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Conservation of *Hibiscus acetosella* germplasm by seed cryopreservation

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Abstract

Hibiscus acetosella (Malvaceae) is a shrub of great importance for landscaping, food and medicinal purposes. The objective of this study was to preserve *H. acetosella* germplasm by seed cryopreservation. Half of the seed batch was scarified and the other half was kept intact. Cryopreservation occurred by immersion in liquid nitrogen for 1 hour. Moisture content (MC%), germination percentage (G%), germination speed index (GSI), normal seedling formation (NS%), shoot length (SL), dry matter (DM), biometry and plant survival were evaluated after treatment. MC% ranged between 7.7% and 6.65% in intact and scarified seeds, respectively. Scarification raised G% and GSI compared to intact seeds. Intact and scarified seeds had 100% and 70% NS%, respectively, when not cryopreserved. Cryopreservation reduced NS% to 62% and 12.75%, respectively. The highest SL was observed in intact and non-cryopreserved seeds, with an average of 10.21 cm in height. However, the cryopreservation of intact seeds reduced SL by about 50%, and scarification led to a further reduction, either with (3.32 cm) or without (2.47 cm) cryopreservation. Seedlings from intact and non-cryopreserved seeds showed higher DM in relation to seedlings from cryopreserved seeds. The association of cryopreservation and scarification further reduced DM. The cryopreservation of intact seeds yielded 100% survival at the end of the acclimatization process. However, cryopreservation of scarified seeds reduced the survival percentage to 15%. Changes in color were observed for seeds scarified and subjected to cryopreservation. Thus, cryopreservation is considered an efficient technique for the conservation of intact *H. acetosella* seeds in the long term.

Keywords: *In vitro* conservation; Medicinal plants; Mechanical scarification; *Vinagreira roxa*.
Abbreviations: MC%_moisture content; G%_germination percentage; GSI_germination speed index; NS%_percentage of normal seedling formation; SL_shoot length; DM dry matter.

Introduction

The genus *Hibiscus* L. is the largest in the family Malvaceae, and consists of about 300 species (Rocha and Neves, 2000), which are appreciated for the beauty of their flowers and also for their medicinal properties (Ojeda *et al.*, 2010; Tsumbu *et al.*, 2012). Among the species of the genus *Hibiscus* L., *Hibiscus acetosella* Welw., a shrub native to Africa, popularly known in Brazil as *vinagreira roxa*, *groselheira* or *quiabo-roxo* (Sakhanokho and Kelley, 2009), stands out. This species, which has botanical similarities with the medicinal plant *Hibiscus sabdariffa*, is mainly exploited for food purposes, and it is consumed fresh, as salads, teas and jellies (Lorenzi, 2008).

According to Tsumbu et al. (2011) and (2012), *H. acetosella* has aroused special interest in its medicinal properties due to its great content of polyphenols, coumarins and flavonoids, which act as antioxidants and anti-inflammatory substances, as well as caffeic acid. This secondary metabolite is also involved in antioxidant (Özyurt et al., 2004), anti-inflammatory (Toyoda et al., 2009) and anticancer activities (Borrelli et al., 2002; Wang et al., 2005), besides the modulation of heart rate and blood pressure (Tan et al., 2005). Due to its extensive exploitation for its medicinal potential, efforts are justified for the conservation of *H. acetosella*. In addition, preserving the genetic diversity of this species, through appropriate germplasm conservation techniques, is necessary for the maintenance of biological material and management of this resource in the future (Lopes et al., 2013; Pandey et al., 2015).

According to Goldfarb et al. (2011), seed germplasm conservation is the most viable form of storage, since it is a more organized system and requires small spaces. Among the *ex situ* germplasm conservation techniques, cryopreservation stands out as a long-term conservation method. This technique is efficient in preserving biological

material at ultra-low temperatures (-196 °C) for an indeterminate time (Engelmann, 2011). This procedure ensures the viability of storing the material in small spaces, where it is protected from contamination and requires minimal maintenance (Wang et al., 2011). In addition, it is an alternative to preserve species that are maintained in field collections, botanical gardens, nature reserves or *in vitro* conservation systems, with plants under controlled growth conditions (Prudente et al., 2015). However, one of the challenges of cryopreservation is to lead to freezing by preventing the formation of ice crystals inside the tissues, which can reduce seed viability (Engelmann, 2011).

In cryopreservation studies, it is necessary to evaluate the viability of cryopreserved seeds, which can be performed using standardized germination tests (Brazil, 2013). However, these tests are subject to experimental errors related to the application of techniques (Pinto, 2015). Therefore, in the search for automation and speed in seed evaluation processes, the GroundEye® L800 equipment was developed, aiming to capture and analyze seed images by processing data through histograms, graphs and others that facilitate the image analysis of cryopreserved seeds and seedlings. Thus, biometric evaluations using the GroundEye® L800 equipment aid in the detection and measurement of changes caused by cryopreservation and replace biochemical and physiological methods of measuring damage, reducing the subjectivity of the evaluation process (Pinto, 2015). Therefore, through the use of this tool, this study consolidates this image analysis technique to evaluate the vigor and viability of cryopreserved seeds.

