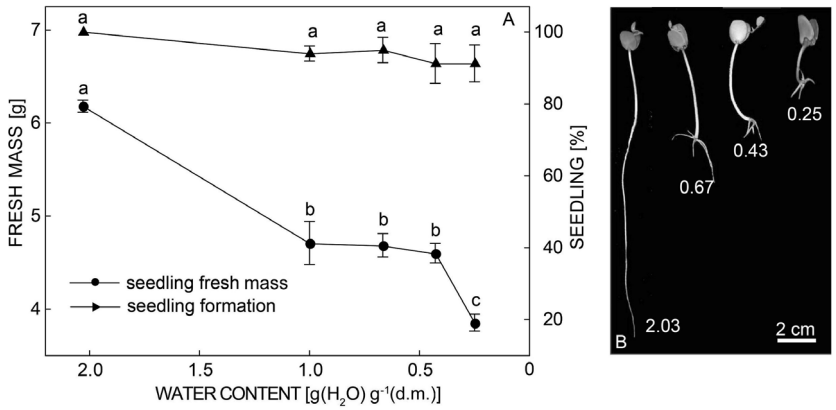

Fig. 4. Seedling formation and growth in *Leucaena* seeds subjected to different dehydration stresses. *A* - Seedling formation (*triangles*) and fresh mass (*circles*) upon different dehydration stresses. Fresh mass was measured 7 d after rehydration. Means ± SEs, $n = 4$; different letters represent significant differences at $P \leq 0.05$ between data points in the same curve. *B* - Appearance of dehydrated and hydrated *Leucaena* seedlings after 7 d. After dehydration to 0.67 g(H_2O) g^{-1}(d.m.), most seedlings developed secondary roots to compensate for the loss of their primary root.

germinated seeds, independently of the developmental stage, had high survival rates when dried to water content higher or equal to 0.25 g(H_2O) g^{-1}(d.m.) (Fig. 3*D*), a gradual reduction in seedling fresh mass was evident (Fig. 4*A*) showing a gradual loss of DT along drying. The point where DT begins to be lost, taking into account the imbibition time and primary root length after radicle protrusion, varies from species to species. Nevertheless, other factors such as onset of the cell cycle may be relevant for seed desiccation tolerance. It has been suggested that nuclear DNA content is directly related to sensitivity to stress responses and phase transitions on seeds where a higher or lower DNA content can positively or negatively influence the sensitivity to a stress or speed of seed germination (Saracco *et al*. 1995, Lemontey *et al*. 2000, Sliwinska 2003, Faria *et al*. 2005, Atif *et al*. 2013). The flow cytometry analysis of *Leucaena* dissected root tips before, during, and after germination indicates the existence of nuclei 2C, 4C, and 8C (Fig. 5 and Fig. 1 Suppl.). Most of the nuclei measured during imbibition and after radicle protrusion had 2C DNA content (Fig. 5*A*) indicating that most of the evaluated cells were at the G1 phase of the cell cycle. The root tips isolated from the dry seeds displayed the lowest 4C DNA content indicating, as expected, small changes in the cell replication profile. The increase in sensitivity to dehydration observed in the *Leucaena* seeds under and after the visible germination coincided with an increase in 4C DNA content in primary root cell nuclei (Fig. 5*B*). These data indicate that at the end of maturation, two blocks may be acting on the cell cycle. The first works maintaining cells in the G1/S phase (2C DNA, a pre-synthetic phase of the cell cycle), whereas the second prevents G2 phase cells (4C DNA) from progressing into mitosis. Similar results were found by Faria *et al*. (2005) who demonstrated that radicles from mature embryos of *Medicago truncatula* also contain relatively high 4C DNA content (45 %). Although some species show a high 4C DNA content, this is not a prevalent situation found in embryos of mature orthodox seeds. Generally, the majority of cells in mature quiescent embryos have 2C DNA content reflecting their retention in the G1 phase of the cell cycle (Deltour 1985, Bino *et al*. 1993, De Castro *et al*. 2000). Similarly as in *Leucaena*, an increase in 4C DNA content also marks germination progress in seeds of *Lycopersicon esculentum*, *Capsicum annuum*, *Pinus banksiana*, *Beta vulgaris*, *Coffea arabica, Hordeum vulgare*, and many other species (Sliwinska 2009).

