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Loss and re-establishment of desiccation tolerance in the germinated seeds of *Sesbania virgata* (Cav.) (Pers.)

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ABSTRACT. This research aimed to investigate the cellular alterations during the loss and re-establishment of desiccation tolerance (DT) in germinated *Sesbania virgata* seeds. The loss of DT was characterized in germinated seeds with increasing radicle lengths (1, 2, 3, 4 and 5 mm) when subjected to dehydration in silica gel, followed by rehydration. To re-establish DT, the germinated seeds were incubated for 72h in polyethylene glycol (PEG, -2.04 MPa) with or without ABA (100 µM) before dehydration in silica gel. Cell viability was assessed by seedling survival, and DNA integrity was evaluated by gel electrophoresis. Seeds with 1 mm radicle length survived dehydration to the original moisture content (MC) of the dry seed (approximately 10%). PEG treatment was able to re-establish DT, at least partially, with 2, 3 and 4 mm but not in 5 mm radicle lengths. Germinated seeds treated with PEG+ABA performed better than those treated only with PEG, and DT was re-established even in germinated seeds with a 5 mm radicle length. Among the PEG-treated germinated seeds dehydrated to 10% MC, DNA integrity was maintained only in those with a 1 mm radicle length.

Keywords: abscisic acid, cytological alteration, osmotic stress.

Perda e restabelecimento da tolerância à dessecação em sementes germinadas de *Sesbania virgata* (Cav.) (Pers.)

RESUMO. Esta pesquisa objetivou investigar as alterações celulares durante a perda e o restabelecimento da tolerância à dessecação (TD) em sementes germinadas de *Sesbania virgata*. A perda da TD foi caracterizada em sementes germinadas com 1, 2, 3, 4 e 5 mm de comprimento de radícula, submetidas à desidratação em sílica gel seguida de reidratação. Para restabelecer a TD, as sementes germinadas foram incubadas por 72h em PEG (-2,04 MPa) com e sem ABA (100 µM) antes da secagem em sílica gel. A viabilidade celular foi avaliada pela sobrevivência de plântulas e a integridade do DNA foi avaliada por meio de eletroforese em gel. Sementes com 1 mm de radícula sobreviveram à secagem até o teor de água original (aproximadamente 10%). O tratamento com PEG foi eficiente para restabelecer a TD, parcialmente, em sementes com 2, 3 e 4 mm, exceto com 5 mm de radícula. Sementes germinadas tratadas com PEG+ABA apresentaram melhor desempenho em relação às sementes sem ABA, sendo que a TD foi restabelecida em sementes com 5 mm de radícula. Dentre as sementes tratadas com PEG e secas até 10% de teor de água, a integridade do DNA foi mantida em sementes com 1 mm de radícula.

Palavras-chave: ácido abscísico, alteração citológica, estresse osmótico.

Introduction

Desiccation tolerance corresponds to the ability to survive under intense protoplasmatic dehydration, a phenomenon that is common in the plant kingdom with regard to pteridophytes, lichens, pollen and the seeds of many angiosperms. In orthodox seeds, desiccation tolerance (DT) is acquired during the first half of the maturation process (BOUDET et al., 2006; LEPRINCE; BUITINK, 2010).

Imbibing orthodox seeds pass from a desiccation-tolerant to a desiccation-intolerant stage as germination progresses. The cells of such seeds remain uninjured when subjected to mild dehydration, but further dehydration causes cell death (BOUBRIAK et al., 1997). With the completion of germination, the emerged radicle is typically the first organ to lose the ability to tolerate dehydration, followed by the hypocotyl and cotyledons. Because of their sensitivity to dehydration, germinated orthodox seeds are similar to recalcitrant seeds (BOUDET et al., 2006) and can be used as a tool in studies of recalcitrance. However, research on recalcitrant seeds faces a number of obstacles, with the very limited time of

availability of fresh seeds being the main hindrance. Indeed, the main advantage of the use of orthodox seeds is the continuous supply of experimental material. Many processes at the physiological, cellular and molecular levels that occur during the loss of DT in germinated orthodox seeds may be similar to those responsible for the desiccation sensitivity exhibited by recalcitrant seeds (FARRANT, 2010).

