Physiological and cytological aspects of *Inga vera* subsp. *affinis* embryos during storage

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The remarkably short storability of recalcitrant seeds has prevented their inclusion in programs of *ex situ* conservation. The causes of their desiccation sensitivity and rapid decline in viability during storage are not fully elucidated yet. In this study the highly recalcitrant, fully viable embryos of the tree species *Inga vera* subsp. *affinis* were stored under various conditions and analyzed physiologically and cytologically at intervals, in order to obtain more insights in their loss of viability during storage. Embryos stored fully hydrated at 5°C fully lost viability in 18 d. Sealed storage of partially dehydrated embryos slightly prolonged their viability, with 95% germination being attained after 14 d of storage. However, after 30 d of storage, viability was completely lost. Storage of hydrated embryos in a solution of polyethylene glycol (PEG) at -1.7 MPa water potential was capable of maintaining high germinability until 30 d of storage. When added to the PEG solution, abscisic acid showed a strong temperature-dependent interaction, with a positive effect on the longevity of embryos stored at 20°C and a negative effect on embryos stored at 5°C. In any case, embryo viability could be maintained no longer than 50 d. Embryos from seeds collected from the same trees in the following year showed better storability and still attained 45% germination after 62 d of storage. Analysis of cellular alterations during storage and viability loss of the embryos showed disappearance of starch granules and various damages to the cells, such as cell wall folding and cytoplasm fragmentation.

Key-words: abscisic acid, polyethylene glycol, recalcitrant seeds, seed conservation

Aspectos fisiológicos e citológicos de embriões de *Inga vera* subsp. *affinis* durante o armazenamento: A capacidade reduzida de armazenamento apresentada pelas sementes recalcitrantes tem impedido sua inclusão em programas de conservação *ex situ*. As causas da rápida queda da viabilidade durante o armazenamento ainda não estão totalmente elucidadas. Visando, portanto, a um melhor entendimento desse comportamento, embriões de sementes recalcitrantes de *Inga vera* subsp. *affinis* foram armazenados sob várias condições e avaliados periodicamente com relação a aspectos fisiológicos e citológicos. Embriões armazenados sem secagem, a 5°C, perderam totalmente a viabilidade em 18 dias. Embriões parcialmente desidratados apresentaram 95% de germinação após 14 dias de armazenamento em sacos plásticos selados. Entretanto, após 30 dias de armazenamento, a viabilidade foi totalmente perdida. O armazenamento de embriões sem secagem em solução de polietileno glicol (PEG) a um potencial hídrico de -1,7 MPa foi capaz de manter uma alta capacidade de germinação até 30 dias de armazenamento. Quando adicionado à solução de PEG, o ácido abscísico mostrou uma forte interação com a temperatura de armazenamento, com um efeito positivo na longevidade dos embriões armazenados a 20°C, porém um efeito negativo nos embriões armazenados a 5°C. No entanto, em nenhuma condição, a viabilidade dos embriões pôde ser mantida por mais de 50 dias. Embriões de sementes coletadas nas mesmas árvores no ano seguinte mostraram uma melhor capacidade de armazenamento, alcançando 45% de germinação após 62 dias de armazenamento. A análise de alterações celulares durante o armazenamento dos embriões mostrou o desaparecimento de grânulos de amido e vários danos às células, como dobras na parede celular e fragmentação do citoplasma.

Palavras-chave: ácido abscísico, conservação de sementes, polietileno glicol, sementes recalcitrantes,

INTRODUCTION

In recent years, awareness concerning the loss of plant diversity has captured worldwide attention and germplasm conservation has become necessary as a means of maintaining species diversity to prevent genetic erosion (Marzalina and Krishnapillay, 1999). While it is relatively easy to store orthodox and intermediate seeds in the long- and medium-term, respectively (Roberts, 1973; Ellis et al., 1990), one of the greatest challenges for those who deal with seed banks is the conservation of recalcitrant seeds. Recalcitrant seeds are shed with high moisture content, high metabolic activity and their viability is lowered or even totally lost when moisture content is reduced to a certain relatively high value. The refinement of storage techniques for intermediate and recalcitrant seeds is among the key storage research subjects for the future (Smith et al., 2003).

The bad storability of recalcitrant seeds has been widely reported, but the mechanisms involved in seed death during storage are not yet fully elucidated. In general, germinability has been the only parameter used to characterize the loss of viability (e.g., Goldbach, 1979; Tompsett, 1984, 1985; Farrant et al., 1993; Hong and Ellis, 1998; Tompsett and Pritchard, 1998). Although essential for characterizing the recalcitrant behavior, decrease in germination rates does not reveal *per se* the causes of the poor storability, since it is the end point of several processes occurring within the stored seed, which ultimately lead to seed death.

