

Original Article

## *In vitro* germination and reserve mobilization of *Vriesea friburgensis* Mez

### Germinação *in vitro* e mobilização de reservas de *Vriesea friburgensis* Mez

J. P. Corredor-Prado<sup>a</sup> , O. F. Santos-Amaya<sup>b</sup>  and R. Pescador<sup>c\*</sup> 

<sup>a</sup>Universidad de Sucre, Departamento de Biología y Química, Sincelejo, Sucre, Colombia

<sup>b</sup>Universidad de Pamplona, Departamento de Agronomía, Pamplona, Norte de Santander, Colombia

<sup>c</sup>Universidade Federal de Santa Catarina – UFSC, Centro de Ciências Agrárias, Programa de Pós-graduação em Recursos Genéticos Vegetais, Florianópolis, SC, Brasil

#### Abstract

Studies on the germination and establishment of plants are key pieces to understanding the reproductive success of plants. This work aimed to describe *in vitro* germination and reserve mobilization in the bromeliad *Vriesea friburgensis* through morphological, histochemical, and biochemical analysis. The conditions used in this study for the *in vitro* germination are adequate. From the third day of *in vitro* inoculation, a uniform germination of 98% was obtained, exhibiting a high physiological quality of the seeds and a high potential to produce seedlings (94%). There is early reserve mobilization, which began in the imbibition phase. The accumulated reserves in the endosperm cytoplasm are degraded by hydrolytic enzymes provided by the aleurone layer. It is possible that compounds in the cell walls of the endosperm contribute to a lesser extent in mobilization. Additionally, it was observed that starch accumulation in the cotyledon increases when the seedling has formed. Results from this study provide insights for future studies on ecology, seed technology, and conservation in this species. This study contributes to the limited knowledge of the dynamics of reserves during germination and seedling establishment in Bromeliaceae. To the best of our knowledge, this is the first study with this approach in the genus *Vriesea*.

**Keywords:** cotyledon, embryo, endosperm, seed, seedling.

#### Resumo

Estudos sobre germinação e estabelecimento de plantas são peças-chave para entender o sucesso reprodutivo das plantas. Este trabalho teve como objetivo descrever a germinação *in vitro* e a mobilização de reservas na bromélia *Vriesea friburgensis* por meio de análises morfológicas, histoquímicas e bioquímicas. As condições utilizadas neste estudo para a germinação *in vitro* são adequadas. A partir do terceiro dia de inoculação *in vitro*, obteve-se germinação uniforme de 98%, apresentando alta qualidade fisiológica das sementes e alto potencial de produção de plântulas (94%). Há uma mobilização precoce de reservas, iniciada na fase de embebição. As reservas acumuladas no citoplasma do endosperma são degradadas por enzimas hidrolíticas fornecidas pela camada de aleurona. É possível que compostos nas paredes celulares do endosperma contribuam em menor grau na mobilização. Além disso, observou-se que o acúmulo de amido no cotilédone aumenta com a formação da plântula. Os resultados deste estudo fornecem informações para estudos futuros sobre ecologia, tecnologia de sementes e conservação desta espécie. Este estudo contribui para o conhecimento limitado da dinâmica das reservas durante a germinação e estabelecimento de plântulas em Bromeliaceae. Até onde sabemos, este é o primeiro estudo com esta abordagem no gênero *Vriesea*.

**Palavras-chave:** cotilédone, embrião, endosperma, plântula, semente.

## 1. Introduction

The family Bromeliaceae constitutes one of the most ecologically diverse, and species-rich clades of flowering plants native to the Neotropics (Givnish et al., 2011). However, this diversity has been negatively affected by habitat loss and fragmentation, climate change, invasive species, and commercialization for ornamental purposes (Ladino et al., 2019). Bromeliaceae contains 3742 species (Gouda and Butcher, 2023) distributed among eight subfamilies (Givnish et al., 2011). Tillandsioideae subfamily has

the widest geographical distribution and includes the genus *Vriesea* Lindl. with 214 species (Gouda and Butcher, 2023).

*Vriesea friburgensis* Mez occupies epiphytic, saxicolous, or terrestrial habits. It is a medium-sized bromeliad, reaching more than 2 m of height during the reproductive stage with beautiful inflorescences which provide an ornamental potential (Reitz, 1983). Typical inflorescences have a central axis with several branches on each side and one flower on each side of the lateral axis (Reitz, 1983).

\*e-mail: rosete.pescador@ufsc.br

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This species occurs from Northeast Brazil to Paraguay and northern Argentina (Costa et al., 2023; Reitz, 1983; WFO 2023).