The feasibility of re-establishing DT in germinated orthodox seeds through osmotic stress has been demonstrated for some species (BRUGGINK; VAN DER TOORN, 1995; BUITINK et al., 2003). Osmotic treatment is normally performed with polyethylene glycol (PEG) with or without the addition of abscisic acid (ABA). The application of PEG+ABA has also been used to stimulate embryo maturation in conifers, as it is possible to simulate the natural water stress that occurs in the final phases of embryo maturation in these trees (STASOLLA et al., 2003).

DT phenomena in any system can be assessed by the extension of cell survival upon rehydration. The dynamics of the loss of viability in the dehydrated state and the essential biophysical characteristics have been reported, including the weakening of the vitreous cytoplasmic state combined with the hydrolysis of sugars and the induction of oxidative processes (PUKACKA; RATAJCZAK, 2005), resulting in lipid, protein and DNA damage (APEL; HIRT, 2004).

Studies at the cellular level in germinated orthodox seeds during dehydration and after rehydration may help to better understand the mechanisms that control DT and sensitivity in seeds. *Sesbania virgata* (Fabaceae) was chosen for the present study: a shrub that grows up to 6 m in height and 25 cm trunk diameter (ARAÚJO et al., 2004) that is useful in land reclamation (KOLB et al., 2002; RODRIGUES et al., 2003) due to its rapid root proliferation and adaptability. *S. virgata* grows in the South, Southeast and Central-Western regions of Brazil and presents a more intense period of flowering in January, April, September and October and fruit shedding in January, October and November.

Studies at the levels addressed herein may provide a clue as to which technological, physiological and molecular strategies should be adopted to re-establish DT in sensitive seeds. Accordingly, the objective of this study was to investigate the cytological alterations during the loss and re-establishment of DT in *S. virgata* seeds.

Material and methods

Seed collection and processing

Ripe fruits were collected manually from approximately 40 seed-trees in Ijaci (21º 10'S - 44º 54'W), southern Minas Gerais State, Brazil. Seed processing consisted of breaking the fruits using a rubber hammer and separating the seeds from the fruit debris in sieves.

Moisture content of the seeds

The seed moisture content was assessed in 4 replications of 2 g each by oven-drying at 103ºC for 17h (BRASIL, 2009) and is expressed as a percentage of the moisture content on a fresh weight basis.

Germination test

The coat dormancy presented by *S. virgata* was overcome through chemical scarification with concentrated sulfuric acid for 40 min. The seeds were then washed in running water for 10 min, sterilized with sodium hypochlorite at 2% for 2 min., washed again and sown on moistened filter paper in germination boxes. The experiment was performed in incubators at 25ºC and constant white light (BRASIL, 2009). Four replications of 25 seeds were used.

Imbibition curve

Five replications of 10 seeds were used; the were seeds first scarified with concentrated sulfuric acid and imbibed under the same conditions described above. The seeds were weighed at intervals of 6h for 3 days.

Assessment of desiccation tolerance loss after germination

Germinated seeds with 1, 2, 3, 4 and 5 mm radicle lengths were subjected to dehydration at 25ºC in the dark in sealed plastic boxes with activated silica gel on the bottom, which generated a relative humidity of 8%. Successive weighing during dehydration was performed to monitor the decreasing in moisture content (SACANDÉ et al., 2005). After dehydration, the seeds were pre-humidified in a humid chamber (100% RH) for 24h at 25ºC in the dark and then rehydrated under the same conditions used for germination. The germinated seeds that developed into normal seedlings were considered desiccation tolerant. Four independent experiments with 25 germinated seeds for each radicle length were performed.

Re-establishment of DT by incubation in PEG and PEG+ABA

Germinated seeds with 1, 2, 3, 4 and 5 mm radicle lengths were placed in Petri dishes with a filter paper on the bottom that was moistened with 20 mL PEG 8000 solution (380 g dissolved in 1 L water, according to MICHEL; KAUFMANN, 1973) with or without ABA (100 μM) at 5ºC for 72h. At this temperature, the PEG concentration used provides an osmotic potential of -2.04 MPa, which did not permit radicle growth. The germinated seeds were then washed in running water to remove the PEG solution residue and superficially dried on a paper towel for 10 min. The moisture content (MC) of the seeds was assessed as described above. The seeds were dehydrated in silica gel at 20ºC/60% RH, and samples were collected at 10 percent decrease until reaching the original seed moisture content; the samples were pre-humidified and rehydrated as described above. Four independent experiments with 25 germinated seeds for each radicle length were performed.