Given the above, the objective of this study was to preserve *H. acetosella* germplasm by seed cryopreservation, verifying the impact of vitrification and scarification on seed viability and seedling development of the species, as well as consolidating the image analysis technique by the GroundEye® L800 equipment to evaluate the vigor of cryopreserved seeds.

Results

Moisture content and seed germination

The moisture content (MC%) found in *H. acetosella* seeds prior to the cryopreservation treatment ranged from 7.7% to 6.65% for intact and scarified seeds, respectively. This result evidences a significant reduction (p<0.05) for the MC% of scarified seeds, when compared to seeds with intact integument.

Scarification significantly increased germination percentage (G%) and germination speed index (GSI) in relation to intact seeds, indicating that this treatment can optimize the germination of *H. acetosella* seeds. However, cryopreservation of intact and scarified seeds yielded distinct results, so that, for intact seeds, cryopreservation increased G% and GSI, while for scarified seeds, there was a reduction in germination percentage and speed (Table 1). In addition, cryopreservation can also be used to overcome dormancy, optimizing germination in intact *H. acetosella* seeds, although it showed results lower than those of scarification.

Growth and initial seedling development

When not cryopreserved, intact and scarified *H. acetosella* seeds had 100% and 70% normal seedlings (NS%), respectively. On the other hand, seed cryopreservation significantly reduced NS% to 62% and 12.75% in intact and scarified seeds, respectively (Table 1). This demonstrates that both seed cryopreservation and mechanical scarification may cause abnormalities in *H. acetosella* seedlings (Fig. 1D).

The highest shoot length (SL) of *H. acetosella* seedlings was observed in intact and non-cryopreserved (control) seeds, with an average of 10.21 cm in height (Table 1). However, cryopreservation of intact seeds significantly reduced SL by about 50%, and scarification led to a further reduction, either with (3.32 cm) or without (2.47 cm) cryopreservation (Fig. 1D).

H. acetosella seedlings from intact and non-cryopreserved seeds (control) showed significantly higher dry matter (DM) than those from cryopreserved seeds. The association of cryopreservation with scarification further reduced DM (Table 1). These results corroborate the reduction in NS% and SL verified for cryopreserved and scarified *H. acetosella* seeds. Nevertheless, through histological comparison of normal and abnormal plant stems from intact and cryopreserved seeds, no tissue disorganization of colenchyma, vascular bundle, xylem and phloem after cryopreservation was observed (Fig. 1E-H). These results emphasize the cryopreservation viability of intact seeds for the conservation of *H. acetosella* germplasm, allowing a survival percentage of 100% at the end of the acclimatization process. However, cryopreservation of scarified seeds significantly reduced survival percentage to 15% (Table 1).

Biometric characterization

In order to reduce the subjectivity of physiological evaluations and optimize the measurement of seed viability through biometric analysis, the GroundEye® L800 equipment detected a significant color variation between seed scarification and cryopreservation (Table 2). In general, there was a change in staining, mainly in scarified seeds submitted to cryopreservation.

Unlike the results obtained for the staining of *H. acetosella* seeds, biometric analysis using the GroundEye® L800 showed that the main geometric difference occurred for the scarification treatment, compared to the seeds maintained with intact integument (Table 3). However, for cryopreserved and non-cryopreserved seeds, there was a significant difference only for shape complexity and convex distances.

Regarding seed texture, the biometric changes verified by the GroundEye® L800 also showed significant differences between the scarification treatment and seeds maintained with intact integument, except for the Haralick variance (Table 4). Thus, the geometry and texture of *H. acetosella* seeds were more influenced by scarification than by cryopreservation, which shows that cryopreservation does not cause significant interference in the natural characteristics of the seed.

Table 1. Germination percentage (G%), germination speed index (GSI), normal seedling formation (NS%), shoot length (SL), dry matter (DM) and survival percentage (Surv) of intact and scarified *H. acetosella* seeds submitted to cryopreservation at the end of the *ex vitro* acclimatization period.

		Evaluated germination and growth parameters					
		G%	GSI	NS%	SL(cm)	DM(g)	Surv%
Intact	Non-cryopreserved	31 d	4.05 d	100,0 a	10.21 a	14.77 a	100 a
	Cryopreserved	50 c	7.68 c	62.0 c	5.34 b	9.69 b	100 a
Scarified	Non-cryopreserved	92 a	21.95 a	70.0 b	2.47 c	10.87 b	100 a
	Cryopreserved	85 b	19.38 b	12.75 d	3.32 c	5.55 c	15 b

*Means followed by the same letter in the column do not differ statistically by the Scott-Knott test (p <0.05).

