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RESEARCH PAPER

Changes in DNA and microtubules during loss and re-establishment of desiccation tolerance in germinating *Medicago truncatula* seeds

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Abstract

Desiccation tolerance (DT) in orthodox seeds is acquired during seed development and lost upon imbibition/germination, purportedly upon the resumption of DNA synthesis in the radicle cells. In the present study, flow cytometric analyses and visualization of microtubules (MTs) in radicle cells of seedlings of *Medicago truncatula* showed that up to a radicle length of 2 mm, there is neither DNA synthesis nor cell division, which were first detected in radicles with a length of 3 mm. However, DT started to be lost well before the resumption of DNA synthesis, when germinating seeds were dried back. By applying an osmotic treatment with polyethylene glycol (PEG) before dehydration, it was possible to re-establish DT in seedlings with a radicle up to 2 mm long. Dehydration of seedlings with a 2 mm radicle, with or without PEG treatment, caused disassembly of MTs and appearance of tubulin granules. Subsequent pre-humidification led to an almost complete disappearance of both MTs and tubulin granules. Upon rehydration, neither MTs nor tubulin granules were detected in radicle cells of untreated seedlings, while PEG-treated seedlings were able to reconstitute the microtubular cytoskeleton and continue their normal development. Dehydration of untreated seedlings also led to an apoptotic-like DNA fragmentation in radicle cells, while in PEG-treated seedlingss DNA integrity was maintained. The results showed that for different cellular components, desiccation-tolerant seedlings may apply distinct strategies to survive dehydration, either by avoidance or further repair of the damages.

Key words: Cell cycle, desiccation tolerance, DNA content, DNA integrity, *Medicago truncatula*, microtubules.

Introduction

Desiccation tolerance (DT) in plants can be considered as the ability to rehydrate successfully after the removal of 80–90% of protoplasmic water, leading to moisture content (MC) below 0.3 g g^{-1} (or 23% on a wet basis), when the hydration shell of molecules is lost (Oliver *et al.*, 2000; Hoekstra *et al.*, 2001). There are three criteria that a plant or plant structure must meet in order to survive such severe loss of protoplasmic water: (i) limitation of the damage suffered by the cells during desiccation to a repairable level; (ii) maintenance of its physiological integrity in the dry state; and (iii) mobilization of repair mechanisms upon rehydration aiming to revert the damages caused by desiccation and/or rehydration (Bewley, 1979).

In orthodox seeds, DT is acquired during seed development, enabling them to withstand maturation drying, when

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more than 90% of the water may be lost (Adams and Rinne, 1980). DT is maintained after shedding, allowing further drying of seeds, when MC may be diminished to *c*. 0.05 g g^{-1}, without loss of viability. When dried seeds are imbibed, DT remains unchanged for some time, so they can be dried back to their original MC without irreversible damage. However, if seeds are allowed to imbibe longer, DT is gradually lost. The point at which DT starts to be lost varies among species if analysed in terms of imbibition time (Senaratna and McKersie, 1983, 1986; Hong and Ellis, 1992; Reisdorph and Koster, 1999; Koster *et al.*, 2003; Ren and Tao, 2003) or protruded radicle length (Lin *et al.*, 1998; Leprince *et al.*, 2000; Pukacka, 2001; Buitink *et al.*, 2003). However, if germination is assessed regarding the activation of the cell cycle, loss of DT coincides, irrespective of species, with the resumption of cell division (Berrie and Drennan, 1971; Osborne *et al.*, 2002) or, more frequently, DNA synthesis (Sargent *et al.*, 1981; Dasgupta *et al.*, 1982; Deltour, 1985; Osborne and Boubriak, 1994; Osborne, 2000; Boubriak *et al.*, 2000). It has been shown that cells in the G_2 phase of the cell cycle, with duplicated DNA, are more sensitive to stress than cells that are still in the G_1, pre-synthetic phase (Deltour, 1985; Sliwinska, 2003). It has been suggested, however, that although DNA replication is a suggestive developmental marker for the loss of DT in germinating seeds, it is not necessarily the reason (Sargent *et al.*, 1981).

Another component of the cell cycle that may be involved in the loss of DT during germination is the microtubular cytoskeleton, which is markedly sensitive to desiccation stress (Sargent *et al.*, 1981). Microtubules (MTs) are elongated tubular structures, made of α and β-tubulin, which in plant cells play an important part in cell elongation, determination of the division site, chromosome separation, and cytokinesis (Alberts *et al.*, 2002; Wasteneys and Galway, 2003). In desiccation-sensitive seeds MTs can be irreversibly deranged by dehydration (Berjak and Pammenter, 2000). Microtubule reassembly is among the cellular repair processes that have been linked to DT (Oliver, 1996; Mycock *et al.*, 2000).

The maintenance of the genetic information carried by the DNA is essential to cell survival upon dehydration and rehydration (Osborne *et al.*, 2002). The stability of the DNA on dehydration and the ability for its repair on rehydration is a prominent feature displayed by desiccation-tolerant seeds (Boubriak *et al.*, 1997). Seeds that are allowed to germinate until (or beyond) the point where they become desiccation-sensitive, can experience irreversible DNA degradation when subjected to dehydration. When cells are confronted with environmental stresses, they can either die passively (accidental cell death) or can self-destruct (programmed cell death), depending on the stress type and intensity (Danon *et al.*, 2000). Self-destruction is orchestrated by an active mechanism known as apoptosis, the biochemical hallmark of which is the cleavage of the DNA at internucleosomal sites by endonucleases, generating oligonucleosomal fragments. This DNA fragmentation can be detected by the formation of DNA ladders on agarose gels (Stein and Hansen, 1999). For cell death processes other then apoptosis, a smear of broken DNA, instead of a clear fragmentation pattern is seen (Wang *et al.*, 1998).

The genetic information for DT is certainly present in the genome of plants that bear orthodox seeds. In those plants, the restriction of DT to specific stages of seed development is due to differences in the control of gene expression (Bartels and Salamini, 2001). The cellular protection system exhibited by those seeds may be induced in vegetative tissues by environmental cues related to drying (Oliver *et al.*, 2000). The feasibility of the re-establishment of DT in seedlings originated from orthodox seeds by applying an osmotic stress, as shown by Bruggink and van der Toorn (1995) and Buitink *et al*. (2003), has appeared as an outstanding tool for studies on the mechanisms of desiccation tolerance and sensitivity in seeds. By using such an approach, the present study aimed to investigate the relationship of the DNA (relative content and integrity) and microtubule configurations with the loss and re-establishment of desiccation tolerance in germinating seeds of *Medicago truncatula* Gaertn. cv. Jemalong A17.

