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RESEARCH NOTE

Cell changes during the re-induction of desiccation tolerance in germinated seeds of *Sesbania virgata (*Cav.) Pers.[1]

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ABSTRACT - During germination, orthodox seeds lose their ability to tolerate desiccation resembling recalcitrant seeds. This research aimed to investigate the cell changes during the re-induction of the desiccation tolerance (DT) in *Sesbania virgata* germinated seeds with 1, 3 and 5 mm long radicles. 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. Cell viability was assessed through TUNEL test in dry radicles and transmission electron microscopy in both fresh and dry radicles. The positive-TUNEL confirmed the DNA degradation, through the green fluorescence of the cell nuclei from 5 mm radicle length and the ultra structural evaluations detected loss of cellular content integrity in 3 and 5 mm cell radicles that did not survive dehydration to 10%.

Index terms: cell viability, transmission electron microscopy, TUNEL test.

Mudanças celulares durante a re-indução da tolerância à dessecação em sementes germinadas de *Sesbania virgata* (Cav.) Pers.

RESUMO – Durante a germinação, as sementes ortodoxas perdem sua habilidade de tolerar a dessecação assemelhando-se com as sementes recalcitrantes. Este trabalho objetivou investigar as mudanças celulares que ocorrem durante a re-indução da tolerância à dessecação (TD) em sementes germinadas de *Sesbania virgata* com 1, 3 e 5 mm de comprimento de radícula. Para restabelecer a TD, as sementes germinadas foram incubadas durante 72 h em solução de polietilenoglicol (PEG, -2,04 MPa), posteriormente foram desidratadas em sílica gel (10% de teor de água) e reidratadas. A viabilidade celular foi avaliada pelo teste de TUNEL e através de microscopia eletrônica de transmissão em radículas que foram submetidas à secagem ou não. O TUNEL-positivo confirmou a degradação do DNA por meio da fluorescência dos núcleos nas células da radícula com cinco mm de comprimento e as avaliações ultraestruturais detectaram a perda da integridade do conteúdo celular nas radículas com 3 e 5 mm de comprimento e que não sobreviveram à desidratação a 10% de teor de água.

Index terms: viabilidade celular, microscopia eletrônica de transmissão, teste de TUNEL.

Introduction

The integrity of DNA and cell structures during severe water loss in seeds is predicated on the seeds survival and their capacity of resume their growth after rehydration, suggesting that eventual DNA degradation is linked to death in seeds (Osborne and Boubriak, 1997; Faria et al., 2005). Studies at cellular level in germinated orthodox seeds during dehydration and following rehydration may help to better understand the mechanisms that control desiccation tolerance (DT) and sensitivity in seeds (Masetto et al., 2015). Thus, assessment of DNA integrity may also help to explain DT and sensitivity, since DNA stability during dehydration or the ability of its repairment upon rehydration is an essential component of the

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tolerance mechanisms (Boubriak et al., 1997). This can be done through techniques such as transmission electron microscopy and TUNEL (*Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling*) test, in which modified nucleotides (dUTP) are incorporated to extremities of DNA fragments by the *terminal deoxynucleotide transferase* enzyme (El-Maarouf-Bouteau et al., 2011)

It has previously been shown that there was a relationship between the loss of DNA integrity and loss of DT in 3 and 5-mm-long radicles of *Sesbania virgata* (Cav.) (Pers.) (Fabaceae) - a shrub useful in land reclamation - when dehydrated to 10% moisture content, and the cytological assessment of the radical meristem provided evidence of the occurrence of cell death in the 3 and 5-mm-long radicles, which did not survive dehydration (Masetto et al., 2015). Although the authors indicated that, this study seeks to understand the nature and extent of damage from intracellular dehydration during the re-establishment of DT in *S. virgata* germinated seeds.