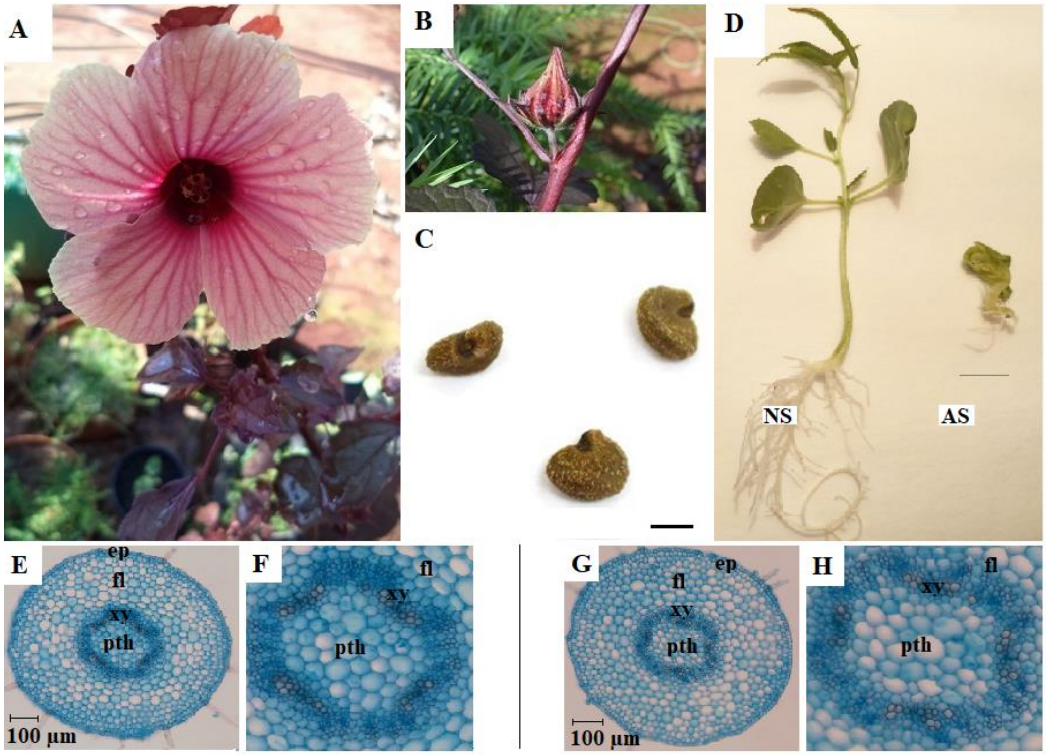

Fig 1. Flowering of *H. acetosella* parent plants set in greenhouse (A); *H. acetosella* fruit (B); freshly collected *H. acetosella* seeds (C); Normal (NS) and abnormal (AS) *H. acetosella* seedlings from intact and cryopreserved seeds (D); General aspect of the NS stem organization from intact and cryopreserved *H. acetosella* seeds (E-H), evidencing the epidermis = ep, xylem = xs, phloem = fl and pith = pth. Bars: 0.5 cm.

Table 2. Biometric changes related to the color of intact and scarified *H. acetosella* seeds submitted to cryopreservation.

Biometry: Color	Intact		Scarified		CV (%)
	Non-cryopreserved	Cryopreserved	Non-cryopreserved	Cryopreserved	
Below Otsu: CIELab: a	2.20 a	2.22 a	2.43 a	1.97 b	16.69
Above Otsu: Blue Channel	64.0 a	63.0 a	61.0 b	67.0 a	8.66
Above Otsu: CIELab: a	2.20 a	2.24 a	2.45 a	1.95 b	23.6
CIELab: a	2.19 a	2.23 a	2.46 a	1.91 b	18.28
CIELab: Dispersion	4.72 b	5.08 b	5.47 a	5.40 a	11.67
CIELab: Dispersion of b	3.22 b	3.47 b	3.81 a	3.58 a	12.49
CIELab: Dispersion of L	2.58 b	2.81 b	2.97 a	3.19 a	14.16
Matrix concentration	1.77 a	2.03 a	1.66 b	1.44 b	28.79
Red dominance	0.01 a	0.00 b	0.01 a	0.00 b	18.79
Saturation	0.14 b	0.16 b	0.18 a	0.14 b	27.69
Matrix variance	20.0 b	18.0 b	21.9 a	25.5 a	25.16

Table 3. Biometric changes related to the geometry of intact and scarified *H. acetosella* seeds submitted to cryopreservation.