A negative correlation between the 8C DNA content and the capacity of germinating *Leucaena* seeds to tolerate desiccation was also observed (Fig. 5*C*). Apparently, the seeds of *Leucaena* underwent extra cycles of endoreduplication and accumulated a greater DNA content in their cells. This observation may indicate that endoreduplication cycles may interfere with tolerance to certain abiotic stresses. Endoreduplication is well documented in endosperm and cotyledons of developing seeds, but its physiological significance is poorly understood. Endoreduplication principally influences cell size and elongation (*e.g.*, seed development and germination), nutrient storage (*e.g.*, cotyledons) and metabolic activity (Atif *et al*. 2013, Dante *et al*. 2014, Edgar *et al*. 2014). The association of endoreduplication with stress tolerance or sensitivity is less evident. Nevertheless, some studies support an idea that certain stresses, like osmotic stress acting *via* abscisic acid, could act by inhibiting endoreduplication (Wang *et al*. 1998, Setter and Flannigan 2001). On the other hand, a recent review by Ochatt (2015) collected information that supports a role for abscisic acid in inducing a cell division arrestment, whereas still allowing DNA replication in developing embryos, thus promoting

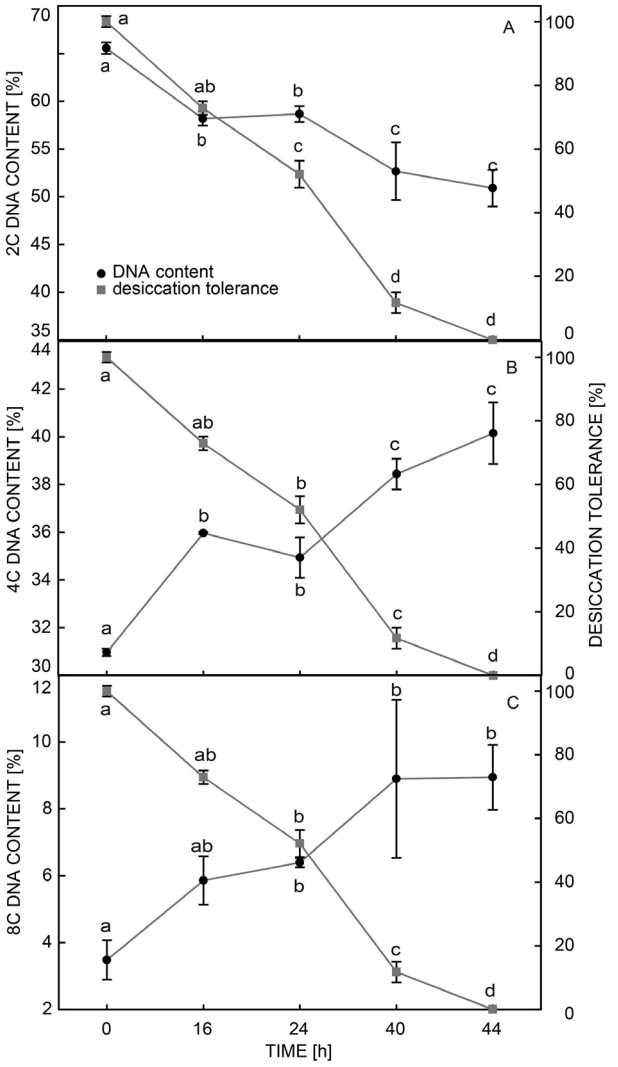

Fig. 5. Nuclear DNA content and desiccation tolerance in *Leucaena* seeds. Nuclear DNA content 2C (*A*), 4C (*B*), and 8C (*C*) of excised *Leucaena* radicles (*circles*) *versus* desiccation tolerance (*squares*) of *Leucaena* seeds during and after germination. At a time point of 44 h, seeds had a 1 mm radicle. Desiccation tolerance is expressed as the percentage of seeds that showed primary root survival. Means ± SEs, $n = 4$; different letters represent significant differences at $P \leq 0.05$ between data points in the same curve.

endoreduplication. In addition, it has been shown that the expression of *CCS52*, a gene that controls the cell cycle switch, was up-regulated in *Medicago truncatula* calli subjected to salt stress and coincided with the onset of endoreduplication and the acquisition of tolerance to water and ionic stresses (Elmaghrabi *et al*. 2013). Together, those findings indicate a positive role for endoreduplication and stress tolerance at least in embryogenic calli and during seed development. In *Leucaena*, endoreduplication apparently indicates progression of germination and could be a compensatory response to associated stresses experienced during germination (*e.g.*, oxidative stress caused by radicle protrusion). Consequently, a greater genome copy number caused by increased endoreduplication at the end of germination might be detrimental in the case of severe stresses like desiccation, thus contributing to a reduced capacity to tolerate drying observed in the *Leucaena* germinated seeds.

