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RESEARCH NOTE

In situ DNA fragmentation during the re-establishment of desiccation tolerance in germinated seeds of *Cedrela fissilis* Vell.[1]

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ABSTRACT – Dehydration is a necessary procedure prior to exposing seeds to long term storage, but this is associated with metabolism-linked injury mediated by cell injury. In order to assess cellular alterations during re-establishment of desiccation tolerance (DT) in *C. fissilis* germinated seeds and their relation to DNA damage, we verified the occurrence of DNA fragmentation through the TUNEL test and its evidence through the cytological analyses. To re-establish DT, germinated seeds were incubated for 72 h in polyethylene glycol (PEG, -2.04 MPa) before dehydration in silica gel (at 10% moisture content) followed by rehydration. The moisture content changes during the reestablishment of the desiccation tolerance was accomplished. (DT)TdT-dUPT terminal nick-end labeling (TUNEL) was used to assess rates of cell death. TUNEL staining was performed using Click-iT-TUNEL Alexa Flour imaging assay. The TUNEL test showed a consistent DNA fragmentation in the 2 and 5 mm long radicles. Moreover, nuclear and chromosomal alterations were observed in the 5 mm meristematic root cell cycle, contributing to the identification of diagnostic markers of cell death.

Index terms: cell injury, moisture content, TUNEL test.

Fragmentação de DNA *in situ* durante o restabelecimento da tolerância à dessecação em sementes germinadas de *Cedrela fissilis Vell.*

RESUMO – A desidratação é um procedimento necessário antes de expor as sementes ao armazenamento a longo prazo, mas isso está associado à lesão ligada ao metabolismo mediada por lesão celular. A fim de avaliar as alterações celulares durante o restabelecimento da tolerância à dessecação (TD) em sementes germinadas de C. *fissilis* e sua relação com danos no DNA, verificamos a ocorrência de fragmentação do DNA através do teste de TUNEL e suas evidências por meio da análise citológica. Para restabelecer o TD, as sementes germinadas foram incubadas por 72 h em polietilenoglicol (PEG, -2,04 MPa) antes da desidratação em sílica gel (a 10% de umidade) seguida de reidratação. O teor de água foi alterado durante o restabelecimento da tolerância à dessecação. Foi utilizado o rotulador TDT-dUPT (TUNEL) para avaliar as taxas de morte celular. A coloração TUNEL foi realizada usando o teste de imagem Click-iT-TUNEL Alexa Flour. O teste TUNEL mostrou uma fragmentação de DNA consistente nas radículas com 2 e 5 mm de comprimento. Além disso, alterações nucleares e cromossômicas foram observadas no ciclo celular meristemático de 5 mm, contribuindo para a identificação de diagnósticos de marcadores de morte celular.

Termos para indexação: lesão celular, teor de água, teste de TUNEL.

Introduction

Understanding of the basis of seed desiccation sensitivity starts with knowledge of processes and mechanisms involved in the acquisition and maintenance of desiccation tolerance in orthodox seeds and studying whether or not these occur in recalcitrant types (Berjak and Pammenter, 2013). In orthodox seeds, metabolism is reduced to very low levels

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and the embryo enters a state of quiescence, although remains responsive to environmental signals (Finch-Savage and Bassel, 2015; Leprince et al., 2017). In this way, even desiccation-tolerant seeds in the quiescent state may be affected by cellular deterioration when subjected to long-term storage. Cell damages arise from the combination of transitions between desiccation and rehydration, coupled with low cellular repair activities in the dried state. Together, these events lead to an accumulation of damage to macromolecules, including proteins, membrane lipids, and DNA (Waterworth et al., 2015).

Damage to DNA structure has been reported during ageing or drying of both desiccation-tolerant and intolerant seeds (Masetto et al., 2008; Kranner et al., 2011; Masetto et al., 2014; 2015; Dresch et al., 2015), suggesting that DNA fragmentation is linked to death, or that DNA degradation continues under standard seed storage conditions. However, changes of DNA that cause death (i.e. unrepaired breaks) are minor, compared to the damage that occurs after death (Walters et al., 2006). In this way, although the physiological and biochemical disorders that lead to growth inhibition of the root and of the whole plant are relatively well known (Farrant and Moore, 2011; Gechev et al., 2012; Challabathula et al., 2016), the alterations of DNA status, which represent the direct cause of seeds survival under abiotic stress and/or their capacity of resume their growth after rehydration, have not been explored to any great extent in seeds.

