CROP SCIENCES

Post-seminal development and cryopreservation of endemic or endangered bromeliads

SIMONE S.S. SILVA, EVERTON H. SOUZA, FERNANDA V.D. SOUZA, DANIELA A.S. MAX, MONICA L. ROSSI & MARIA A.P.C. COSTA

Abstract: *Vriesea bahiana*, *Hohenbergia castellanosii* and *Encholirium spectabile* are endemic Brazilian species that are considered endemic or endangered. Development of strategies to conserve these species is important to prevent irreversible genetic erosion. The objective of this study was to evaluate the post-seminal development and seed cryopreservation of three endemic or in danger of extinction bromeliad species in Brazil, to obtain a protocol that can safeguard the genetic variability of these species. In the seed cryopreservation assay, we evaluated five desiccation periods. The seeds in the cryotubes were taken from the desiccator and immediately plunged into liquid nitrogen. For the analysis of post-seminal development, seeds in different germination stages were collected and evaluated by light and scanning electron microscopy. *Vriesea bahiana* seeds frozen in liquid nitrogen presented almost 100% germination, indicating dormancy break of this species. *Vriesea bahiana* can be cryopreserved with 5.9% water content after being dried for 24 hours. *Hohenbergia castellanosii* and *E. spectabile* seeds did not need to be desiccated before being cryopreserved. The most relevant morphological traits for differentiation of genera and subfamilies of Bromeliaceae are the shape and type of seed appendages. In this study, all three species presented well-differentiated size and shape of their structures.

Key words: *Encholirium spectabile*, *Hohenbergia castellanosii*, *Vriesea bahiana*, Bromeliaceae, Seed desiccation, Germplasm conservation.

INTRODUCTION

The family Bromeliaceae Juss. contains a large number of species, and is considered to be one of the most diversified families in the world, with 78 genera and 3,659 species (Gouda et al. 2021), of which 1,178 are endemic to Brazil (Forzza et al. 2020). Despite being present in all the country's ecosystems, the greatest diversity of species is found in the Atlantic Forest biome (Forzza et al. 2012, 2013). Some of these species grow in very restricted environmental conditions and occur only as small and isolated populations (Benzing 2000). Besides this, many bromeliads are considered to be endangered due to severe fragmentation caused by destruction of habitat by farming and logging, as well as heavy exploitation of these plants for ornamental purposes (Jorgensen 2004, Pereira et al. 2008).

Despite the ecological importance of the family and its wide diversity, little is known about the propagation structures and germination behavior of seeds in response to environmental factors. Studies of the morphological structures of seeds are very important to differentiate taxonomic groups, as well as to support adequate storage and better understand the germination processes (Moura et al. 2010, Pêgo et al. 2011). Observation of post-seminal development is important to differentiate plant species and to

understand the dynamics of the establishment of natural populations, since the emergence from seeds is a critical stage in their life cycle (Fontenelle et al. 2007, Silva & Môro 2008).

Furthermore, seeds can be ideal structures to store the gene pool of a determined species, to guarantee the conservation of genetic variability (Gosling 2003). Therefore, development of methods for secure storage of seeds contributes to the preservation of genetic diversity, which is crucial in the case of species that are vulnerable or endangered.

Despite the existence of various *ex situ* conservation methods, storage in seed banks is most efficient for a wide range of plant species, mainly due to its easy application and the large number of species that can be preserved in a small space (Gosling 2003). Hence, this is the most common conservation method in the world, such that seed banks represent nearly 90% of all genetic collections (FAO 1996). Although many other structures, types of tissues and even entire plants are preserved in genetic banks, seed banks are the main repositories of genes. The creation of the Svalbard Global Seed Vault (SGSV) in 2008 is the leading example of this method.

Cryopreservation can be used to preserve various types of tissues, including meristematic tips, pollen grains and seeds, among others (Benson 2008, Rodrigues et al. 2014, Kaya et al. 2016). It basically consists of maintenance of plant tissues frozen in liquid nitrogen at ultra-low temperature (-196 °C). Since the metabolism is virtually stopped at this temperature, the process of deterioration is nearly zero, as are the risks of loss through ailments or genetic variations (Walters et al. 2004, Kaya & Souza 2017). In recent decades, significant advances have been made in cryopreservation, providing alternatives for long-term preservation of the valuable genetic resources of many plant

species (Voronkova & Kholina 2010, Kaya et al. 2013, Rodrigues et al. 2014, Ferrari et al. 2016, Pullman et al. 2019, Streczynski et al. 2019).

Nevertheless, additional studies are necessary to determine the best cryogenic storage conditions for seeds, such as cooling and thawing rates, physical and chemical properties, and most importantly, the quantity of water in seeds (González-Benito et al. 2003). In all types of preservation, the moisture of seeds is an important factor: in conventional conservation, too much water can cause deterioration and attract insect attacks, while in cryopreservation, water forms damaging ice crystals upon freezing, which at the extreme can cause cell death (Engelmann 1997). On the other hand, larger reductions in seed water content can trigger automatic lipid oxidation processes and, consequently, generate free radicals that, being very reactive, may inactivate enzymes and alter the integrity of cell membranes as well as causing unrivaled damage and reduction in seed viability (Harrington 1972). Therefore, it is necessary to remove the water through desiccation or to use cryoprotective substances to reduce these cell damages. This water reduction may vary between species and even within the same species.

The need for efficient conservation strategies is relevant for endemic bromeliads species that are vulnerable to anthropic actions. Cryopreservation of seeds obtained from natural populations of these species (*Vriesea bahiana*, *Hohenbergia castellanosii* and *Encholirium spectabile*) can be a valuable alternative, even if only for security duplicates.

Based on this assumption, the objective of this study was to evaluate the post-seminal development and cryopreservation of three endemic bromeliad species or in danger of extinction (*V. bahiana*, *H. castellanosii* and *E. spectabile*) in Brazil, from three sub-families, to

obtain a protocol that can safeguard the genetic variability of these species.

MATERIALS AND METHODS

Genetic material

Seeds of the following species were collected in different locations (Table I). To obtain seed samples representative of the populations, 40 ripe fruits (based on coloration) from different plants of each species were collected at random from the three populations.

Seed germination tests

The seeds were removed from the fruits manually and the appendage (*V. bahiana*) and mucilage (*H. castellanosii* and *E. spectabile*) surrounding the seeds were removed with tweezers and paper towels to prevent proliferation of microorganisms. A digital caliper was used to measure the length, width and thickness of 150 seeds of each species, chosen randomly, according to the method described by Pereira et al. (2008). The weight of 1,000 seeds was determined with a precision analytical balance, according to the Brazilian Seed Analysis Rules (Brasil 2009).

For the germination test, the seeds were first disinfected by immersion in 70% alcohol for one minute and then were rinsed three times in autoclaved distilled water. The seeds were placed to germinate in plastic boxes (Gerbox®) at temperature of 27 ± 2 °C, light intensity of 36 μmol m^{-2} s^{-1} and 16-hour photoperiod. The seeds were evaluated daily for 40 days.

Three germination substrates were used: Germitest® paper; the commercial substrate Vivato®; and washed sand. All were autoclaved beforehand at a temperature of 120 °C for 20 minutes. In the case of Germitest® paper, the seeds were distributed evenly and the paper was moistened with autoclaved water (2.5 times the weight of the paper). For the commercial substrate Vivato® and washed sand, 500 grams of substrate was weighed and moistened with 200 mL of autoclaved water.