Material and Methods

The seed collection and processing was carried out according to Masetto et al. (2015) from about 40 seed-trees, in Ijaci (21º10'S, 44º54'W), South of Minas Gerais State, Brazil. The moisture content of the seeds was assessed in four replications of 2 g each, by oven-drying at 103 ºC for 17 hours (Brasil, 2009). The seeds showed 10% moisture content. For the obtainment of germinated seeds, initially the seeds were scarificated with concentrated sulphuric acid, for 40 min, washed in running water for 10 min, disinfested with sodium hypochlorite at 2% for 2 min, washed again and sown on moistened filter paper in germination boxes. The test was carried out in incubators at 25 ºC and constant white light (Brasil, 2009). The evaluations after re-induction of desiccation tolerance were carried out with germinated seeds with 1, 3 and 5 mm long radicles which were chosen for cell investigations according to the results of the previous experiments of Masetto et al. (2015) as follows: Germinated seeds with 1, 3 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 provides an osmotic potential of -2.04 MPa. Germinated seeds were washed in running water to remove the PEG solution residues and superficially dried on paper towel, for 10 min, following the seeds were dehydrated in silica gel at 20 ºC/60% RH. Samples were dried to the original seed moisture content (10%), pre-humidified (100% UR/ 24 h/ 25 ºC) and rehydrated as described previously. Four independent experiments with 25 germinated seeds of each radicle length were carried out.

The *Terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling* (TUNEL) reaction is used to evaluate the DNA fragmentation by the 3'-OH extremities detection of the DNA strand, by the action of the *Terminal deoxynucleotide transferase enzyme*, through the green fluorescence (APO-BrdU™ TUNEL Assay Kit, Invitrogen, Molecular Probes, with Alexa fluor). Radicles of germinated seeds (1, 3 and 5 mm long) dehydrated in silica to 10% MC after PEG-treatment, were fixed in paraformaldehyde 1% for 12 h. After this period, radicles were dehydrated in alcohol gradient for 1 h each (30%, 50%, 70%, 90% and 100%), fixed in Steedman's wax 37%, using one series of wax:ethanol (v:v) (50:50%, 70:30%, 90:10%) and 100% of wax every 1 h. The radicles were sectioned longitudinally with 10 µm width 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).

For the cellular morphology analysis through electron microscopy transmission, the 1, 3 and 5 mm long radicles from both fresh germinated seeds and after PEG treatment, dehydration in silica gel and rehydration, were analyzed (five germinated seeds per treatment). Samples were fixed in the modified Karnovsky's solution (glutaraldehyde 2.5%; formaldehyde 2% in a cacodylate sodium buffer 0.05M; $CaCl_2$ 0.001M, pH 7.2), washed three times (10 min each) in cacodylate buffer 0.05M, fixed in an aqueous solution of osmium tetroxide 1% for 2 h, at room temperature and washed in Milli-Q water for 15 min. Later, samples were contrasted in uranyl acetate (0.5%) for 12 h, at 4 ºC, and dehydrated in an acetone gradient (25%, 50%, 75%, 90% and 100% three times). Afterwards the material was embedded in an increasing gradient of spurr/acetone solutions 30%/8 h; 70%/12 h and 100% twice, for 24 h each. Samples were then transferred to silicon molds and polymerized in oven at 70 ºC, for 48 h. Sections were made in the meristematic region of the radicles, using a Reichart-Jung ultramicrotome, with 100 nm thickness and contrasted with uranyl acetate, followed by lead citrate (three min). Samples were evaluated on transmission electron microscope (Zeiss EM 109, Carl Zeiss, Jena, Germany), at 80 kV.

Results and Discussion

Images of cells after TUNEL test are presented on Figure 1. Germinated seeds with 1 and 3 mm long radicles showed TUNEL-negative (Figure 1A and Figure 1B, respectively).

The green fluorescent-colored nuclei indicate TUNEL-positive cells as revealed by bright green fluorescent spots (Figure 1C), which means that numerous nuclei were labelled. This corresponds to the total loss of integrity in the genetic material of PEG-treated 5 mm long radicles which did not survived dehydration and was unable to produce normal seedlings, as demonstrated by Masetto et al. (2015). TUNEL analysis was applied to detect a possible collapse of DNA *in situ*, since both programmed and necrotic cell death are characterized by the degradation of nuclear DNA and can be visualized with the TUNEL assay that labels the DNA breaks (Gavrieli et al., 1992; El-Maarouf-Bouteau et al., 2011).