The *terminal desoxynucleotide transferase mediated d-UTP nick-end labeling*, or TUNEL, is a largely used sensitive method to evaluate the nuclear deformation during the apoptosis, in which the DNA degradation occurs, common in tissues that suffer cellular death (Liu et al., 2007). Germinated seeds of *Cedrela fissilis* Vell. (Meliaceae), a native tree from the Brazilian Atlantic Forest, have been used as a model to better understand the dessication tolerance, particularly the cytological assessment of the root tip provided evidence of the occurrence of cell death in the 2 and 5-mm-long radicles, which did not survive dehydration (Masetto et al., 2014). In order to assess cellular alterations during re-establishment of DT in *C. fissilis* germinated seeds and its relation to DNA damage, we verified the occurrence of DNA fragmentation through the TUNEL test and its evidence through the cytological analyses.

Materials and Methods

The seed collection and processing were carried out according to Masetto et al. (2014) from ripe fruits collected at the beginning of their dehiscence from about 20 trees in Lavras, South of Minas Gerais State (21°14'S, 45°00'W).

The evaluations after re-establishment of desiccation tolerance were carried out with germinated seeds with 1, 2 and 5 mm long radicles that were chosen for moisture content and *in situ* DNA fragmentation investigations according to the previous results of Masetto et al. (2014) as follows: the germinated seeds with 1, 2 and 5 mm radicle length were put in Petri dishes with a filter paper on the bottom, moistened with 20 mL of PEG 8000 solutions (380 g dissolved in 1 L water, according to Michel and Kaufmann, 1973) at 5 ºC for 72 h, which provided an osmotic potential of -2.04 MPa. After that, the germinated seeds were washed in running water to remove the PEG solution residues, superficially dried on paper towel for 10 min and dehydrated in silica gel at 20 ºC/60% RH. Samples were dried to the original seed moisture content (10%), pre-humidified (100% RH/ 24 h/25 ºC) and rehydrated as described previously. Four independent experiments with 25 germinated seeds of each radicle length were carried out.

The moisture content changes during dehydration, pre-humification and rehydration were assessed in four replications of 2 g each, by oven-drying at 103 ± 2 ºC for 17 hours (Brasil, 2009) and expressed as percentage of moisture content on a fresh weight basis.

The *terminal deoxynucleotide transferase mediated-dUTP nick-end labeling* or TUNEL's reaction is used to evaluate the DNA fragmentation by the 3'-OH extremities detection of DNA strand, by the action of the terminal deoxynucleotide transferase enzyme, through the green fluorescence. It was used in agreement of the protocol instructions of the "APO-BrdU™ TUNEL Assay Kit" (Invitrogen – Molecular Probes) with Alexa Fluor. The 1, 2 and 5 mm long radicles fresh and PEG treated and dehydrated in silica at 10% of moisture content (three replications each) were fixed in paraformaldehyde at 1% for 12 hours. After this period, radicles were dehydrated in alcoholic gradient for 1 hour each (30%, 50%, 70%, 90% and 100%), fixed in Steedman's wax at 37%, using one series of wax:ethanol (v:v) (50:50%, 70:30%, 90:10%) and 100% of wax every 1 hour. The radicles were sectioned longitudinally with 10 µm thick using microtome and reactions were prepared according to the manufacturer. The images were observed with an epifluorescence microscope, utilizing 500 nm wave length (Olympus BX60).

In accordance to the previous results obtained through the TUNEL´s reaction, the 5 mm long radicles, incubated in PEG 8000, dehydrated in silica gel at 10% MC and rehydrated, were chosen for cytological evaluation. Tips were collected, fixed in Carnoy's solution {methanol:acetic acid – (3:1)} and stored at -20 °C until the slide preparation. The radicle tips were taken from the fixative solution and washed in distilled water (5 minutes). Radicle tips were dried on filter paper and

macerated in an enzymatic solution {2% cellulase (Sigma): 20% pectinase solution (Sigma) diluted in phosphate-citrate tampon pH 4.8} at 37 °C for 6 hours. After that, slides were prepared through the cellular dissociation technique, as described by Carvalho and Saraiva (1993). A total of 1000 cells were counted per slide, with two slides per Petri dish, totalizing ten slides and 10,000 cells analyzed.