Materials and methods

Plant material

Medicago truncatula Gaertn. cv. Jemalong A17 plants were routinely grown in an environmentally controlled growth chamber (16/8 h photoperiod; 170 μmol m^{-2} s^{-1}; 25 °C; 60% RH). Mature pods were collected at shedding, around 30 d after flowering, stored at 20 °C (Journet *et al.*, 2001) and seeds were extracted manually when needed.

Dormancy release and seed germination

Medicago truncatula seeds exhibit a combination of physical (coat-imposed) and physiological dormancy, with the latter lasting for 3–4 months following pod abscission (Journet *et al.*, 2001). In order to overcome these dormancies, seeds were chemically scarified by immersing them in concentrated sulphuric acid for 5–10 min and subjected to cold imbibition (36 h at 4 °C), in the dark, in Petri dishes (9 cm diameter; 250 seeds per dish) with two filter papers (No. 595, Schleicher & Schuell, Germany) moistened with 9 ml distilled water. During cold imbibition Petri dishes were kept shaking on a benchtop shaker (70 rpm). Seeds were transferred to new Petri dishes (9 cm diameter; 50 seeds per dish) with two filter papers moistened with 2.5 ml of distilled water and kept in the dark at 20 °C (modified from Sieberer *et al.*, 2002). Seeds that showed radicle protrusion were considered germinated. To characterize the dormancy release better, the germination of 2-month-old seeds treated with sulphuric acid plus cold imbibition was compared with seeds subjected to only one treatment (acid scarification or cold imbibition) as well as to untreated (control) seeds, in three replications of 50 seeds.

Moisture content (MC) determination

MC was assessed in four replications of 10 seeds (or radicles), by oven-drying at 103 °C for 17 h, according to the International Seed

Testing Association (ISTA, 1996). MC is expressed on a dry weight basis, i.e. in g H_2O g^{-1} dry matter or simply g g^{-1}.

DNA content assessment

Relative DNA content assessment was done by flow cytometry, using suspensions of intact nuclei prepared from radicles excised from dry seeds or seedlings with increasing protruded radicle length (1, 2, 3, and 4 mm). Only the tip (1 mm) of the radicles, which includes the root cap and the meristematic region, was used in the analyses. Each treatment consisted of five replications of 10 radicle tips. Sample preparation was done according to Arumuganthan and Earle (1991) and analyses were performed with a flow cytometer (EPICS XL-MCL, Beckman-Coulter, Miami, FL, USA) equipped with an argon ion laser at 488 nm. Histograms were processed using ModFit LT (Verity Software House, Topsham, ME, USA) for data analysis and correction of the background noise. In each replication 10 000 nuclei were analysed. Statistical analyses were performed with the software SPSS 11.0.1.

Assessment of the loss of desiccation tolerance during germination

Seeds were germinated as described previously, germination was scored at various times and then seeds/seedlings were dehydrated. Dehydration was done over a saturated solution of K_2CO_3 (43% RH) in a closed box with circulating air at 23 °C for 3 d, based on Buitink *et al.* (2003). After dehydration, seeds were pre-humidified in humid air (100% RH) for 24 h at 20 °C to avoid imbibitional damage and then rehydrated (2.5 ml H_2O/Petri dish 9 cm; two filter papers; 20 °C; in the dark). Seeds that germinated and seedlings that continued their normal development were considered desiccation-tolerant. The data of DT (%) were normalized to the maximum germination attained (%) in the germination test. The experiment was replicated three times with 50 seeds being used each time that germination and DT were evaluated.

Re-establishment of desiccation tolerance

To assess the re-establishment of DT in seedlings, they were selected by their radicle length (1, 2, 3, 4, and 5 mm) using a dissection microscope and a metallic ruler with 0.5 mm scale divisions and either dried directly or after 3 d of incubation in a polyethylene glycol (PEG) 6000 solution (355 g PEG dissolved in 1.0 l H_2O). Incubation was done in the dark, in 9 cm Petri dishes containing two filter papers wetted with 7 ml of PEG solution. The incubation temperature of 10 °C which gives a water potential of −1.7 MPa, used by Buitink *et al.* (2003) for the re-establishment of DT in seedlings of *M. truncatula* cv. Paraggio, was not suitable for cv. Jemalong A17 used in the present study because it could not inhibit protruding radicles from continuing growth during incubation. The problem was solved by carrying out the incubation at 5 °C. By decreasing the temperature, the water potential of the PEG solution was slightly lowered to −1.8 MPa. After incubation, seedlings were rinsed thoroughly in distilled water and then dehydrated, pre-humidified, and rehydrated as described before. Seedlings that resumed normal growth after rehydration were considered desiccation-tolerant. Four independent experiments with 50 seedlings each were carried out.

Viability test (tetrazolium test)

Seedlings with a radicle length of 2 mm were dehydrated (with or without previous PEG treatment), pre-humidified, and incubated in a 1% (w/v) solution of 2,3,5-triphenyl tetrazolium chloride (Merck, Darmstadt, Germany), at 20 °C for 18 h in the dark. Stained tissues were considered viable, and unstained white tissues were considered dead (ISTA, 1996). The test was done using three replications of 50 seedlings per treatment.

DNA isolation and electrophoresis to assess DNA fragmentation

Chromosomal DNA was extracted from 2 mm long radicles of seedlings (control and dehydrated with and without PEG treatment) and isolated following a protocol modified from Liu *et al.* (1995). Approximately 40 mg of radicles from hydrated (control) and 20 mg from dehydrated (PEG-treated and untreated) seedlings were ground to a fine powder with a mortar and pestle in liquid nitrogen and mixed with the extraction buffer (0.6 ml NaCl, 100 mM TRIS–HCl pH 7.5, 40 mM EDTA, 4% sarkosyl, and 1% SDS) previously diluted with urea and phenol. Phenol:chloroform was added, the mixture was centrifuged and the aqueous phase collected and mixed with isopropanol. DNA was precipitated by inverting the tubes a few times. After incubation for 10 min at room temperature and centrifugation, the pellet was washed with 80% ethanol and dissolved in TRIS-EDTA (TE) pH 8.0 containing RNase A. Samples (5 μg $lane^{-1}$) of DNA were loaded on a 1% agarose gel stained with ethidium bromide.