Paggi et al. (2013) highlight that *V. friburgensis* is self-sterile and depends on pollinator services to maintain its population fitness and viability through cross-pollination. According to Martinelli et al. (2008) the conservation status of this species is vulnerable. The populations of *V. friburgensis* have been reduced by recent destruction and fragmentation of its natural habitat due to human disturbance and illegal collections (Paggi et al., 2013). Added to this, Duarte et al. (2019) indicate that environmental changes may be detrimental to the initial growth of *V. friburgensis* seedlings due to their limited capacity to handle extreme temperature-triggered oxidative stress. Therefore, it is relevant to understand the characteristics of seed germination that allow the development of *ex-situ* conservation and production protocols necessary to mitigate the effects of these threats (Flores-Palacios et al., 2015; Pereira et al., 2010). In this regard, *in vitro* seed germination can be considered the best approach to producing a large number of seedlings (preserving genetic diversity) and also a powerful tool for genetic improvement (Koufan et al., 2022). In addition, *in vitro* studies may function as a tool for extensive studies in bromeliads (Corredor-Prado et al., 2016, 2019).

Notably, germination and seedling emergence are the most critical transitions in plant development, which may compromise their survival (Larson et al., 2015). Since seedlings do not possess complete photosynthetic and mineral-uptake systems during germination, the energy required for physiological activities is mainly provided through the mobilization of primary reserves (Yu et al., 2014). For instance, lipids, proteins, or carbohydrates contribute to the germination potential of the embryo and to the establishment of seedlings (Zaynab et al., 2021). Therefore, the chemical composition of seeds and the deposition and mobilization of reserves influence their germination and vigor, constituting fundamental information for seed production technology (Bewley et al., 2013). Despite this, the study of germination and seedling establishment are aspects that have been little studied or are not sufficiently detailed in bromeliads, especially in aspects related to anatomy and histochemistry (Chilpa-Galván et al., 2018; Kowalski et al., 2021; Lidueña-Peréz et al., 2022; Pereira et al., 2008). Most of the existing works refer to morphological aspects (Chilpa-Galván et al., 2018; Martelo-Solorzano et al., 2022; Pereira et al., 2008, 2009; Scatena et al., 2006; Silva et al., 2021; Silva and Scatena 2011; Tillich, 2007), and a few include anatomical analyses. The studies by Kowalski et al. (2021) and Silva et al. (2021) evaluated various species of epiphytic, rupicolous, and terrestrial bromeliads. However, they focused the anatomical description on the emerged embryonic tissues and the formed seedlings. Only the study by Cecchifioridi et al. (2001) included histochemical and ultrastructural analyses to describe the mobilization of reserves from the endosperm during seedling development in the genus *Tillandsia*. To the best of our knowledge, no other study has addressed aspects related to the consumption of reserves during germination/postgermination events in bromeliads. Considering the diversity, the ecological importance, and the constant threats for the bromeliads,

studies with this approach can be regarded as acritical factor to understanding the processes involved in successful reproduction. In view of this, the objective of this study was to describe *in vitro* germination and reserve mobilization in the bromeliad *Vriesea friburgensis* through morphological, histochemical, and biochemical analysis.

## 2. Materials and Methods

### 2.1. Plant material

*Vriesea friburgensis* var. *paludosa* seeds were extracted from mature fruits collected from plants of the bromeliads collection of the Center for Agricultural Sciences, Federal University of Santa Catarina, Brazil. After plumose appendage removal, seeds were disinfected with 70% ethanol for 2 min and 1% sodium hypochlorite for 25 min with a drop of Tween 20 per 100ml of solution. Finally, the seeds were rinsed three times in sterile water and inoculated into test tubes (22 mm x 150 mm) containing 12 ml of culture medium. The medium used was composed of the saline formulation MS (Murashige and Skoog, 1962), supplemented with Morel vitamins (Morel and Wetmore, 1951) and sucrose (30 g L<sup>-1</sup>). The medium was gelled with 7.5g L<sup>-1</sup> of Agar-agar (Sigma®), and the pH was adjusted to 5.5 before autoclaving for 15 min at 121°C and 131 KPa. Cultures were maintained in a growth room at 25 ± 2 °C and 16 h day<sup>-1</sup> photoperiod, with a light intensity of 50–60 μmol m<sup>-2</sup> s<sup>-1</sup>.

### 2.2. Germination and post-seminal development

Four replicates of 50 seeds were used to evaluate the germination percentage [(number of germinated seeds/number of seeds) \*100] and the percentage of seedlings formed [(number of normal seedlings/number of seeds) \*100]. The number of germinated seeds was recorded daily, and the final germination percentage was determined after eight days. Was calculated the number of days taken for the first seed to germinate (T0) and the number of days to reach 50% of final/maximum germination (T50) according to Coolbear et al. (1984). The germination rate index (GR) was calculated according to Maguire (1962):  $GR = \sum (Gi/ni)$ , where Gi = the number of germinated seeds and ni = day of count. The rupture of the seed coat and the emergence of the cotyledonary sheath were the criteria used to define germination. The criteria adopted for the seeding stage were the full expansion of the first leaf and the appearance of the second leaf. The final percentage of seedlings formed was recorded after 28 days. The post-seminal development was observed daily and microphotographs were taken using a camera coupled to a stereoscope (Olympus® SZH-ILLB).