Results and Discussion

The moisture content (MC) alterations from radicles during PEG incubation are presented on Figure 1. There was a remarkable reduction in the MC in radicles 1, 2 and 5 mm length during the first 6 hours of PEG incubation. The 1 mm radicle reduced eight percentage points of MC; 2 mm radicle length presented a reduction of nine percentage points and the 5-mm radicle length showed a reduction of 12 percentage points. After the first hours of PEG incubation, there was a subtle reduction of MC. After 72 hours, 1 and 2 mm radicle length presented, equally, 42% MC, while the 5-mm radicle length presented 52% MC (Figure 1A).

Changes in MC of the radicles submitted or not to the PEG treatment and dehydrated in silica gel are shown on Figure 1B. Both untreated and PEG treated radicles showed a similar pattern of MC reduction during the first 6 hours of dehydration in silica gel. As had already been observed for the moisture content from radicles, the 6 hours following dehydration, permitted noticing PEG-treated seedlings MC decrease at a constant until the end of the 24 h dehydration time, while the untreated radicles decrease moisture content during the whole drying period.

Notably, survival curves as a function of water content during drying showed an important loss of viability/integrity when the cells were dried below 10% MC, as seen by Masetto et al. (2014). Aside from the osmotic treatments improve the rate of water loss even in terms of method of drying, according to Leprince and Buitink (2010), there is also a need to understand the cause of damages occurring during drying. Understanding why these germinated seeds do not tolerate different levels of drying could be helpful to understand desiccation tolerance. The underlying mechanisms of the death of germinated orthodox seeds are less understood than the empirical description of the radicle length and the moisture content attained (Masetto et al., 2016). This is supported by the comparative analysis of the changes in MC of the PEG-treated and untreated *C. fissilis* radicles, during pre-humidification (for 24 h) and the first 24 h of rehydration were similar (Figure 1C).

Along the first 12 h of pre-humidification, MC increased rapidly, and it was observed by the increase of about 4 percentage points in the radicles MC. It was observed that, during the first 24 hours of rehydration, the PEG-treated 1 mm long radicles showed a remarkable increase in water absorption when compared to untreated radicles of the same length. The other radicles (2 and 5 mm) had a similar behavior during the pre-humidification and rehydration period, showing, at the end of 48 hours, 35% MC average (Figure 1C). Despite their sensitivity to drying, the PEG+ABA treated 1 mm long radicles were able to trigger a response involving 100% survival (Masetto et al., 2014), possibly validating further insights between survival and death which could lead to investigate