Immunohistochemical detection of the microtubular cytoskeleton

Radicles were excised from seeds/seedlings, both before imbibition (dry seeds) and after germination, with lengths of 1, 2, 3, and 4 mm. Radicles (2 mm long) were also excised from seedlings after each of the following steps, with or without PEG treatment: dehydration, pre-humidification, and rehydration. The type of fixation used depended on the moisture content of the tissue. Radicles excised from dry seeds/seedlings (both before imbibition and after germination followed by dehydration) were chemically fixed in water-free methanol+0.1% glutaraldehyde for 4 h, at 20 °C. Radicles excised from undried seedlings were plunged into liquid propane (cooled down in liquid nitrogen) and transferred to cryo-tubes containing frozen freeze-substitution medium (water-free methanol+0.1% glutaraldehyde), also cooled down in liquid nitrogen. The cryo-tubes were put in a freeze substitution unit (FreasySub, Cryotech Benelux, Schagen, The Netherlands) for 78 h. The next steps were similar for both chemically and cryo-fixed samples. The fixative medium was replaced by ethanol (series of increasing concentrations), followed by embedding in butylmethylmethacrylate (BMM) and UV polymerization at −20 °C for 48 h, according to Baskin *et al*. (1992). Five roots were analysed for each treatment. Longitudinal sections (3 μm thick) were placed on slides and BMM was removed by washing in acetone. The slides were then rinsed in phosphate buffered saline (PBS) and sections were blocked in 100 mM hydroxyl tetra ammonium chloride (HAC) and in 26 mM bovine serum albumin (BSA). Next, they were incubated with the primary antibody (mouse anti-α-tubulin [Sigma, Zwijndrecht, The Netherlands], diluted 1:200 v/v), followed by the secondary antibody (goat anti-mouse IgG conjugated with fluorescein-5-isothiocyanate – FITC [Molecular Probes, Leiden, The Netherlands], diluted 1:200 v/v). As a control, slides without the first antibody were used for every treatment. A confocal laser scanning microscope (Biorad MRC-600) and an epifluorescence microscope (Nikon Labophot) were used for the visualization of microtubules.

Results

Medicago truncatula *seed germination: dormancy release and imbibition*

Germination of seeds without pre-germination treatment (control) remained around 5% until 11 d, increasing afterwards and reaching its maximum (95%) at 17 d of

imbibition. Seeds subjected to chemical scarification or cold imbibition germinated faster than the control, with a better performance by acid-treated seeds, suggesting that in the combined dormancy present in *M. truncatula* seeds, the physical (seed coat) dormancy is stronger than the physiological (embryo) dormancy. The most efficient treatment was achieved by the combination of acid scarification and cold imbibition that allowed the start of germination after a few hours of imbibition at 20 °C, reaching the maximum (96%) within 1 d (Fig. 1).

The imbibition curve of seeds chemically scarified and subjected to cold imbibition is shown in Fig. 2. Phase 1 of imbibition, characterized by a rapid increase in fresh weight, occurred in the first 9 h. Between 9 h and 40 h of imbibition, the gain in fresh weight was very small, characterizing the plateau or phase 2 of imbibition. Visible germination (radicle protrusion) started after 42 h of imbibition (or 6 h after transfer to 20 °C). After this point, germinated seeds (seedlings) entered phase 3, resuming the increase in fresh weight (Fig. 2). Although seeds germinated only after transfer to 20 °C, they were also able to do so at 4 °C, when kept at this temperature for 4 d (not shown).

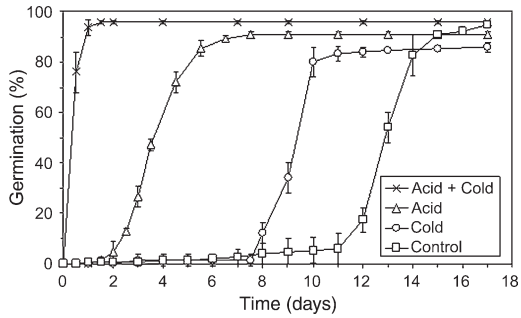

Fig. 1. Germination of *Medicago truncatula* seeds at 20 °C after various treatments for dormancy release: chemical scarification with sulphuric acid; cold imbibition (4 °C) for 36 h; chemical scarification plus cold imbibition; and control. Each data point is the mean of three replications of 50 seeds. Bars represent standard deviation.

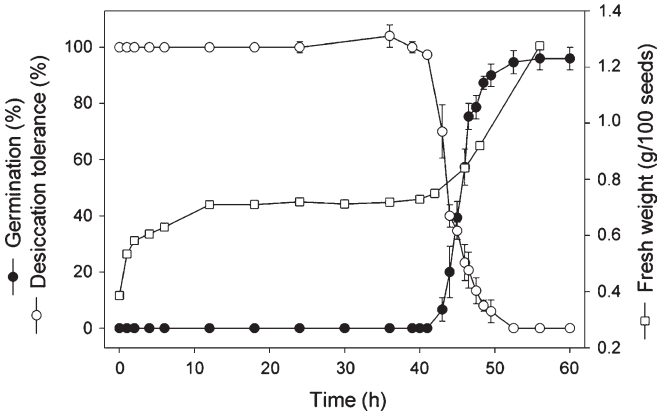

Fig. 2. Imbibition curve, germination, and loss of desiccation tolerance of chemically scarified *Medicago truncatula* seeds. Seeds were imbibed for 36 h at 4 °C and then transferred to 20 °C. Desiccation tolerance was determined after drying of the imbibed/germinated seeds, followed by pre-humidification and rehydration. Seeds that germinated or seedlings that resumed radicle growth and normal development were considered desiccation-tolerant. Each data point is the average of three independent experiments of 50 seeds/seedlings. Bars represent standard deviation.

DNA content

Flow cytometric analyses of nuclear DNA contents in radicles of mature dry seeds of *M. truncatula* revealed a high 4C DNA content (45%), which remained unchanged during germination and radicle growth until 2 mm. With further growth the relative content of 4C nuclei increased significantly, reaching 63% in 3 mm long radicles, levelling off afterwards, with 65% of 4C DNA in 4 mm long radicles (Fig. 3). The 4C DNA content in 2 mm long radicles remained unchanged during incubation in PEG for 3 d (not shown).