Because recalcitrant seeds are not desiccation-tolerant they must be stored moist (Greggains et al., 2000). However, even in moist conditions, their longevity is short, varying from a few weeks to a few months, depending on the species (Roberts and King, 1980a). One of the possible causes of viability loss of stored recalcitrant seeds is that they remain metabolically active during storage (Pammenter et al., 1994), thereby exhausting the seed's food reserves. Intracellular damages have been reported for hydrated recalcitrant seeds during storage and include degeneration of plastids, disruption of nuclear morphology, spatial disorientation of organelles and collapse of cell wall (Drew et al., 2000).

If metabolism is an underlying cause of the short storage life span, then reducing metabolic rate should extend longevity (Berjak and Pammenter, 2003). One possible approach is to store the seeds in an osmotic medium, such as polyethylene glycol (PEG) with or without the addition of germination inhibitors, such as abscisic acid (ABA) (Tompsett, 1985). Besides slowing down the metabolism, ABA can also induce desiccation tolerance in sensitive seeds and tissues, such as germinated seeds of cucumber (Bruggink and van der Toorn, 1995). Many of the physiological and biochemical changes caused by ABA in developing embryos can also be induced by low osmotic potentials (Bewley and Black, 1994). In cucumber seedlings, chilling tolerance was enhanced by exposing them to osmotic or heat stress, which induced the synthesis of dehydrin-like and heat-shock proteins respectively (Kang et al., 2005). Since these two protein families have been associated with desiccation tolerance (Delseny et al., 1994), it is possible that both osmotic and heat stress can also enhance desiccation tolerance in sensitive seeds. Osmotic stress (PEG) is also capable of re-establishing desiccation tolerance in germinated seeds of cucumber (Bruggink and van der Toorn, 1995; Leprince et al., 2000) and *Medicago truncatula* (Buitink et al., 2003; Faria et al., 2005). Intermittent heat-shock (HS) treatment has been used to improve low-temperature fruit storage of recalcitrant species, such as mango and avocado (Marcellin, 1992). Incubation in PEG, slow drying and heat-shock, independently, were able to increase longevity in primed seeds (Bruggink et al., 1999). It seems therefore that these techniques have the potential to decrease the desiccation sensitivity shown by recalcitrant seeds and/or increase their longevity.

Inga vera subsp. *affinis*, seeds of which were used in this study, is a leguminous tree species typical of the riparian forests in south-eastern Brazil, and one of the most important species for ecological restoration of those ecosystems. Its seeds are recalcitrant and have one of the worst storabilities known (Bilia et al., 1999; Barbedo and Cicero, 2000; Faria et al., 2004; Andréo et al., 2006). In order to develop storage protocols for recalcitrant seeds a better knowledge of the events associated with storage is mandatory. In the present study the physiological and cytological aspects of *I. vera* embryos during storage under different conditions were characterized.

MATERIAL AND METHODS

Plant material: By the time of their natural dispersion, mature fruits of *I. uruguensis* (a botanical synonym of *I. vera* subsp. *affinis*) become yellowish (Figliolia and Kageyama, 1994). Mature seeds of *Inga* were thus obtained from yellowish, soft fruits hand-harvested in January 2004 and January 2005 from 20 adult, healthy trees, growing in riparian forests along the *Rio Grande* at the surroundings of the city of Lavras (21º09'S, 44º53'W, 808 m a.s.l.), Minas Gerais State, Brazil. The climate at the site is classified as a transition between Cwb and Cwa, characterized by hot and humid summers with mild and dry winters, according to Köppen's classification (Köppen, 1936). On the same day of fruit collection seeds were manually extracted, processed and sampled for the experiments described below. Seeds were covered by fleshy, aqueous sarcotesta and embryos were fully developed. During processing the seeds, the sarcotesta was removed, which caused removal of the inner layers of the seed coat as well, ending up with the naked embryo. Removal of the sarcotesta was an absolute prerequisite since it is rich in sugars (Figliolia and Kageyama, 1994) which results in excessive growth of microorganisms during storage (Zuniga, 1996) and during germination tests (Bilia et al., 1998).

Assessment of moisture content: Moisture contents (MC) of the embryos were determined gravimetrically on four replications of five embryos by oven drying them for 17 h at 103ºC (ISTA, 1996). Moisture contents were calculated as $g\ H_2O\ g^{-1}$ DM (hereafter $g\ g^{-1}$). Embryos stored in PEG were opened by separating the cotyledons and washed rapidly before MC determination, in order to remove PEG residues.