The germination criterion was the emergence of the first root (1 mm minimum length), as described by Pereira et al. (2008). The germination percentage, germination speed index (GSI) and mean germination time (MGT) were calculated by the following formulas: G(%) = (N/A) × 100, where N = number of germinated seeds and A = total number of seeds; GSI = Σ(Gi/ni), where Gi = number of germinated seeds and

Table I. Information about collection sites and risk assessment of the species studied in this work.

Species	Collection site	Coordinate	Collection date	Risk assessment
Vriesea bahiana Leme	Santa Terezinha - BA	12° 51' 08.19'' S 39° 28' 34.32'' W	March 2017	Endemic (Bahia)*
Hohenbergia castellanosii L.B.Sm. & Read	Nilo Peçanha - BA	13° 42' 21.78'' S 39° 0' 46.26'' W	April 2017	Endemic (Bahia)* Endangered**
Encholirion spectabile Mart. ex Schult. & Schult. f	Milagres - BA	12° 52' 18.19'' S 39° 51' 16.78'' W	April 2017	Endemic (Northeast)*

* Forzza et al. (2020) ** Forzza et al. (2013).

ni = number days until germination; and MGT = (ΣGi/ni)/ ΣGi).

The experimental design was completely randomized in a 3 x 3 factorial scheme (species x substrates), with four repetitions, where each repetition consisted of 25 seeds, for a total of 100 seeds per treatment. The biometric data, germination percentage and GSI were submitted to analysis of variance and the means were compared by the Tukey test (p <0.01), with the germination data first being transformed into arc sin ($\sqrt{x/100}$) for normalization and homogenization of the variances. All the analyses were performed with the SAS program (SAS Institute 2010).

Seed water content

The water content of the seeds was evaluated using four samples of 25 seeds of each species, applying the fresh weight method by heating to 105 °C for 24 h, according to the Brazilian Seed Analysis Rules (Brasil 2009).

Post-seminal development

For analysis of post-seminal development, 100 seeds of each species were distributed evenly on two sheets of Germitest® paper, according to the method described above.

The post-seminal development was observed daily and seeds in the different phases were collected with aid of a stereoscopic microscope and fixed for observation with a scanning electron microscope and light microscope. The criteria applied for classification of plantlets as normal followed the recommendations of Pereira et al. (2008) and Silva & Scatena (2011): root development with total expansion of the first leaf and appearance of the second leaf. For young plants, the total expansion of the second leaf and appearance of the third leaf were considered.

For analysis in the different post-seminal development phases, the plantlets were fixed in modified Karnovsky's solution (Karnovsky 1965) [glutaraldehyde (2%), paraformaldehyde (2%), CaCl2 (0.001 M), sodium cacodylate buffer (0.05 M), at pH 7.2], for 48 hours and then were processed for scanning electron microscopy (SEM) or light microscopy (LM).

For the morphological analyses by SEM, the samples were dehydrated in a graded ethylic series (35-100%) and then dried to critical point with liquid CO_2 (Leica EM CPD 300, Balzers, Germany). The specimens were mounted on metal supports and sputtered with gold for 180 seconds (Leica EM ACE 600). The images were captured with a JEOL JSM-IT300 LV scanning electron microscope (Tokyo, Japan) at 20 kV coupled to a digital camera.

For anatomical characterization by LM, the plantlet samples were dehydrated in a graded ethylic series (35-100%), infiltrated and embedded using the Historesin kit (hydroxyethyl methacrylate, Leica, Heidelberg). The resin was polymerized at room temperature for 48 hours, and serial histological sections (4-5 μm) were obtained with a Leitz, model 1516 rotary microtome. The sections were mounted on histological slides and stained with acid fuchsin (1%), followed by toluidine blue (0.05%) (Feder & O'Brien 1968). The slides were analyzed and photographed with a B x S1 fluorescence microscope system (Olympus Latin America, Inc.).

Cryopreservation of seeds

The cryopreservation and seed viability tests were conducted with five time intervals (2, 4, 6, 12 and 24 hours). The seeds were distributed in three lots in 2 mL cryotubes and placed in a desiccator containing active silica gel. One lot was investigated to determine the water content after the different time periods, by weighing on

an analytical balance. The second lot was used as a control (LN-) and the third to determine the effects of cryopreservation (LN+). The control group consisted of seeds retrieved from the desiccator and allocated directly in a Gerbox® box on Germitest® paper under the same incubation conditions as in the germination tests.

For cryopreservation, the seeds in the cryotubes were taken from the desiccator and immediately plunged into liquid nitrogen (-196 ℃), where they remained for 24 hours. Then they were thawed at room temperature for 30 minutes and placed to germinate in the same conditions described above.

The seeds were monitored daily and the emergence of the first root (1 mm) was also the germination criterion. The germination percentages were determined as described before.

The experimental design was completely randomized in a 3 x 5 factorial scheme (species x drying times). Four repetitions were used for each treatment, with each repetition consisting of 25 seeds, for a total of 100 seeds per treatment. The data were submitted to analysis of variance and the means were compared by the Tukey test (p < 0.01), with the germination data previously transformed into arcsine ($\sqrt{x/100}$). The analyses were performed with the SAS program (SAS Institute 2010).

RESULTS AND DISCUSSION

The analysis of variance showed that the bromeliad species behaved differently from each other, but without interacting significantly with the substrates regarding germination percentage. For the germination speed index and mean germination time there was significant interaction between the two factors.

The highest germination percentages were achieved by *H. castellanosii*, with variation from 74% in Vivato® substrate to 72% in washed sand (Table II). For *V. bahiana*, the germination percentages varied from 80% in Vivato® substrate to 46% in the Germitest® paper, while for *E. spectabile*, the variation was from 69% in the Germitest® paper to 45% in washed sand (Table II). In general, there was variation between substrates within each species, which can be related to the great plasticity of bromeliads. For greater practicality of the tests and experimental conditions, we decided to use Germitest® paper for the other cryopreservation experiments.

The highest germination speed index (GSI) values were observed on Germitest® paper for the species *E. spectabile* and *H. castellanosii*, with 15.63 and 12.54, respectively, thus resulting in the shortest mean germination times, since these two variables are inversely related: the higher the GSI is, the lower the MGT will be. Among the three substrates, Germitest® paper enabled higher GSI values than the others for all three bromeliad species, followed by Vivato®, while washed sand led to the lowest GSI values, irrespective of species studied (Table II).

With respect to the mean germination time, the species *V. bahiana* planted in Vivato® substrate took the longest time, more than 30 days, to complete germination, while the shortest germination times were recorded with use of Germitest® paper for all three species. Of the three substrates, washed sand produced the longest germination times, except for *V. bahiana*, as mentioned above (Table II).

The start and the peak of germination of all three species varied according to substrate (Figure 1). For *V. bahiana*, the first seeds germinated on washed sand, starting on day 3, and peak germination (maximum germination achieved) occurred on day 8, followed by Germitest® paper, for which germination began

Table II. Germination percentage, germination speed index and mean germination time of three bromeliad species in different substrates.