The relation between the progress of germination and loss of desiccation tolerance

In order to relate the course of germination with the loss of DT, seeds were chemically scarified, imbibed (cold imbibition followed by imbibition at 20 °C) and, at various times, germination and DT evaluated. Germination, as assessed by radicle protrusion, did not occur during the 36 h of cold imbibition, but was observed after 7 h of imbibition at 20 °C (43 h of total imbibition time). From this point germination raised sharply until its maximum (96%) by 56 h of total imbibition time (Fig. 2). Before the start of the germination, i.e. until 43 h of imbibition, DT remained

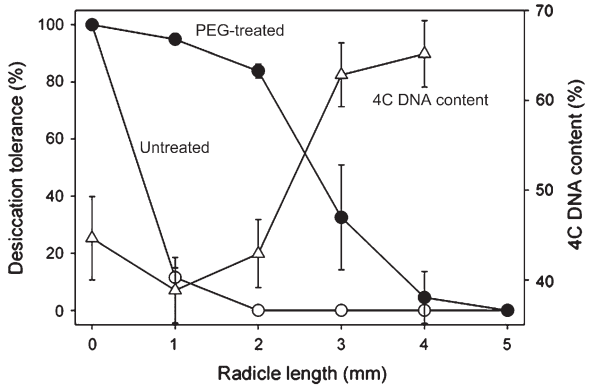

Fig. 3. Protruded radicle length and desiccation tolerance in *Medicago truncatula* seedlings with (closed symbols) and without (open symbols) previous incubation in PEG, and 4C DNA content of radicle cells from dry seeds and seedlings. DT was determined after drying the seedlings with or without PEG treatment, followed by pre-humidification and rehydration. Seedlings that resumed radicle growth and normal development upon rehydration were considered desiccation-tolerant. Each data point is the average of four independent experiments of 50 seedlings. Bars represent standard deviation. For flow cytometry each data point is the average of five replications of 10 radicle tips. Bars represent standard deviation.

unchanged, at 100%, dropping fast afterwards, inversely related to the progress of germination (Fig. 2).

Typically, even for a homogeneous batch of seeds, germination did not occur uniformly. Consequently, at any given time-point of the course of germination after 43 h (Fig. 2), the population of seeds is comprised of germinated (at different stages) and non-germinated seeds. Thus, to characterize the loss of DT to the progress of the germination in a more accurate way, seeds were put to germinate, selected by their protruded radicle length, and tested again for DT. The results show that right after visible germination, when the protruded radicle length was 1 mm, only 12% of the seedlings was still desiccation tolerant, i.e. able to resume normal growth after being dehydrated and rehydrated. Seedlings with a radicle length of 2 mm or longer lost DT completely (Fig. 3). Seedlings that did not resume radicle growth, frequently showed growth of the cotyledons and, to a lesser extent, also of the hypocotyl. However, the longer the radicle before dehydration, the less frequent the growth of cotyledons and hypocotyl (not shown).

Re-establishment of desiccation tolerance in seedlings by incubation in PEG

In order to relate re-establishment of DT to the progress of germination, seedlings of *M. truncatula* with radicle lengths ranging from 1 mm to 5 mm were incubated in PEG solution, dehydrated, pre-humidified, and rehydrated. Seedlings that resumed radicle growth after dehydration and rehydration and showed normal development were considered desiccation tolerant. The results in Fig. 3 show that DT could be substantially re-induced (84%) in seedlings with a radicle length of up to 2 mm. From 2 mm onwards there was an abrupt drop in DT, decreasing in value to 33% at 3 mm and to near zero at 4 mm (Fig. 3). As had already been observed for untreated seedlings, PEG-treated seedlings that did not resume radicle growth frequently showed elongation of the cotyledons and, to a lesser extent, also of the hypocotyl (up to 3 cm). This was mainly observed when the protruded radicle before dehydration was short, i.e. 1–2 mm (not shown). Thus, the radicle appeared to be the more desiccation-sensitive part of the seedling, followed by the hypocotyl and cotyledons.

Seed viability

Although seedlings with a radicle of 2 mm did not resume radicle growth after dehydration (without PEG), pre-humidification, and rehydration (Fig. 3), their radicles were turgid and apparently healthy during the first days following rehydration. Therefore, a tetrazolium test was performed in order to assess biochemically the viability of both untreated and PEG-treated seedlings (2 mm long radicles) after dehydration and pre-humidification. All untreated seedlings showed dark-red stained cotyledons and unstained radicles (Fig. 4A), indicating that cotyledon cells survived dehydration whereas radicle cells did not. Nevertheless 10% of these seedlings showed a dark-red stained hypocotyl, indicating that cells in that region were still alive. In 100% of the PEG-treated seedlings the cotyledons were also dark-red stained, while different situations were observed in the radicle and hypocotyl. In 33% of them, both radicle and hypocotyl were dark-red stained (Fig. 4B); in 36% a dark-red staining of the hypocotyl occurred and a partial staining (dark- or light-red) of the radicle, normally in the tip (Fig. 4C); 12% showed dark-red stained hypocotyls and light-red stained radicles (Fig. 4D); 8% remained with white, unstained radicles and hypocotyls (Fig. 4E); and 11% showed unstained radicle and dark-red stained hypocotyl (Fig. 4F).

Changes in moisture content (MC) of the radicles during incubation in PEG, dehydration, pre-humidification, and rehydration

During incubation in PEG, MC of 2 mm long radicles decreased steadily in the first 9 h, from 3.74 g g^{-1} to 2.35 g g^{-1}, and then slowly until 72 h (2.24 g g^{-1}) (Fig. 5A). In terms of percentage, 37% of the water was removed in the first 9 h and, by the end (72 h), 40% of the water had been lost. During dehydration the rate and extent of the water loss were similar in radicles of both PEG-treated and

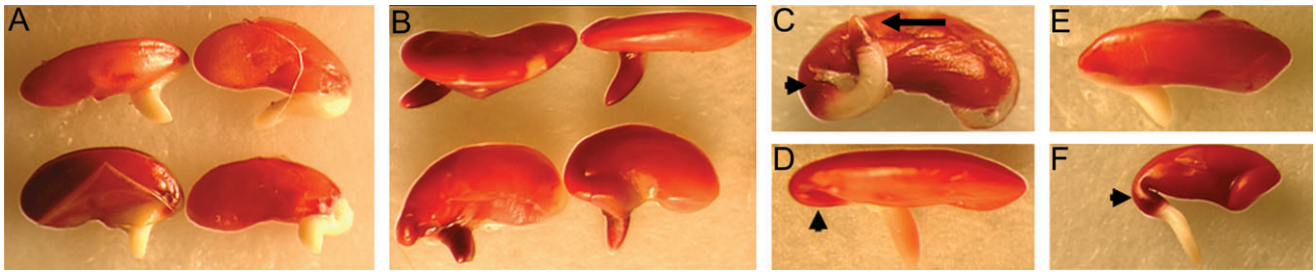

Fig. 4. Tetrazolium test performed on seedlings (2 mm long radicles) of *Medicago truncatula* following dehydration (with or without previous PEG treatment) and pre-humidification. (A) Untreated seedlings showing unstained radicles and dark-red stained cotyledons. (B–F) PEG-treated seedlings. Cotyledons of all seedlings stained dark-red. (B) Radicle and hypocotyl totally dark-red stained; (C) radicle partially stained (arrow) and hypocotyl (arrowhead) stained; (D) radicle light-red stained and hypocotyl (arrowhead) stained; (E) radicle and hypocotyl unstained; (F) radicle unstained and hypocotyl (arrowhead) stained.

untreated seedlings, which rapidly lost, respectively, 82% and 93% of the water in the first 2 h of drying (Fig. 5B, inset). By the end (72 h) the radicle MCs of PEG-treated and untreated seedlings were 0.20 g and 0.15 g g^{-1}, respectively (Fig. 5B), showing no statistical difference between them and with the original (dry seed) radicle MC (0.19 g g^{-1}). During pre-humidification (for 24 h) and the first 24 h of rehydration, changes in MC were again similar in radicles from both PEG-treated and untreated seedlings. During pre-humidification MC increased at a constant and low rate to around 1.3 g g^{-1} (Fig. 5C, 0–24 h) and rehydration quickly increased MC, with the highest rates occurring in the first hour. After 24 h of rehydration, the radicle MCs of PEG-treated and untreated seedlings were 6.21 and 5.72 g g^{-1}, respectively (Fig. 5C).