Germination tests: Due to the ovoid shape of the embryos, they were germinated (partially buried) on moistened sand in plastic boxes (11 x 11 x 3.5 cm), at 30ºC and constant light (Faria et al., 2004). Four replications of 20 embryos were used. Germination was scored daily, with visible growth of the radicle as the criterion for germination. Before being set to germinate, all embryos were surface sterilized by washing in a solution of sodium hypochlorite (1%) for 10 min, followed by thorough rinsing in distilled water.

Embryo storage: The storability of *I. vera* embryos was assessed in 15 treatments, distributed over two experiments, as described below.

Storage Experiment 1: Embryos from seeds collected in January 2004 were placed in a bench top humidity chamber (Hotpack model 435314, Warminster, PA, USA), with running air (43% relative humidity and 25°C) and were partially dried, from 1.35 to 1.02 $g\ g^{-1}$. The embryos were then packed in aluminized plastic bags (light- and air-protected; 150 embryos per bag). Air was removed from the packages by using a vacuum pump until the plastic pressed moderately hard against the embryos. The bags were sealed and stored at 5 and 20°C, in a refrigerator and an incubator, respectively. This protocol is further referred to as 'sealed storage'. After one week a heat-shock was applied to half of the embryos stored at 5°C, by transferring the bags to an incubator at 30ºC and keeping them at this temperature for 24 h, after which they were transferred back to 5°C. Experiment 1 was thus composed of three treatments (Table 1). Non-stored embryos (control, kept at 20°C) and embryos stored for 14 and 30 d were sampled for germination test, MC assessment and cytological studies.

Storage Experiment 2: Embryos from seeds collected in January 2004 were stored with their original moisture content (1.35 $g\ g^{-1}$), in a single layer in plastic trays (40 x 25 x 8 cm; 350 embryos per tray) containing a solution of polyethylene glycol (PEG 8000; Sigma-Aldrich, Zwijndrecht, The Netherlands) at -1.7 MPa water potential, enough to slightly cover the embryos. Solutions of PEG were prepared with two abscisic acid (ABA; Sigma-Aldrich, Zwijndrecht, The Netherlands) concentrations (0 and 100 mM), based on Barbedo and Cícero (2000). The trays were covered with a plastic sheet to decrease the evaporation and stored at 5 and 20°C, in a refrigerator and an incubator, respectively, in the dark. After one week, a heat-shock was applied to half of the embryos stored at 5°C, by transferring the trays to an incubator at 30°C, in the dark for 24 h, after which they were transferred back to 5°C. Two weeks after the beginning of storage, half of the stored embryos was removed from the PEG solution, washed, blot-dried, air dried (as in Experiment 1) to *ca.* 0.64 $g\ g^{-1}$, to test for a possible decrease in desiccation sensitivity. The value of 0.64 $g\ g^{-1}$ is around the critical MC for *I. vera* embryos, which ranges from 0.54 $g\ g^{-1}$ (Bilia et al., 1999) to 0.7 $g\ g^{-1}$

Table 1. Storage of *Inga vera* embryos after partial dehydration (Experiment 1) or in PEG solution (Experiment 2), with or without ABA, heat-shock or dehydration in combination with sealed storage, as indicated by the plus and minus signs, respectively.

Treatment	Temperature of storage (^{o}C)	ABA	Heat-shock	Air-dried to 0.64 g g^{-1} after 14 d of storage + sealed storage
Experiment 1 (partial dehydration: 1.35 → 1.02 g g^{-1} + sealed storage)				
1	5	-	+	-
2	5	-	-	-
3	20	-	-	-
Experiment 2 (moist storage in PEG)				
4	5	-	+	+
5	5	-	+	-
6	5	-	-	+
7	5	-	-	-
8	5	+	+	+
9	5	+	+	-
10	5	+	-	+
11	5	+	-	-
12	20	-	-	+
13	20	-	-	-
14	20	+	-	+
15	20	+	-	-

(Faria et al., 2004). The dried embryos were then put into aluminized plastic bags (sealed conditions, as in Experiment 1) and transferred back to their original temperatures (5 or 20°C). The other half of the stored embryos remained in PEG solutions. Experiment 2 was therefore composed of 12 treatments (Table 1). Non-stored embryos (control) and embryos stored for 14, 30 and 50 d were sampled for germination test, moisture content assessment and cytological studies (light and scanning electron microscopy). Based on the results of 2004, more seeds were collected in 2005 and treatments 7, 11, 13 and 15 (Table 1) were repeated. Non-stored embryos (control) and embryos stored for 30 d (treatments 7, 11, 13 and 15) and 62 d (treatment 15) were sampled for germination test, MC assessment and transmission electron microscopy.