	Germitest® paper	Vivato® Substrate	Washed sand
	Germination (%)		
V. bahiana	46 b	80 a	69 a
H. castellanosii	73 a	74 a	72 a
E. spectabile	69 a	49 b	45 b
CV (%)	7.45		
	Germination Speed Index (GSI)		
V. bahiana	4.86 cA	1.86 cB	1.69 cB
H. castellanosii	12.54 bA	6.25 aB	3.22 aC
E. spectabile	15.63 aA	5.53 bB	2.97 bC
CV (%)	1.28		
	Mean Germination Time (MGT) days		
V. bahiana	9.74 cA	31.67 bC	23.20 aB
H. castellanosii	6.38 bA	12.93 aB	25.63 bC
E. spectabile	4.96 aA	12.92 aB	22.51 aC
CV (%)	0.26		

Means followed by different lowercase letters in the column and uppercase letters in the row for each variable analyzed differ from each other at 1% probability by the Tukey test.

on day 6 and peaked on day 11. For this species, the slowest treatment was with Vivato®, in which germination started on day 10 and peaked on day 14.

In the case of *H. castellanosii,* the start of germination occurred on day 2 and peaked on day 5 on washed sand, while in the Germitest® paper, germination started on day 3 and peaked on day 8. Finally, in commercial substrate Vivato®, germination starting on day 9 and peaking on day 15 (Figure 1b).

In the case of *E. spectabili*, the start of germination occurred on day 2 and peaked on day 5 in the Germitest® paper, while in the washed sand, germination started on day 6 and peaked on day 11. Finally, commercial substrate Vivato® again produced the slowest germination, beginning on day 26 and peaking on day 34 after inoculation (Figure 1b).

The lower germination percentages of *V. bahiana*, irrespective of the substrate, corroborate the pattern reported for other species of the genus *Vriesea* in natural conditions (Droste et al. 2005). However, in controlled conditions of temperature and water content, these values can be elevated (Mercier & Guerreiro Filho 1990).

Germination is one of the least studied aspects of plants of the family Bromeliaceae (Barbosa 2005, Winkler et al. 2005), and confirmation of what factors are most influential for *ex situ* germination remains an open question for the majority of species.

However, the germination test we conducted allows some relevant inferences regarding the great adaptability of bromeliads in general. The results obtained demonstrate the germinative capacity of these species in terrestrial
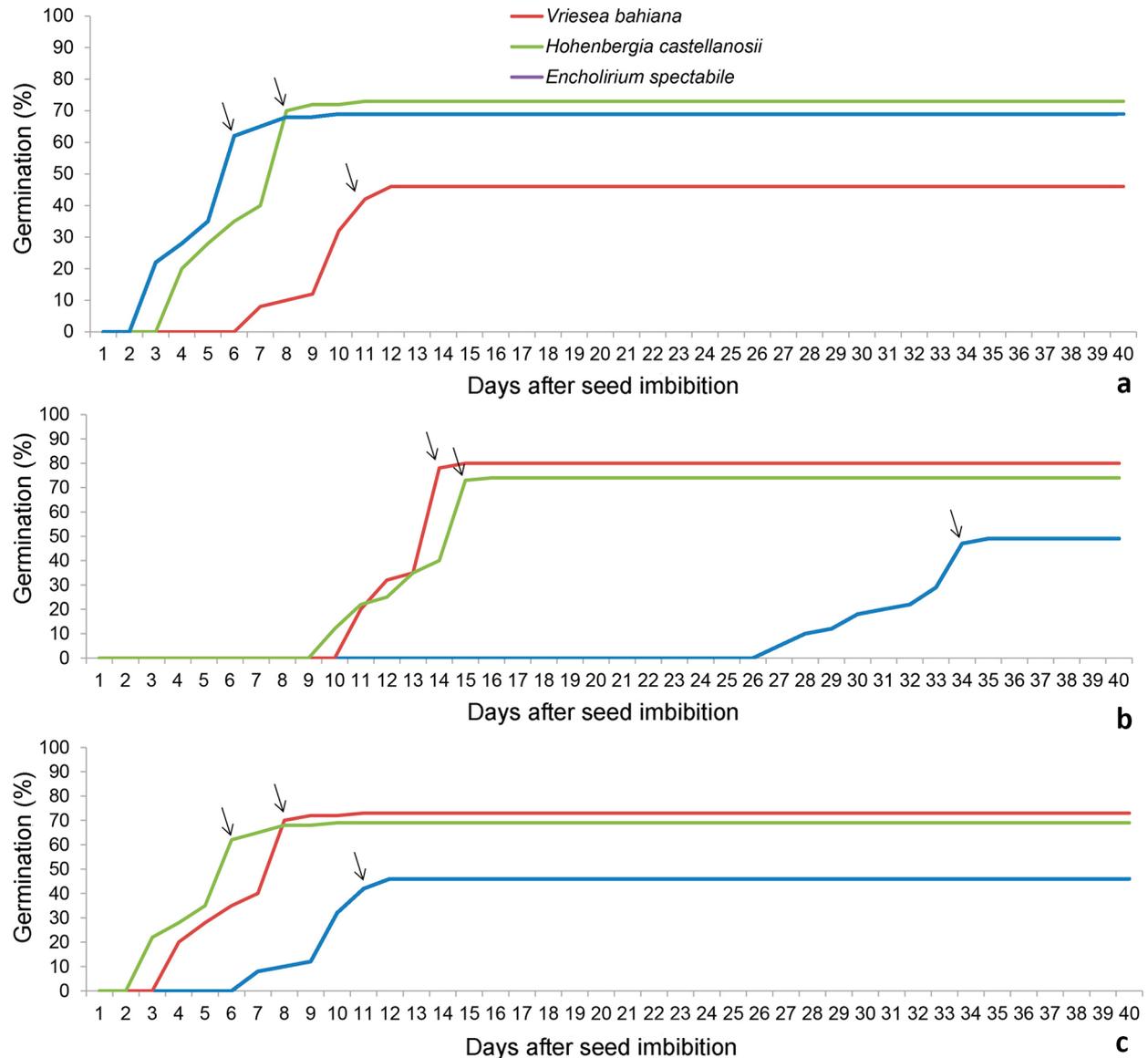
Figure 1. Cumulative germination percentage after moistening seeds of the three bromeliad species. a) Germitest® paper; b) Vivato® substrate; and c) washed sand. Arrows indicate the peak germination.

conditions, as observed in the commercial substrate. In contrast, in washed sand, which mimics conditions in the wild, although the germination was slower, it was very efficient, with percentages close to those of the species in other substrates.

Morphoanatomy of post-seminal development

The thousand-seed weights were 1.53 g for *V. bahiana*, 2.82 g for *H. castellanosii* and 2.63 g for

E. spectabile. This measure, which is generally used to calculate the sowing density and the weight of experimental samples, can also be considered an indicator of seed quality and state of maturity and health (Brasil 2009).

The *V. bahiana* seeds are small (7.2 mm long, 0.9 mm wide and 0.4 mm thick), lightweight, filiform, inserted in thin capsule-like fruits, with light brown color when immature and dark brown when mature. The seeds have long

whitish plumose appendages (Figure 2a) on one end that enable them to be easily carried by the wind. The plumose appendages also enable the seeds to attach to various surfaces, such as tree trunks and bark, assuring successful dispersion (Benzing 2000).

