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Re-induction of desiccation tolerance in germinated cowpea seeds

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ABSTRACT

The objective of this work was to re-induce desiccation tolerance (DT) in cowpea germinated seeds and to investigate the mechanisms related to the re-induction of DT in this species. For this, germinated seeds with primary roots with length of 1, 2, 3 and 4 mm were treated with osmotic solution of polyethylene glycol 6000 (PEG) of −1.7 MPa at 10 °C for 24, 48 and 72 h. Those seeds were dried, rehydrated and evaluated regarding the re-induction of DT. We studied mitosis events and performed scanning electron microscopy (SEM) in primary roots of untreated and PEG-treated germinated seeds. Furthermore, we quantified the glucose, sucrose and raffinose contents during the re-induction of DT. The re-induction of DT in cowpea germinated seeds with primary roots with length up to 2 mm was successfully obtained through an osmotic treatment of PEG during 24 h. The PEG treatment favored the maintenance of cellular structure integrity, the arrest of cell cycle and the accumulation of sucrose in the primary roots. Those mechanisms may contribute to re-induction DT in germinated cowpea seeds.

1. Introduction

Cowpea [*Vigna unguiculata* L. (Walp)] is a highly nutritious legume species. Cowpea is rich in proteins, dietary fiber, micronutrients, minerals, vitamins, antioxidants properties and phytochemicals (Kadam et al., 1985; Phillips et al., 2003; Siddhuraju and Becker, 2007; Kapravelou et al., 2015). Furthermore, cowpea is an important food source and it is a strategic crop in tropical and subtropical regions (Torres et al., 2015) being an essential component of the production systems in dry areas of the tropics, especially in some parts of Asia, United States, Middle East and Central and South America, where it is widely consumed (Singh et al., 2002).

Although cowpea is adapted to adverse environmental conditions, its productivity can be affected by abiotic stresses, especially low water availability caused by periods of drought and high temperatures (Silva et al., 2012). Events such as these can cause the death of germinated seeds, which have already lost the ability to tolerate desiccation (Buitink et al., 2003), and can cause reduction in plant stand directly effecting crop productivity.

Desiccation tolerance (DT) is the ability that some organisms, including microorganisms, animals and plants, have to tolerate loss of more than 90% of their total water content and to resume metabolism and normal development after rehydration without accumulation of lethal or irreversible damage (Colville and Kranner, 2010). DT is an important attribute allowing the seeds to support dehydration and to survive a wide range of stressful environmental conditions that would be detrimental to adult plants such as extreme temperatures and extreme drought (Colville and Kranner, 2010; Leprince and Buitink, 2010).

To date many mechanisms have been described as being involved in DT such as the presence of antioxidant systems, amphipathic molecules and oleosinas, the accumulation of protective molecules such as LEAs (Late embryogenesis Abundant Proteins) and HSPs (Heat-Shock Proteins), the accumulation of non-reducing sugars and oligosaccharides of the raffinose series, and the coordinated shutdown of metabolism (Hoekstra et al., 2001). The response to desiccation also comprises a complex matrix of protective mechanisms against mechanical stresses caused by the removal of water. These mechanisms include cell wall, membranes, and cytoskeleton modifications, chromatin compaction (Dekkers et al., 2015) and changes in the molecular mobility within the cells during drying (Walters, 2015).

During seed imbibition, the increase in water availability permits the resumption of metabolic processes that lead to radicle protrusion and progressive loss of DT (Buitink et al., 2003). However, after radicle protrusion, there is a small developmental window in which desiccation tolerance can be re-established by osmotic treatment with polyethylene glycol (PEG) and/or abscisic acid (Buitink et al., 2003, 2006; Maia et al., 2011, 2014). The PEG treatment inhibits radicle growth, down-regulates genes involved in energy metabolism and up-regulates genes related to anti-oxidant activity, ABA biosynthesis and genes involved in the biosynthesis of many molecules such as non-reducing

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sugars and LEA proteins, contributing to the re-induction of DT (Buitink et al., 2003, 2006; Lü et al., 2007; Maia et al., 2011).