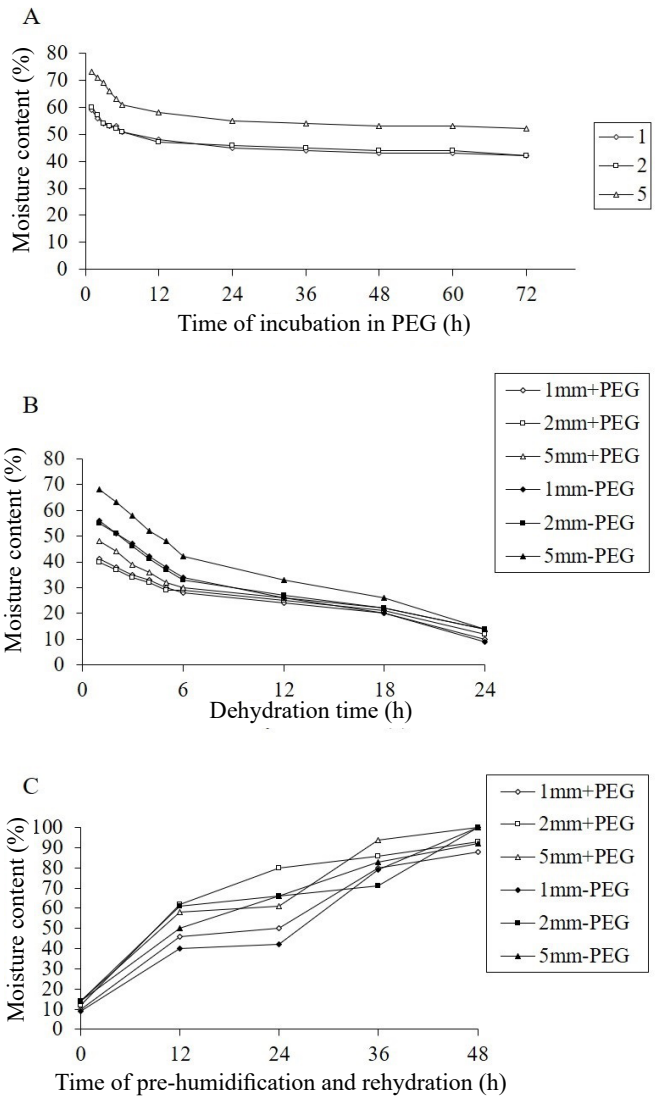

Figure 1. Changes in moisture content (MC) of *Cedrela fissilis* radicles during incubation in PEG (A), dehydration (B), pre-humidification and rehydration during 24 h (C).

the changes in gene expression or protein abundance related to the hydration level at which tissues can survive (Leprince and Buitink, 2010).

It is well known that cellular macromolecules are protected by the reduced mobility of water and the capability of the cytoplasm to form a 'glassy' state (Bewley et al., 2013). However, the germinated seeds showed high moisture content (Figure 1A), and although the incidence of damage may be reduced with PEG treatment, as seen by Masetto et al. (2014), possibly, is the capacity for repair damages accumulated during dehydration has the potential to limit the cell survival after rehydration, according to the radicle length and the moisture content range.

In this way, the TUNEL test is largely applied to detect DNA degradation *in situ* and cellular death occurrence, resulting from the cell passive or programmed death. Additionally, to the results obtained from the DNA electrophoresis and from the cellular viability evaluation as seen by Masetto et al. (2014), TUNEL test detected the occurrence of DNA injury in 1, 2 and 5 mm long radicles dehydrated, characterized by the green fluorescence of nucleus or TUNEL-positive (Figures 2A, 2B and 2C, respectively).

Meristems nucleus from 1 mm long radicles, despite the fact of presenting a partial desiccation tolerance, as seen by Masetto et al. (2014), also presented loss of cellular viability after dehydration to 10% MC, characterized by TUNEL-positive in less intensity (Figure 2A). Figures 2B and 2C correspond to the meristem nuclei from 2 and 5 mm long radicles, respectively, and are highlightened by the green color, related to the loss of viability after dehydration. In this way, the DNA degradation observed in the present study was not related to internucleosomal fragmentation, as can be seen by the lack of DNA laddering in the gel electrophoresis preparations (Masetto et al., 2014), being probably related to necrotic cell death. In this context, TUNEL detected necrotic cells which multiple free DNA ends generated especially after oxidant and toxic injury by activated endonucleases inserted labeled dUTP and it could be detected by fluorescence microscopy (Kelly et al., 2003).

In this way, cellular alterations, like cells in necrosis (Figure 3) were also confirmed in 5 mm long radicles. The inability to survive after drying and rehydration may be due to inhibition of DNA synthesis or a blocking of cells as a result of damage in cell-nuclei. Particularly, DNA damage arises as a consequence of endogenous ROS (reactive oxygen species), metabolic by-products, and breaks induced during DNA replication. Maintenance of genome integrity is critically important to prevent mutation prior to re-initiation of cell-cycle activity in the embryonic meristems (Waterworth et al., 2015). This is in agreement with our TUNEL results which showed a significant increase in DNA strand breaks, specially from 5 mm long radicles, as seen also in meristematic root cells, like cell in necrosis (Figure 3F). According to Huang et al. (2005), TUNEL test is based on the detection of DNA fragments through modified nucleotides incorporation (dUTP) by the Terminal deoxynucleotide transferase enzyme, whose nucleotides are fluorescent pigment highlighted; and the literature successfully reports the use of TUNEL test as a marker of cell death in roots submitted to abiotic stress in several species, such as barley (Katsuhara, 1997), corn (Xiong et al., 2006) and rice (Liu et al., 2007).

The data obtained from *C. fissilis* TUNEL-positive nuclei and the cellular alterations like necrosis are strongly related with the DNA degradation, as seen in Masetto et al. (2014) with the structural changes determined through the necrotic cells with compromised plasma membranes. This explains the cell injury events occurring in *C. fissilis* germinated seeds in

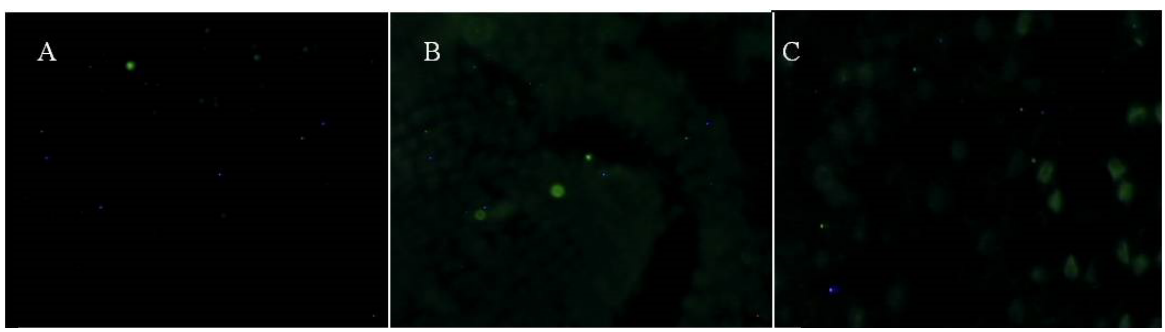

Figure 2. Representative images of TUNEL stained nuclei extracted from *Cedrela fissilis* germinated seeds with 1 (A), 2 (B) and 5 (C) mm long radicles, after incubation in PEG (-2.04 MPa), dehydration in silica gel to 10% MC and rehydration. Green spots correspond to the TUNEL-positive nuclei.

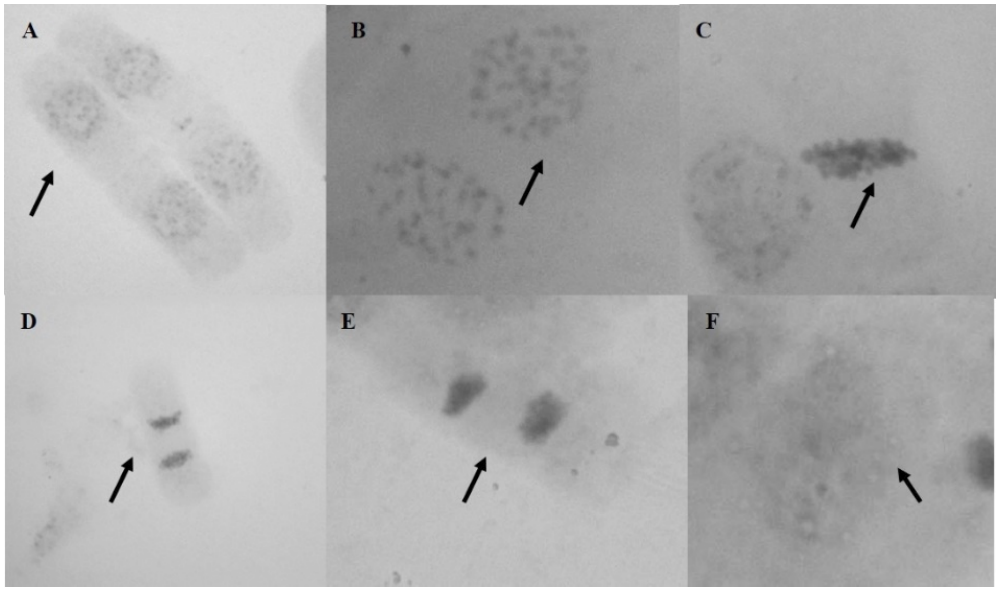

Figure 3. Meristematic root cells (5-mm) in different mitosis phases and abnormalities probably induced by silica gel-dehydration following rehydration. Normal cells in: A (arrow) - interphase, B (arrow) - prophase, C (arrow) - metaphase, D (arrow) – anaphase, E (arrow) – telophase and F (arrow) – cell in necrosis.

the dry state and probably why these germinated seeds could not be resume their growth during rehydration.

Conclusions

DNA damage and cellular necrosis during drying are associated with radicle length and moisture content of *C. fissilis* germinated seeds.

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