In the longitudinal section of the seed, at the very start of germination it was possible to observe the presence of a haustorial cotyledon inside the seed coat, in contact with the endosperm (Figure 2b, c). The hypocotyl was also observed, although it was very small, between the cotyledon sheath and primary root
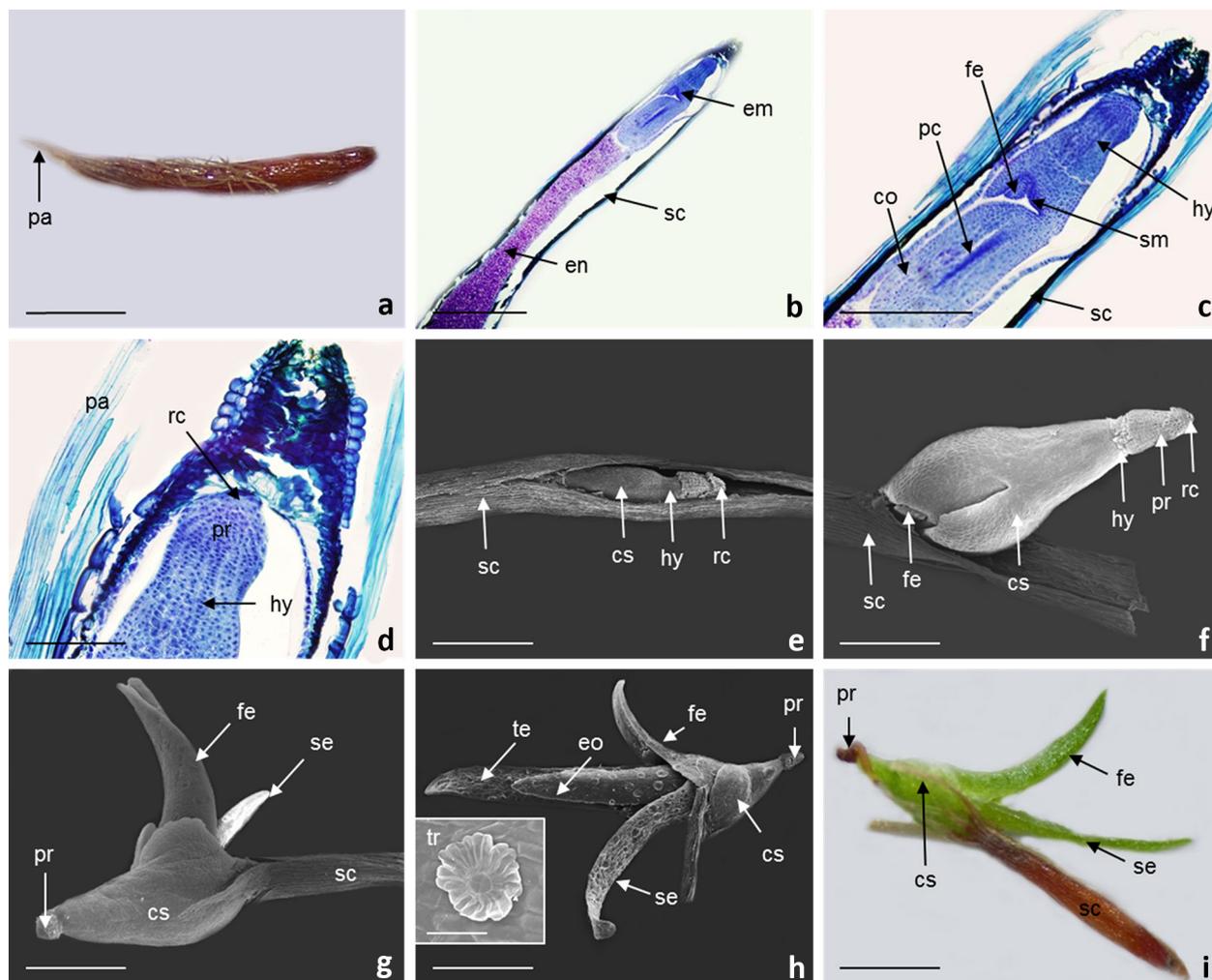

Figure 2. Seed morphology and post-seminal development of *Vriesea bahiana*. a) Mature seed, showing a plumose appendage. b- i) Phases of post-seminal development observed by light microscopy (b, c, d, i) and scanning electron microscopy (e, f, g, h). b- d) Start of germination, where it is possible to observe the presence of the haustorial cotyledon inside the seed coat, in contact with the endosperm, along with a well-defined procambium and small vestigial primary root and the presence of the root cap. e) Germination observed on day 7 from the end of the plumose appendage, with emergence of the still rudumentary primary root and exposure of the cotyledon sheath. f) Germination on day 12, showing the emergence of the first eophyll. g- i) Germination on day 15, showing the second eophyll and first scale-like trichomes on the surface of the leaf epidermis, more intense in the other eophylls. (co) cotyledon; (cs) cotyledon sheath; (em) embryo; (en) endosperm; (eo) eophyll; (fe) first eophyll; (hy) hypocotyl; (pa) plumose appendage; (pc) procambium; (pr) primary root; (rc) root cap; (sc) seed coat; (se) second eophyll; (sm) shoot tip meristem; (te) third eophyll; (tr) trichome. Bars: a- d = 0.2 cm; e- i = 500 μm.

(Figure 2c, d). It was not possible to distinguish the root meristem from the other anatomical structures. The shoot meristem was well defined and wrapped with the first eophyll (Figure 2c).

Germination started on day 7 from the end of the plumose appendage, with extrusion of the still rudimentary primary root and exposure of the cotyledon sheath (Figure 2e). On day 12, the first eophyll appeared (Figure 2f), followed by the second eophyll on day 15 (Figure 2g). At this stage, the first scale-like trichomes emerged on the surface of the leaf epidermis, intensifying on the other eophylls (Figure 2g- i). The second eophyll had similar anatomical characteristics to the first. Overlap of the first and second eophylls was also observed, forming a tank, a characteristic of this species (*V. bahiana*) (Figure 2g- i). At the end of 35 days, the young plantlet had a large number of trichomes (Figure 2h, i), structures involved in the absorption of the water and nutrients necessary for survival.

The characteristics observed in these seeds and plantlets were also noted by Mantovani & Iglesias (2005) for the species *Vriesea neoglutinosa* Mez, by Pereira et al. (2008) for *V. heterostachys* (Baker) L.B.Sm. and *Alcantarea imperialis* Harms, and by Silva & Scatena (2011) for *Tillandsia adpressiflora* Mez. The differences between these works and the results presented here are mainly related to the time intervals of the phases.

In *T. adpressiflora* (Tillandsioideae), the start of germination was marked by the emergence of the cotyledon, the same pattern of post-seminal development found for other species of the genus *Tillandsia* (Silva & Scatena 2011). For other species of the sub-family Tillandsioideae, such as *V. heterostachys* and *A. imperialis*, the first structures to emerge are the cotyledon sheath and rudimentary primary root (Pereira et al. 2008). This trait can be considered derived and has taxonomic importance for this sub-family.

The *H. castellanosii* seeds are small (1.8 mm long, 1.2 mm wide and 1.4 mm thick), but are heavier than the others. They are oval, inserted in elliptical berries, smooth and without any type of appendage, and are covered by a mucilaginous substance (Figure 3a). According to Benzing (2000) and Scatena et al. (2006), the mucilage in bromeliad seeds serves as protection against desiccation.

Hohenbergia castellanosii had epigeal germination, which started on day 4 after moistening, with emergence of the primary root and elongation of the hypocotyl region with rupture of the seed coat (Figure 3b, c). The morphological observations revealed a cylindrical haustorial cotyledon inside the seed coat in contact with the endosperm (Figure 3c). The root cap was enveloped by the cylindrical primary root and was well developed (Figure 3c). The cotyledon was not separated from the seed coat, and the haustorium remained inside (Figure 3b- i). According to Garwood (1996), this structure corresponds to the haustorial cotyledon and is responsible for absorbing and transferring reserves from the endosperm for growth of plantlets.