### 2.3. Scanning electron microscopy

Representative samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) plus 0.2 M sucrose overnight. The material was post-fixed with 1% osmium tetroxide for 4 h. The samples were dehydrated in ethanolic series, dried in the CO<sub>2</sub> critical point dryer (EM-CPD-030, Leica, Heidelberg, Germany), and then sputter-coated with gold prior to examination.

The samples were examined under SEMJSM 6390 LV (JEOL Ltd., Tokyo, Japan) at 20 kV.

#### 2.4. Fluorescence microscopy

Representative samples of fresh material were longitudinally cut free-hand, mounted on slides, and analyzed using a UV light-emitting diode (wavelength of 405 nm) in the Epifluorescent Microscope (Olympus BX 41) equipped with the Image Capture Q Capture Pro 5.1 Software. (Qimaging Corporation, Austin, TX, USA).

#### 2.5. Light microscopy

Seed samples collected at 0, 2, 4, and 18 days of culture were fixed in 2.5% paraformaldehyde in 0.2 M (pH 7.2) sodium phosphate buffer overnight. The samples were dehydrated in ethanol-aqueous solutions and infiltrated with Histo-resin (Leica®, Heidelberg). Sections (5 µm) were obtained with a manually rotating microtome (Slee Technik®). Histochemical tests applied: Toluidine Blue O (TBO) to identify phenols and acidic polysaccharides (O'Brien et al., 1964); Lugol to identify starch grains (Johansen, 1940); Periodic Acid-Schiff (PAS) to identify neutral polysaccharides (Gahan, 1984); and Coomassie Brilliant Blue (CBB) to identify proteins (Gahan, 1984). Some sections were double stained with PAS + CBB. Sections were analyzed with a microscope (Olympus® BX-40).

#### 2.6. Protein content

The extraction of proteins was performed following the method of Carpentier et al. (2005) with modifications. Briefly, a 500 mg sample was powdered with liquid nitrogen. Then it was homogenized with 5.0 mL of extraction buffer (50 mM Tris-HCl pH 8.5, 5 mM EDTA, 100 mM KCl, 1% w/v DTT, 30% w/v sucrose, and 1 mM PMSF) and 5.0 mL of buffer-saturated phenol (pH 8.0). The homogenates were centrifuged for 30 min at 10,000 × g at 4°C. The phenolic phase was recovered, homogenized with 5.0 mL of extraction buffer, and centrifuged again. The phenolic phase was precipitated with 100 mM of ammonium acetate in methanol (1:5 v/v) for 12h at -20°C. The proteins were solubilized in 0.3 mL of buffer (7 M urea, 2 M thiourea, 3% CHAPS, 2% IPG-buffer, 1.5% DTT). Proteins were quantified using the 2-D Quant Kit® (GE Healthcare, Uppsala, Sweden).

#### 2.7. Starch content

Samples of 500 mg were ground to powder with liquid nitrogen and subsequently submitted to an 80% ethanol extraction (70°C for 5 min). The extracts were centrifuged at 3,000 rpm (20°C for 10 min) and filtered through fibreglass. The extraction was repeated three times. One milliliter of cold distilled water and 1.3 mL of 52% perchloric acid were added to the pellet and kept on ice bath with agitation. Subsequently, 2 mL of water was added, and the material was centrifuged at 3,000 rpm for 15 min. The extraction was repeated, and the final volume was adjusted to 10 mL with distilled water. The starch content was estimated by the phenol-sulfuric method (Dubois et al., 1956) using glucose as a standard as proposed by McCready et al. (1950). The absorbance was measured at 490 nm.