Biometry: Geometry	Intact		Scarified		CV (%)
	Non-cryopreserved	Cryopreserved	Non-cryopreserved	Cryopreserved	
Thinning	0.80 a	0.80 a	0.78 b	0.77 b	2.15
Circularity	0.77 a	0.78 a	0.74 b	0.74 b	3.77
Circularity by form	0.38 a	0.38 a	0.36 b	0.35 b	4.76
Circularity by FFCg	7.17 a	7.28 a	6.97 b	6.58 b	10.28
Circularity by FFCm	0.78 a	0.79 a	0.76 b	0.76 b	2.51
Shape complexity	3.18 aA	3.14 aB	3.15 bA	3.11 bB	1.67
Compression	8.88 a	0.88 a	0.87 b	0.86 b	1.91
Shape convexity	0.93 a	0.93 a	0.92 b	0.92 b	0.80
Minimum diameter	0.27 a	0.26 a	0.25 b	0.26 b	4.81
Convex distances	124 bB	133 bA	139 aB	152 aA	11.69
Sphericity of form	15.75 b	15.71 b	16.15 a	16.33 a	2.14
Sphericity of the modified form	0.28 a	0.29 a	0.28 b	0.28 b	3.48
Extension	0.75 a	0.75 a	0.73 b	0.73 b	4.00
Number of corners by Harris	76.0 b	76.0 b	80.0 a	89.0 a	17.45
Aspect ratio	0.71 a	0.72 a	0.68 b	0.67 b	5.15
Change ratio	0.62 a	0.62 a	0.59 b	0.59 b	6.28
Circumscribed rectangles: Side opposite to the smallest edge	0.36 b	0.36 b	0.37 a	0.38 a	5.24
Shape strength	0.973 a	0.974 a	0.972 b	0.965 b	0.61
Elliptical variance	53.52 b	53.17 b	54.94 a	54.82 a	3.59

*Means followed by the same letter in the row do not differ statistically by the Scott-Knott test (p <0.05). Lower-case letters compare the effect of scarification, and upper-case letters compare the effect of cryopreservation.

Tabela 4. Biometric changes related to the texture of intact and scarified *H. acetosella* seeds submitted to cryopreservation.

Biometry: Texture	Intact		Scarified		CV (%)
	Non-cryopreserved	Cryopreserved	Non-cryopreserved	Cryopreserved	
Haralick: Contrast	14.9 b	15.7 b	16.5 a	20.4 a	28.88
Haralick: Dissimilarity	2.61 b	2.62 b	2.74 a	3.07 a	14.06
Haralick: Energy	0.07 a	0.07 a	0.06 b	0.06 b	14.4
Haralick: Entropy	5.90 b	5.96 b	6.07 a	6.24 a	4.16
Haralick: Homogeneity	0.36 a	0.36 a	0.35 b	0.32 b	9.24
Haralick: Variance	60.4 bB	70.1 bA	79.4 aB	96.7 aA	30.79
Run Length: LRE	1.65 a	1.68 a	1.63 b	1.55 b	6.92
Run Length: SER	0.88 b	0.88 b	0.89 a	0.90 a	1.66

*Means followed by the same letter in the row do not differ statistically by the Scott-Knott test (p <0.05). Lower-case letters compare the effect of scarification, and upper-case letters compare the effect of cryopreservation.

Discussion

According to Roberts (1989), seeds with naturally low moisture content, such as those obtained in this study for *H. acetosella*, are of orthodox physiological character. Moisture content (MC%) refers to the amount of water in the seed in relation to its total mass, which is a function of relative air humidity and ambient temperature (Almeida et al., 2006). In this context, in cryopreservation studies for seeds of recalcitrant and orthodox species, the determination and modulation of seed MC% are determining factors, since one of the great challenges of this technique is to carry out cryopreservation without the formation of ice crystals (Engelmann, 2011). When formed inside the tissues, ice crystals cause physical damage to the cells, leading to, for ple, rupture of the cell membrane system, besides loss of selective permeability and compartmentalization of cellular organelles (Panis and Lambardi, 2005). Thus, the water content in the seeds has different effects on the germination process, and can influence both germination percentage and uniformity (Schwember and Bradford, 2010). The increase in germination percentage (G%) in scarified seeds corroborates the higher germination speed index (GSI) observed in this treatment, since scarification favors imbibition, shortening the first germination phase and leading to the development of the hypocotyl in a shorter time period (Morais et al., 2017). These results are in agreement with those found for species of the genus *Hibiscus*, since they generally have a hard integument, which establishes a mechanical barrier to water entry into the seed, reducing germination (Amaro et al., 2013). According to Albuquerque et al. (2016), seed germination begins with imbibition and ends with endosperm rupture, through embryo expansion and endosperm weakening. Thus, the impermeability of the integument is the main cause of seed dormancy (Souza et al., 2012; Jayasuriya et al., 2013), which prevents a rapid germination due to the low water absorption by the seeds soon after their dispersion. Given the above, these results demonstrate a probable physical dormancy in *H. acetosella*