Cytological analysis: For all cytological studies, three to five embryonic axes per treatment were excised from the embryos and fixed by immersion for 24 h in a modified Karnovsky solution (2.5% glutaraldehyde, 2% formaldehyde in 50 mM sodium cacodylate buffer, 1 mM CaCl$_2$, at pH 7.2). Thereafter different protocols were followed, depending on the microscopy technique, as described below.

Scanning Electron Microscopy (SEM): Samples were transferred to a cryo-protectant solution (30% (v/v) aqueous glycerol) for 30 min and sectioned longitudinally in liquid nitrogen using a scalpel blade. Sections were transferred to a 1% aqueous solution of osmium tetroxide for 1 h at room temperature and subsequently dehydrated in an increasing series of acetone solutions. Dehydrated samples were critical-point dried. Specimens were mounted on aluminum stubs, sputter-coated with gold and observed with a scanning electron microscope (LEO Evo40 XVP – Leo Electron Microscopy, Cambridge, UK). Observations were always made in the meristematic region of the radicle.

Transmission Electron Microscopy (TEM): Samples were rinsed three times with 50 mM cacodylate buffer, post-fixed in a 1% aqueous solution of osmium tetroxide, rinsed three times with MQ H_2O, stained in uranyl acetate (0.5%) and subsequently dehydrated in an increasing series of acetone solutions. The dehydrated tissue was gradually infiltrated with Spurr resin/acetone and embedded in pure Spurr resin and polymerized at 68°C for 48 h. Thin sections (< 100 nm) were cut in a Reichert-Jung (ultracut) ultramicrotome equipped with a diamond knife, picked up on copper slot grids, and allowed to dry onto Formvar-coated aluminum racks (Rowley and Moran, 1975). Sections were post-stained with uranyl acetate followed by lead citrate (3 min each) and examined using a transmission electron microscope (Zeiss EM 109, Carl Zeiss, Jena, Germany). Observations were made of cells in the radicle meristem.

RESULTS

Moisture content
Experiment 1 – Embryos stored partially dried: During 30 d of storage, MC showed a slight increase, if any, in the three treatments, from about 1.05 to 1.25 g g⁻¹ (Figure 1A).

Experiment 2 – Embryos stored moist in PEG: The MC of the embryos at the beginning of the storage experiment performed in 2004 was 1.35 g g⁻¹. Embryos removed from PEG at 14 d of storage, dehydrated to 0.64 g g⁻¹ and stored again in plastic bags did not show subsequent significant changes of MC (data not shown). In embryos that were not dried and remained in PEG, MC varied differently among treatments, increasing or decreasing, depending on the treatment (Figure 2A) and sometimes on the seed batch (data not shown). The MC of seeds stored at 5°C (+ ABA, with or without HS) was consistently and significantly lower than the MC of seeds stored in the absence of ABA. In contrast, ABA led to significantly higher MC at 20°C. Both the ABA effects at 5°C and at 20°C were visible after 14, 30 and 50 d of storage (Figure 2A). In 2005 no significant effects of ABA on MC were observed (data not shown).
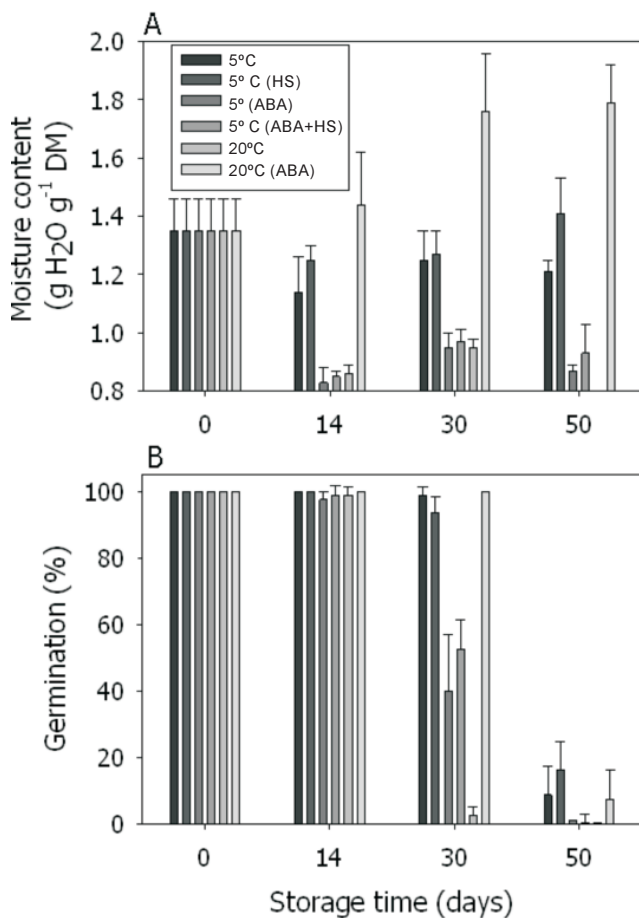
Germination