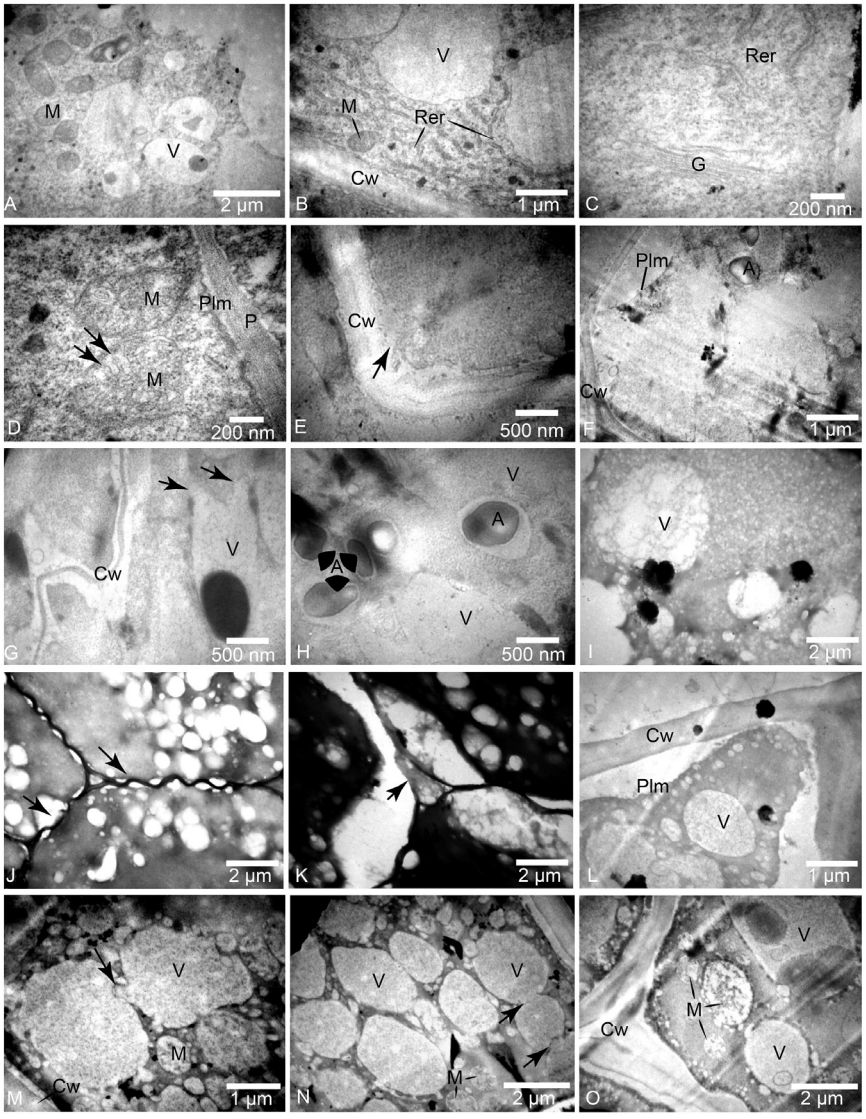

Fig. 6. Ultrastructural changes in *Leucaena* seeds subjected to different dehydration stresses. Transmission electron micrographs of 3 mm long primary roots excised from fresh [2.03 g(H2O) g^{-1}(d.m.)] (*A*,*B*,*C*,*D*) and dried to 0.43 g(H_2O) g^{-1}(d.m.) (*E*,*F*,*G*,*H*) or 0.09 g(H_2O) g^{-1}(d.m.)] (*I*,*J*,*K*), and from radicles excised directly from non-germinated dried seeds further dried to 0.07 g(H_2O) g^{-1}(d.m.) (*L*,*M*,*N*,*O*). Mitochondria (M), vacuoles (V), cell wall (Cw), Golgi body (G), rough endoplasmic reticulum (Rer), plasmalemma (Plm), and amyloplast (A) are shown. *Arrows* indicate mitochondrial cristae (*D*), plasmallemma rupture (*E*), vacuole rupture (*G*), convoluted cell wall (*J*), cell wall rupture (*K*), and vacuole fusion (*M*).