Different species respond differently the re-establishment of DT and the main factors that can influence are the incubation time, temperature and osmotic potential of the solution (Buitink et al., 2003). Thus, uncharacterized species such as cowpea require the development of specific protocols for the re-establishment of DT in order to assess whether they can also be used as models in the studies of DT. Therefore, here we investigated the possibility to re-establish DT in cowpea germinated seeds submitted to various treatments and characterized the mitotic events and the ultrastructural changes associated to the re-establishment of DT in this species.

2. Material and methods

Commercial cowpea seeds from the cultivar BRS Guariba were placed to germinate in paper rolls moistened with 2.5 times the volume of water in relation to the dry mass of the paper, at constant temperature of 25 °C, in the dark. Seeds that exhibit radicle protrusion of at least 1 mm long were considered germinated. At different time intervals, the percentage of germination was determined by using four independent replicates of 50 seeds to obtain the germination curve. To assess desiccation tolerance, germinated seeds were dried in a constant atmosphere of 42% relative humidity, obtained with the use of saturated solution of K_2CO_3 in a closed chamber at 25 °C, in the dark, based on Buitink et al. (2003), during 72 h, and placed to rehydrate in the same conditions described for the germination experiment. In parallel, three independent replicates of 20 seeds were placed to imbibe at different intervals of time, to determine the water content during the imbibition. The water content was evaluated gravimetrically and the percentage of seed moisture content was expressed on wet basis.

Germinated seeds with 1, 2, 3 and 4 mm of length of primary root were collected between 21 and 23 h of imbibition. Following, the primary roots were treated with osmotic solution of polyethylene glycol 6000 (PEG) with osmotic potential (ψ_w) of −1.7 MPa at 10 °C (Buitink et al., 2003, 2006). The germinated seeds with different lengths of primary roots were placed in Petri dishes (9 cm diameter) containing 20 ml of the PEG solution. The Petri dishes were sealed and kept in the dark, at 10 °C, for 24, 48 and 72 h. After the treatment the germinated seeds were submitted to drying (42% RH, 25 °C) based in Buitink et al. (2003), for 72 h. The water content during the drying was monitored gravimetrically, using three independent replicates of 20 seeds. After drying, four independent replicates of 50 seeds were rehydrated in filter paper at constant temperature of 25 °C, in the dark. At the same time, it was established a control treatment, which consisted of drying of the germinated seeds with primary root with lengths of 1, 2, 3 and 4 mm untreated with solution of PEG 6000. Germinated seeds were considered tolerant to desiccation when the primary roots resumed growth after re-hydration (Buitink et al., 2003, 2006; Maia et al., 2011), resulting in normal seedlings.

The analyses of the mitotic events and of the mitotic index (MI) were carried out following the methodology described by Iwata-Otsubo et al. (2016) with modifications. We analyzed cells of the procambium region of primary roots of germinated seeds with 1, 2, 3 and 4 mm long, untreated and treated with PEG (−1.7 MPa) at 10 °C for 24 h. For that, we used 1 mm cuts from the primary root distal end that were fixed in Carnoy solution (i.e., 3:1 v/v, ethanol - glacial acetic acid) for at least 24 h at 7 °C. After fixation, the primary roots sections were washed three times in distilled water during 5 min and incubated for enzymatic digestion (i.e., 30 μl of pectinase - 2% and cellulase - 4% + 20 μl of pectolyase - 5% at pH 4.8) during 4 h at 37 °C.

After digestion, the sections were dissected and the procambium region of 0.5 mm from the distal end was isolated and stained with aceto-orcein at 5%. Samples were observed with optical microscope. The number of interphase cells in each mitosis phase and cytogenetic altered cells were counted. For each treatment, 2000 cells were counted, i.e., samples consisted of four 500 cells independent replicas from at least 8 primary roots. The mitotic index (MI) was calculated as the total number of cell in division phase divided by the total number of cells analyzed multiplied by 100 (Menezes et al., 2014). For the scanning electron microscopy (SEM) studies we used radicles from dry seeds, primary roots with 2 mm long untreated and without drying, primary roots with 2 mm long untreated and subjected to drying (42% RH, 25 °C, for 72 h), primary roots with 2 mm long treated with PEG (−1.7 MPa, 10 °C, 24 h) and without drying, and primary roots with 2 mm long treated with PEG and subjected to drying. Radicles and primary roots were fixed using the modified Karnovsky fluid (2.5% glutaraldehyde and 2.5% paraformaldehyde) for at least 24 h and 0.5 mm from the distal end cryofracture was performed. Then, the cuts were washed three times – 10 min each, in 0.05 M cacodylate buffer, post-fixation in 1% osmium tetroxide for 4 h at ambient temperature, washed with distilled water and dehydrated during 10 m at different acetone concentrations (25, 50, 75, 90 and 100%). Samples were dried to CO_2 critical point and covered with gold in a BAL-TEC, SCD-050. Electromyographies were obtained using a LEO-EVO 40 XVP scanning electron microscope.