#### 2.8. Statistical analyses

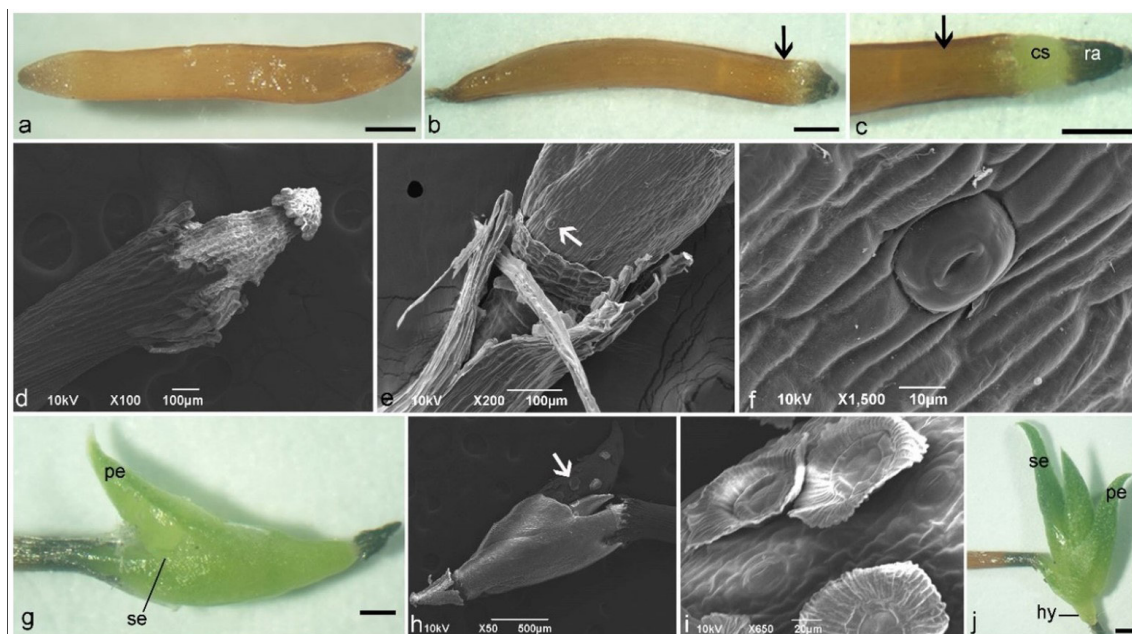
Data obtained of total proteins and starch content were subjected to analysis of variance and subsequently to Tukey's Honestly Significant Difference procedure for mean separation ( $P < 0.05$ ) using the PROC GLM procedure of SAS.

### 3. Results

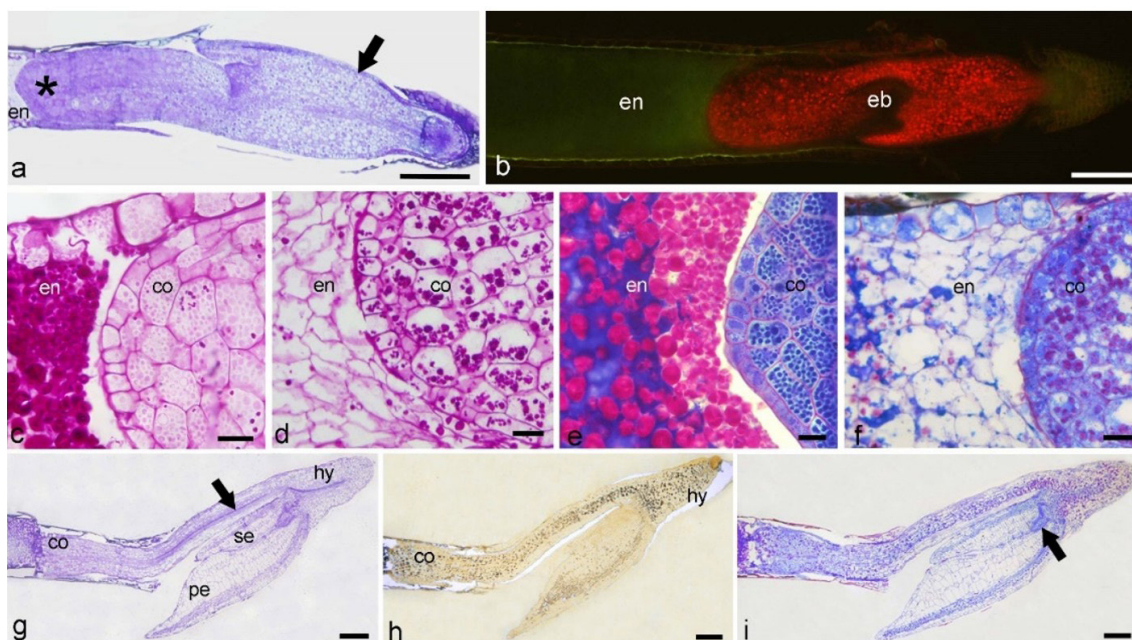
The average percentage of germination and seedling formation was  $98 \pm 1.2\%$  and  $94 \pm 2.0\%$ , respectively. Seeds of *V. friburgensis* began to germinate at 3 days ( $T_0=3$ ) and reached the highest germination percentage at 6 days. The  $T_{50}$  value was 4.24 days, and the GR value was 10.81. On day 2, after sowing, the seeds were swollen due to the imbibition process (Figure 1a-b). When the seed germinated (Day 4), the cotyledonary sheath and root apex emerged, while the distal portion of the cotyledon remained within the seed (Figure 1c-d). Stomata were observed in the emerged cotyledon sheath (Figure 1e-f). The first eophyll appeared after 12 days of culture. The seedlings were formed after 18 days of culture (Figure 1g). Eophylls are lanceolate with obtuse apex. Peltate trichomes are visible on the surface of the first and second eophyll (Figure 1h-i). The rosette shape was defined by day 28 upon the emergence of the third leaf (Figure 1j). A reduced hypocotyl was observed and in some seedlings, the formation of adventitious roots began. The cotyledon does not detach from the seed coat, which corresponds to a cryptocotyledonary germination.