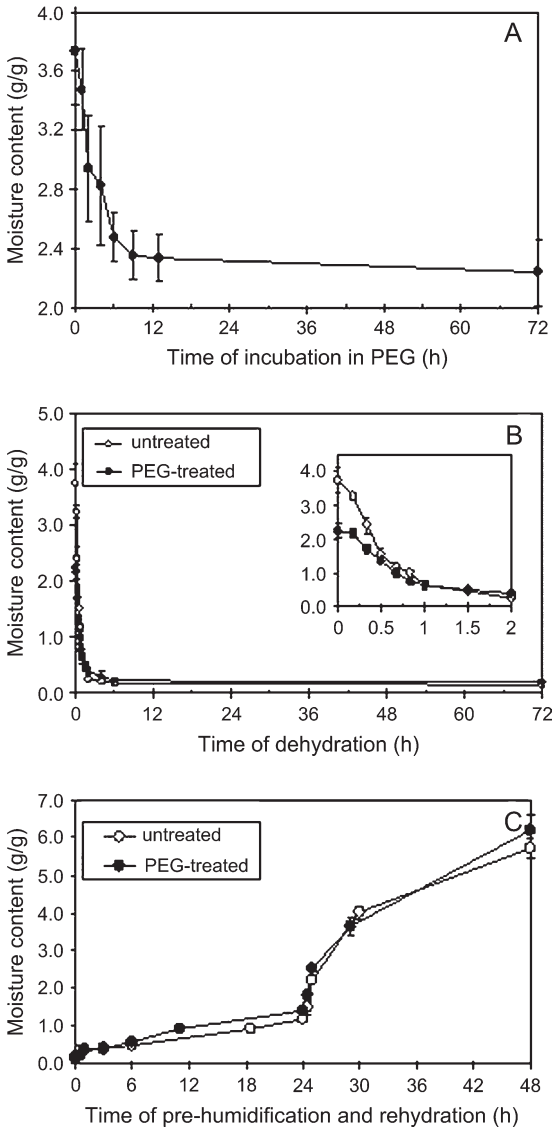

Fig. 5. Changes in the moisture content (g H_2O g^{-1} dry matter) of the radicles (2 mm long) of seedlings during (A) incubation in PEG; (B) dehydration, and (C) pre-humidification (for 24 h) followed by rehydration.

Detection of DNA fragmentation in seedlings subjected to dehydration

Analysis of DNA integrity in 2 mm long protruded radicles revealed DNA degradation in radicles excised from seedlings subjected to dehydration (Fig. 6; lanes 2 and 3), while control seedlings (not dehydrated) showed intact DNA. DNA fragmentation was much stronger in untreated than in PEG-treated seedlings, with the laddering pattern composed of multimers of about 200 bp.

Microtubular cytoskeleton in radicles before and after germination

Radicles of dry seeds and of seedlings were analysed for microtubular cytoskeleton configurations in order to characterize their changes during radicle growth and to relate them to the loss of DT. In dry seeds a high level of fluorescence was detected in the form of granules, indicating that, at that stage, tubulin was present in granules in the cytoplasm, instead of being assembled into MTs (Fig. 7A). In seedlings with a radicle length of 1 mm (Fig. 7B) and 2 mm (Fig. 7C) abundant cortical microtubular arrays were observed with MTs transversely oriented to the direction of cell elongation. In 3 mm long radicles cortical MTs (Fig. 7D) and the first mitotic MTs were detected (Fig. 7E), indicating the start of cell division. In 4 mm long radicles the number of cells entering the M phase of the cell cycle was much higher than in 3 mm long radicles, with all the mitotic MT configurations (preprophase band,

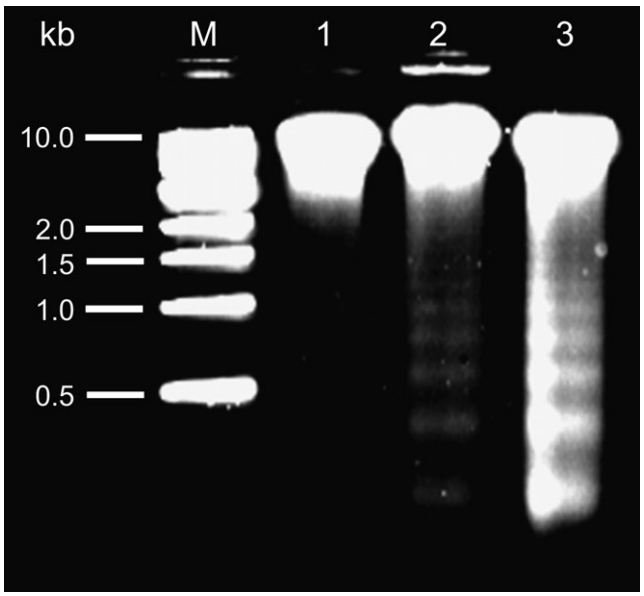

Fig. 6. Agarose gel of genomic DNA extracted from 2 mm long radicles of seedlings of *Medicago truncatula*. (M) Marker with the band lengths shown in the left; (1) control (not subjected to dehydration); (2) PEG-treated seedlings (dehydrated after incubation in PEG); and (3) untreated seedlings (dehydrated without previous incubation in PEG). DNA samples (5 μg) were loaded on a 1% agarose gel stained with ethidium bromide.