Experiment 1 – Embryos stored partially dried (at sealed conditions): After 14 d of storage of partially

dehydrated embryos at sealed conditions, germination dropped from 100% to less than 20% for those stored at 20°C and remained high (over 90%) for those stored at 5°C, regardless of HS treatments. However, after 30 d of storage, all seeds failed to germinate, irrespective of the storage temperature (Figure 1B). Embryos did not germinate during storage nor did they show any sign of degradation upon macroscopic inspection.

Experiment 2 – Embryos stored moist in PEG: After 14 d of storage in PEG, germination in all treatments was still high (≈ 95%; Figure 2B). In all treatments in which embryos were removed from PEG, dehydrated to 0.64 g g⁻¹ after 14 d of storage and stored again in plastic bags at
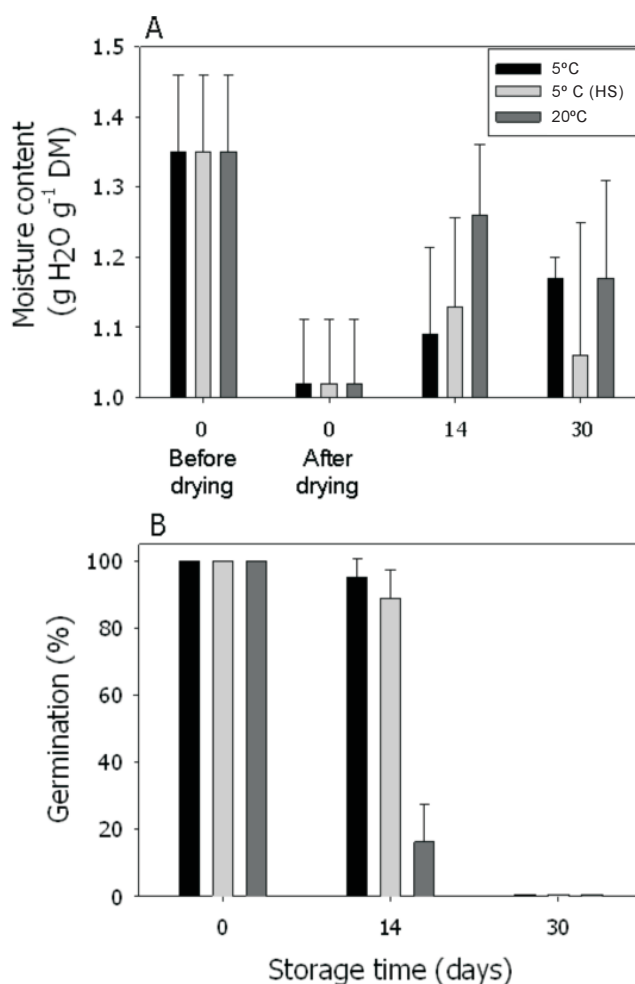
Figure 1. Moisture content (**A**) and germination (**B**) of partially dried *Inga vera* embryos after sealed storage (Experiment 1). Averages are from four replications of 20 embryos. Bars represent SD.

Figure 2. Moisture content (**A**) and germination (**B**) of *Inga vera* embryos (2004) after storage in PEG solution (Experiment 2) at different temperatures, with or without ABA or heat-shock (HS). Averages are from four replications of 20 embryos. Bars represent SD.

sealed conditions, there was an almost total loss of viability (less than 10% of germination) in the assessment done at 30 d of storage (data not shown). The only treatment not subjected to dehydration and sealed storage (Figure 2B) that also showed an almost complete loss of viability at 30 d, was that of embryos stored at 20°C: under these conditions metabolic activity is also supposed to be high. In the same evaluation, embryos stored at 20°C with ABA attained maximum germination (100%) like fresh, non-stored embryos (Figure 2B). High germination (over 94%) was also observed in embryos stored at 5°C (without ABA, regardless of HS treatments). Embryos stored at 5°C with ABA attained 53% (with HS) and 40% (without HS) of germination in the evaluation done 30 d after the beginning of the storage. There was a