Microscopy has proved to be a useful tool to investigate the extent of damage to seeds under various stresses. Here, the samples obtained from 3 mm long primary roots of *Leucaena* [fresh, 2.12 g(H_2O) g^{-1}(d.m.), and dried to 0.43 and 0.07 g(H_2O) g^{-1}(d.m.)] as well as from radicles excised directly from non-germinated dry

seeds were analyzed. In general, fully hydrated radicle cells displayed a high number of well-differentiated mitochondria, strong profiles of rough endoplasmic reticulum, and a prominent Golgi apparatus (Fig. 6*A*,*B*,*C*,*D*) indicating the presence of an active metabolism. The plasmalemma was also intact and adhered to the cell wall (Fig. 6*D*). As soon as the germinated seeds were progressively dehydrated, their radicle cells became vacuolated and were extensively damaged (Fig. 6*E*,*F*,*G*,*H*). Most of the organelles disappeared, few mitochondria, apparently degraded, were observed (data not shown), and amyloplasts became less electron-dense (Fig. 6*H*). Cytoplasm contraction led by the reduction of water content caused the cell membrane to separate from the cell wall and the collapse of its trilaminar structure (Fig. 6*E*,*F*). When dried to 0.07 g(H_2O) g^{-1}(d.m.), the primary root cells experienced an extreme damage and total collapse of their intracellular structure. The cytoplasm was electron-dense, indistinct and, in some cells, it became granular (Fig. 6*I*). Besides, many cells had numerous vacuoles of a small size, almost no organelles were discernible. The cell membrane was disrupted and detached from the cell wall, which was thin convoluted, and ruptured at some points (Fig. 6*J*,*K*). Opposite, radicle cells of non-germinated seeds (DT) taken directly from storage and dried to 0.07 g(H_2O) g^{-1}(d.m.) showed a relatively organized intracellular space (Fig. 6*L*,*M*,*N*,*O*). Although those radicles were completely dry, it was still possible to observe some organelles such as mitochondria with poorly developed cristae (Fig. 6*M*,*N*,*O*) and the presence of large vacuoles (Fig. 6*M*,*N*). The plasmalemma was detached in relation to the cell wall, however, no extensive damage to its trilaminar structure was present (Fig. 6*L*).

Apparently, two strategies to avoid further damage took place in *Leucaena* seeds. First, under the intermediate dehydration level, most of the organelles related to an active metabolism, such as the Golgi apparatus, rough endoplasmic reticulum, and mitochondria, were degraded. The dismantlement of these organelles might serve to arrest development and to minimize oxidative damage caused by reactive oxygen species production. Second, under the lower water content, mechanical protection strategies, such as cell wall convolution, cell membrane displacement, and intense vacuolization, had to take place to prevent cell collapse. As observed in the radicle tips of the non-germinated dry *Leucaena* seeds, the displacement of the cell wall membrane caused by cytoplasm retraction in response to dehydration could be linked to the ability to tolerate drying. This response ensured membrane integrity maintenance during the dry stage. On the other hand, when germinated seeds were dried to 0.07 g(H_2O) g^{-1}(d.m.), the ultrastructural damage they experienced compromised their tolerance mechanisms. As suggested by Öpik (1985), cell wall and membrane folding during drying may be linked to the prevention of tension between the cell membrane and the cell wall. Additionally, filling the intracellular space with vacuoles may also be connected to cellular structure maintenance *via* preventing cytoplasm retraction, cell membrane detachment, and cell wall collapse (Pampurova and Van Dijck 2014). This mechanism has been described in mesophyll cells of two resurrection plants, *Xerophyta humilis* and *Craterostigma wilmsii*. In both species, vacuolization occurred in response to drying and the prevention of cell wall membrane detachment and collapse was associated with this strategy (Farrant 2000).

To sum up, we have explored the dynamics of loss of DT in *Leucaena* seeds. According to our results, the *Leucaena* seeds lost the ability to tolerate drying at the end of germination at the point when the embryonic axis started to elongate, and the radicle protruded. The loss of DT was associated with changes in nuclear DNA status in the cells of the radicle. Cells with higher ploidy levels were less tolerant to desiccation. Contrary to what was observed in the *Leucaena* dry seeds, the intensity and extent of ultrastructural damage observed in the dried germinated *Leucaena* seeds were severe enough to overcome the resilience of its primary root cells and consequently their ability to resume normal growth. In the future, the pattern of loss of DT observed in the seeds of *Leucaena* might be used to elucidate structural, biochemical, genetic, and ontogenetic events associated with desiccation sensitivity.

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