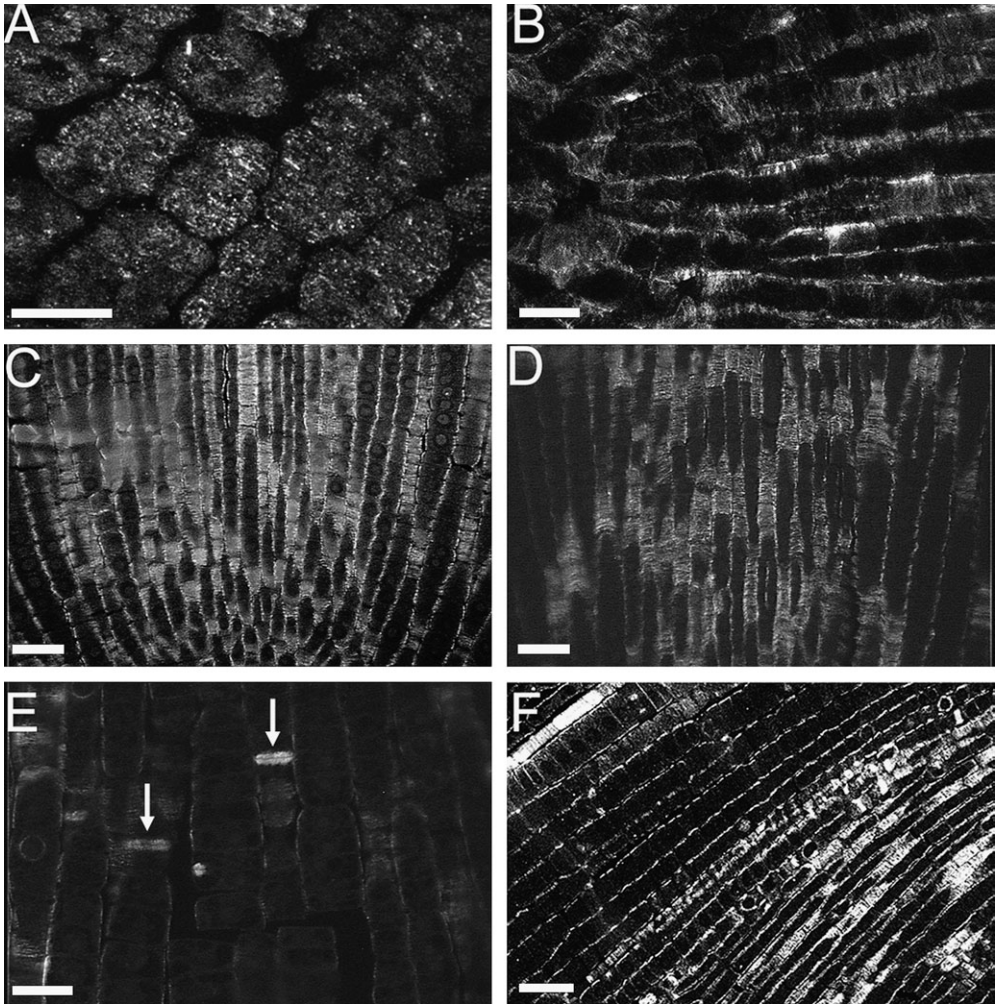

Fig. 7. Fluorescence micrographs of radicle cells of dry seeds and seedlings of *Medicago truncatula*, labelled with α-tubulin antibody and with a fluorescent secondary antibody. (A) Dry seed showing abundant fluorescent granules; (B) 1 mm protruded radicle with well-established cortical microtubular cytoskeleton arrays oriented perpendicularly to cell (and radicle) elongation; (C) 2 mm protruded radicle showing the same situation as the previous figure; (D, E) 3 mm protruded radicle: presence of cortical microtubules (D) and first appearance of mitotic configurations (E, arrows pointing to phragmoplast arrays), indicating the presence of cell division; and (F) 4 mm protruded radicle, with abundant cortical and mitotic microtubules. Bars (A–E) 25 μm, (F) 100 μm.

spindle, and phragmoplast) displayed. As for the shorter radicles (1–3 mm), cells with cortical microtubular cytoskeleton were also abundantly present (Fig. 7F).

Effects of dehydration, pre-humidification, rehydration, and PEG-treatment of M. truncatula *seedlings on the microtubular cytoskeleton*

PEG-treated and untreated seedlings with a 2 mm radicle were used to study the effect of dehydration, pre-humidification and rehydration on the microtubular cytoskeleton in radicle cells. Incubation in PEG for 3 d caused no changes in the microtubular cytoskeleton (compare Fig. 8A and B). Dehydration of both untreated and PEG-treated seedlings dismantled partially the well-established microtubular cytoskeleton and led to the appearance of tubulin granules (Fig. 8C, D). Pre-humidification worsened the situation, with no MTs and only a few granules of tubulin being detected in both untreated (Fig. 8E) and PEG-treated (Fig. 8F) seedlings. Further, when pre-humidified untreated seedlings were rehydrated for 24 h, some tubulin granules could still be detected, but this was rather a rare event and the prevalent situation was that of a total absence of MTs and tubulin granules (Fig. 8G). However, a different situation occurred in PEG-treated seedlings, which exhibited, after 24 h of rehydration, a rebuilt, functional microtubular cytoskeleton in the radicle cells, with both interphase and mitotic configurations (Fig. 8H).

Discussion

The radicle length at which seedlings from orthodox seeds lose DT varies among species, with 2 mm being reported for