clear relationship between MC (Figure 2A) and germination after 30 d of storage (Figure 2B). In the final evaluation, at 50 d, the only treatments still showing some germination, albeit at low levels, ranging from 8 to 16%, were storage at 5°C without ABA and at 20°C with ABA (Figure 2B). Again, there were similarities with the MC at 50 d of storage. When part of the experiment was carried out again in 2005 to generate samples for TEM visualization, the results of storability were in general better than in the previous year. The four treatments that were repeated (5°C, 5°C + ABA, 20°C and 20°C +ABA) attained over 80% germination after 30 d of storage in PEG (data not shown). Embryos stored at 20°C with ABA reached 45% germination after 62 d of storage. Because the storage experiment made in 2005 was based on the results of the previous year, only a limited number of embryos was stored enough for evaluation (germination, moisture content and TEM visualization) at 30 d (5°C, 5°C + ABA and 20°C) and 62 d (20°C + ABA) of storage. For this reason the experiment could not be extended to determine when embryos from 2005 would completely lose viability.

Cytological observations in stored embryos: SEM observations suggested that the abundance of starch granules in radicle cells declined as storage progressed (Figure 3). For example, most cells from embryonic axes of non-stored fresh embryos contained abundant starch granules (Figure 3A) whereas cells after 50 d of storage at 20°C in PEG + ABA generally appeared empty (Figure 3B).

Micrographs (TEM) of axis cells of the fresh control embryos generally showed that full-grown *Inga* seeds are still metabolically active, as can be expected for such a very recalcitrant species. The typical picture emerges of a metabolically active and viable cell, with polysomes and a round nucleus with dense nucleolus and peripherally arranged heterochromatin (Figure 4A). Mitochondria and amyloplasts (Figure 4B) were present in large numbers. Axis cells of embryos stored moist in PEG showed various changes, including confluence of lipid droplets resulting in large lipid masses in the cytoplasm and loss of cellular structure (Figure 4C). More severely damaged cells appeared totally devastated with cell wall folding, fragmented cytoplasm and amorphous nuclei (Figure 4D). In the same tissues and under the same storage conditions both healthy appearing cells and cells
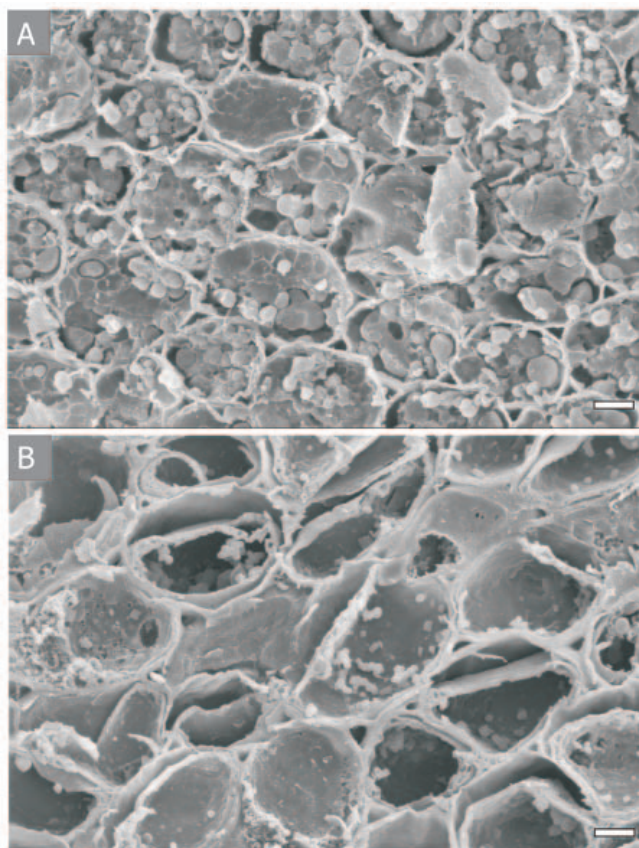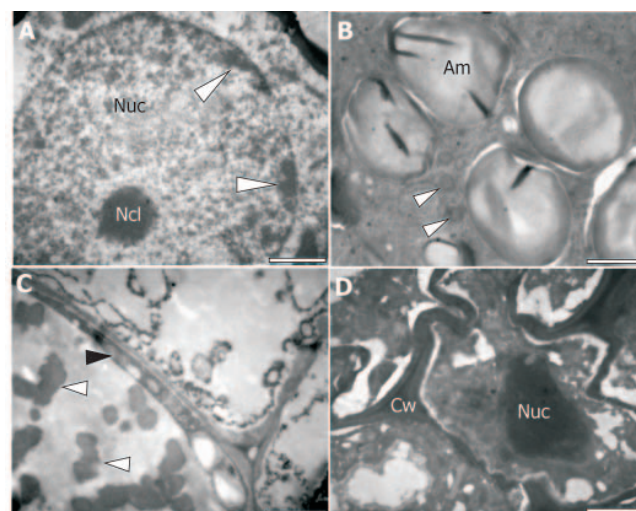
Figure 3. Scanning electron micrographs of embryonic axes from stored *Inga vera* embryos. (**A**) Control, non-stored, fresh embryos (100% germination); (**B**) embryos stored in PEG + ABA at 20°C for 50 d (7.5% germination). Bars = 10 μm.