The analysis of the internal structure of germinated seeds (day 4) allowed to observe the cell extension of the embryo that causes the rupture of the integument and the part of the cotyledon that remains inside the seed coat in contact with the endosperm (Figure 2a). The chlorophyll inflorescence of the embryo evidenced its metabolic reactivation (Figure 2b). Few starch grains were found in the cotyledon at the time of germination. However, there was a more significant accumulation on day 18 when the seedling was formed (Figure 2c-f). At germination, there was a high protein content in the endosperm and cotyledon (Figure 2e), but when the seedling was formed the content was reduced (Figure 2f). After the rupture of the integuments, cell division in the shoot apical meristem, and tissue differentiation led to seedling formation. The cotyledon does not show cell division and therefore does not grow towards the endosperm, always occupying the same space (Figure 2g-i).

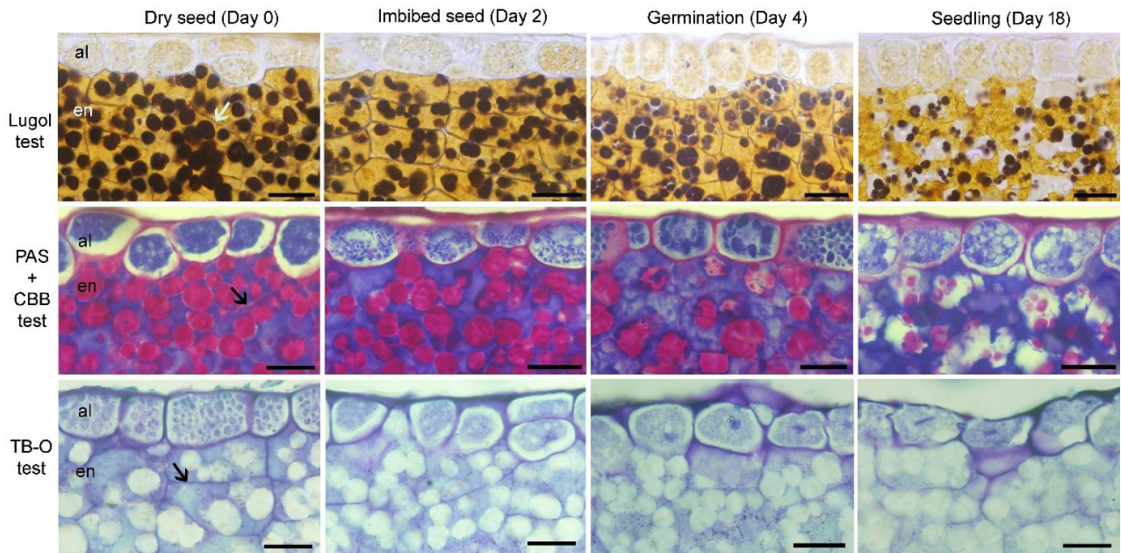
As the seeds germinated and the seedlings established, histochemical tests showed a gradual decrease in the starch grains and proteins in the endosperm (Lugol and PAS+CBB test; Figure 3). Faint alterations in the staining indicate a slight and simultaneous decrease of these compounds throughout the endosperm (Figure 3). However, there was visibly higher consumption in the endosperm region adjacent to the cotyledon. During the seedling development, this digestion zone increases so much that the endosperm cells appear empty and only the cell walls are visible (Figure 2d-f). Also, a decrease in protein content in the cytoplasm of cells of the aleurone layer was identified. When the seedlings were formed, the aleurone layer showed a well-developed vacuolar apparatus (PAS+CBB test; Figure 3).