On day 8, the emergence was noted of a leafy cupuliform cotyledon sheath and first eophyll with the presence of glandular trichomes (Figure 3e- g), besides many absorbent hairs on the root collar and base of the hypocotyl as well as on the primary root (Figure 3d). Glandular trichomes and sparse stomata appeared on the first eophyll, which increased in quantity as the plantlet developed (Figure 3g). On day 18, the hypocotyl and first eophyll were elongated and the second eophyll had emerged, along with the start of formation of adventitious roots (Figure 3h, i). The overlapping cupuliform leaves formed rosettes (Figure 3h, i).

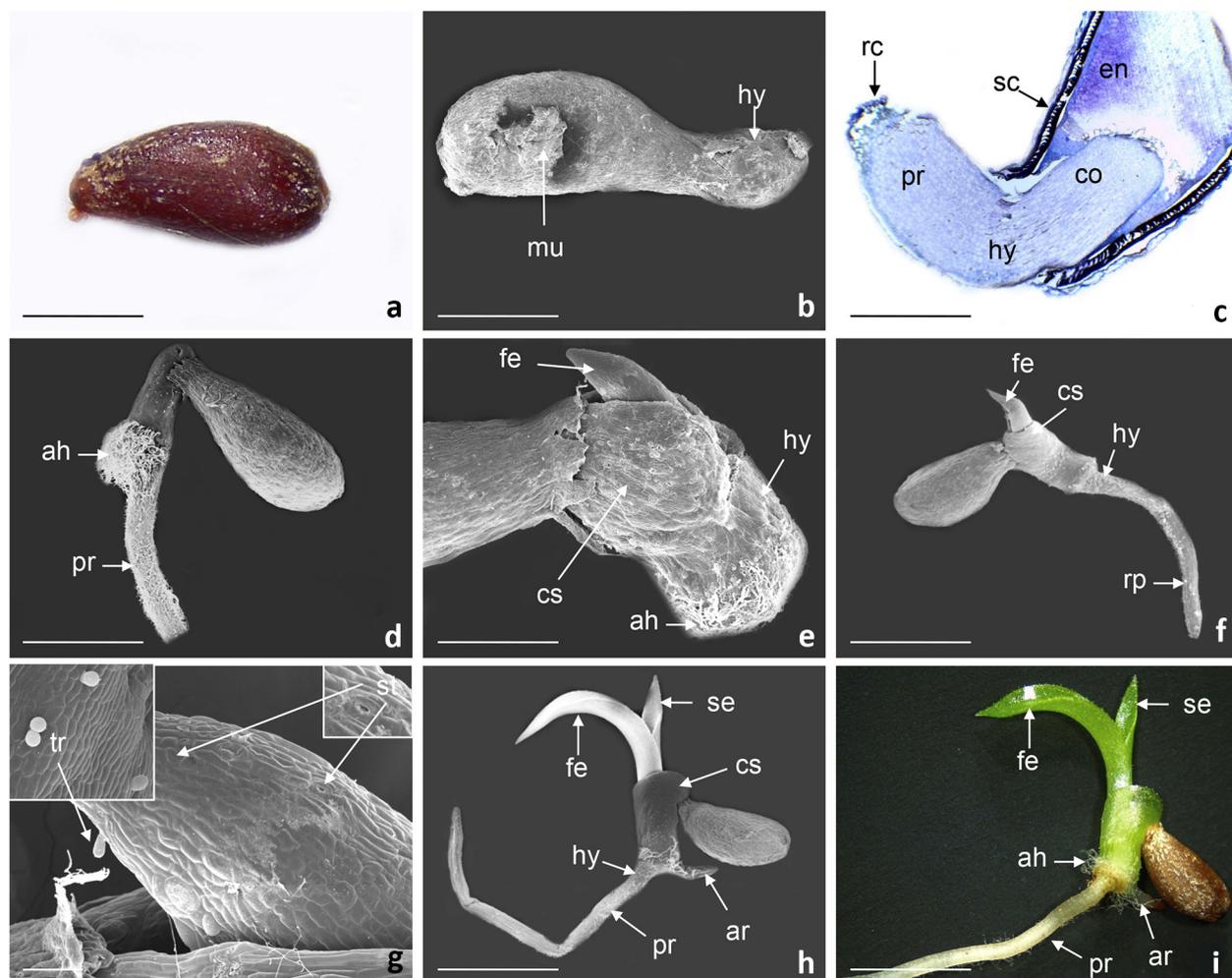The seeds of *E. spectabile* are small (8.2 mm long, 10.0 mm wide and 0.1 mm thick), in

Figure 3. Seed morphology and post-seminal development of *Hohenbergia castellanosii*. a) Mature seed and surrounding mucilaginous substance; b- i) Phases of post-seminal development observed by light microscopy (c, i) and scanning electron microscopy (b, d, e, f, g, h). b, c) Emergence of the primary root and elongation of the hypocotyl region by the rupture of the seed coat. c) Cylindrical haustorial cotyledon inside the seed coat in contact with the endosperm and root cap around the well-developed cylindrical primary root. d) Numerous absorbent hairs on the root collar and base of the hypocotyl, as well as on the primary root. e- g) On day 8, the emergence was noted of a leafy cupuliform cotyledon sheath and the first eophyll, with the presence of glandular trichomes. h, i) On day 18, the hypocotyl and first eophyll were elongated and the second eophyll had emerged, along with the start of formation of adventitious roots. Overlapping cupuliform leaves forming rosettes were also observed. (ah) absorbent hairs; (ar) adventitious root; (co) cotyledon; (cs) cotyledon sheath; (en) endosperm; (fe) first eophyll; (hy) hypocotyl; (mu) mucilage; (pr) primary root; (rc) root cap; (sc) seed coat; (se) second eophyll; (st) stomata; (tr) trichome. Bars: a, c, i = 2 mm; b- h = 500 µm.

the form of flattended oval disks, inserted in septicidal capsule fruits, and have light brown color, becoming darker in the region around the embryo. They have a winged membranous appendage, enabling easy transport by wind or water (Figure 4a).