Figure 1. Epifluorescence micrographs of TUNEL assays performed on cells from 1 (A), 3 (B) and 5 (C) mm long radicles of *Sesbania virgata* germinated seeds, after incubation in PEG (-2.04 MPa), dehydration in silica gel to 10% MC and rehydration. Bright green spots correspond to the TUNEL-positive nuclei.

According to the cytological factors influencing the re-establishment of DT, the transition from fresh germinated radicles to dehydrated and rehydrated ones was accomplished by structural changes in the cells of radicle meristem (Figure 2). In fresh 1, 3 and 5 mm long radicles (Figures 2A, 2C and 2E, respectively) there was a perfect delimitation of the cell wall, small vacuole and presence of lipid bodies and starch granules. After dehydration and rehydration of 3 and 5 mm long radicles (Figure 2D and Figure 2F, respectively) there was loss of cell wall integrity and cellular content, and disappearance of starch granules. Thus, cytosolic constituents that spill into extracellular space through damaged plasma membrane may provoke injury responses (Proskuryakov et al., 2003).

In despite of these results, only 5 mm long radicles showed Tunel-positive (Figure 1 C), indicating the total loss of nuclei viability provoking 100% of dead seedlings (Masetto et al., 2015). Possibly, the loss of cell compartmentalization in 3 mm long radicles indicates low cellular repair activities in the desiccated state (Figure 2D) but still not lead to a total accumulation of damage to DNA (Figure 1B), suggesting ability at least, partially, to resume their growth after dehydration following rehydration, as demonstrated in 47% seedlings (Masetto et al., 2015).

Findings concerning the loss of desiccation tolerance during the seeds germination have already been reported in germinated seeds of *Medicago truncatula* (Buitink et al., 2003; Faria et al., 2005), *Tabebuia impetiginosa* (Vieira et al., 2010) and *Cedrela fissilis* (Masetto et al., 2014), where DT could be re-established applying a mild osmotic shock with polyethylene glycol. Mature seeds of *S. virgata* are desiccation tolerant (survive the loss of most of their water content), but they become desiccation sensitive while progressing to germination. In any way, the maintenance of DNA integrity and the cell compartments of the root tissue seems to be important prerogatives to re-establish the normal seedlings development after the primary root dehydration. Alterations in DNA caused by several factors affect nucleus and ultimately the entire cell leading to compromised function of the organism (Kraner et al., 2011; Atale et al., 2014; Dresch et al., 2015).

The germinated seeds of *S. virgata* restrict their resumption of growth according to the PEG treatment, the radicle length and the moisture content attained. According to Masetto et al. (2015) the occurrence of a non-programmed event of the cell death in 3 and 5 mm long radicle of *S. virgata* dehydrated at 10% MC may be a regulated cellular response to stress. This behaviour indicates that the maintenance of genetic information is fundamental to DT and cell survival after dehydration and rehydration.

Desiccation sensitivity of PEG-treated 5 mm long radicles has clearly induced the cellular death and indicates that this may be an important mechanism of intolerance to water stress in *S. virgata* germinated seeds. Considering the results of cytological assays, the data obtained from TUNEL are strongly related with the DNA degradation, as seen in Masetto et al. (2015) and with the structural changes (Figure 2F). Cellular death in the primary root of *Stenocereus gummosus*

and *Pachycereus pringlei* was also detected by TUNEL (Shishkova and Dubrovsky, 2005). Although the nuclei TUNEL-positive may be considered a cell death indicator in *S. virgata* germinated seeds, other cytological factors may be assessed, such as the ultra-structural characterization of cellular death in plants, which indicates a progressive loss of cellular compartmentalization and organization (DeBono and Greenwood, 2006).