DNA extraction and electrophoresis to assess DNA integrity

The extraction of DNA from dehydrated and pre-humidified seeds with 1, 3 and 5 mm radicle lengths was performed according to the CTAB protocol. The samples were ground into powder in liquid nitrogen and transferred to a 2 mL microtube. A 700 µL aliquot of 2X CTAB (pure water, 1 M TRIS-HCl [pH 7.5], 5 M NaCl, 0.5 M EDTA [pH 8.0] and 2 g CTAB) pre-warmed at 65ºC was added and incubated at 65ºC for 30 min. A 600 µL aliquot of chloroform-isoamyl alcohol (24:1) was then added, and the samples were inverted for 5 min. and centrifuged at 12,000 rpm at room temperature for 10 min. The supernatant was transferred to a new microtube, and 450 µL cold isopropanol was added. The microtubes were incubated at -20ºC for 24h to precipitate the DNA and then centrifuged for 10 min. at 12,000 rpm at 4ºC. The supernatant was discarded and 100 µL 70% ethanol was added to each sample; after 10 min., the microtubes were centrifuged again at 4,000 rpm at 4ºC for 10 min. to remove the CTAB residue. The tubes were then inverted on filter paper to dry the pellet, which was later dissolved in 50 µL TE (pH 8.0, 10 mM TRIS-HCl and 1 mM EDTA).

For electrophoretic analysis, 5 µL of each DNA sample was separated on a 1% agarose gel and stained with ethidium bromide. The 1 Kb Plus DNA Ladder (1 µg μL^{-1}) was used as a marker. The gel was visualized under ultraviolet light and photographed using EDAS 290 (Kodak®) equipment.

Cytological assessment of radicles after re-establishment of desiccation tolerance

According to the results of previous experiments, germinated seeds with 1, 3 and 5 mm long radicles were chosen for cytological evaluation. The radicles were excised from both freshly germinated seeds and after PEG incubation, dehydration in silica gel and rehydration. The radicle tips were fixed in Carnoy's solution (methanol:acetic acid – [3:1]) and stored at -20ºC. The tips were washed twice in distilled water (5 min. each), dried on filter paper and macerated in an enzyme solution (2% cellulase [Sigma]: 20% pectinase [Sigma] diluted in citrate-phosphate buffer, pH 4.8) at 37ºC for 6h. The slides were then prepared using the cellular dissociation technique described by Carvalho and Saraiva (1993) and stained with 5% Giemsa for 12 min. The slides were evaluated using a light microscope (Leica) connected to a computer. The cellular morphology of radicle meristems was compared before and after drying, analyzing 5 slides for each treatment and evaluating 200 cells per slide.

Results and discussion

The imbibition curve of *Sesbania virgata* seeds is shown in Figure 1. After 48h of imbibition, 98% of the seeds presented radicle protrusion, results that are in agreement with Tonini et al. (2007). Figure 2A shows the data related to the formation of normal seedlings after the dehydration of germinated seeds with different radicle lengths in silica gel to decreasing moisture contents, followed by rehydration. Only seeds with 1 mm long radicles maintained a high percentage of survival (95%) when dehydrated to the original MC (approximately 10%). For the other radicle lengths tested (2 to 5 mm), there was no survival when dehydrated below 20% MC (Figure 2A).

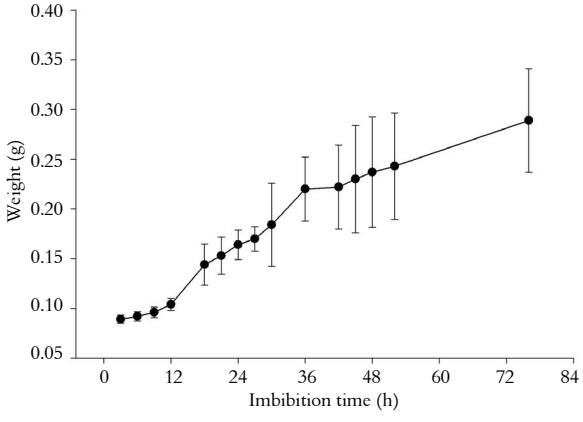

Figure 1. Imbibition curve of *Sesbania virgata* seeds.