The glycose, sucrose and raffinose contents in dry and imbibed seeds for 16 h and in primary roots with 2 mm long treated with PEG (−1.7 MPa, 10 °C), for 8, 16 and 24 h were estimated using a high performance liquid chromatography technique, according to the AOAC/CR-0093 method (AOAC, 2005). For each stage 200 radicles or primary roots (about 1.2 g) were collected, frozen in liquid nitrogen and stored at −80 °C. Before the analytical the water content of the samples was evaluated gravimetrically. The sugar content was normalized in relation to the dry mass.

The percentage of DT and MI were submitted to ANOVA. The DT analysis used was a 4 × 4 factorial design (0, 24, 48 and 72 h of PEG treatment × 1, 2, 3 and 4 mm of primary root length), with 4 replications per treatment. Similarly, the MI analysis used a 2 × 4 factorial design (treated and untreated seeds × 1, 2, 3 e 4 mm root length). The MI analysis used a 2 × 4 factorial design, with 4 replications per treatment. The analysis of sugar content used 6 treatments (0 h, 16 h of imbibition in water, 2 mm untreated and 2 mm treated in PEG for 8, 18 and 24 h) with 3 replications per treatment. Averages were compared using Tukey's test ($p < 0.05$).

3. Results

The imbibition curve did not follow the three-phase model proposed by Bewley et al. (2013) and it was not possible to precisely identify the transition between the imbibition phases II and III (Fig. 1). Although the phases of imbibition are related to the events that occur during seed

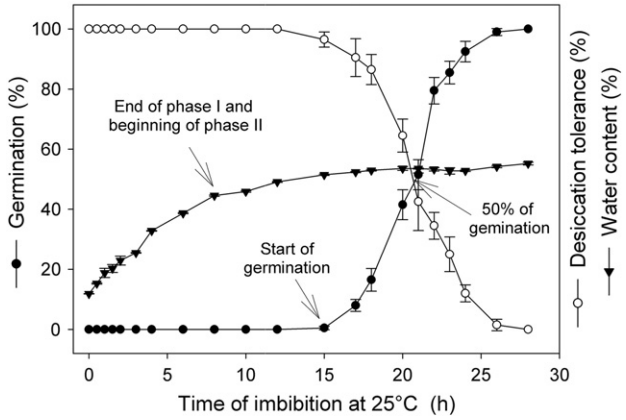

Fig. 1. Imbibition curve, germination and desiccation tolerance and of cowpea seeds imbibed in water at 25 °C. Means ± SDs, $n = 3$ (imbibition curve) or 4 (germination and desiccation tolerance); bars indicate standard deviation.

germination, in some species this phases are not clearly defined and are not perceptible in the imbibition pattern (Nonogaki et al., 2010).

Approximately, 15 h after imbibition germination with visible radicle protrusion started and DT was reduced from 100% to 96.5%. Around 21 h of imbibition, when the germination percentage reached 50% and the seeds reached 52% of water content, the DT was reduced to 42%. As germination proceeds DT decreased and was completely lost after 28 h of imbibition (Fig. 1). Untreated seeds with primary root of 1 mm maintained high ability to tolerate desiccation and did not differ from PEG-treated seeds. The treatment with PEG for 24 and 48 h induced DT in seeds with primary root with 2 and 3 mm long but was not effective for untreated primary roots and primary roots treated with PEG for 72 h (Fig. 2).