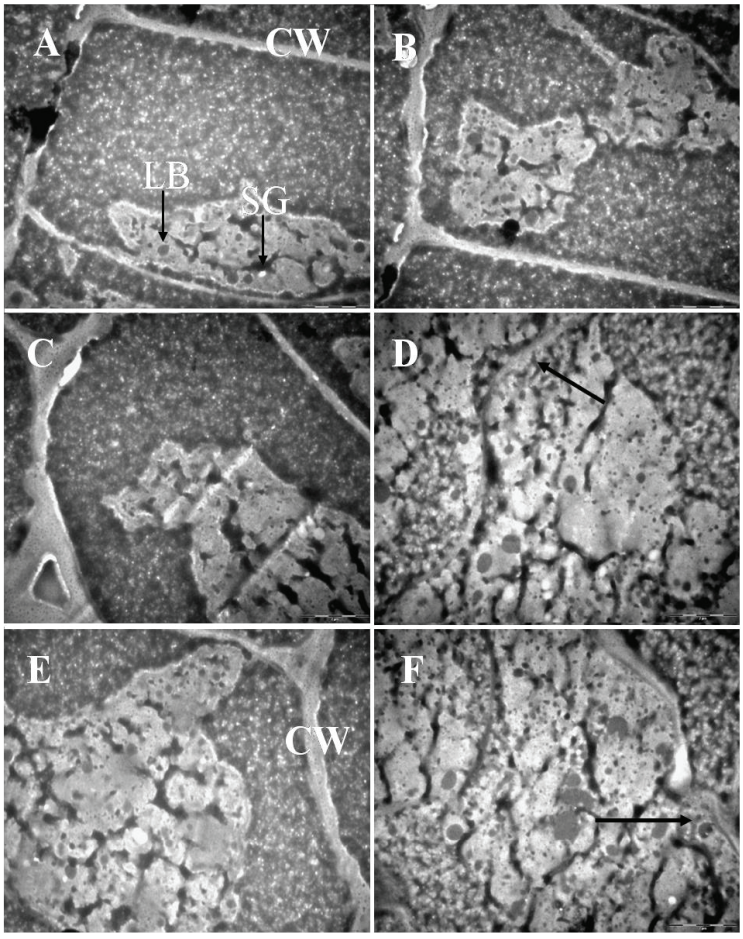

Figure 2. Images of meristematic cells obtained by Transmission Electron Microscopy of *S. virgata* germinated seeds with 1 (A-B), 3 (C-D) and 5 (E-F) mm long radicle before dehydration (A, C, E) and after dehydration and rehydration (B, D, F). Emphasis on the cell wall (CW) with normal aspect before dehydration (A, E). The arrows in the Figures D and F show the loss of cytoplasm integrity after dehydration and rehydration (D, F). LB = lipid bodies; SG = starch granule.

Many morphological and biochemical changes have been found in vegetal cells that suffer stress and show cellular and nuclear phenotypes that result from DNA damage, among them, cytoplasm and nucleus condensation and shrinkage, as seen in Figures 2D and 2F. The cytoplasm and plasmatic membrane presented morphological aberrations. The cytoplasm becomes extremely vesicular and vacuolar, with floccose appearance and deformed (Gunawardena et al., 2001; Errakhi et al., 2008). The loss of cell compartmentalization and green fluorescent-colored nuclei assert the desiccation sensitivity in 5 mm long radicles of *S. virgata*.

According to the Figures 2D and 2F, possibly, in the germinated seeds with 3 and 5 mm long radicles, respectively, the cells die and release their content, which include highly harmful lysosomal proteolytic enzymes that affect other cells and start a cascade of cell-death event, in the surroundings (Atale et al., 2014), clearly marking the method of cell death as necrosis, according demonstrated in an agarose gel by Masetto et al. (2015).

The underlying mechanisms of orthodox germinated seeds death are less understood than the empirical description of the radicle length and the moisture content attained. In any

way the ability of orthodox germinated seeds to withstand severe desiccation generally depends on the integrity of nucleic acids. However, the loss of nucleic acid integrity together with other cell deteriorative phenomena may also be interpreted as the cause for further non overcoming severe dehydration and eventually death of germinated seeds.

Conclusions

The nuclei TUNEL-positive may be considered a cell death indicator in *S. virgata* germinated seeds, besides the progressive loss of cellular compartmentalization and organization evidence the occurrence of a non-programmed cell death.

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