seeds imposed by the integument, since seeds that received prior scarification, optimized and standardized germination. In addition, dormancy overcoming due to cryopreservation verified for *H. acetosella* seeds in this study corroborates Rocha et al. (2009), who tested seeds of different cotton cultivars and concluded that cryopreservation was also able to increase G% by overcoming dormancy. According to Pinto et al. (2015), it is possible to evaluate seed physiological quality from the image analysis performed by the GroundEye® L800 equipment. Its use for the automation of seed analysis can facilitate decisions on batch destination or arrangement, and it is possible to apply this information to cryopreserved seed batches. The use of automated image analysis of seeds and seedlings is associated with the need for fast and accurate methods to evaluate their viability (Kikuti, 2012; Dell'aquila, 2009). The color changes caused by scarification and cryopreservation in *H. acetosella* seeds could be detected by the GroundEye® L800 equipment in the biometric analysis, by thresholding, which is one of the tools that leads to image segmentation, and is characterized by recognizing and distinguishing an image from its background (Gonzalez et al., 2010). According to Bewley et al. (2013), seed darkening may be an indication that there was integument oxidation. In this context, the observation of color differences, together with the knowledge of several seed characteristics, allow the determination of the presence, location and nature of the damage (Costa et al., 2003) and can be adapted to cryopreservation research. Therefore, this equipment shows techniques that allow the evaluation of storage deterioration by color analysis (Yagushi et al., 2014), which may or may not be associated with the cryopreservation procedure. In addition to color changes, texture attributes have been one of the main characteristics used in the classification of digital images (Aksoy and Haralick, 1998) and, in this study, they could be verified by the GroundEye® L800 through Haralick descriptors (Felipe and Traina, 2003). Although there is no consensus on the definition of texture, it can be referred to as a set of certain patterns existing on physical surfaces, perceptible to the human eye and that bring great amount of information about the nature of the surface, such as softness, roughness and regularity (Alves et al., 2006). In this context, it can be inferred that the texture differences detected by the GroundEye® L800 are related to surface modifications in *H. acetosella* seeds, due to the cryopreservation treatment. However, further studies are still required to relate the physiological parameters of seed viability assessment with the biometric data provided by the GroundEye® L800.

The reduction in the percentage of normal seedlings (NS%), shoot length (SL) and dry matter (DM) of *H. acetosella* obtained in this study are in accordance with Santos et al. (2004), who demonstrated that scarification and cryopreservation can also cause tissue damage inside the integument, in addition to external modifications. This is common in cryopreservation studies, since seed cryopreservation may cause the formation of ice crystals, damaging cell membranes and causing irreversible tissue damage (Panis and Lambardi, 2005). This type of damage can reduce the amount of xylem and phloem, which impairs water transport, causing a reduction in mineral supply for shoot growth and development. However, by histological comparison, it was possible to verify that, for *H. acetosella*, cryopreservation did not damage internal tissues, maintaining the integrity of the conducting vessels, colenchyma and epidermis, even for seedlings considered abnormal. These results corroborate those of Meletti et al. (2011), who also observed a significant difference in shoot length (SL) for passion fruit (*Passiflora edulis*) seedlings submitted to cryopreservation, compared to seedlings kept at 25 °C. This means that, although cryopreservation is applicable to germplasm conservation of several plant species, its use may cause a significant reduction in SL, NS% and DM of plants. *H. acetosella* seeds can be cryopreserved and stored in germplasm banks for an indeterminate time, since the seeds maintain their viability and vigor, even after cryopreservation (Goldfarb et al., 2011). However, mechanical scarification is unnecessary to avoid a reduction in NS% (12.75%), SL (3.32 cm) and DM (5.55 g). Despite the reduction in seedling size observed in this study, cryopreservation did not alter the survival rate of plants from intact seeds during the acclimatization process. These results corroborate those found by De Faria et al. (2017), who used cryopreserved *Physalis angulata* L. seeds, and obtained 100% plant survival at the end of the acclimatization process. After the germination of cryopreserved seeds, plant acclimatization is a crucial step to verify the efficiency of this technique, since one of the objectives of cryopreservation, besides conservation, is the possibility of restoration of the material in the field. During transplanting, plants are susceptible to several stress sources (Shinohara and Leskovar, 2014), which were tolerated by *H. acetosella* seedlings from intact and cryopreserved seeds, and it did not occur for scarified and cryopreserved seeds.

Materials and methods

Plant material and treatments

H. acetosella L. (Malvaceae) seeds were collected in January 2017 from plants located in Southern Minas Gerais, Brazil, and homogenized. Viable seeds were separated by density in the water, so that denser seeds were collected manually to ensure batch uniformity. Subsequently, the initial seed moisture content (MC%) was determined, using 5 samples with 25 seeds each, according to the rule for seed analysis (Brasil, 2013).

Half of the seed batch was manually scarified with sandpaper (No. 100) until partial exposure of the endosperm, and the other half was maintained with the integument intact (control). All seeds were disinfected within a laminar flow chamber by immersion in 70% alcohol for 1 minute and 2.5% (v/v) commercial sodium hypochlorite for 10 minutes, followed by three washes in autoclaved distilled water. For cryopreservation, cryotubes (2.5 mL) were used, remaining immersed in liquid nitrogen for 1 hour (De Faria et al., 2017). All seeds were then thawed in a water bath at 38 °C for 3 minutes. Seed sowing occurred in gerbox containing a double layer of germitest paper moistened with 5 mL of distilled and autoclaved water.