Figure 4. Transmission electron micrographs of embryonic axes from non-stored (**A, B**) and stored (**C, D**) *Inga vera* embryos. (**A**) Round nucleus (Nuc) with dense nucleolus (Ncl) and heterochromatin arranged peripherally (arrowheads); (**B**) amyloplasts (Am) and mitochondria (arrowheads) with normal appearance; (**C**) confluence of lipid droplets resulting in large masses in the cytoplasm (white arrowheads) and in the extraprotoplasmic space (black arrowhead); (**D**) more severely damaged cell, showing cell wall (Cw) folding, cytoplasm degradation and amorphous nucleus (Nuc). Bars: **A** = 5 μm; **B-D** = 10 μm.

damaged to various degrees were observed next to each other. Because of the limited number of observations we could not correlate the extent of the damage or the number of damaged cells to the viability of the whole embryo, as assessed by the germination tests.

DISCUSSION

Just after processing, MC of the embryos of *I. vera* was 1.35 g g^{-1}, which corresponds to a water potential close to 0 MPa (Faria et al., 2004). During storage in PEG, with a water potential of -1.7 MPa, it was expected that in all treatments embryos would lose water to the osmotic medium, thus becoming to some extent dehydrated. This was indeed the case in embryos stored in PEG at 5°C with ABA, at 5°C with ABA and HS and at 20°C without ABA or HS. However, a significant increase in MC was observed with storage in PEG + ABA at 20°C after 20 and 50 d of storage. Intense respiration of stored recalcitrant seeds, which generates water, can increase MC during storage, Pritchard, 1998). In our experiments the unfavourable effect of oxygen depletion during sealed storage may have masked a possible positive effect of partial dehydration on the storability.

The longest effective storage period for *Inga* embryos was reported by Andréo et al. (2006) working with non-dried embryos stored in PEG. They showed that the embryos were still able to reach 75% germination after 90 d of storage either at -1.6 or -2.4 MPa water potentials. In the present work, for the embryos stored moist in PEG, effects of the treatments were apparent after 30 d of storage. Abscisic acid completely prevented the loss of viability of embryos stored at 20°C but displayed a moderately negative effect on the viability of embryos stored at 5°C. The efficacy of ABA in extending the

storage life span of recalcitrant seeds depends on maturity status (Barbedo and Cícero, 2000) and whether or not the seeds are responsive to it (Berjak and Pammenter, 2003). Our results suggest that the efficacy of ABA is also dependent on the storage temperature. The use of exogenous ABA in recalcitrant seeds to improve storability has generally shown positive results for various species [e.g., *Melicoccus bijugatus* and *Eugenia brasiliensis* (Goldbach, 1979); *Avicennia marina* (Pammenter et al., 1997), *Inga uruguensis* (Barbedo and Cícero, 2000) and *Acer saccharinum* (Marshall et al., 2000)]. Absence of ABA effect was reported for *Virola guatemalensis* (Gonzalez and Fisher, 1997). Therefore, in addition of being dependent of the conditions of the experiments (seed maturity status, ABA concentration and storage temperature), effect of ABA on recalcitrant seed storage seems to be species-specific.

The HS treatment caused no significant effect on the storability at 5°C of moist-stored *I. vera* embryos. Incubation in PEG, slow drying and HS independently increased longevity of primed seeds of *Impatiens walleriana* (Bruggink et al., 1999). The heat-shock treatment was most effective at the highest MC of these seeds. Intermittent HS treatment has also been used to improve fruit storability of (recalcitrant) species, such as mango and avocado (Marcellin, 1992). This treatment was applied to reduce or prevent cold damage to the fruits by allowing repair processes to proceed at the intermittent elevated temperature. Although these treatments suggest having the potential to decrease the desiccation sensitivity shown by recalcitrant seeds and/or increase their longevity, their effectiveness remains to be demonstrated.

Re-establishment of desiccation tolerance in germinated *Impatiens walleriana* and *Medicago truncatula* seeds was accompanied by an increase in sucrose content (Bruggink et al., 1999; Buitink et al., 2003). The likely source of sucrose synthesis is starch, which is a common food reserve in seeds. Thus, apart from a role as energy source in the metabolically active embryos during moist storage, starch hydrolysis could also produce the sucrose necessary for the reduction of desiccation sensitivity. However, in the present study there was no direct relationship between disappearance of starch and reduction of desiccation sensitivity, at least at the moisture content reached (0.64 g g^{-1}) after

dehydration of stored embryos. Seeds of *Castanea sativa* have a high content of sucrose but are recalcitrant, suggesting that although sucrose may play a protective role in desiccation tolerance, it is not *per se* sufficient to confer survival to dehydration (Steadman et al., 1996) In many species starch is the last reserve material to be degraded during germination (Werker, 1997). Because recalcitrant embryos are in a germination mode of development it seems plausible that they use starch to maintain their energy metabolism during moist storage and that low temperatures slow down metabolism. Starch mobilization has also been reported in the integument of early developing *Brassica napus* seeds, when it undergoes programmed cell death (Wan et al., 2002). Based on the SEM images, disappearance of starch granules correlated with the decline of viability during storage of *I. vera* embryos. Abscisic acid did not affect starch degradation but it affected viability negatively at 5°C and positively at 20°C when stored in PEG. Thus, low temperature and ABA may affect viability by different mechanisms.

Various features observed in embryonic cells from the control treatment, such as numerous mitochondria and polysomes and well organized rER are indicators of ongoing metabolism and progress to germination as might be expected in newly-shed recalcitrant seeds (Berjak et al., 1993; Kioko et al., 1998). Most of these characteristics were also observed in stored embryos, but with the simultaneous occurrence of sub-cellular damages that have been associated with viability loss during storage of recalcitrant seeds (Berjak et al., 1993). A correlation between the extent of cellular damages and viability seems obvious but is difficult to demonstrate. This correlation may depend on cell type, the number of cells within the axis with non-repairable damage, as well as the number of non-viable embryos within the population that is used for the viability assessment.

Seeds collected in 2005 and stored in PEG performed better than those of 2004 as they were still able to germinate for 45% after 62 d of storage. In one single species the characteristics of mature seeds may vary from year to year within provenances, due to environmental factors and plant reproductive strategies. The best known examples are related to seed production (Kelly and Sork, 2002; Greene and Johnson, 2004), seed mass (Leishman et al., 2000) and seed dormancy (Baskin and

Baskin, 1998). However there is a scarcity of such studies concerning desiccation sensitivity and storability. Tompsett and Pritchard (1993) showed that germination of mature *Aesculus hippocastanum* seeds collected from the same trees in two successive years decreased from 77 to 17% when dried to a MC of *ca.* 0.43 g g^{-1}. Such a year-to-year variation was also shown in relation to the storability of recalcitrant seeds of *Machilus kusanoii*: after four months of wet storage, germination of mature seeds decreased from *ca.* 100 to below 10%, while in the next year, seeds collected from the same tree and stored for the same period under the same conditions, maintained germinability close to 100% (Chien and Lin, 1997). It is likely that in the present study the different results of storability of *I. vera* embryos attained with seeds collected in 2005, compared to 2004, were due to this natural variation.

For germplasm conservation purposes, seeds need to be stored for periods longer than those from sowing to sexual maturity of the plant (Roberts and King, 1980a,b). *Inga vera* trees start to bear fruits three to four years after planting, which is much longer than the storability achieved in the present study. Therefore, there is still an urgent need to develop techniques that lengthen substantially the viability of stored *I. vera* and recalcitrant seeds in general. Since recalcitrant seeds usually lack developmental arrest after maturation, storage protocols should aim at suppressing metabolic activity during moist storage. From our results it appeared that this will not easily be achieved by partial dehydration in combination with sealed storage. Storage under controlled atmosphere, common in long-term conservation of fresh fruits, may prove successful. Treatments with osmotic agents, such as PEG, or HS, which have proven successful in re-induction of desiccation tolerance or extension of longevity of germinated orthodox seeds (Bruggink and van der Toorn, 1995; Bruggink et al., 1999; Faria et al., 2005) were not effective. Polyethylene glycol may be used as a means to slow down metabolism rather than inducing desiccation tolerance. Application of growth regulators, such as ABA requires further study since it seems that ABA is able to extend storability of *I. vera* embryos, depending on the storage temperature. In addition, it is possible that mature recalcitrant seeds have completely lost unique seed traits such as desiccation tolerance and should therefore be regarded as vegetatively growing plants rather than seeds.

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