DT was gradually reduced along with the growth of the primary root (Fig. 2), however seeds treated with PEG for 24 and 48 h showed significant rates of re-induction of DT. This indicates that cowpea seeds have a broad developmental window after visible germination and before seedling establishment where they can still experience some periods of drought and still survive. Germinated seeds treated in PEG for 24 h displayed higher percentages of re-induction of DT specially in primary root with 4 mm, with 20% of increment in relation to untreated seeds, suggesting that this treatment is the most effective to re-induce DT in this species (Fig. 2). The largest difference of DT among primary roots untreated and treated with PEG for 24 h was observed in seeds with 2 mm of primary root, which explains the choice of this stage for further analysis of mitotic events, ultrastructural modifications and sugars metabolism.

The drying rate was similar for untreated seeds and treated with PEG, with intense water loss during the first 12 h of drying. The drying reduced the water content of the seeds in >90% (Fig. 3), featuring the desiccation state, according Colville and Kranner (2010).

To check whether the cell cycle is influencing the capacity of cowpea germinated seeds to re-establish DT we assessed the mitotic indexes of germinated seeds with different primary root lengths. Most cells in division were in prophase and the number of cells in metaphase, anaphase and telophase, when present, was very low (Table 1). We did not observe cellular abnormalities, such as micronuclei or condensed nuclei. In untreated seeds the MI increased with the growth of the primary root from 1 to 2 mm and from 2 to 4 mm, while for seeds treated with PEG the MI only increased when the primary root reached 4 mm (Fig. 4).

The cells of the procambium region (Fig. 5A) of the apex of radicle of not imbibed seeds (Fig. 5B) showed cell walls with irregular shape and cellular content contracted, which resulted in the separation between

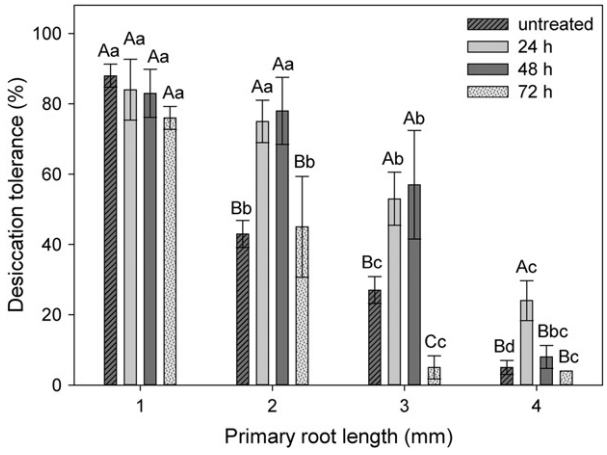

Fig. 2. Desiccation tolerance in germinated cowpea seeds untreated and treated with PEG (−1.7 MPa, 10 °C) for 24, 48 and 72 h. Means ± SDs, $n = 4$; different capital letters represent significant differences at $P \leq 0.05$ between primary roots with the same length; lowercase letters represent significant differences at $P \leq 0.05$ between primary roots with different lengths for the same time of incubation in PEG.

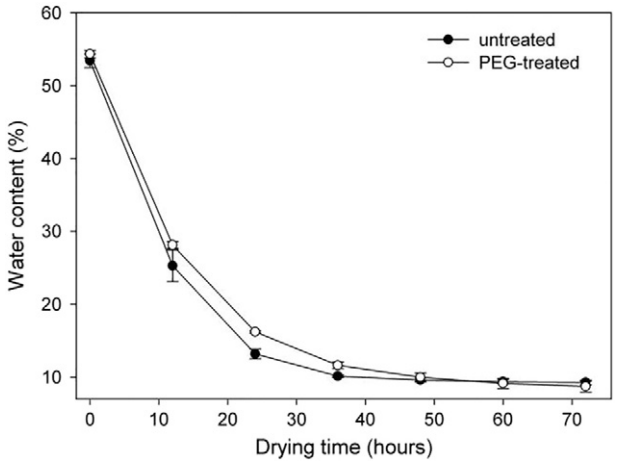

Fig. 3. Drying curves of cowpea seeds germinated with primary root length of 2 mm, treated and untreated in PEG solution (1.7 MPa at 10 °C) for 24 h. Means ± SDs, $n = 3$; bars indicate standard deviation.

the protoplast and the cell wall featuring the aspect of withered plant cells.