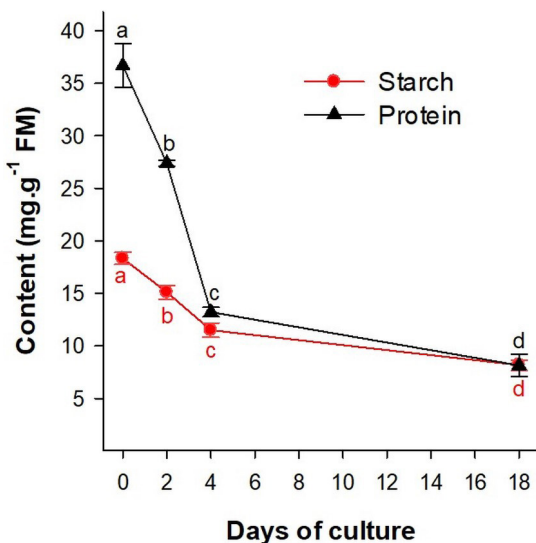
**Figure 1.** *In vitro* germination and post-seminal development of *Vriesea friburgensis*. (a) Seed before inoculation in the culture medium (Day 0); (b) Swollen seeds due to the imbibition process (Day 2). Arrow indicates the location of the embryo; (c, d) Seed germination. Rupture of the seed integument. Visible the cotyledonary sheath and the root apex (Day 4). Arrow indicates the portion of the cotyledon that remains within the seed; (e) Arrow indicates stomata in the cotyledon sheath (Day 8); (f) Stoma detail; (g, h) Seedling (Day 18). Arrow indicates the peltate trichomes; (i) Peltate trichomes detail; (j) Rosette-shaped young plant (Day 28). The plumose appendages were removed for the photographs. cs: cotyledonary sheath; hy: hypocotyl; pe: primary eophyll; ra: root apex; se: secondary eophyll. Bars a,b,c,g,j= 5µm.



**Figure 2.** Longitudinal sections during germination and post-seminal development of *Vriesea friburgensis*. (a-c,e) germination (day 4); (d,f-i) seedling (day 18). (a) cell extension of the embryo (arrow) and the cotyledon that remains inside the seed coat (asterisk); (b) autofluorescence in the embryo; (c) in germination, grains of starch accumulated in the endosperm and few grains accumulated in the cotyledon; (d) in the seedling, starch granules increase in the cotyledon and decrease in the endosperm; (e) in the germination, proteins present in the endosperm and the cotyledon; (f) in the seedling, there is a reduction of protein in the cotyledon and endosperm; (g) differentiation of vascular tissue (arrow) in seedling; (h) greater accumulation of starch in the cotyledon and hypocotyl; (i) greater accumulation of proteins in the shoot apical meristem (arrow). co: cotyledon; eb: embryo; en: endosperm; hy: hypocotyl; pe: primary eophyll; se: secondary eophyll. a,g TBO test; c,d PAS test; e,f,i PAS+CBB test; h Lugol test. Bars: a,b,g-i= 250µm; c-f= 20µm.



**Figure 3.** Dynamics of primary reserves in the endosperm of *Vriesea friburgensis* seeds during germination and seedling formation. Histochemical tests in longitudinal sections. al: aleurone layer; en: endosperm. Arrow indicates the cell wall in the endosperm. Bars= 25 $\mu$ m.



**Figure 4.** Total proteins and starch content during germination and post-seminal development of *Vriesea friburgensis*. Data (mean  $\pm$  SE) followed by different letter are significantly different (Tukey's HSD,  $P < 0.05$ ) between the days evaluated.  $n=3$ .

On the other hand, endosperm cell walls reacted positively to PAS and TB-O, revealing the presence of polysaccharides. Before seed sowing (Day 0), the endosperm presented well-defined and intensely stained cell walls. However, the intensity decreased during germination and seedling formation. A change in the integrity of the walls was visualized as rigid to flaccid.

When the seedling was formed, not all the available reserves in the endosperm cytoplasm had been consumed (Figure 3). In the seedling, a greater accumulation of starch grains was observed in the cotyledon and hypocotyl, while

the proteins were more visible in the shoot apical meristem. Lower accumulations of these compounds were observed in eophylls (Figure 2g-i). The quantitative analyzes corroborated the significant decrease in starch and protein during the days evaluated. The highest contents were registered in mature seeds (Day 0): starch 18.3 mg g<sup>-1</sup> FM and protein 36.7 mg g<sup>-1</sup> FM. Reserve mobilization began during imbibition and continued until seedling formation (Figure 4).

#### 4. Discussion

Our results corroborate that the germination of *V. friburgensis* is favored by *in vitro* techniques, obtaining a high percentage (98%) in a culture medium with normal MS formulation and Morel vitamins. This evidence high viability of the seeds and corroborates that *in vitro* germination helps considerably to overcome the limitations present in environmental conditions. Previous works reported lower germination percentages for this species. Paggi et al. (2013) obtained 76.9% germination using a culture medium with 1/2-MS salts and vitamin B5. In a study carried out by Kievitsbosch (2011), a culture medium containing 1/4-MS of macronutrients, micronutrients and vitamins MS, coconut water (10mL L<sup>-1</sup>), and banana extract (60g L<sup>-1</sup>) was used. This author found higher germination percentages in *in vitro* conditions (77.7%) than in greenhouses (66.9%). Certainly, seeds grown in suitable *in vitro* culture media and under controlled conditions provide the optimal nutrients and environment to promote germination and post-seminal development (Mazri et al., 2022). Several *Vriesea* species present low germination percentages in the natural environment (Mekers 1977; Mercier and Kerbauy 1995). However, high *in vitro* germination percentages were obtained in *V. cacuminis* (95%) (de Resende et al., 2016), *V. incurvata* (95%) (Sasamori et al., 2016), *V. philippocoburgii* (99%), and *V. reitzii* (100%) (Pradella et al., 2022).

According to Kievitsbosch (2011), *in vitro* germination of *V. friburgensis* is faster than *ex vitro* germination: germination began between the third and fourth day after inoculation *in vitro*; the appearance of the first eophyll occurred on the 15<sup>th</sup> day, and the formation of seedlings occurred at 20<sup>th</sup> day. Likewise, Kowalski et al. (2021) evaluated the germination by placing seeds in Petri dishes with filter paper moistened with distilled water. Although the authors did not indicate the germination percentage, they reported that the formation of seedlings required a longer time: germination between the fourth and fifth day after soaking the seed, first eophyll observed between the 19<sup>th</sup> and 22<sup>nd</sup> days, and second eophyll between the 24<sup>th</sup> and 29<sup>th</sup> days. Our results indicate that the formation of *V. friburgensis* seedlings is faster. The cultivation conditions would be influencing so that the appearance of the first aeophyll and the formation of seedlings occurs in less time. Regarding the T0 value (=3), we consider this data as an estimate of latency. Meireles et al. (2007), indicate that the solutions used during seed asepsis can act in seed coat scarification, increasing its permeability to water, oxygen and solutes, and in the removal or oxidation of germination inhibiting compounds. Therefore, the disinfection process used in our study could also contribute to shortening the germination time. On the other hand, temperature control affects the speed of water absorption by the seeds and can change the total percentage, speed, and uniformity of germination (Bewley et al., 2013; Nonogaki et al., 2010). Additionally, Ferreira et al. (2020) indicate that aspects related to seed harvesting, subsequent handling, storage, and experimental conditions may influence temporal behavior related to germination.

The germination of *V. friburgensis* was marked by the emergence of the cotyledonary sheath. Studies report the same pattern of post-seminal development for other species of *Vriesea* genus (Corredor-Prado et al., 2016, 2020; Pereira et al., 2008, 2009) and other genera of Tillandsioideae subfamily (Chilpa-Galván et al., 2018; Martelo-Solorzano et al., 2022; Scatena et al., 2006; Silva and Scatena, 2011; Tillich, 2007). Germination is a complex process that starts with imbibition, where the seed restores metabolism, completes essential cellular events to allow embryo emergence, and prepares for subsequent seedling growth (Nonogaki et al., 2010). According to Bewley et al. (2013) the mobilization of the major reserves within seed storage tissues occurs following the completion of germination to provide nutrients for the growing seedling. We verify that the endosperm of *V. friburgensis* stores a large amount of reserves and plays an active role during mobilization. However, when the seeds were still in the imbibition phase, histochemical tests indicated changes in the composition of the endosperm. A decrease in reserve compounds was corroborated with biochemical quantifications, indicating that in this species, the mobilization of reserves occurs early. Likewise, ultrastructural analyses in soaked *Tillandsia* seeds indicated a reduction in the size of starch granules, fragmentation of protein bodies, and the presence of hydrolytic enzymes in the endosperm (Cecchifiordi et al., 2001). The beginning of the mobilization of reserves during imbibition is probably related to the repair of cellular structures before germination (Buckeridge et al., 2004).

Several studies verified the early mobilization of reserves during imbibition (Mazzottini-dos-Santos et al., 2017; Ferreira et al., 2020). According to Sabelli and Larkins (2009), upon seed imbibition, aleurone cells activate a gene expression program that results in the synthesis of proteolytic and hydrolytic enzymes, which cause digestion of cell walls and mobilization of proteins and starch stored in the cytoplasm of endosperm cells. The presence of the aleurone layer in bromeliad seeds has been previously reported (Cecchifiordi et al., 2001; Corredor-Prado et al., 2014; Lidueña-Peréz et al., 2022; Magalhães and Mariath 2012; Martelo-Solorzano et al., 2022). Therefore, the alteration of the protein bodies of the aleurone layer in *V. friburgensis* during imbibition would be related to the beginning of its activity. Cecchifiordi et al. (2001) verified the presence of hydrolytic enzymes (acid phosphatase) in the aleurone layer of soaked *Tillandsia* seeds. These authors also identified a well-developed vacuolar apparatus in the aleurone layer when the seedlings were formed.

Tissue protrusion through the structures surrounding the embryo is the usual event that terminates germination and marks the beginning of seedling growth. This occurs because of cell extension, which may or may not be accompanied by cell division (Bewley et al., 2013). In *V. friburgensis* we identified only the cell extension in the embryo as the cause of the rupture of the integuments, like what was found in *Tillandsia* seeds (Cecchifiordi et al., 2001). Subsequently, the intense consumption of proteins and starch indicated their direct participation as nutritional reserves in the energy supply for germination and post-seminal development. The highest decrease in endosperm reserves in the region close to the embryo has also been described in *Tillandsia* seeds. However, the endosperm of these seeds also presented lipid reserves (Cecchifiordi et al., 2001). These authors identified ultrastructural modifications in the cotyledon epidermis correlated with haustorial function. Studies with species from other botanical families relate a similar pattern of reserve degradation through histochemical analysis (Carvalho et al., 2022; Ferreira et al., 2020; Mazzottini-dos-Santos et al., 2017; Oliveira et al., 2020) and biochemical quantifications (Carvalho et al., 2022; Mello et al., 2022; Bicalho et al., 2016). The values of starch and protein content found in this study are similar to those reported during the formation of *V. reitzii* seedlings (Corredor-Prado et al., 2016, 2020).

Starch mobilization occurs due to enzymes ( $\alpha$ -amylase,  $\beta$ -amylase, and starch phosphorylase) that break down its carbon chains into smaller structures (maltose and glucose), which are used for energy metabolism and cellulose biosynthesis (Bewley et al 2013). On the other hand, proteins are broken down into amino acids for energy generation and biosynthesis of new proteins and enzymes (Tan-Wilson and Wilson 2012). In addition to starch, another less common form of carbohydrate reserves is the hemicelluloses stored in the secondary cell walls of the endosperm (Bewley et al., 2013). According to Oliveira et al. (2020) these compounds are mobilized during germination, although they are less usually described. In this study, we identified a slight change in the intensity of staining by PAS and TBO in the endosperm cell walls during the formation of seedlings.

This suggests that the hemicelluloses released from the cell wall could contribute to a lesser extent to these initial developmental processes. However, more detailed analyzes are necessary. In cereals, the cell walls of starchy endosperm and aleurone are rich in arabinoxylans (hemicellulose) that are degraded by enzymes synthesized and released from the aleurone layer (Butardo Junior and Sreenivasulu, 2016). Several works also reported alterations in the endosperm cell-wall through histochemical analysis during the formation of seedlings (Mazzottini-dos-Santos et al., 2017; Oliveira et al., 2020). According to Oliveira et al. (2020) and Mazzottini-dos-Santos et al. (2017) cytoplasmic reserves such as proteins and lipids are mobilized more rapidly than cell-wall compounds.

Corredor-Prado et al. (2014) found uniformly distributed starch grains in the embryo of mature seeds of *V. friburgensis*. We also visualized starch grains in the embryo during germination (day 4), and the amount of these grains increased markedly when seedlings were formed. This could result from the accumulation of sugars in their tissues that come from the mobilization of reserves from the endosperm. To maintain osmotic balance in the seedling, the excess would be converted back to the starch reserve. Through histochemical analysis, other studies have also demonstrated an increase in the number of starch grains in the embryo before germination and during seedling formation (Bicalho et al., 2016; Cecchifiordi et al., 2001; Ferreira et al., 2020; Mazzottini-dos-Santos et al., 2017). For the establishment of seedlings in *V. friburgensis*, we observed that the total consumption of the reserves accumulated in the endosperm was unnecessary. While in the genus *Tillandsia* the endosperm reserves are almost completely consumed (Cecchifiordi et al., 2001). This may be related to the fact that the space occupied by the endosperm in the seeds may vary according to the genus. In general, in *Vriesea* the endosperm occupies a greater percentage of the volume of the seed compared to *Tillandsia* (Chilpa-Galván et al., 2018; Magalhães and Mariath, 2012; Martelo-Solorzano et al., 2022).

In view of the scarce knowledge about the anatomical and histochemical aspects during the germination and formation of seedlings in bromeliads, this work contributes to the knowledge of these aspects for the species *V. friburgensis*. Our results indicate that the *in vitro* conditions used for germination are effective, despite being a more expensive technique. The seeds have a high physiological quality and, consequently, a high potential to produce seedlings. The rapid germination is related to the hydrolytic enzymes supplied by the aleurone layer, which allows for early mobilization of the reserve compounds present in the cytoplasm (proteins and starch) and in the cell walls (hemicelluloses) of the endosperm, making available the necessary energy for germination and seedling establishment.

To preserve genetic diversity within this species, *in vitro* seed germination may be considered the best approach for micropropagation for ornamental purposes or for reintroduction into its natural habitat. The results presented constitute useful tools for future studies on ecology, seed technology, and conservation in this species.

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