The germination started on day 3 after moistening, with rupture of the seed coat and emergence of the primary root covered by the root cap (Figure 4b, c). There was positive geotropism of the primary root and the cotyledon, along with elongation of the s hypocotyl.
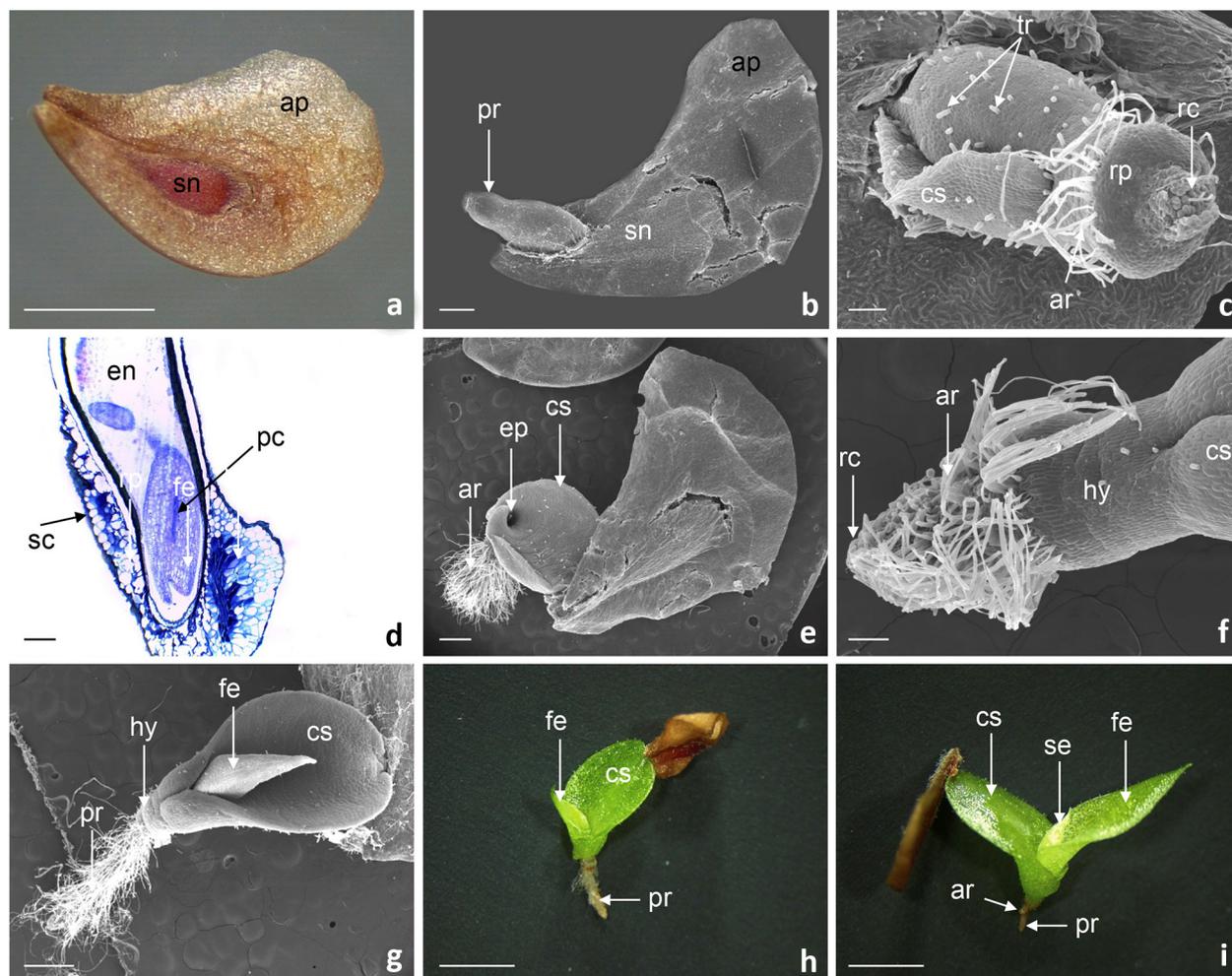
Figure 4. Seed morphology and post-seminal development of *Encholirion spectabile*. a) Mature seed, showing the seminiferous nucleus and winged appendage. b- i) Phases of the post-seminal development observed by light microscopy (d, h, i) and scanning electron microscopy (b, c, e, f, g). b, c) Germination on day 3 after moistening, with rupture of the seed coat and emergence of the primary root covered by the root cap. c) Day 5, with emergence of the leafy cupuliform cotyledon sheath and presence of numerous tector and glandular trichomes on the cotyledon sheath, along with absorbent hairs in the well-delineated root collar region. d) Day 8, development of the first eophyll with lanceolate shape. e- g) Day 12, with the first eophyll already showing the shape of a small tank. h, i) Day 15, showing emission of the second eophyll and start of formation of adventitious roots. In this phase, the primary root is short with many absorbent hairs. (ap) appendage; (ar) adventitious roots; (cs) cotyledon sheath; (en) endosperm; (fe) first eophyll; (hy) hypocotyl; (pr) primary root; (pc) procambium; (rc) root cap; (se) second eophyll; (sc) seed coat; (sn) seminiferous nucleus; (tr) trichome. Bars: a- d = 0.2 cm; e- h = 500 µm.

On day 5, the emergence was noted of the leafy cupuliform cotyledon sheath. Large numbers of tector and glandular trichomes were observed on the cotyledon sheath, along with absorbent hairs in the region of the well-delineated root collar (Figure 4c). The haustorial cotyledon was observed inside the seed coat in direct contact with the endosperm (Figure 4d).

On day 8, the development began of the first eophyll with lanceolate shape (Figure 4e, g). On day 12, the first eophyll already had the shape of a small tank (Figure 4g, h). On day 15, the plantlet emitted the second eophyll and started the formation of adventitious roots (Figure 4i). At this stage, the primary root was short with many absorbent hairs. Our observations are

similar to those of Silva & Scatena (2011) for the species *Dyckia duckey* and *D. racemosa*.

Cryopreservation of the seeds

In the three species evaluated, the water content of the seeds declined with increasing desiccation time on silica gel, as expected in this type of test (Table III). The differences between the original water contents of each species corresponded to what was described previously about the size. *Hohenbergia castellanosii* seeds are much smaller (1.8 mm x 1.2 mm) than *V. bahiana* and *E. spectabille* (7.2 mm x 0.9 mm and 8.2 mm x 10.0 mm, respectively).

With respect to germination in the absence freezing in liquid nitrogen (LN-), the non-dried (control) seeds presented higher germination percentages than those dried on silica gel. In

Table III. Water content, germination percentage after desiccation and freezing in liquid nitrogen of seeds of *Vriesea bahiana*, *Encholirium spectabile* and *Hohenbergia castellanosii*.

Drying Time	Vriesea bahiana	Hohenbergia castellanosii	Encholirium spectabile
Water contente of seeds (%)			
Control	7.5	2.2	7.2
2h	6.8	2.2	7.2
4h	6.8	2.2	7.2
6h	6.7	2.1	6.9
12h	6.3	1.7	6.8
24h	5.9	1.5	6.1
CV (%)	22.12 ^{ns}		
Germination (%) (LN-)			
Control	52 aC	83 aA	74 aB
2h	45 bcC	84 aA	72 aB
4h	30 dB	56 bA	60 cA
6h	33 dC	50 bB	69 bA
12h	42 cB	37 cB	67 bA
24h	43 cB	26 dC	68 bA
CV (%)	4.89 **		
Germination (%) (LN+)			
Control	61 cdA	65 aA	67 aA
2h	65 cA	61 aA	64 abA
4h	52 eAB	48 bB	57 bcA
6h	54 deA	41 bB	52 cA
12h	85 bA	45 bB	37 dC
24h	95 aA	40 bB	27 eC
CV (%)	4.14 **		

Mean values followed by equal lowercase letters in the column and uppercase letters in the row do not differ by the Tukey test. ** $p \leq 0.01$; ^{ns} = not significant.

line with previous results, *V. bahiana* had the lowest germination percentage (52%) compared to *H. castellanosii* (83%) and *E. spectabille* (74%).

However, as the drying time increased, the species presented different reactions. The germination rate of the *H. castellanosii* seeds fell drastically with increased drying time, from 84% for 2 h to 26% for 24 h of exposure to silica gel, which can be related to their very small size and also by the low seed water content. In contrast, for *E. spectabille* the decline of germination percentage was less, from 72% after 2 h to 68% after 24 h, indicating this species is more tolerant to desiccation than the other studies.