Evaluated characteristics

After sowing, the following parameters were evaluated during the first seven days of cultivation: germination percentage (G%), counting the number of seeds with root

protrusion of ± 2 mm; germination speed index (GSI), according to McGuire (1962); percentage of normal seedling formation (NS%) (plants with well developed shoot and roots) and seedling dry matter (DM) in each treatment. DM was determined by weighing all germinated seedlings in each replicate with subsequent drying in a forced air oven at 105 °C for 24 hours. After this period, the material was removed from the oven and placed in a desiccator for 30 minutes for cooling, and then weighed again, calculating the difference between the two weighings on a dry matter basis (Nery et al., 2009).

At 15 days of *in vitro* cultivation, shoot length (SL) was evaluated with the aid of a digital caliper, and acclimatization was carried out by transferring 25 normal plants from each treatment to tubes (250 mL) containing Tropstrato® substrate. The tubes were wrapped with transparent plastic packaging to maintain relative humidity, and their tips were cut at 7-day intervals until completely removed at 21 days. The tubes containing the seedlings were kept in a growth room at a temperature of 25 °C and irradiance of 43 µmol m^{-2} s^{-1}. After 30 days of the beginning of acclimatization, seedling survival percentage was evaluated.

Histological comparisons

Histological comparisons were made in hypocotyls collected from normal and abnormal plants from intact and cryopreserved seeds, and fixed in 70% (v/v) ethanol. The cross-sections were hand-made and, subsequently, they were clarified in 5% (v/v) commercial sodium hypochlorite solution over a period of 3-5 minutes and washed three times with distilled water. Sections were stained with a mixture (9:1) of Astra Blue (1%) and Safranine (1%), mounted on 1:1 (v/v) glycerol:water, according to Kraus and Arduin (1997). Provisional and semi-permanent slides were prepared according to Johansen (1940) and observations were made using an Axiophot microscope equipped with DIC optics (Zeiss®, Oberkochen, Germany); a Powershot A640 digital camera (Canon®, Tokyo, Japan) was used for photographic documentation.

Biometric analysis

For the biometric analysis of scarified and cryopreserved seeds, 15 seeds of each treatment were collected, and high resolution camera images were captured in the GroundEye® L800 equipment (Pinto et al., 2015), evaluating changes in color, geometry and texture. The color model used in the equipment was the CIELab (L*, a*, b*), which orders and describes all colors according to a triangular system: luminance coordinate (L*) and two color coordinates, a* for red/green and b* for yellow/blue (Hoffmann, 2017), in addition to the Otsu method, based on the search for a threshold value that minimizes the intraclass variance of two pixel groups in an image. For texture description, the equipment used the Haralick methodology (1979), based on second-order statistics, which defines characteristics from the calculation of matrices called “co-occurrence matrices”, consisting of a count of how many different combinations of gray levels occur in an image, in a particular direction.

Statistical analysis

All experiments were conducted in a completely randomized design with 5 replicates of 25 seeds each for MC%, 4 replicates of 25 seeds each for G%, GSI, NS%, SL and DM, and 15 seeds of each treatment for biometric analyses. In addition, for acclimatization, 25 normal plants of each treatment were used, totaling 100 plants. Data were submitted to analysis of variance and means were compared by the Scott-Knott test (p<0.05), using the statistical software R Core Team (2011), version 3.4.0, via the ExpDes.pt package.

Conclusions

Cryopreservation is considered an efficient technique for the conservation of intact *H. acetosella* seeds in the long term. However, mechanical scarification of *H. acetosella* seeds, associated with cryopreservation, is not recommended, due to the low survival rate.

It is possible to use the GroundEye® L800 equipment to perform biometric analyses, reducing subjectivity and allowing to standardize the evaluation methodology of cryopreserved seeds.

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References

Aksoy S and Haralick RM (1998) Textural features for image database retrieval. Proceedings. IEEE Workshop on Content-Based Access of Image and Video Libraries (Cat. No.98EX173), 45-49.

Albuquerque E, Santos DDC, Neto JJDSB (2016) Crioarmazenagem de sementes de manga (*Mangifera indica* L.). A Barriguda: Revista Científica. 6: 01-10.

Almeida CA, Gouveia JPG, Almeida FAC, Silva FLH (2006) Avaliação da cinética de secagem em frutos de acerola. Bioterra. 6: 145-151.

Alves WAL, Araújo SAD, Librantz AFH (2006) Reconhecimento de padrões de texturas em imagens digitais usando uma rede neural artificial híbrida. Exacta. 4: 325-332.

Amaro HTR, David AMSS, Neta ICS, Alves DD, Silva FG (2013) Avaliação fisiológica de sementes e crescimento de plântulas de vinagreira. Com Sci. 4: 96-102.

Bewley JD, Hilhorst HWM, Bradford KJ, Nonogaki H (2013) Seeds: physiology of development, germination and dormancy. 3rd ed. Springer, 392.

Borrelli F, Izzo AA, Di Carlo G, Maffia P, Russo A, Maiello FM, Capasso F, Mascolo N (2002) Effect of a propolis extract and caffeic acid phenethyl ester on formation of aberrant crypt foci and tumors in the rat colon. Fitoterapia. 73: 38-43.

Brazil. Ministry of Agriculture, Livestock and Food Supply (2013) Rules for seed testing /Ministry of Agriculture, Livestock and Supply. Agricultural Defense Secretariat. Mapa/ACS.

Costa NP, Mesquita CM, Maurina AC, Neto JBF, Krzyzanowski FC, Henning AA (2003) Qualidade fisiológica, física e sanitária de sementes de soja produzidas no Brasil. Rev Bras Sem. 25: 128-132.

De Faria CVN, Paiva R, Freitas RT, Figueiredo JRM, Silva DPC, Reis MV (2017) Criopreservação de sementes de *Physalis angulata* L. por meio da desidratação em sílica gel. Plant Cell Cult Micropropag. 12: 27.

Dell'Aquila A (2009) Digital imaging information technology applied to seed germination testing: A review. Agri Sustain Develop. 29: 213–221.

Engelmann F (2011) Cryopreservation of embryos: an overview. Plant embryo culture: methods and protocols. 155-184.

Felipe JC, Traina, AJM (2002) Utilizando características de textura para identificação de tecidos em imagens médicas. In: II Workshop de Informática Médica. Anais do II Workshop de Informática Médica, 4.

Goldfarb M, Duarte MEM, Mata MERC (2011) Armazenamento criogênico de sementes de pinhão manso (*Jatropha curcas* L.) Euphorbiaceae. Biotemas. 23: 27-33.

Gonzalez RC, Woods RE, Eddins SL (2010) Morphological reconstruction. Digital Image Processing using MATLAB, MathWorks.

Haralick RM (1979) Statistical and structural approaches to texture. Proceedings of the IEEE. 67: 786-804.

He JM, Ma XG, Zhang Y, Sun TF, Xu FF, Chen YP, Liu X, Yue M (2013) Role and interrelationship of Gα protein, hydrogen peroxide, and nitric oxide in ultraviolet B-induced stomatal closure in Arabidopsis leaves. Plant Physiol. 161: 1570-1583.

Hoffmann G CIELab Color Space. August 2017, [online] Available: http://docs-hoffmann. de/cielab03022003.pdf.

Jayasuriya KMG, Wijetunga ASTB, Baskin JM, Baskin CC (2013) Seed dormancy and storage behaviour in tropical Fabaceae: a study of 100 species from Sri Lanka. Seed Sci Res. 23: 257-269.

Johansen DA (1940) Plant microtechnique. 2. ed. Macgraw-Hill. 532.

Kikuti ALP (2012) Testes de vigor em sementes de alface. Hort Bras. 30: 44-50.

Kraus J and Arduin M (1997) Manual básico de métodos em anatomia vegetal. Seropédica. EDUR.

Lopes KP, Almeida FAC, Carvalho JMFC, Bruno RLA (2013) Criopreservação de eixos embrionários zigóticos de algodoeiro. R. Bras. Eng. Agríc. Ambiental. 17: 291-298.

Lorenzi H (2008) Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas do Brasil. 4 ed. Instituto Plantarum. 1: 384.

McGuire JW, Chiu JSY, Elbing AO (1962) Executive incomes, sales and profits. The American Economic Review. 52: 753-761.

Meletti LMM (2011) Avanços na cultura do maracujá no Brasil. Rev. Bras. Frutic. 33: 83-91.

Morais LF, Almeida JCC, Nepomuceno DD, Morenz MJF, Deminicis BB, Carvalho CAB, Silvestre MF (2017) Efeito da mastigação sobre a sobrevivência de sementes de leguminosas forrageiras tropicais e germinação. Arch Zootec. 66: 131-135.

Nery MC, Carvalho MLM, Oliveira JA, Kataoka VY (2009) Beneficiamento de sementes de nabo forrageiro. Ver Bras Sem. 31: 36-42.

Ojeda D, Jiménez-Ferrer E, Zamilpa A, Herrera-Arellano A, Tortoriello J, Alvarez L (2010) Inhibition of angiotensin convertin enzyme (ACE) activity by the anthocyanins delphinidin-and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa*. J ethnopharmacol. 127: 7-10.

Özyurt H, Söğüt S, Yildirim Z, Kart L, Armutçu F, Temel I, Özen S, Uzun A, Akyol O (2004) Inhibitory effect of caffeic acid phenethyl ester on bleomycine-induced lung fibrosis in rats. Clin Chim Acta. 339: 65-75.

Pandey R, Sharma N, Agrawal A, Gupta S, Jain A, Tyagi RK (2015) *In vitro* conservation and cryopreservation of vegetatively propagated crop germplasm. Management of Plant Genetic Resources, National Bureau of Plant Genetic Resources. 197.

Panis B and Lambardi M (2005) Status of cryopreservation technologies in plants (crops and forest trees). The Role of Biotechnology. 43-54.

Pinto CAG, Carvalho MLM, Andrade DB, Leite ER, Chalfoun I (2015) Image analysis in the evaluation of the physiological potential of maize seeds1. Rev Ciên Agron. 46: 319-328.

Prudente DO, Nery FC, Paiva R, Santos PAA, Nery MC, Paiva PDO (2015) *In vitro* germination and cryopreservation of *Zinnia elegans* seeds. Ornamental Horticulture. 21: 243-250.

R Development Core Team (2011) R: a language and environment for statistical computing. reference index version 3.4.0 (ExpDes.pt package). R Foundation for Statistical Computing, Vienna, Austria.

Roberts EH (1989) Seed storage for genetic conservation. Plants Today. 2: 12-17.

Rocha JF and Neves LDJ (2000) Anatomia foliar de *Hibiscus tiliaceus* L. e *Hibiscus pernambucensis* Arruda (Malvaceae). Rodriguésia. 51: 113-131.

Rocha MS, Mata MERMC, Carvalho JMFC, Lopes KP (2009) Crioconservation of cotton seed. R Bras Eng Agríc Ambiental. 13: 312-318.

Sakhanokho HF and Kelley RY (2009) Influence of salicylic acid on *in vitro* propagation and salt tolerance in *Hibiscus acetosella* and *Hibiscus moscheutos* (cv 'Luna Red'). Afr J Biotechnol. 8: 1474-1481.

Santos TO, Morais TGDO, Matos VP (2004) Escarificação mecânica em sementes de chichá (*Sterculia foetida* L.). Rev Árvore. 28: 1-6.

Schwember AR and Bradford KJ (2010) A genetic locus and gene expression patterns associated with the priming effect on lettuce seed germination at elevated temperatures. Plant Mol Biol. 73: 105-118.

Shinohara T and Leskovar D (2014) Effects of ABA, antitranspirants, heat and drought stress on plant growth, physiology and water status of artichoke transplants. Scientia Horticulturae. 165: 225-234.

Souza TV, Voltolini CH, Santos M, Paulilo MTS (2012) Water absorption and dormancy-breaking requirements of physically dormant seeds of *Schizolobium parahyba* (Fabaceae–Caesalpinioideae). Seed Sci Res. 22: 169-176.

Tan J, Ma Z, Han L, Du R, Zhao L, Wei X, Hou D, Johnstone BH, Farlow MR, Du Y (2005) Caffeic acid phenethyl ester possesses potent cardioprotective effects in a rabbit model of acute myocardial ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol. 289; 2265-2271.

Toyoda T, Tsukamoto T, Takasu S, Shi L, Irano N, Ban H, Kumagai T, Tatematsu M (2009) Anti-inflammatory effects of caffeic acid phenethyl ester (CAPE), a nuclear factor-κB inhibitor, on Helicobacter pylori-induced gastritis in Mongolian gerbils. Int J Cancer. 125: 1786-1795.

Tsumbu CN, Deby-Dupont G, Tits M, Angenot L, Franck T, Serteyn D, Mickalad AM (2011) Antioxidant and antiradical activities of *Manihot esculenta* Crantz (Euphorbiaceae) leaves and other selected tropical green vegetables investigated on lipoperoxidation and phorbol-12-myristate-13-acetate (PMA) activated monocytes. Nutrients. 3: 818-838.

Tsumbu CN, Dupont GD, Tits M, Angenot L, Frederich M, Kohnen S, Mickalad AM, Serteyn D, Franck T (2012) Polyphenol content and modulatory activities of some tropical dietary plant extracts on the oxidant activities of neutrophils and myeloperoxidase. Int J Mol Sci. 13: 628-650.

Wang D, Xiang DB, He YJ, Li ZP, Wu XH, Mou JH, Xiao HL, Zhang QH (2005) Effect of caffeic acid phenethyl ester on proliferation and apoptosis of colorectal cancer cells *in vitro*. World J Gastroenterology. 11: 4008-4012.

Wang X, Catt S, Pangestu M, Smith PT (2011) Successful in vitro culture of pre-antral follicles derived from vitrified murine ovarian tissue: oocyte maturation, fertilization, and live births. Reproduction. 141: 183-191.

Yagushi JT, Costa DS, Neto JDBF (2014) Saturated salt accelerated aging and computerized analysis of seedling images to evaluate soybean seed performance. J Seed Sci. 36: 213-221.