After imbibition, the cells from untreated primary roots and not subjected to drying displayed round shape presented well-defined intercellular spaces and presence of considerable starch grains (Fig. 5C). After drying, the intercellular spaces in the primary roots of untreated seeds were reduced, being visible only among cells with larger volume. Although the cellular content was not retracted and in some cases showed shape with some irregularities. Although apparently in minor amount, it was observed the persistence of starch grains after drying (Fig. 5D).

The cells of the primary roots of seeds treated with PEG (Fig. 5E) present shape and structure similar to that observed in root cells of untreated germinated seeds (Fig. 5C). However, we observed an apparent reduction in starch grains in primaries roots treated with PEG (Fig. 5E). After drying, the cells of the primary roots of seeds treated with PEG have kept the conformation and integrity of the cell wall and showed small depressions after the degradation of starch grains (Fig. 5F).

During re-induction of DT by PEG solution, the water content of the primary root was on average 66% (i.e., period of 8 to 24 h of PEG-treatment), presenting small variations (Fig. 6). This shows that during the re-induction of DT there was movement a loss and gain of water in the primary roots, probably due to the action of osmotic adjustment mechanisms of the cells caused by the PEG solution.

The sucrose and raffinose were present in all samples analyzed. However, sucrose content was always higher than the content of raffinose (Fig. 7). We did not detect the presence of glucose in the primary roots of cowpea seeds. After 16 h of imbibition, the contents of sucrose and raffinose were reduced in relation to the contents observed in the

Table 1
Number of cells at different stages of division in primary roots of cowpea seeds germinated, untreated and treated with polyethylene glycol 6000 solution (−1.7 MPa at 10 °C) for 24 h.

Primary root length	Interphase	Prophase	Metaphae	Anaphase	Telophase	Total
Untreated seeds						
1 mm	1919	80	1	0	0	2000
2 mm	1884	116	0	0	0	2000
3 mm	1876	122	1	0	1	2000
4 mm	1846	147	2	3	2	2000
Seeds treated with PEG						
1 mm	1906	94	0	0	0	2000
2 mm	1880	120	0	0	0	2000
3 mm	1878	119	1	1	1	2000
4 mm	1830	166	3	1	0	2000

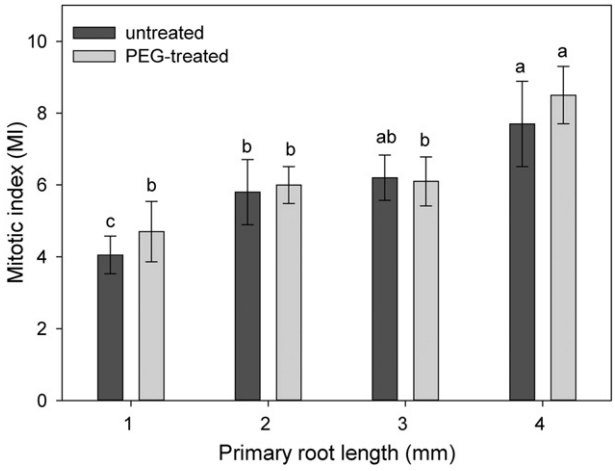

Fig. 4. Mitotic index (MI) in primary roots of seeds cowpea germinated untreated and treated with PEG (−1.7 MPa, 10 °C, 24 h). Means ± SDs, $n = 4$; different letters represent significant differences at $P \leq 0.05$ between primary roots with the different lengths untreated or treated with PEG; bars indicate standard deviation.

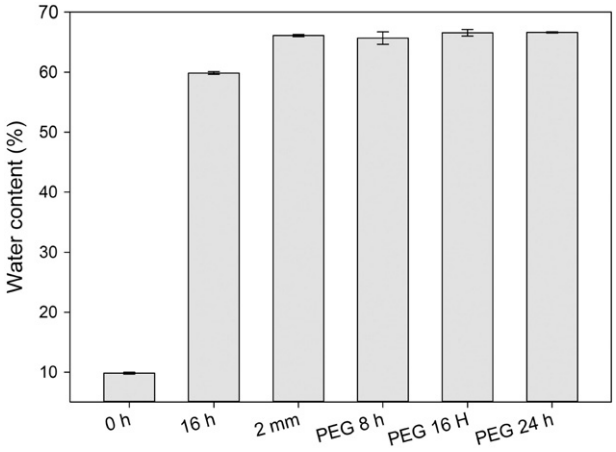

Fig. 6. Water content in radicles of seeds not imbibed (0 h) and imbibed in water for 16 h, and in primary roots of germinated of cowpea seeds with 2 mm length untreated and treated in PEG solution (1.7 MPA to 10 °C) for 8, 16 and 24 h. Means ± SDs, $n = 3$; bars indicate standard deviation.