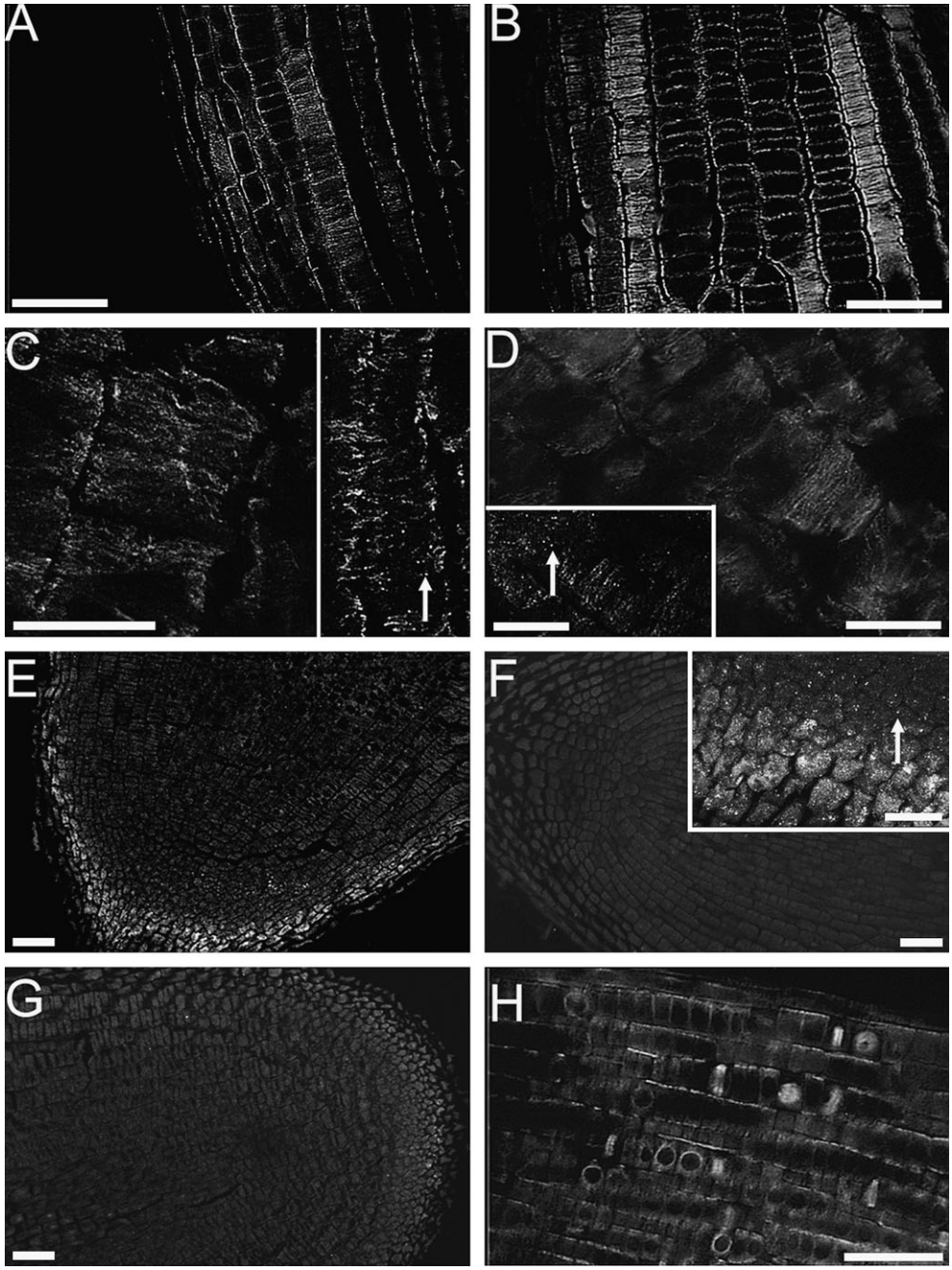

Fig. 8. Fluorescence micrographs of radicle cells of seedlings of *Medicago truncatula*, with a radicle length of 2 mm, subjected to dehydration (with or without PEG-treatment), pre-humidification and rehydration. Sections were labelled with α-tubulin antibody and with fluorescent secondary antibody. (A) Control (before dehydration) showing abundant cortical MTs; (C, E, G) untreated seedlings. (C) Dehydration led to a decrease in the abundance of MTs and appearance of tubulin granules (arrow); (E) after dehydration and pre-humidification, MTs disappeared totally and tubulin granules could hardly be detected; (G) after dehydration, pre-humidification, and 24 h of rehydration, the total absence of MTs and tubulin granules. (B, D, F, H) PEG-treated seedlings. (B) After incubation in PEG, the situation of the microtubular cytoskeleton remained unchanged, compared with the control; (D) after dehydration, a decrease in the abundance of MTs and appearance of tubulin granules (arrow) was detected; (F) after dehydration and pre-humidification, although some cells still exhibited tubulin granules (inset, arrow), the general picture was of total absence of MTs and tubulin granules; (H) after dehydration, pre-humidification, and 24 h of rehydration, the normal situation was restored, with cortical MTs being seen throughout the radicle cells. In addition, mitotic MTs were also observed. Bars (A, B, E, F, G, H) 50 μm; (C) (both pictures), (D, F) (inset) 25 μm.

tomato (Lin *et al*., 1998) and *M. truncatula* cv. Paraggio (Buitink *et al*., 2003), 1 mm for okra and mung bean (Lin *et al*., 1998), and 0.5 mm for snow pea and cucumber (Lin *et al*., 1998). Besides the expected variation among species, the different experimental procedures adopted, especially the drying rate, are certainly another cause for the differences found. Decrease in DT before radicle protrusion appears to be a relatively rare event. It has been reported for

coffee seeds (Ellis *et al.*, 1991) and was found in the present study. For instance, at 44 h of imbibition, 20% of the seeds had germinated (Fig. 2), suggesting that at least the 80% that had not germinated could still tolerate desiccation. However, only 40% were still desiccation tolerant. The drying condition used to assess DT (43% RH; corresponding to a water potential of −115 MPa) which led to a very fast dehydration of the seeds certainly diminished the chances of *de novo* synthesis of protective components. It has been shown that slow water loss may allow protective changes to occur, not only in germinating (Sun, 1999) but also in developing orthodox seeds (Kermode and Finch-Savage, 2002), in somatic embryos (Senaratna *et al*., 1989) and in the whole plant (Oliver *et al.*, 1998), enabling them to withstand subsequent severe dehydration. In the present study such a slow (and limited) water loss was achieved by subjecting the seedlings to a mild osmotic stress through incubation in PEG solution (–1.8 MPa), resulting in re-establishment of DT at high rates in seedlings with a protruded radicle length of up to 2 mm. In a study with *M. truncatula* cv. Paraggio, Buitink *et al.* (2003) showed that during incubation in PEG, synthesis of possibly protective substances, such as sucrose and a dehydrin, occurred cumulatively until 24 h, although water was lost only in the first 6 h.

Radicles of untreated dried seedlings (2 mm long radicles) had lost their viability, confirming the absence of DT. In PEG-treated seedlings (also with 2 mm long radicles) 45% of the radicles were totally stained, and 36% only partially. The sum of these values (81%) is very close to the 84% of DT shown by these seedlings, suggesting that the partially stained radicles should also be considered viable.

Radicles of dry mature *M. truncatula* seeds contained relatively high 4C DNA content (45%). This suggests that, by the end of seed maturation, there are two blocks acting in the cell cycle: one at the G_2/M boundary, excluding those cells with 4C nuclei progress to mitosis, and another at G_1/S keeping the cells with 2C nuclei at the pre-synthetic phase. However, this is not the prevalent situation in orthodox seeds, in which the quiescent embryo normally exhibits most (or all) cells with a 2C DNA content, reflecting a stringent arrest of the cell cycle at the pre-synthetic G_1 phase (Deltour, 1985; Bino *et al.*, 1993).