Finally, the *V. bahiana* seeds' germination rate decreased up to drying time of 6 h (33%) and then increased until 24 h, to 43%. This behavior of *V. bahiana* accentuated more after cryopreservation (LN+), with the highest germination rates among the three species, which were 85% after 12 h and 95% after 24 h of drying. Similar results were also related by Tarré et al. (2007), who studied bromeliads of the genus *Encholiriun* and found that the germinability of *E. magalhaestii* and *E. subsecundum* seeds increased after freezing in liquid nitrogen. These authors stated that seeds from those species display an orthodox behavior.

The majority of species have seeds that tolerate desiccation and water levels in the range from 2% to 5%, or even lower, and are called "orthodox". In turn, when the seeds tolerate desiccation and water content around 10% to 13%, they are called "intermediate" and have low viability at lower water content. Finally, seeds that do not tolerate water content in the range of 15% to 20% are classified as "recalcitrant" and present a significant decline in viability when stored at low temperatures, where desiccation is essential (Fonseca & Freire 2003). Although by definition the drying of recalcitrant seeds

reduces there viability, considerable variation in the sensitivity to desiccation has been reported in the literature. From our results, it can be inferred that *H. castellanosii* and *E. spectabile* and *V. bahiana* seeds presented orthodox behavior. Therefore, these seeds do not need any desiccation for cryopreservation. On the other hand, *V. bahiana* seeds can be cryopreserved with 5.9% water content after being dried for 24 hours, with maintenance of 95% germinability.

Low water content of seeds is fundamental for successful cryopreservation (Benson 2008, Engelmann 2011, Rodrigues et al. 2014, Kaya et al. 2016) because this avoids the formation if ice crystals with consequent damages to the seeds. Marcos Filho (2015) reported that the water content of seeds between 10% and 12% allows maintenance of germination after a storage period of six to eight months for the majority of species, and that below these levels, the seeds may or may not lose viability. Nevertheless, what we observed was highly variable behavior, depending on the species, which might be related to the seed size, as described previously. The largest seeds were those of *E. Spectabile* (8.2 mm long, 10.0 mm wide and 0.1 mm thick), which can explain the larger quantities of water and thus the greater difficulty of removing it in the desiccation times investigated here.

These observations are consistent with the hypothesis that orthodox seeds are subject to immediate adverse effects on their viability when they have low water content and are exposed to liquid nitrogen (Vertucci 1989, Pritchard 2004), which we observed for *H. castellanosii*. Despite the presence of some reports that seed viability can be maintained indefinitely by cryopreservation, Walters et al. (2004) refuted the idea that all metabolism ceases in orthodox seeds, and found immediate adverse effects on viability when seeds are excessively dried. Prudente et al. (2015), analyzing seeds of *Zinnia*

elegans Sessé & Moc., also frozen in liquid nitrogen for 24 hours after desiccation times of 1,2 or 3 hours, with water content of 9%, did not obtain germination percentages higher than 60%. For this reason, it is important to define the desiccation limit for each species, because excessive desiccation can reduce the germination potential as a consequence of damage to the embryo's metabolism (Pammenter et al. 1998, Walters et al. 2004, Berjak et al. 2012).

Results similar to ours with low water content were reported by Ferrari et al. (2016), working with *E. spectabile* and water content of 8.4%. These authors observed that the germination rates after immersion in liquid nitrogen also were not higher than 70%. Rodrigues et al. (2014), studying Bromeliaceae species from different sub-families, found that in general 7% water content is ideal for cryopreservation of the seeds, achieving high regeneration percentages. The authors suggested that the seeds had orthodox behavior for storage, because they were able to survive desiccation and freezing. Since the initial water content of these seeds was low, their germination was impaired by longer drying times.

CONCLUSIONS

Morphoanatomic differences in post-seminal development were observed in the three species. The hypocotyls of the plantlets varied from short to long or little developed.

Vriesea bahiana seeds can be cryopreserved with 5.9% water content after being dried for 24 hours, with maintenance of 95% germinability.

Hohenbergia castellanosii and *E. spectabile* seeds did not need to be desiccated before being cryopreserved.

REFERENCES

BARBOSA JG. 2005. Germinação de sementes e sobrevivência das plântulas de *Tillandsia geminiflora* Brongn, em diferentes substratos. Acta Sci Agron 27: 165-170.

BENSON EE. 2008. Cryopreservation theory. In: Reed BM (Ed), Plant cryopreservation: a practical guide. Springer, New York, USA, p. 15-32.

BENZING DH. 2000. Bromeliaceae: profile of an adaptive radiation. UK: Cambridge University Press, Cambridge, 690 p.

BERJAK P, PAMMENTER NW & WESLEY-SMITH J. 2012. The effects of various parameters during processing for cryopreservation on the ultrastructure and viability of recalcitrant zygotic embryos of *Amaryllis belladonna*. Protoplasma 249: 155-169.

BRASIL. 2009. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. Regras para análise de sementes. Brasília, DF: MAPA: SDA, 395 p.

DROSTE A, MACHADO AS, MATOS AV & ALMEIDA JW. 2005. *In vitro* culture of *Vriesea gigantea* and *Vriesea philippocoburgii*: two vulnerable Bromeliads native to southern Brazil. Braz Arch Biol Techn 48: 717-722.

ENGELMANN F. 1997. Importance of desiccation for the cryopreservation of recalcitrante seed and vegetatively propagated species. Plant Gen Resour 112: 9-18.

ENGELMANN F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro* Cell Dev - Pl 47: 17-25.

FAO. 1996. Agricultural production: primary crops. Disponível em: <http://www.fao.org>. Acesso em: 17 de abril de 2018.

FEDER N & O'BRIEN TP. 1968. Plant microtechnique: some principles and new methods. Am J Bot 5: 123-142.

FERRARI EAP, COLOMBO RC, FARIA RT & TAKANE RJ. 2016. Cryopreservation of seeds of *Encholirium spectabile* Martius ex Schultes f. by the vitrification method. Rev Cienc Agron 47: 172-177.

FONSECA SCL & FREIRE HB. 2003. Sementes recalcitrantes: problemas na pós-colheita. Bragantia 62: 297-303.

FONTENELLE ACF, ARAGAO WM & RANGEL JHA. 2007. Biometria de frutos e sementes de *Desmanthus virgatus* (L) Willd Nativas de Sergipe. Rev Bras Bioc 5: 252-254.

FORZZA RC ET AL. 2012. New Brazilian floristic list highlights conservation challenges. BioScience 62: 39-45.

FORZZA RC ET AL. 2013. Bromeliaceae. In: Martinelli G & Moraes MA. Livro vermelho da flora do Brasil. Rio de Janeiro: Andrea Jakobsson & Instituto de Pesquisas do Jardim Botânico do Rio de Janeiro, p. 315-397.

FORZZA RC ET AL. 2020. Bromeliaceae in Flora do Brasil 2020. Jardim Botânico do Rio de Janeiro. Disponível em: <http://reflora.jbrj.gov.br/reflora/floradobrasil/FB66>. Acesso em: 09 March 2021.

GARWOOD NC. 1996. Functional morphology of tropical tree seedlings. In: Swaine MD (Ed), The ecology of tropical forest tree seedlings. Paris, Man and the Biosphere series, p. 59-129.

GONZÁLEZ-BENITO ME, SALINAS P & AMIGO P. 2003. Effect of seed moisture content and cooling rate in liquid nitrogen on legume seed germination and seedling vigour. Seed Sci Techol 31: 423-434.

GOSLING PG. 2003. Viability testing. In: Smith RD, Dickie JB, Linington SL, Pritchard HW & Probert RJ (Eds), Seed conservation turning science into practice. Kew: Royal Botanic Gardens, p. 445-481.