radicles of seeds not imbibed. This reduction became more pronounced for the sucrose content in primary roots with 2 mm of untreated seed (Fig. 7). The sucrose content in the primary roots of germinated seeds increased from 8.29 to 16.5 mg g^{-1} DM after 24 h of treatment with PEG while the raffinose content increased from 6.68 to 9.02 mg g^{-1} DM (Fig. 7).

The ratio sucrose/raffinose, which was 1.6:1 in radicles from not imbibed seeds decreasing from 1.2:1 in the primary roots of germinated seeds with 2 mm of length. During the treatment with PEG there was an accumulation of sucrose and raffinose and the ratio sucrose/raffinose in the primary roots increased, reaching the proportion of 1.8:1 after 24 h of treatment (Fig. 7).

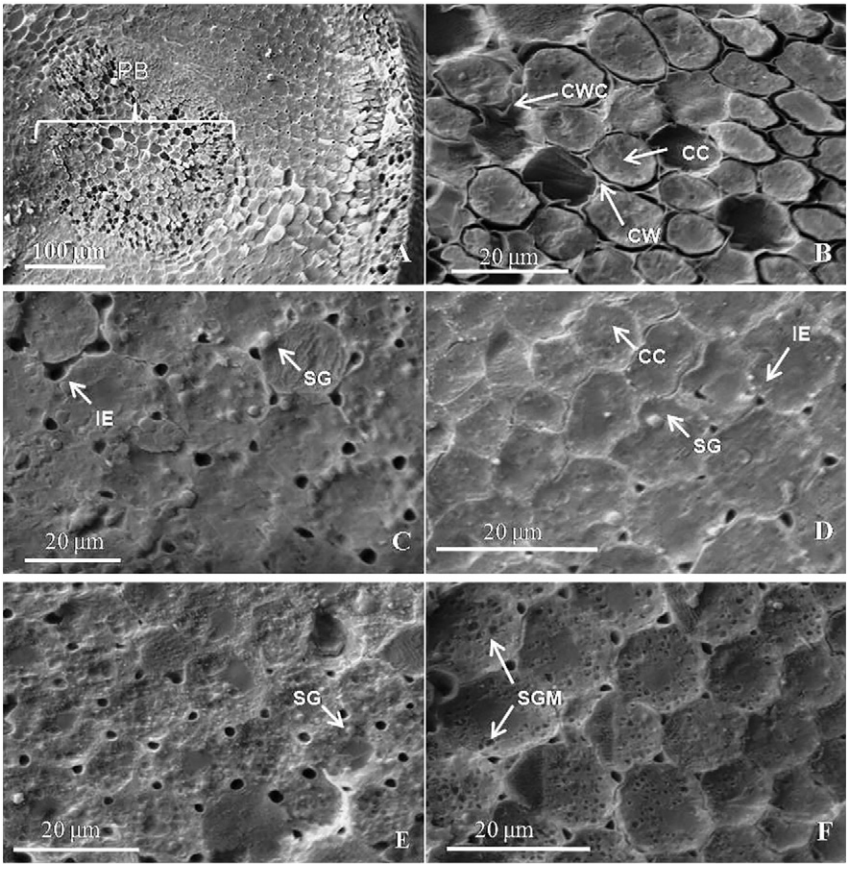

Fig. 5. Scanning electron micrography (SEM) of radicle and primary root of cowpea. Transversal section of the procambium region from the far end of the radicle apex of dry seed (A). Longitudinal section of the the radicle of dry seeds (B). Longitudinal section of primary root with 2 mm long untreated with PEG and without drying (C). Longitudinal section of primary root with 2 mm long untreated and subjected to drying (D). Longitudinal section of primary root with 2 mm long treated with PEG (−1.7 MPa, 10 °C, 24 h) without drying (E). Longitudinal section of primary root with 2 mm long treated with PEG and subjected to drying (F). Cell content (CC), loss of cell content (LCC), cell wall (CW), cell wall curvature (CWC), intercellular space (IE), starch grain (SG), starch grain mark (SGM), procambium (PB).

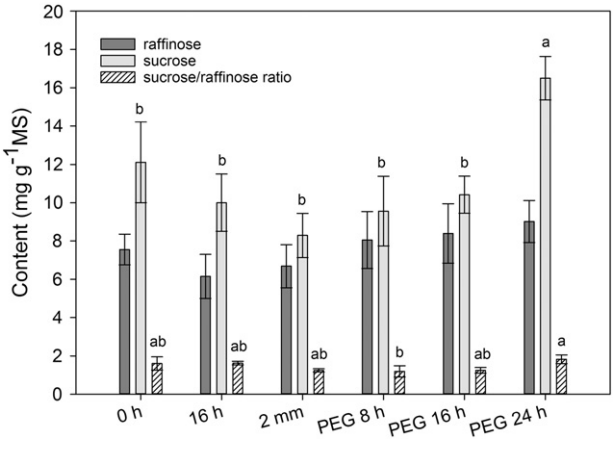

Fig. 7. Sucrose content and raffinose in radicles of cowpea seeds not imbibed (0 h) and imbibed for 16 h, and in primary roots with 2 mm long untreated and treated with PEG (−1.7 MPa, 10 °C). Means ± SDs, $n = 3$; bars indicate standard deviation. Different letters in the same column type represent significant differences for each sugar analyzed at $P \leq 0.05$ in different samples.

4. Discussion

Previous reports have shown that osmotic treatments can efficiently re-induce DT in germinated seeds of several species. In these studies, we show that the success of the re-induction of DT depended on in the development of a right protocol that provided the conditions required to necessary to restore DT (e.g. water potential of the osmotic solution, time and temperature of incubation, drying conditions of the seeds after osmotic treatment). Some physiological mechanisms, such as the accumulation of protective molecules, cell changes, gene expression and cell cycle events, among others, were related to the re-induction of DT. However, the contribution of these mechanisms diverge among the species (Buitink et al., 2003, 2006; Faria et al., 2005; Vieira et al., 2010; Maia et al., 2011, 2014).

In this study, we demonstrated that the re-induction of DT in germinated seeds of cowpea can be successfully obtained by treating the germinated seeds with an osmotic solution. The period of 24 h of treatment (−1.7 MPa, 10 °C) was the most effective treatment to re-induce DT. Here we also showed that the maintenance of cell structures, the arrest of the cell cycle and the accumulation of sucrose and raffinose during the osmotic treatment are mechanisms that may contribute to the re-induction of DT in cowpea germinated seeds.

During PEG treatment the primary roots of germinated seeds are subjected to mild water stress (Ashraf and Foolad, 2005), which produces a mediated memory of proteins, transcription factors and epigenetic changes (Bruce et al., 2007). The osmotic treatment also induce an increase in the synthesis of abscisic acid in roots of corn (Lü et al., 2007), a phytohormone which is associated with various protective mechanisms, among which the relaxation of the cell membrane, allowing reduction in cytoplasmic volume without the occurrence of ultrastructural damages (Creelman and Mullet, 1991).

Differently from other species (Buitink et al., 2003; Maia et al., 2011), cowpea germinated seeds treated with PEG kept their water content as high as the water content found for untreated germinated seeds. This result suggests that to re-establish DT in the primary roots treated with PEG do not necessarily need to reduce their water content. On the other hand, the osmotic potential imposed by the PEG solution should be sufficiently negative to paralyze the imbibition of water and block primary root growth to activate the osmotic adjustment and protection mechanisms necessary for the re-induction of DT.

Considering that there was no difference for the MI among primary roots of untreated seeds and treated with PEG with the same length (Fig. 3), it is possible to infer that treatment with PEG inhibited the progress of the cell cycle and growth of the primary root, favoring the re-induction of DT. According to Costa et al. (2015), the re-establishment of desiccation tolerance may be divided in two phase. In the first phase there is induction of events related to protection (such as LEA proteins) and arrest of growth while the second phase is related to events that promotes adaptation to stress and contribute to survival in the dry state.