The damage caused by the desiccation of sensitive plant tissues includes the breakdown of the cytoskeleton, membrane injury, changes in pH, solute crystallization and protein denaturation (BLACK; PRITCHARD, 2002), potentially leading to the death of the tissue, organ or organism. Regarding germinated seeds or seedlings, the developmental stage after which they cannot tolerate desiccation varies with the species. For instance, this stage occurs at a radicle length of 2.7 mm in *Medicago truncatula* cv. Paraggio (BUITINK et al., 2003).

In the present work, the germinated seeds with a 1 mm-long radicle that were dehydrated to 10% MC, followed by rehydration, showed some darkening of the primary root tissues, suggesting necrosis. Although these primary roots did not resume their growth, adventitious roots emerged around the basal portion of the hypocotyl, leading to the development of normal seedlings at a high rate and enabling these seeds to be classified as desiccation tolerant. Adventitious roots also formed with other radicle lengths yet with lower frequency. The formation of adventitious roots in germinated seeds subjected to water stress was also observed by Vieira et al. (2010) when re-inducing desiccation tolerance in the seeds of *Tabebuia impetiginosa*, a tree species native to Brazil. Gutterman and Gozlan (1998) reported that *Hordeum spontaneum* seedlings with 40-50-mm-long primary roots survived dehydration, and adventitious roots also formed.

The results of the re-establishment of desiccation tolerance in germinated *Sesbania virgata* seeds by PEG incubation before dehydration are presented in Figure 2B. PEG treatment substantially increased desiccation tolerance in the germinated seeds with 2, 3 and 4 mm-long radicle, attaining 50, 47 and 14% normal seedlings after dehydration to 10% MC, followed by rehydration. Regarding the germinated seeds of a 5 mm radicle length, PEG treatment was efficient in increasing the desiccation tolerance only when the seeds were dried to 40% MC, whereas further drying (30% MC) resulted in the total loss of viability. Radicle death was characterized by softening and flaccidity of the terminal portions of the primary root.

The survival rates of germinated seeds with different radicle lengths subjected to PEG+ABA treatment before drying are presented in Figure 2C. This treatment led to positive results for the re-establishment of desiccation tolerance in *S. virgata* germinated seeds with a radicle length up to 5 mm. The survival rates (normal seedlings) were above 60%, even with the reduction of the MC to 10%. PEG treatment plus ABA was also more efficient than PEG alone in re-establishing desiccation tolerance in the germinated seeds of *Tabebuia impetiginosa* with 3 mm-long radicles (VIEIRA et al., 2010).

The presence of ABA in the isolated endosperms of *S. virgata* suggests that this hormone is strongly involved in the control of reserve mobilization and indirectly involved in seedling growth. Under natural conditions, the ABA concentration decreases with the germination while the cotyledons are developing, permitting the mobilization of galactomannans, with the consequent entrance of water that was retained during imbibition for embryo growth (POTOMATI; BUCKERIDGE, 2002).

The application of exogenous ABA is related to the accumulation of reserve protein transcripts in seeds that germinate precociously (REN; BEWLEY, 1999), the routes of translation that increase desiccation tolerance (BECKETT et al., 2005) and increases in soluble sugars, which are associated with desiccation tolerance. The increase in solutes could stimulate the tolerance to desiccation, possibly by stabilizing cell structures and protecting metabolic functions against water stress (WANG et al., 2002). Alternatively, osmotic treatment can inhibit radicle growth until ABA accumulates and manifests its functions, as suggested by Buitink et al. (2003).

The application of exogenous ABA may also inhibit the mobilization of reserves during seed germination, which is presumably due to the induction of factors by ABA that prevent the transcription and/or translation of hydrolase (LIN et al., 1998). In addition to the acquisition of desiccation tolerance, another important effect is the reduction of cell damage due to metabolic protection, as shown for *Medicago sativa* somatic embryos (SREEDHAR et al., 2002).

In the present study, the primary root showed more desiccation sensitivity than the hypocotyl. The same behavior has been reported in many species, such as cucumber and tomato (LIN et al., 1998), *Medicago truncatula* (BUITINK et al., 2003) and *Tabebuia impetiginosa* (VIEIRA et al., 2010).

Based on our results, germinated seeds with 1, 3 and 5 mm-long radicles were selected, treated with PEG and dehydrated in silica gel for cytological assessment. These radicle lengths were chosen because the corresponding percentages of normal seedlings differed widely (100, 47 and 0%, respectively).

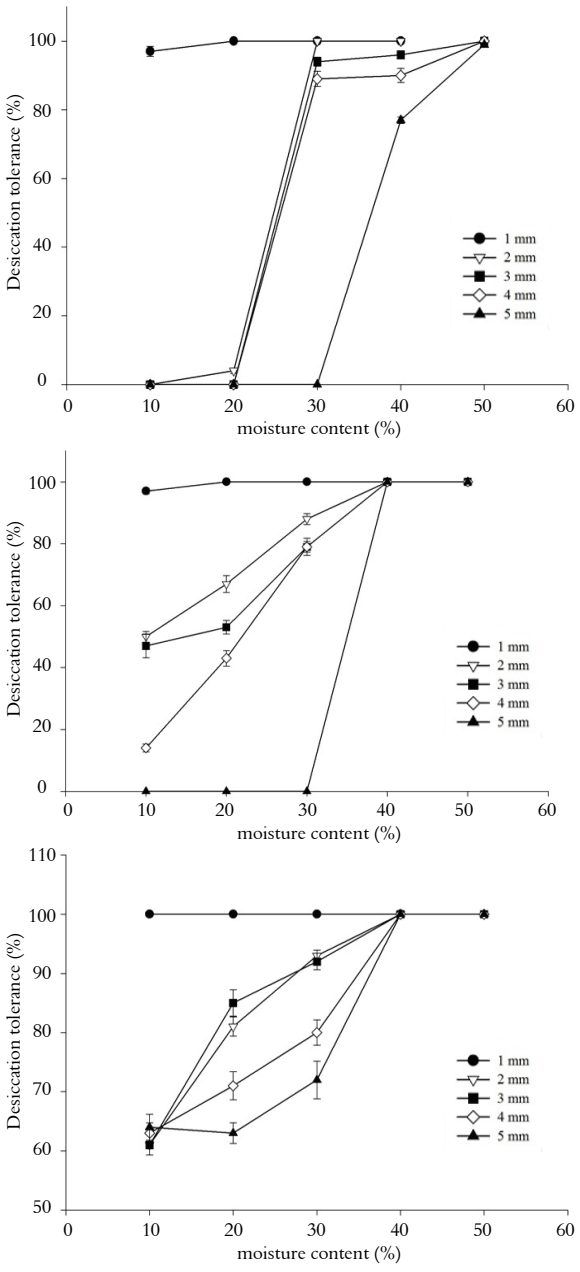

Figure 2. A - Desiccation tolerance in *S. virgata* seeds with different radicle lengths dehydrated in silica gel to different moisture contents. B - Desiccation tolerance of *S. virgata* seeds with different radicle lengths subjected to PEG treatment and dehydrated in silica gel to different moisture contents. C - Desiccation tolerance of *S. virgata* seeds with different radicle lengths subjected to PEG+ABA treatment and dehydrated in silica gel to different moisture contents.

The changes in MC of the 1, 3 and 5 mm-long radicles during PEG treatment, dehydration in silica gel and pre-humidification and rehydration are presented in Figure 3A, B and C, respectively. MC decreased significantly during the first 6h of PEG incubation and at a lower rate thereafter. After 72 hours, the MC values of the 1, 3 and 5 mm-long radicles were 39, 45 and 50%, respectively (Figure 3A).

The changes in MC of the germinated seeds (treated or not with PEG) during dehydration in silica gel are presented on Figure 3B. The rate of water lost in the first 6h was similar for both the PEG-treated and non-treated germinated seeds; after 24 hours of dehydration, the PEG-treated and non-treated seed MC values had decreased to approximately 10-15%. After 24h of pre-humidification and 24h of rehydration MC of the seeds increased to approximately 30-40% (Figure 3C).

DNA integrity in the radicles (1, 3 and 5 mm long) of germinated seeds after PEG treatment, dehydration in silica gel to 10% MC, pre-humidification and rehydration was assessed through visualization on agarose gels. The DNA of the 1-mm-long radicles remained intact, whereas that of the 3- and 5-mm-long radicles appeared increasingly degraded (Figure 4). These results coincide with the desiccation tolerance of PEG-treated germinated seeds (Figure 2B), with a 1-mm radicle length approaching 100% of normal seedling formation, 3 mm attaining 47% and 5 mm unable to produce normal seedlings. It is possible that the DNA in the cells from the 3- and 5-mm-long radicles subjected to dehydration suffered irreversible damage that rendered them unable to perform vital functions.

The total DNA degradation pattern (Figure 4) showed the passive death or necrosis of the *S. virgata* radicle cells, which has previously been reported in fresh desiccation-sensitive *Eugenia pleurantha* seeds when subjected to severe dehydration (7% MC) (MASETTO et al., 2008). The maintenance of the genetic information is fundamental to desiccation tolerance and cell survival after dehydration and rehydration (OSBORNE et al., 2002). Faria et al. (2005) suggested that PEG incubation may induce the synthesis of nuclear proteins that perform a DNA-protective role.

Necrosis is different from programmed cell death. Programmed cell death is a physiological process that depends on energy and is programmed genetically, involving regulatory genes, signaling pathways and distinct characteristics expressed morphologically. In contrast, necrosis is non-physiological, is dissociated from morphogenetic events and is related to development, being directly involved with cell turgidity and lysis and lixiviation of the cellular content, without obvious changes in DNA (for instance, without chromatin condensation and DNA fragmentation) (KRISHNAMURTHY et al., 2000; XU et al., 2004). Necrosis can occur up to a certain developmental stage in germinated seeds.

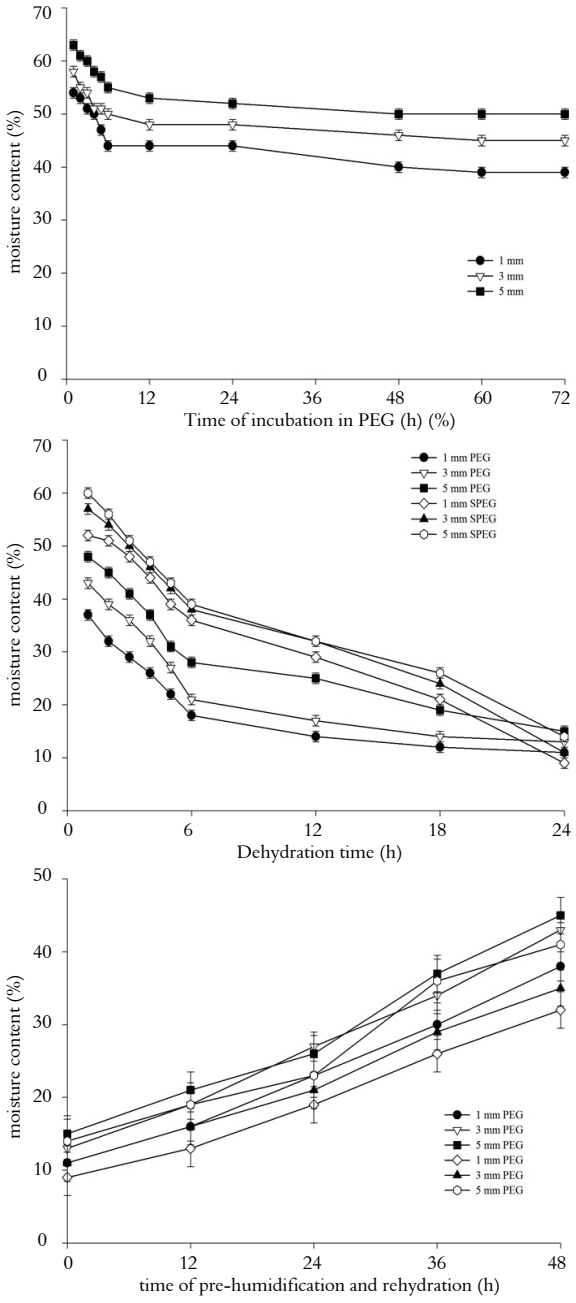

Figure 3. Changes in the moisture content (MC) of *S. virgata* radicles during incubation in PEG (A), dehydration (B), pre-humidification and rehydration for 24h (C).

The results concerning DNA integrity are in accordance with the data obtained from the assessment and quantification of living cells in the 1, 3 and 5-mm-long radicles after PEG treatment, dehydration in silica gel, pre-humidification and rehydration (Figure 5). The nuclei of the radicle meristem presented a reduced size and very dark color (not shown). Cellular death in the 1, 3 and 5-mm-long radicles was 10, 26 and 67%, respectively. The integrity of the cellular structure during severe dehydration is still a phenomenon without explanation in many biological systems (RÖHRIG et al., 2006).

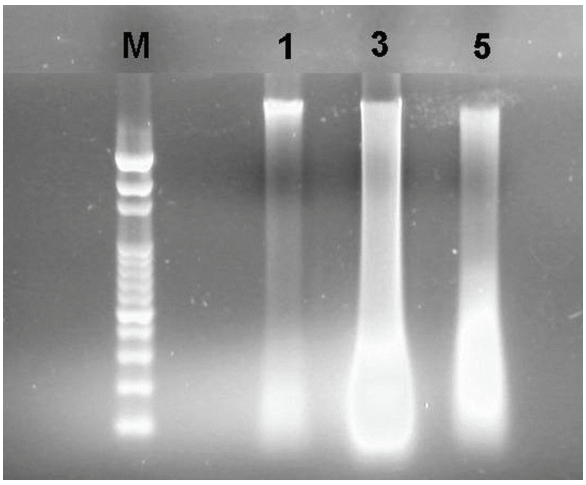

Figure 4. Agarose gel (1%) with DNA extracted from *S. virgata* radicles after PEG treatment and dehydration. M: 1-kb Plus DNA Ladder marker, 1: 1-mm-long radicle, 3: 3-mm-long radicle and 5: 5-mm-long radicle.

Many morphological and biochemical similarities have been found in plant cells that undergo apoptosis, including cytoplasm and nuclear condensation and shrinkage, formation of apoptotic bodies containing DNA and genomic DNA degradation (XU; HANSON, 2000). DNA degradation and the occurrence of cell death indicate desiccation sensitivity in the 3 and 5-mm-long radicles of *S. virgata*. This finding also corroborates the usefulness of assessing germinated orthodox seeds to evaluate recalcitrance phenomena, as germinated *S. virgata* seeds constitute an interesting system for the study of desiccation tolerance and sensitivity in seeds. Furthermore, the results showed that DT re-establishment by PEG and PEG+ABA in *S. virgata* germinated seeds relied on the radicle length.

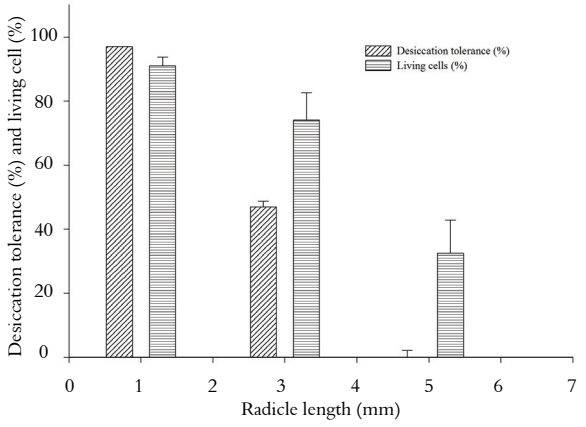

Figure 5. Desiccation tolerance and living cells from 1, 3 and 5-mm-long radicles of germinated *Sesbania virgata* seeds after PEG treatment, dehydration in silica and pre-humidification.

Conclusion

PEG and PEG+ABA treatment were efficient to re-induce DT in the germinated seeds of *Sesbania virgata*.

There was a relationship between the loss of DNA integrity and DT in 3 and 5-mm-long radicles when dehydrated to 10% moisture content.

The cytological assessment of the radicle meristem provided evidence of the occurrence of cell death in the 3 and 5-mm-long radicles, which did not survive dehydration.

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