The relationship between the progress of the cell cycle and stress sensitivity in plants has not yet been clarified, although it has been known for a long time that cells in G_2 are more stress-sensitive than cells in G_1 (Sybenga, 1972). Several studies relating cell cycle and stress resistance in seeds have shown that cells in G_1 are more resistant to desiccation, cold- or heat-shock, storage, and radiation (reviewed by Deltour, 1985; Saracco *et al.*, 1995; Sliwinska, 2003). Deltour (1985) hypothesized that nuclei with a 2C content might be more stress-resistant by offering a smaller target for mutation-inducing factors than those with a 4C content. However, very high 2C nuclei contents have also been found in mature embryos of intermediate and recalcitrant seeds, such as coffee (da Silva, 2002), neem (*Azadirachta indica*) (Sacandé, 2000), *Castanea sativa* (Bino *et al.*, 1993), and *Inga vera* (Faria *et al.*, 2004). Furthermore, seeds of the related tree species *Acer platanoides* (desiccation-tolerant) and *A. pseudoplatanus* (desiccation-sensitive) are shed with a similar 4C DNA content in the radicles (38% and 37%, respectively) (Finch-Savage *et al.*, 1998). Thus, it appears that, in mature seeds, DT is not correlated with the arrest of the cell cycle at any particular DNA content.

In seedlings from orthodox seeds, DNA content and DT normally show a high correlation (Sargent *et al.*, 1981; Dasgupta *et al.*, 1982; Deltour, 1985; Osborne and Boubriak, 1994; Osborne, 2000; Boubriak *et al*., 2000), although the resumption of DNA synthesis is unlikely to be the only effective agent in inducing the change from the tolerant to the intolerant state (Dasgupta *et al.*, 1982). As DNA replication is, in general, a late event during germination, other processes may be more tightly linked to the loss of DT, with DNA content playing only an additive role in the increasing stress sensitivity upon germination (Saracco *et al.*, 1995; Boubriak *et al.*, 1997). In PEG-treated seedlings the greatest drop in DT occurred simultaneously with the increase in 4C DNA content.

A second significant decrease in DT of PEG-treated seedlings (from 33% to 5%) was observed between radicle lengths of 3 mm and 4 mm. The DNA content remained unaltered but a great number of cells had entered the M phase of the cell cycle. Dividing cells are less tolerant to desiccation than those that are elongating (Dasgupta *et al*., 1982).

Programmed cell death has been shown to occur in different plant organs and tissues during normal development (e.g. senescence of leaves and post-germinative megagametophyte cell death) or induced by pathogens and stress (Danon *et al.*, 2000; He and Kermode, 2003). Dehydration of desiccation-sensitive seeds (both recalcitrant and germinating orthodox seeds) may lead to the fractionation of DNA (Osborne and Boubriak, 1994; Boubriak *et al.*, 2000). In the present study, directly dried seedlings with 2 mm long radicles displayed degradation of nuclear DNA, which was visualized by the formation of DNA ladders. The fragment lengths were multiples of approximately 200 bp. These multimers, with lengths of 170 to 200 bp, are generated by the cleavage of the chromatin by endonucleases at internucleosomal sites (Stein and Hansen, 1999). In desiccation-tolerant seeds some DNA damage that occurs during dehydration or dry storage may be repaired when water is again available (Osborne, 2000). However, DNA laddering is an indicator of the endpoint of the apoptotic process and cannot be reversed (Boubriak *et al.*, 2000). It appears thus that the weak signal of laddering shown by PEG-treated seedlings possibly comes from the 16% that did not survive dehydration. To our knowledge this is the first time that

DNA laddering is shown to occur during drying of intolerant plant tissue.

It has been suggested that compounds such as sugars and LEA proteins may act as protecting factors, stabilizing cellular structures during drying (Crowe and Crowe, 1986; Dure, 1997). It is also known that mild stresses can trigger the synthesis of protective substances in plant tissues (Farnsworth, 2000) and seedlings (Buitink *et al.*, 2003). It can thus be speculated that PEG incubation induced the synthesis of nuclear proteins with a protective role of the DNA. It is thought that nuclear desiccation-induced proteins, such as QP47, isolated from *Pisum sativum* seeds, protect DNA during desiccation (Chiatante *et al.*, 1995). Besides the synthesis of protectants, loss of water may also cause reversible conformational changes in the DNA, altering the recognition of specific base-sequence domains by enzymes (Osborne and Boubriak, 1994; Osborne *et al.*, 2002), thereby hindering the action of the nucleases, although this is yet to be proven in plant cells.

There were no MTs in radicle cells of dry *M. truncatula* seeds. Only granules of tubulin were detected, as in seeds of tomato (de Castro, 1998) and coffee (da Silva, 2002). Upon germination free tubulin assembled into a cortical microtubular cytoskeleton in cells of protruded radicles with a length of 1 mm and 2 mm and, afterwards, together with mitotic MTs. Hence, cell elongation alone was sufficient for radicle protrusion and early radicle growth, with cell division being additionally required later.

When desiccation-sensitive tissues are exposed to water potentials below −2 MPa, dehydration may lead to the loss of membrane organization, cellular integrity, and degradation of macromolecules (Osborne and Boubriak, 1994). Around −5 MPa there is a general trend towards contraction or dismantling of organelles (Walters *et al*., 2002). In the present study, seedlings were exposed to much more severe dehydration conditions (43% RH; −115 MPa) and the consequence in both untreated and PEG-treated seedlings was a decrease in abundance of MTs and appearance of tubulin granules. The dismantling of the cytoskeleton in seeds caused by dehydration has also been reported for recalcitrant (desiccation-sensitive) seeds, such as *Quercus robur* (Mycock *et al*., 2000), *Trichilia dregeana* (Gumede *et al.*, 2003) and *Inga vera* (Faria *et al.*, 2004). The subsequent pre-humidification of the dried seedlings resulted in the total disappearance of the MTs and a great reduction of tubulin granules. Again, the decay of the microtubular cytoskeleton was comparable in both untreated and PEG-treated seedlings. The difference between untreated and PEG-treated seedlings only appeared when the pre-humidified seedlings were rehydrated: PEG-treated seedlings were able to reconstruct a functional microtubular cytoskeleton and continue normal development, while untreated seedlings showed a total absence of MTs and tubulin granules, and, consequently, the ability to resume normal growth. It is clear that the ability of PEG-treated seedlings to survive dehydration, as far as the microtubular cytoskeleton is concerned, did not rest on its protection during dehydration, but on its reconstruction upon rehydration.

Desiccation-tolerant organisms must rely on one or both of the following strategies: avoidance of the accumulation of desiccation-induced damage, and the activation of repair mechanisms upon rehydration (Buitink *et al.*, 2002). The present study showed that both strategies were distinctly applied by seedlings in which DT was re-established by PEG treatment. Nuclear DNA was kept intact during dehydration, whilst MTs were dismantled and later rebuilt upon rehydration.

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