The loss of desiccation tolerance has been associated with the start of germination and activation of the cell cycle (Berrie and Drennan, 1971; Saracco et al., 1995; Osborne et al., 2002; Faria et al., 2005). Germinating seeds usually become desiccation sensitive when the radicle cells enter in the G2 phase of the cell cycle (Saracco et al., 1995; Faria et al., 2005). Although the activation of the cell cycle have been repeatedly related to the loss of desiccation tolerance in germinating seeds this relation is not yet clear (Faria et al., 2005). In tomato seeds, for example, the activation of the cell cycle as factor for loss of DT continues to be questionable (Dekkers et al., 2015).

The decrease in the sucrose and raffinose contents in the radicles from seeds imbibed in water for 16 h in comparison with dry radicle coincided with the initial phase of loss of DT, when the capacity to tolerate desiccation was reduced to approximately 93% (Figs. 1 and 7). This result indicates that the loss of DT in germinating cowpea seeds may be related to the reduction of sucrose and raffinose contents. The PEG treatment induced the accumulation of sucrose in cowpea primary roots (Fig. 7) suggesting that the accumulation of this soluble sugar is a physiological response related to the re-induction of DT. Raffinose and sucrose also works as reserves in the embryo and are used as energy sources during early germination and during imbibition (Downie and Bewley (2000); Buckeridge et al., 2004). The degradation of the starch grains in the cells of the primary root treated with PEG (Fig. 5E) coincided with the increase in the accumulation of sucrose and raffinose (Fig. 7). Thus, we may say that these mechanisms of osmotic adjustment of cells in cowpea germinated seeds, contributing to the protection of cellular structures against the tensions caused by low osmotic potential during PEG treatment and against the removal of water during the desiccation. Pluskota et al. (2015), have observed increase in sucrose content and raffinose in roots of pea seedling after treatment with polyethylene glycol solution (PEG 8000). In addition, sucrose accumulation during the PEG treatment was also observed in primary roots of germinated seeds of *Medicago truncatula*, contributing to the re-induction of DT (Buitink et al., 2003). According Pluskota et al. (2015), the accumulation of organic osmolytes in response to osmotic stress, allow the cells to osmotically adjust in order to keep the cell turgor.

The loss of desiccation tolerance has been associated with the ratio sucrose/rafinose (Koster, 1991; Brenac et al., 1997; Cherussery et al., 2015). During the development of maize embryos the DT was not acquired in the absence of raffinose, being established only when the ratio of sucrose/raffinose was <20:1 (Brenac et al., 1997). The increase in the ratio of sucrose/raffinose in cocoa seeds (with 100% germination) from 8:1 to ratios superior than 15:1 was associated with the total loss of seed viability during desiccation (Cherussery et al., 2015). In our work, the ratio sucrose/raffinose in the radicles of dry cowpea seeds (1.6) was reduced when the seeds germinated and the primary roots reached 2 mm long (1.2) (Fig. 6), coinciding with the reduction of TD (Fig. 2). After 24 h of incubation in PEG the relationship sucrose/raffinose increased (1.8) (Fig. 6) and the DT was re-established (Fig. 2). Thus, it is probable that the re-induction of DT in germinated seeds of cowpea by PEG treatment influences the accumulation of sucrose and raffinose in primary roots, instead of changes in the ratios of sucrose/raffinose.

According to our results, the treatment with PEG (−1.7 MPa, 10 °C) for 24 h is efficient to re-induce desiccation tolerance in cowpea germinated seeds. Treatment with PEG contributes to the maintenance of cell structure and inhibits the progress of the cell cycle, favoring the re-induction of desiccation tolerance. The accumulation of sucrose and raffinose are mechanisms of response to treatment with PEG, which are related to the re-induction of desiccation tolerance in germinated seeds of

cowpea. The pattern of re-induction of DT observed in the seeds of cowpea might be used to elucidate physiological, ultrastructural, biochemical, and citogenetic events associated with desiccation tolerance.

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