GOUDA EJ, BUTCHER D & GOUDA K. 2021. Encyclopaedia of Bromeliads Version 4. Disponível em: <http://bromeliad.nl/encyclopedia/>. Acesso em: 9 March 2021.

HARRINGTON JF. 1972. Seed storage and longevity. In: KOSLOWSKI TT. Seed biology. New York: Academic Press, p. 145-245.

JORGENSEN B. 2004. Sustainable trade in ornamental horticulture. Acta Hortic 630: 119-123.

KARNOVSKY MJ. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27: 137-138.

KAYA E, ALVES A, RODRIGUES L, JENDEREK M, HERNANDEZ-ELLIS M, OZUDOGRU A & ELLIS D. 2013. Cryopreservation of *Eucalyptus* Genetic Resources. Cryoletters 34: 608-618.

KAYA E & SOUZA FVD. 2017. Comparison of two PVS2-based procedures for cryopreservation of commercial sugarcane (*Saccharum* spp.) germplasm and confirmation of genetic stability after cryopreservation using ISSR markers. *In Vitro* Cell Dev - Pl 53: 410-417.

KAYA E, SOUZA V, GÖKDOĞAN EY, CEYLAN M & JENDEREK M. 2016. Cryopreservation of citrus seed via dehydration followed by immersion in liquid nitrogen. Turkish J Biol 41: 1-7.

MANTOVANI A & IGLESIAS RR. 2005. Quando aparece a primeira escama? Estudo comparativo sobre o surgimento de escamas de absorção em três espécies de bromélias terrestres de restinga. Rodriguésia 56: 73-84.

MARCOS FILHO J. 2015. Fisiologia de sementes de plantas cultivadas. 2ª ed., Londrina: Abrates, 659 p.

MERCIER H & GUERREIRO FILHO O. 1990. Propagação sexuada de algumas espécies de bromélias nativas da Mata Atlântica. Hoehnea 17: 9-26.

MOURA EF, VENTRELLA MC & MOTOIKE SY. 2010. Anatomy, histochemistry and ultrastructure of seed and somatic embryo of Acrocomia aculeata (Arecaceae). Sci Agric 67: 375-495.

PAMMENTER NW, GREGGAINS V, KIOKO JI, WESLEY-SMITH J, BERJAK P & FINCH-SAVAGE WE. 1998. Effects of differential drying rates on viability retention of *Ekebergia capensis*. Seed Sci Res 8: 463-471.

PÊGO RG, NUNES UR & MASSAD MD. 2011. Qualidade fisiológica de sementes e desempenho de plantas de rúcula no campo. Cienc Rural 41: 341-1346.

PEREIRA AR, PERERIRA TS & ANDRADE ACS. 2008. Morfologia de sementes e do desenvolvimento pós- seminal de espécies de Bromeliaceae. Acta Bot Bras 22: 1150-1162.

PRITCHARD HW. 2004. Classification of seed storage types for ex situ conservation in relation to temperature and moisture. In: Guerrant EO, Kayri H & Mike M (Eds). Ex situ plant conservation. London: Island Press, p. 139-161.

PRUDENTE DO, NERY FC, PAIVA R, SANTOS PAA, NERY MC & PAIVA PD. 2015. *In vitro* germination and cryopreservation of *Zinnia elegans* seeds. Ornamental Hortic 21: 243-250.

PULLMAN GS, BAI K, HANE M, RULAND D, CRUSE-SANDERS JM, BOYD RS & JOHNSON S. 2019. Seed cryopreservation and micropropagation of the federally threatened species, Price's potato-bean (*Apios priceana* B.L. Robins.). *In Vitro* Cell Dev - Pl Online 1-11.

RODRIGUES ARP, FORZZA RC & ANDRADE ACS. 2014. Physiological characteristics underpinning successful cryopreservation of endemic and endangered species

of Bromeliaceae from the Brazilian Atlantic Forest. Bot J Linn Soc 176: 567-578.

SAS INSTITUTE INC. 2010. SAS/ STAT user´s guide: statistics. Version 9.1.3. ed. Cary, NC.

SCATENA VL, SEGECIN S & COAN AI. 2006. Seed Morphology and Post- Seminal Development of *Tillandsia L.* (Bromeliaceae) from the "Campos Gerais", Paraná, Southern Brazil. Braz Arch Biol Tech 49: 945-951.

SILVA BMS & MÔRO FV. 2008. Aspectos morfológicos do fruto, da semente e desenvolvimento pós-seminal de faveira (*Clitoria fairchildiana* R. A. Howard. - Fabaceae). Rev Bras Sem 30: 195-201.

SILVA IV & SCATENA VL. 2011. Morfologia de sementes e estádios iniciais de plântulas de espécies de Bromeliaceae da Amazônia. Rodriguésia 62: 263-272.

STRECZYNSKI R, CLARK H, WHELEHAN LM, ANG ST, HARDSTAFF LK, FUNNEKOTTER B, BUNN E, OFFORD CA, SOMMERVILLE KD & MANCERA RL. 2019. Current issues in plant cryopreservation and importance for ex situ conservation of threatened Australian native species. Aust J Bot 67: 1-15.

TARRÉ E, PIRES BBM, GUIMARÃES APM, CARNEIRO LA, FORZZA RC & MANSUR E. 2007. Germinability after desiccation, storage and cryopreservation of seeds from endemic *Encholirium* Mart. ex Schult. & Schult. f. and *Dyckia* Schult. & Schult. f. species (Bromeliaceae). Acta Bot Bras 21: 777-783.

VERTUCCI CW. 1989. The effects of low water contents on physiological activities of seeds. Physiol Plantarum 77: 172-176.

VORONKOVA NM & KHOLINA AB. 2010. Conservation of endemic species from the Russian Far East using seed cryopreservation. The Biol Bull 37: 581-586.

WALTERS C, WHEELER L & STANWOOD PC. 2004. Longevity of cryogenically stored seeds. Cryobiology 48: 229-244.

WINKLER M, HÜLBER K & HIETZ P. 2005. Effect of canopy position on germination and seedling survival of epiphytic bromeliads in a Mexican Humid Montane Forest. Ann Bot 95: 1039-1047.

How to cite

SIMONE S.S. SILVA[1]
https://orcid.org/0000-0002-6671-1804

EVERTON H. SOUZA[1]
https://orcid.org/0000-0002-8593-5010

FERNANDA V.D. SOUZA[2]
https://orcid.org/0000-0002-2591-0911

DANIELA A.S. MAX[1]
https://orcid.org/0000-0002-8947-7651

MONICA L. ROSSI[3]
https://orcid.org/0000-0003-2057-9470

MARIA A.P.C. COSTA[1]
https://orcid.org/0000-0003-1666-711X

[1] Universidade Federal do Recôncavo da Bahia, UFRB, Centro, 44380-000 Cruz das Almas, BA, Brazil

[2] Embrapa Mandioca e Fruticultura, s/n, Caixa Postal 007, Centro, 44380-000 Cruz das Almas, BA, Brazil

[3] Centro de Energia Nuclear na Agricultura, CENA, Av. Centenário 303, São Dimas, 13400-970 Piracicaba, SP, Brazil

Correspondence to: **Everton Hilo de Souza**
E-mail: hilosouza@gmail.com

Authors contribution

SSSS, DASM and MLR: data curation, formal analysis, investigation, methodology, visualization, writing—original draft. FVDS, EHS, MAPCC: conceptualization, formal analysis, funding acquisition, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing.