CC BY

Research Article

Optimization of the tetrazolium test for neotropical orchids: influence of preconditioning and temperature[1]

Jéssica Celeste Mônico Ramos[2], Leandro de Oliveira Miranda[2], Luan Marlon Ribeiro[2], Rayner Bueno Peinado[2], Marcos Vinnicius Braga Machado de Queiroz[2], Jackeline Schultz Soares[2], José Carlos Sorgato[2]

ABSTRACT

Orchids face significant conservation challenges due to habitat loss and illegal collection, making *ex situ* conservation strategies essential. This study aimed to optimize the tetrazolium test for four Orchidaceae species (*Cattleya forbesii*, *C. wittigiana*, *Encyclia argentinensis*, and *Ionopsis utricularioides*). The experiment followed a completely randomized design, in a 3 × 3 factorial arrangement (six replications), evaluating preconditioning (distilled water for 24 h, 10 % sucrose for 24 h, and control) and incubation temperatures (water bath at 40 ± 0.5 ºC for 24 h, oven at 40 ± 2 ºC for 24 h, and room temperature at 25 ± 2 ºC for 24 h). Estimated viability was validated by *in vitro* germination. At room temperature, the distilled water and sucrose yielded comparable results for *C. forbesii*, *C. wittigiana*, and *I. utricularioides*; whereas, for *E. argentinensis*, the sucrose resulted in the highest estimate. The tetrazolium test overestimated germination across all taxa, suggesting it detects metabolic activity rather than the complex sequence of events required for germination. The optimization was species-specific; however, the combination of distilled water and room temperature is recommended as a practical protocol for the studied species, with sucrose as an alternative when optimizing the test for *E. argentinensis*.

KEYWORDS: *Ex situ* conservation, germplasm, Orchidaceae, seed viability.

RESUMO

Otimização do teste de tetrazólio para orquídeas neotropicais: influência do pré-condicionamento e da temperatura

As orquídeas enfrentam desafios significativos de conservação devido à perda de habitat e coleta ilegal, tornando-se essenciais estratégias de conservação *ex situ*. Objetivou-se otimizar o teste de tetrazólio para quatro espécies de Orchidaceae (*Cattleya forbesii*, *C. wittigiana*, *Encyclia argentinensis* e *Ionopsis utricularioides*). O experimento foi conduzido em delineamento inteiramente casualizado, em esquema fatorial 3 × 3 (seis repetições), avaliando-se o pré-condicionamento (água destilada por 24 h, sacarose 10 % por 24 h e controle) e temperaturas de incubação (banho-maria a 40 ± 0.5 ºC por 24 h, estufa a 40 ± 2 ºC por 24 h e temperatura ambiente de 25 ± 2 ºC por 24 h). A viabilidade estimada foi validada por meio de germinação *in vitro*. Sob temperatura ambiente, a água destilada e sacarose apresentaram resultados comparáveis para *C. forbesii*, *C. wittigiana* e *I. utricularioides*; enquanto, para *E. argentinensis*, a sacarose resultou na maior estimativa. O teste de tetrazólio superestimou a germinação em todos os táxons, evidenciando que o teste detecta atividade metabólica, e não a sequência complexa de eventos necessários para germinação. A otimização foi espécie-específica; no entanto, a combinação de água destilada e temperatura ambiente é recomendada como protocolo prático para as espécies estudadas, com a sacarose como alternativa na otimização do teste para *E. argentinensis*.

PALAVRAS-CHAVE: Conservação *ex situ*, germoplasma, Orchidaceae, viabilidade de sementes.

INTRODUCTION

The Orchidaceae family is the largest and most diverse among angiosperms, accounting for approximately 8 % of their total diversity (Hwang et al. 2024). In Brazil, the family is represented by 2,663 species distributed across all phytogeographic domains – Amazon Rainforest, Caatinga, Cerrado, Atlantic Forest, Pampa, and Pantanal. Among these, the Cerrado (Brazilian Savanna) stands out as one of the richest in orchid diversity, hosting 651 species across 110 genera (FFB 2026).

Despite this significant richness, native orchids have been under pressure from habitat loss

[1] Received: Mar. 13, 2026. Accepted: July 02, 2026. Published: July 27, 2026. DOI: 10.1590/1983-40632026v5685473.

[2] Universidade Federal da Grande Dourados, Faculty of Agricultural Sciences, Dourados, MS, Brazil. *E-mail/ORCID*: jessica_monico13@hotmail.com/0000-0002-3987-6726; leandrobiouems@gmail.com/0000-0001-7437-5786; luanribeiro@ufgd.edu.br/0000-0002-7278-7696; raynerbueno@icloud.com/0009-0007-5632-4038; marcosqueiroz@ufgd.edu.br/0009-0009-5549-4742; jackelinesoares@ufgd.edu.br/0000-0001-9497-2975; josesorgato@ufgd.edu.br/0000-0003-0001-5467.

Editor: Luis Carlos Cunha Junior/Data Availability Statement: Research data are only made available by authors upon request.

and fragmentation, as well as predatory collection, factors that contribute to population decline and increase extinction risk for various taxa (Fay et al. 2025, IUCN 2025). Among native species, *Cattleya forbesii* Lindl. is endemic to Brazil, occurring in the Atlantic Forest; *C. wittigiana* (Barb. Rodr.) van den Berg is found in the Cerrado and Atlantic Forest, listed as "Critically Endangered" (CR) by CITES (2023), and "Endangered" (EN) by Cncflora (2012a); *Encyclia argentinensis* (Speg.) Hoehne occurs in the Amazon, Cerrado, and Atlantic Forest; and *Ionopsis utricularioides* (Sw.) Lindl. is native to the Cerrado, Amazon, and Atlantic Forest, with population reductions across its biomes (Cncflora 2012b, FFB 2021a, FFB 2021b, WFO 2025). On a global scale, approximately 2,123 orchid species are listed on the IUCN Red List, 274 of which are classified as "Critically Endangered", highlighting the magnitude of the conservation challenge for this family (Fay et al. 2025, IUCN 2025).

Orchidaceae species exhibit a highly specialized life cycle, with minute seeds containing little to no nutritional reserves, a characteristic that limits germination under natural conditions. Under natural conditions, the germination process depends on association with specific mycorrhizal fungi, a requirement that increases population vulnerability and contributes to low regeneration in disturbed areas (Jolman et al. 2022, Nongdam et al. 2023, Soares et al. 2023, Smiderle et al. 2025). Thus, the intrinsic characteristics of the seeds, combined with anthropogenic pressures and extraction, reinforce the need for conservation strategies and germplasm management, especially for threatened species (Hosomi et al. 2017, Mercado et al. 2020). In this context, the use of seed banks is a promising long-term *ex situ* conservation strategy, enabling the maintenance of genetic variability and supporting reintroduction efforts and seedling production (Nakum et al. 2025).

The minute size of orchid seeds imposes additional methodological challenges for tetrazolium testing. Orchid seeds are among the smallest in the plant kingdom, typically measuring 0.05-0.30 mm in length and 0.01-0.15 mm in width, with an embryo consisting of only 50-200 undifferentiated cells and occupying a reduced proportion of the total seed volume (Arditti 2022, Lee & Yeung 2023). This reduced embryonic mass, combined with a seed coat that may contain hydrophobic compounds such as suberin, limits the penetration and uniform diffusion of the tetrazolium solution, often resulting in weak, heterogeneous, or difficult-to-interpret staining (Hosomi et al. 2017, Pradhan et al. 2022). These physical constraints justify the need for methodological adjustments to improve the reliability and reproducibility of the tetrazolium test for orchid seeds.

Among the available tests for evaluating seed physiological potential, the tetrazolium test stands out as rapid, simple, and widely used for estimating seed viability. This test is based on the activity of dehydrogenase enzymes associated with respiratory metabolism, particularly malate dehydrogenase: living, metabolically active tissues reduce the colorless tetrazolium salt to form a carmine-red stain, allowing inferences about cellular respiratory activity (Mercado et al. 2020, Ribeiro et al. 2021, França-Neto & Krzyzanowski 2022). In Orchidaceae, however, the application of this test can be challenging, as the minute embryo size, low initial metabolic rate, and limitations related to reagent hydration and penetration may result in weak, heterogeneous, or difficult-to-interpret staining (Hosomi et al. 2017, Mercado et al. 2020, Francisqueti et al. 2024). Therefore, methodological adjustments are necessary to improve reproducibility and accuracy, particularly for stored lots.

Preconditioning strategies have been employed to improve test efficiency by favoring hydration and metabolic reactivation, thereby enhancing tetrazolium uptake and reduction (Mercado et al. 2020, Francisqueti et al. 2024). For species of the *Epidendrum* genus (*E. fimbriatum*, *E. microtum*, and *E. elongatum*), preconditioning at room temperature (25 ± 2 ºC) with deionized water increased test efficiency (Mercado et al. 2020). For *Cattleya nobilior* and *C. walkeriana*, preconditioning in distilled water or a 10 % sucrose solution, combined with conditioning at room temperature, facilitated the visualization of viable seeds (Ribeiro et al. 2021). For *Miltonia flavescens*, the best visualization occurred with 10 % sucrose preconditioning and oven conditioning at 40 ºC; whereas, for *Schomburgkia crispa*, the control treatment (no imbibition) combined with room-temperature conditioning was more efficient (Soares et al. 2021). These results reinforce that the response to preconditioning may be species-specific, possibly reflecting ecophysiological differences and metabolic thresholds for enzymatic reactivation.

Considering the importance of conserving native orchids and the need for rapid, reproducible methods for monitoring stored lots, this study aimed to optimize the tetrazolium test for seeds of four orchid species. We hypothesized that preconditioning (water or sucrose) and temperature modulate metabolic reactivation and reagent diffusion, determining the intensity and clarity of staining. Herein, we propose an adjusted protocol with potential application in seed banks, enhancing the reliability of viability estimates for neotropical species and supporting decisions regarding *ex situ* conservation and seedling production.

MATERIAL AND METHODS

The experiment was conducted at the Universidade Federal da Grande Dourados, in Dourados, Mato Grosso do Sul state, Brazil, in 2025.

Seeds from four Orchidaceae species were used: *Cattleya forbesii* Lindl., *Cattleya wittigiana* (Barb. Rodr.) van den Berg, *Encyclia argentinensis* (Speg.) Hoehne, and *Ionopsis utricularioides* (Sw.) Lindl. (Figure 1). These plants had been cultivated and maintained in an orchid nursery for several years, and the seeds used in this experiment were obtained through manual cross-pollination of these cultivated plants. For each species, four adult mother plants were used as seed donors. Access to the genetic heritage of *E. argentinensis* and *I. utricularioides*, which were originally collected from natural environments, was registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the registration numbers AF963C3 and A21374E, respectively.

Seeds were obtained through manual cross-pollination of mother plants older than 8 years, maintained in a nursery under a double layer of 50 % shading screen. The mean microclimatic conditions in the nursery during capsule development were: irradiance of 235 µmol m^{-2} s^{-1}, temperature of 22.6 ± 5 ºC, and relative humidity of 73.9 ± 10 %.

Closed capsules were harvested at 8 months after pollination, with the exception of *I. utricularioides*, whose capsules were harvested at 6 months. The capsules were collected when early

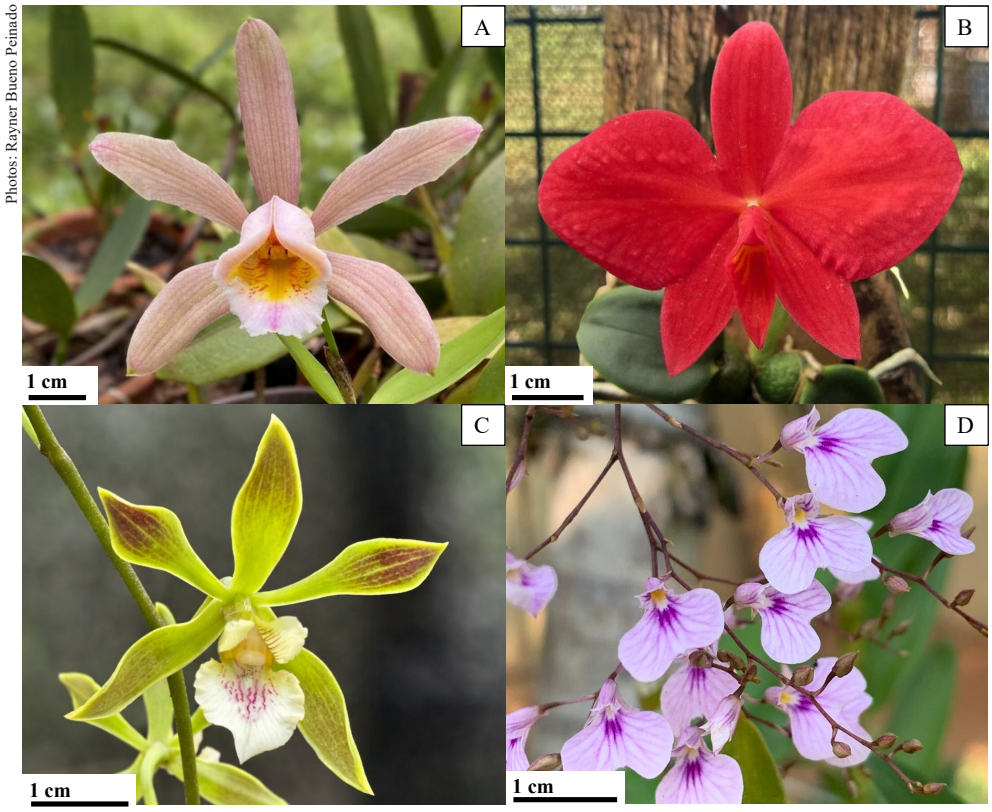

Figure 1. Mother plants of the orchid species *Cattleya forbesii* Lindl. (A), *Cattleya wittigiana* (Barb. Rodr.) van den Berg (B), *Encyclia argentinensis* (Speg.) Hoehne (C), and *Ionopsis utricularioides* (Sw.) Lindl (D).

signs of dehiscence were observed, such as yellowing along the carpel sutures. In the laboratory, seeds were removed from the capsules, manually homogenized, and weighed on a precision analytical scale. To standardize the moisture content, the seeds were kept in a desiccator containing silica gel for 15 days. After desiccation, seeds of each species were packed in aluminum foil envelopes and placed in opaque polypropylene bottles with screw caps containing silica gel as an additional desiccant. The bottles were stored under refrigeration (4 ± 2 ºC) for up to 180 days until the initiation of experiments.

For each species, 36 samples of 0.001 g of seeds were weighed and individually allocated into test tubes (10 mL) for the execution of the tetrazolium test. The experiment was conducted in a completely randomized design, in a 3 × 3 factorial scheme, consisting of three preconditioning protocols (P) and three incubation conditions (C), with six replications per treatment (each test tube constituted one replication). The preconditioning treatments consisted of seed immersion in 3 mL of distilled water for 24 h (P1); immersion in 3 mL of 10 % (w/v) aqueous sucrose solution for 24 h (P2); and control without imbibition (P3). After 24 h, seeds from the treatments P1 and P2 were triple-washed with distilled water to remove residues, and, subsequently, all samples (P1, P2, and P3) received 3 mL of 0.5 % 2,3,5-triphenyltetrazolium chloride aqueous solution (pH 6.5-7.0). The 0.5 % concentration was selected based on Ribeiro et al. (2021). The tubes were then kept in the dark for 24 h under three incubation conditions: thermostated water bath at 40 ± 0.5 ºC (C1); forced-air oven at 40 ± 2 ºC (C2); and room temperature (25 ± 2 ºC; C3). During the incubation period of 24 h, temperature was continuously monitored using a data logger. For the water bath (sensor in the water), the recorded temperature was 40 ± 0.5 ºC; whereas, for the oven (sensor inside the chamber), it was 40 ± 2 ºC, with maximum peaks of up to 43.3 ºC. Both pieces of equipment were pre-stabilized and calibrated.

After 24 hours, the tetrazolium suspensions were diluted with 7 mL of distilled water and homogenized by manual agitation. A 1-mL aliquot was then pipetted for identification and total counting of seeds in a Peters chamber, using a stereomicroscope with zoom (Zeiss™). For each replication, the total number of seeds in the aliquot (TS) and the number of seeds with fully stained embryos (100 % of the embryonic tissue stained, regardless of intensity, from light pink to deep carmine red) were counted (VS). Seeds with partially stained or colorless embryos, as well as empty seeds, were classified as nonviable. The percentage of viable seeds (%VS) was calculated individually for each replication using the formula: %VS = (VS/TS) x 100, where VS is the number of seeds with fully stained embryos (from light pink to deep carmine red), and TS the total number of seeds in the aliquot.

The treatments were photographed using a digital camera coupled to the stereomicroscope, with the AxioVision software version 3.1 (Zeiss™). Seed dimensions (length and width, including the seed coat) were measured from the same micrographs using the 1-mm scale bar as reference. For each species, 20 seeds were randomly selected and measured using the ImageJ software (Fiji, National Institutes of Health, USA). The length corresponded to the longest axis of the seed, and the width was measured at the widest point of the seed body.

To confirm the results, immediately after the test, a 0.005-g seed sample from each species was taken to an aseptic environment and disinfected with 15 mL of 0.8 % sodium hypochlorite solution for 5 min. After this period, the seed suspension was diluted to 50 mL and then triple-washed with sterile distilled water (121 ºC at 1 atm pressure for 20 min). Subsequently, the suspension volume was made up to 50 mL with sterile distilled water. For *in vitro* sowing, 1,000 µL of the seed suspension were inoculated into each flask, with four culture flasks per species.

Thirty milliliters of Murashige & Skoog culture medium at half-strength (½ MS) were used per 200-mL flask. Subsequently, cultures were placed in a growth room with controlled temperature and photoperiod (25 ± 2 ºC; 16 h) and irradiance of 22 µmol m^{-2} s^{-1} provided by 3000K LEDs.

After 45 days of cultivation, the germination percentage was evaluated, with seeds considered germinated if they reached the developmental stage 1 (protocorms). For this, 10 mL of distilled water were added to each culture flask, and, after manual agitation, the seed suspension was poured into a Petri dish. Using a binocular stereomicroscope, the total number of observed seeds (TS) and the number of germinated seeds (GS) was counted. The germination percentage (%G) was calculated as: %G = (GS/TS) x 100, where: GS is the number

of germinated seeds, and TS the total number of observed seeds.

Data for each species were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Bartlett's test). Once assumptions were met, analysis of variance (Anova) was performed in a 3 × 3 factorial scheme using the Sisvar statistical software (Ferreira 2019). In cases where the F-test indicated significance ($p < 0.05$) for individual factors or the interaction, treatment means were compared using the Tukey test at 5 % of significance.

RESULTS AND DISCUSSION

The analysis of variance indicated a significant interaction ($p < 0.05$) between preconditioning protocols (water, sucrose, and control) and incubation conditions (water bath at 40 ºC, oven at 40 ºC, and room temperature) for the percentage of viable seeds (%VS) in all four evaluated species (Figure 2). In practice, this interaction demonstrates that the effect of preconditioning depends on temperature and vice versa; thus, it is not possible to generalize the best treatment by considering only one factor in isolation.

For *Cattleya forbesii*, preconditioning in distilled water yielded the highest estimated viability values, particularly at room temperature (82.99 %) and water bath (80.93 %), with no statistically significant difference between these combinations (Figure 2A). The significant difference between water baths at 40 ºC and room temperature

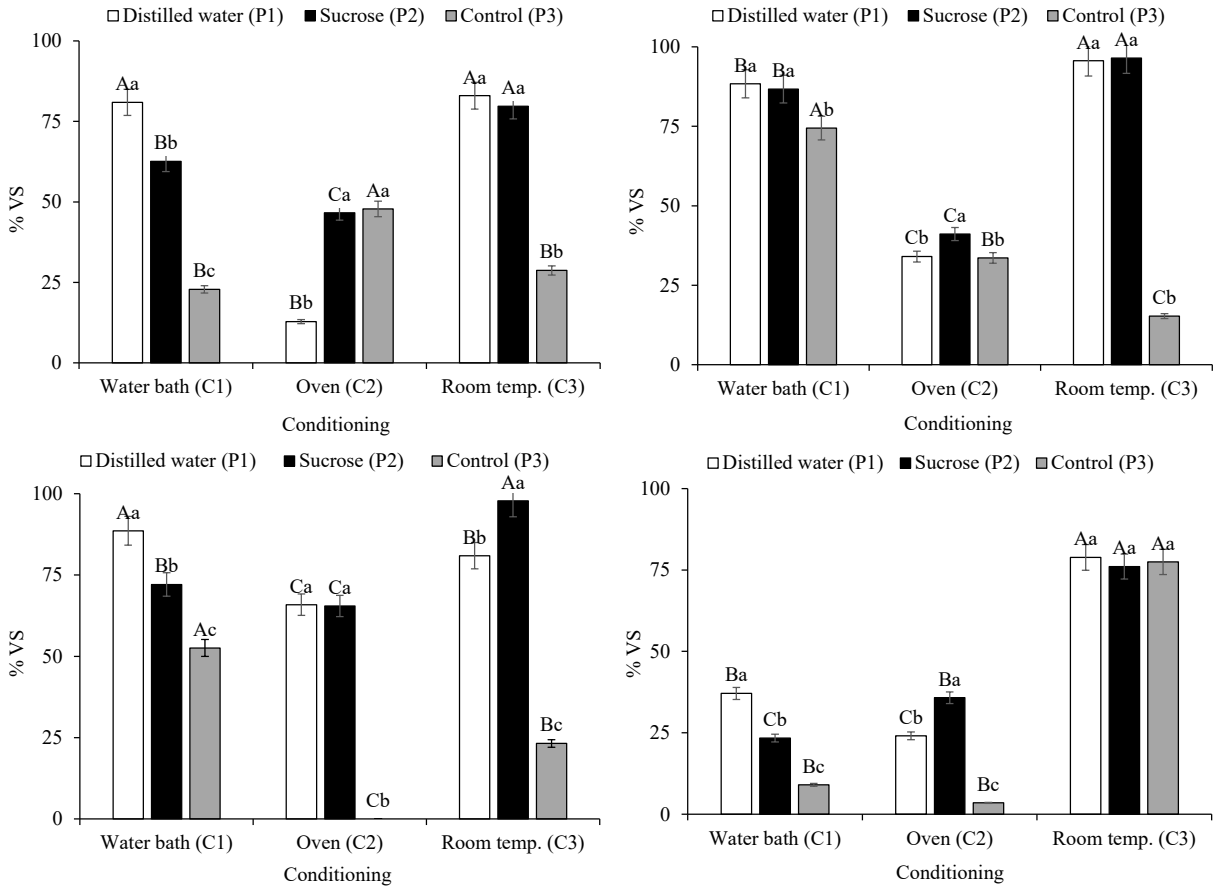

Figure 2. Percentage of viable seeds (%VS) of *Cattleya forbesii* Lindl. (A); *Cattleya wittigiana* (Barb. Rodr.) van den Berg (B); *Encyclia argentinensis* (Speg.) Hoehne (C); and *Ionopsis utricularioides* (Sw.) Lindl. (D), as a function of preconditioning [P1: distilled water (24 h); P2: 10 % sucrose solution (24 h); and P3: no immersion (control)] and incubation conditions [C1: water bath (40 ± 0.5 ºC); C2: oven (40 ± 2 ºC); and C3: room temperature (25 ± 2 ºC)], evaluated by the tetrazolium test. Means followed by the same uppercase letter do not differ within each incubation condition, and means followed by the same lowercase letter do not differ within each preconditioning treatment, according to the Tukey test ($p < 0.05$). Bars represent the standard error of the mean.

under distilled water preconditioning indicates that, once embryonic tissues are adequately hydrated, the enzymatic activity required for tetrazolium reduction proceeds efficiently regardless of moderate temperature variations. This suggests that, for this species, preconditioning in distilled water is the primary determinant of staining quality, whereas thermal incubation plays a secondary role. Notably, the same preconditioning resulted in markedly lower viability when combined with oven incubation at 40 ºC (approximately 15 %). The oven exhibited greater temperature fluctuations (up to 43.3 ºC), which may have caused a partial denaturation of dehydrogenase enzymes, whereas the water bath maintained a stable temperature (40 ± 0.5 ºC).

For *Cattleya wittigiana*, room temperature was the most favorable condition for maximizing estimated viability, highlighting preconditioning in sucrose (96.45 %) and water (95.61 %), both of which differed statistically from the other combinations (Figure 2B). These results indicate that, for this species, preconditioning followed by incubation at room temperature resulted in more adequate staining for test interpretation.

In *Encyclia argentinensis*, the highest %VS was observed when seeds were preconditioned in sucrose and incubated at room temperature (97.79 %). However, this result did not differ statistically from the distilled water treatment associated with the water bath (88.60 %) (Figure 2C), indicating that, for this species, more than one combination can yield an efficient and reliable tetrazolium test reading, albeit with distinct numerical values.

In *Ionopsis utricularioides*, the highest %VS values were observed under incubation at room temperature, regardless of the applied preconditioning (water: 78.87 %; control: 77.51 %; sucrose: 76.04 %), with no statistical difference among these combinations (Figure 2D). This behavior demonstrates that, for *I. utricularioides*, room temperature was the most determining factor for assay efficiency, whereas differences among pretreatments were less pronounced.

The visual evaluation of seeds stained with the tetrazolium test confirmed the presence of specific responses for each species and treatment combination (Figures 3, 4, 5, and 6). In general, variation was observed in the uniformity, intensity, and definition of the carmine-red staining of embryos, as well as in the proportion of seeds with partially stained or colorless embryos, which directly impacts the clarity of test interpretation.

In all four species, treatments resulting in the highest %VS (as indicated in Figure 2) corresponded visually to combinations in which a higher frequency of fully stained embryos was observed, with more pronounced contrast against non-viable seeds. Conversely, in less efficient combinations, embryos with partial or faint staining were more frequent, increasing classification difficulty, especially in small seeds with reduced embryos, a typical characteristic of Orchidaceae.

Validation by *in vitro* germination demonstrated that, for all species, viability values estimated by the tetrazolium test (%VS) were higher than germination percentages (%G) obtained at 45 days. In *C. forbesii*, the highest %VS (82.99 %) corresponded to a %G of 59.16 %. In *C. wittigiana*, %VS and %G values of 96.45 and 72.20 %, respectively, were observed. For *E. argentinensis*, the highest estimated viability (97.79 %) contrasted with a %G of 44.78 %; whereas, for *I. utricularioides*, the highest %VS (78.87 %) was accompanied by only 22.62 % of germination.

Furthermore, the magnitude of this difference varied among species. *C. wittigiana* showed a greater proximity between viability and germination, whereas *E. argentinensis* and *I. utricularioides* showed more marked discrepancies, indicating that a portion of the seeds classified as viable by the tetrazolium test did not complete the germination process under the conditions evaluated at 45 days.

Orchid seed imbibition poses a significant physiological challenge, as the seed surface is equipped with specialized structures for dispersion and protection. These morphoanatomical characteristics vary according to the growth habit of each species, resulting in marked differences in seed coat permeability and solution uptake dynamics. Furthermore, orchid seeds are among the smallest seeds in the plant kingdom (Arditti 2022, Pradhan et al. 2022, Lee & Yeung 2023). Measurements of the seeds used in this study (including the seed coat) revealed species-specific dimensions (Table 1).

Notably, the discrepancy between tetrazolium-estimated viability and actual germination also varied with seed morphology. *I. utricularioides*, which exhibited the most elongated seeds and the narrowest width, showed the largest difference between %VS and %G (78.87 *vs*. 22.62 %). In contrast, *C. wittigiana*, with the shortest seeds

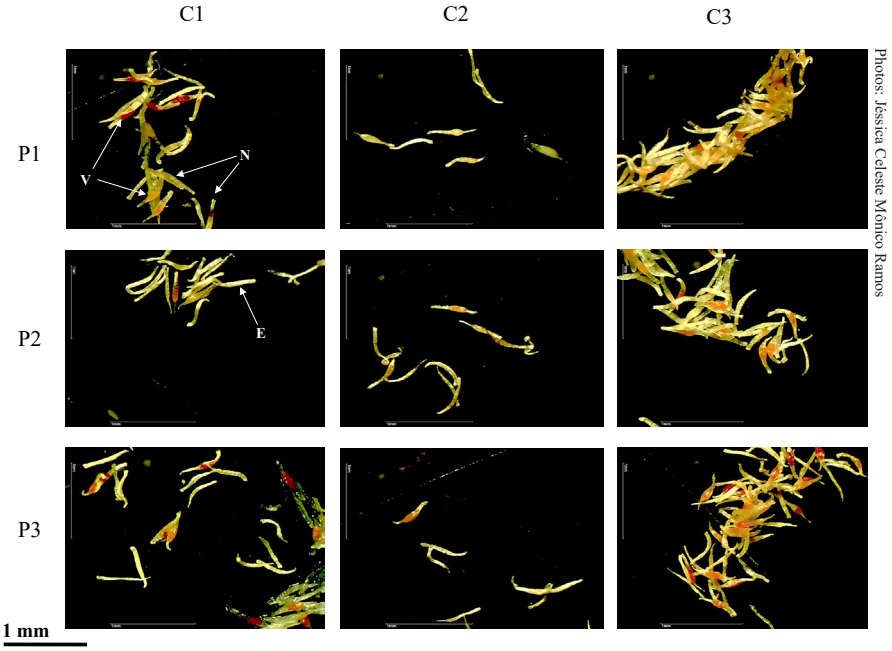

Figure 3. *Cattleya forbesii* Lindl. seeds, as a function of preconditioning [P1: 3 mL of distilled water for 24 h; P2: 3 mL of sucrose solution (10 %) for 24 h; and P3: no soaking (control)] and conditioning [C1: water bath (40 ± 0.5ºC); C2: oven (40 ± 2 ºC); and C3: ambient temperature (25 ± 2 ºC)], in the tetrazolium test. Examples of staining classes considered in the interpretation of the test: fully stained embryos (from light pink to deep carmine red, viable), partially stained embryos, colorless embryos, and seeds without embryos (classes considered non-viable). V, N, and E: viable, non-viable, and empty seed, respectively.

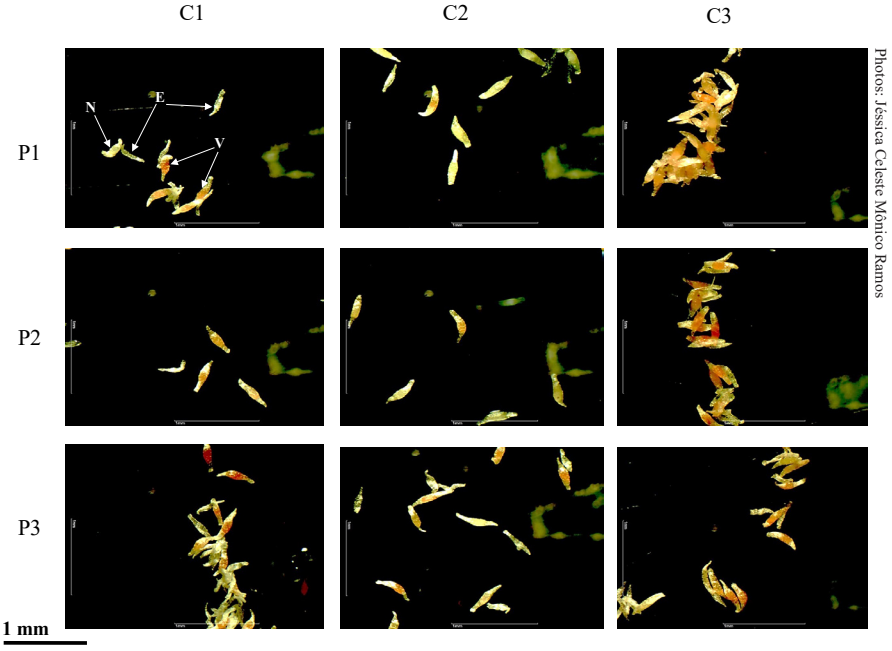

Figure 4. *Cattleya wittigiana* (Barb. Rodr.) van den Berg seeds, as a function of preconditioning [P1: 3 mL of distilled water for 24 h; P2: 3 mL of sucrose solution (10 %) for 24 h; and P3: no soaking (control)] and conditioning [C1: water bath (40 ± 0.5 ºC); C2: oven (40 ± 2 ºC); and C3: ambient temperature (25 ± 2 ºC)], in the tetrazolium test. Examples of staining classes considered in the interpretation of the test: fully stained embryos (from light pink to deep carmine red, viable), partially stained embryos, colorless embryos, and seeds without embryos (classes considered non-viable). V, N, and E: viable, non-viable, and empty seed, respectively.

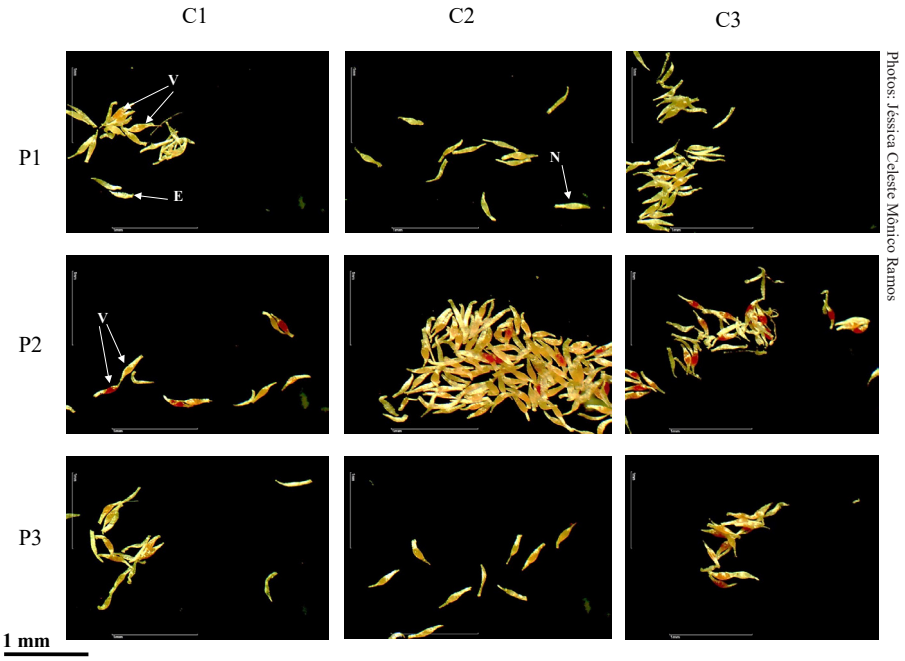

Figure 5. *Encyclia argentinensis* (Speg.) Hoehne seeds, as a function of preconditioning [P1: 3 mL of distilled water for 24 h; P2: 3 mL of sucrose solution (10 %) for 24 h; and P3: no soaking (control)] and conditioning [C1: water bath (40 ± 0.5 ºC); C2: oven (40 ± 2 ºC); and C3: ambient temperature (25 ± 2 ºC)], in the tetrazolium test. Examples of staining classes considered in the interpretation of the test: fully stained embryos (from light pink to deep carmine red, viable), partially stained embryos, colorless embryos, and seeds without embryos (classes considered non-viable). V, N, and E: viable, non-viable, and empty seed, respectively.

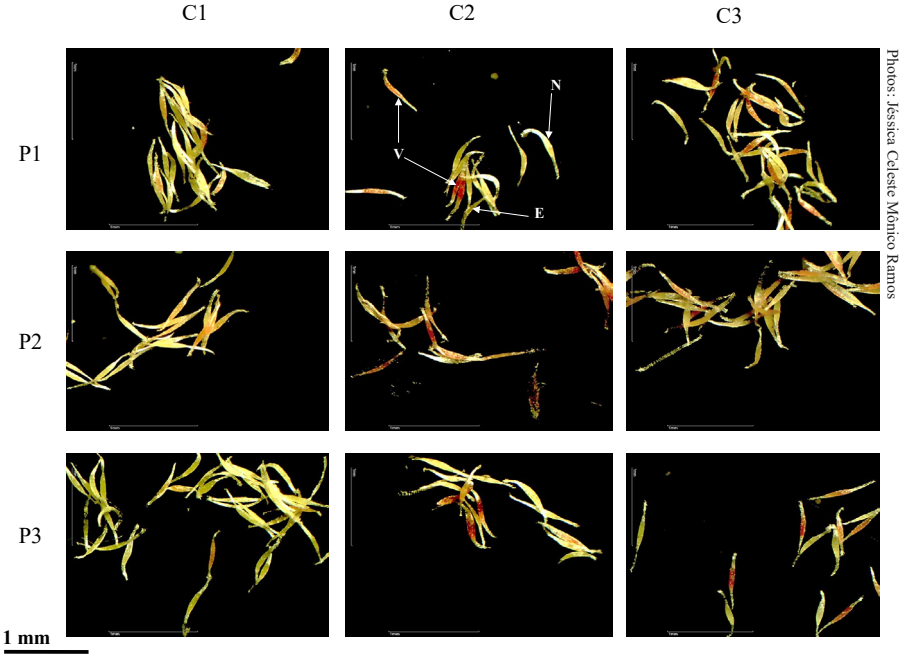

Figure 6. *Ionopsis utricularioides* (Sw.) Lindl. seeds, as a function of preconditioning [P1: 3 mL of distilled water for 24 h; P2: 3 mL of sucrose solution (10 %) for 24 h; and P3: no soaking (control)] and conditioning [C1: water bath (40 ± 0.5 ºC); C2: oven (40 ± 2 ºC); and C3: ambient temperature (25 ± 2 ºC)], in the tetrazolium test. Examples of staining classes considered in the interpretation of the test: fully stained embryos (from light pink to deep carmine red, viable), partially stained embryos, colorless embryos, and seeds without embryos (classes considered non-viable). V, N, and E: viable, non-viable, and empty seed, respectively.

Table 1. Seed dimensions (including seed coat) of four Orchidaceae species used in the tetrazolium test optimization.

Species	n	Length (mm)	Width (mm)	Length:width ratio
Cattleya forbesii	20	0.60 ± 0.08	0.09 ± 0.01	6.8:1
C. wittigiana	20	0.44 ± 0.07	0.10 ± 0.01	4.4:1
Encyclia argentinensis	20	0.40 ± 0.04	0.08 ± 0.01	5.1:1
Ionopsis utricularioides	20	0.73 ± 0.09	0.06 ± 0.01	11.5:1

Values are mean ± standard deviation. n: number of seeds measured per species.

and greatest width, showed the closest agreement (96.45 *vs*. 72.20 %). This pattern suggests that seed morphometry may influence both tetrazolium solution diffusion and the accuracy of embryo staining interpretation, contributing to the overestimation observed in species with more elongated seeds.

In Orchidaceae, the seed coat plays a dual role: mechanical protection and hydration control; in epiphytic species, it tends to present fissures that facilitate exchange; whereas, in terrestrial species, it may be more restrictive (Arditti 2022, Lee & Yeung 2023). This physical barrier is often attributed to the presence of suberin, a waxy, hydrophobic substance that limits the diffusion of water and solutes (Shukla & Barberon 2021, Chen et al. 2022). The minute size of both seed and embryo further limits the surface area available for solution uptake and reagent penetration, amplifying the effect of testa permeability differences among species (Pradhan et al. 2022). Thus, the variation observed in viability estimated by the tetrazolium test among the four studied species may be related to the interplay of structural and functional differences in the seed coat that affect hydration and reagent penetration.

In general, prior imbibition in distilled water or 10 % sucrose solution helped to optimize the visualization of viable seeds during the room-temperature incubation. Physiologically, this preconditioning process allows for gradual tissue hydration and cell membrane reorganization following desiccation and storage. This tissue preparation is fundamental to reducing imbibition damage and improving the infiltration and diffusion of the tetrazolium solution into embryonic tissues, resulting in clearer and more homogeneous staining (Pradhan et al. 2022, Mercado & Delgado 2023). Furthermore, hydration activates the metabolism of dehydrogenase enzymes, which are essential for reducing tetrazolium salt into red formazan (Mercado & Delgado 2023). At room temperature, no statistical difference was detected between distilled water and sucrose for *C. forbesii*, *C. wittigiana*, and *I. utricularioides*, indicating that both preconditioning agents are equally effective in these species. For *E. argentinensis*, however, the sucrose preconditioning resulted in statistically higher viability estimates, suggesting a species-specific response that may be related to osmotic conditioning or to an exogenous carbon source that favors metabolic readiness (López et al. 2025). These findings corroborate studies by Mercado et al. (2020) and Mercado & Delgado (2023), who demonstrated that preconditioning, whether with distilled water or sucrose, improves the tetrazolium staining quality in orchids of the genera *Pleurothallis*, *Spathoglottis*, and *Epidendrum*, although the optimal agent may vary among species.

Regarding thermal incubation conditions, the superiority of room temperature (25 ± 2 ºC) for tetrazolium reading accuracy for all four species indicates that this condition favors a more consistent and interpretable staining, whereas incubation at 40 ºC (water bath or oven) may increase the response variability depending on preconditioning and species. Notably, the marked difference between water bath and oven incubation likely reflects not only the mode of heat transfer, but also differences in thermal stability. The oven exhibited a greater temperature fluctuation (up to 43.3 ºC), which may have caused the partial denaturation of dehydrogenase enzymes. In contrast, the water bath maintained stable temperatures (40 ± 0.5 ºC) within the recommended range for tetrazolium testing (França-Neto & Krzyzanowski 2022). In orchid seeds, the embryo is extremely reduced and possesses low thermal mass; thus, elevated temperatures may induce excessively rapid reaction kinetics, generating heterogeneous staining patterns that hinder interpretation (Oikonomidis & Thanos 2024). The efficacy observed at room temperature here is consistent with studies on *Miltonia flavescens* (Soares et al. 2021) and *Cattleya nobilior* (Ribeiro et al. 2021), reinforcing that thermal adjustment is a key factor for assay reliability.

Moreover, the reduced morphology imposes methodological limitations. The minute size of seeds hinders internal embryo evaluation, and classification necessarily relies on the external staining pattern (Hosomi et al. 2017, Pradhan et al. 2022). In the present study, seeds with uniformly stained embryos, regardless of intensity, were classified as viable. This inclusion of faintly stained embryos likely contributed to the overestimation of %VS relative to %G, as the tetrazolium test detects dehydrogenase activity, indicating metabolic potential, whereas germination requires a coordinated sequence of developmental events that depends on seed vigor and suitable culture conditions (França-Neto & Krzyzanowski 2019, Pradhan et al. 2022). Notably, the discrepancy is influenced by the classification criterion adopted. Studies using more inclusive thresholds, such as pink staining (Hosomi et al. 2017) or more than half of the tissue stained (Mercado et al. 2020), also reported tetrazolium values exceeding germination rates, consistent with our findings. Additional factors such as physiological dormancy or specific *in vitro* culture requirements may further explain why metabolically viable seeds fail to develop into protocorms (Whitehouse et al. 2020, Oikonomidis & Thanos 2024, Silva et al. 2025). Therefore, the tetrazolium test should be interpreted strictly as an indicator of metabolic viability, and the choice of classification criterion must be considered when comparing tetrazolium estimates with germination data.

Given the demand for rapid, reproducible methods for monitoring stored lots, the proposed protocol has potential for use in seed banks, enhancing the reliability of viability estimates for neotropical species and supporting decision-making in *ex situ* conservation and seedling production programs. Furthermore, the observed discrepancy between estimated viability and germination reinforces the need to employ the tetrazolium test strictly as an indicator of viability and to complement it with germination assays when the objective is to infer germination potential under specific culture conditions.

In summary, the results demonstrate that optimizing the tetrazolium test in Orchidaceae depends on the species and the preconditioning and incubation conditions, reflecting the influence of the seed coat and embryonic physiological behavior. The combination of preconditioning and incubation at room temperature offers a technically and operationally simplified solution for germplasm bank monitoring; although, for *E. argentinensis*, sucrose yielded the highest estimates. This distinction is crucial for conservation management, indicating that the refinement of viability protocols must be accompanied by an understanding of the ecophysiological requirements of each species for the full expression of their *in vitro* vigor.

CONCLUSIONS

1. The tetrazolium test was successfully optimized for *Cattleya forbesii*, *C. wittigiana*, *Encyclia argentinensis*, and *Ionopsis utricularioides*, confirming that preconditioning and incubation temperature modulate metabolic reactivation and reagent diffusion in orchid seeds, directly influencing staining intensity, clarity, and test interpretability;
2. Incubation at room temperature (25 ± 2 ºC) provided the most consistent viability estimates across all four species;
3. Distilled water and 10 % sucrose solution yielded comparable results for *C. forbesii*, *C. wittigiana*, and *I. utricularioides*, whereas sucrose preconditioning resulted in the highest viability estimate for *E. argentinensis* (97.79 %);
4. Preconditioning in distilled water combined with room-temperature incubation is recommended as a simplified, low-cost protocol for germplasm bank monitoring of the species studied, with 10 % sucrose as an alternative for *E. argentinensis*.

ACKNOWLEDGMENTS

This study was partly financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (Capes; Finance code 001). The authors are also grateful to the Fundação de Apoio ao Desenvolvimento do Ensino, Ciência e Tecnologia do Estado de Mato Grosso do Sul (Fundect, grant nº 52873.813.11885.12092025) for financial support, and Universidade Federal da Grande Dourados (UFGD) for technical support.

REFERENCES

ARDITTI, J. Orchids: discoverers and firsts. *South African Journal of Botany*, v. 64, n. 27, p. 815-848, 2022.

CENTRO NACIONAL DE CONSERVAÇÃO DA FLORA (Cncflora). *Cattleya wittigiana (Barb. Rodr.) van den Berg in lista vermelha da flora brasileira*. 2012a. Available at: https://cncflora.jbrj.gov.br/ficha/65303. Access on: Jan. 16, 2026.

CENTRO NACIONAL DE CONSERVAÇÃO DA FLORA (Cncflora). *Ionopsis utricularioides (Sw.) Lindl. in lista vermelha da flora brasileira*. 2012b. Available at: https://cncflora.jbrj.gov.br/ficha/11759. Access on: Jan. 16, 2026.

CHEN, A.; LIU, T.; WANG, Z.; CHEN, X. Plant root suberin: a layer of defence against biotic and abiotic stresses. *Frontiers in Plant Science*, v. 13, n. 1, e1056008, 2022.

CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES OF WILD FAUNA AND FLORA (CITES). *Cattleya wittigiana (Barb.Rodr.) van den Berg in appendix II*. 2023. Available at: https://www.speciesplus.net/species#/taxon_concepts/56640/legal. Access on: Jan. 15, 2026.

FAY, M. F.; ANDRIAMAHEFARIVO, L.; BACHMAN, S. P.; BROWN, M. J. M.; CALEVO, J.; CAMPBELL, T.; CHARITONIDOU, M.; DIXON, K. W.; DROISSART, V.; FOREST, F.; HINSLEY, A. E.; KUMAR, P.; BRETON, T. L.; RAJAOVELONA, L. R.; RAMANDIMBISOA, B.; RANKOU, H.; SCHUITEMAN, A.; SHARMA, J.; SIMO-DROISSART, M.; SMIDT, E. C.; STÉVART, T.; SVAHNSTROM, V. J.; VERLYNDE, S.; ZIMMER, H. C. How threatened are orchids?: a review of the state of play and identification of gaps and priorities. *Biodiversity and Conservation*, v. 34, n. 14, p. 5075-5115, 2025.

FERREIRA, D. F. Sisvar: a computer analysis system to fixed effects split plot type designs. *Revista Brasileira de Biometria*, v. 37, n. 4, p. 529-535, 2019.

FLORA E FUNGA DO BRASIL (FFB). *Encyclia argentinensis (Speg.) Hoehne in Jardim Botânico do Rio de Janeiro*. 2021a. Available at: https://floradobrasil.jbrj.gov.br/FB31839. Access on: Jan. 15, 2026.

FLORA E FUNGA DO BRASIL (FFB). *Ionopsis utricularioides (Sw.) Lindl. in Jardim Botânico do Rio de Janeiro*. 2021b. Available at: https://floradobrasil.jbrj.gov.br/FB11759. Access on: Jan. 15, 2026.

FLORA E FUNGA DO BRASIL (FFB). *Orchidaceae in Jardim Botânico do Rio de Janeiro*. 2026. Available at: http://floradobrasil.jbrj.gov.br/. Access on: July 5, 2026.

FRANÇA-NETO, J. B.; KRZYZANOWSKI, F. C. Tetrazolium: an important test for physiological seed quality evaluation. *Journal of Seed Science*, v. 41, n. 3, p. 359-366, 2019.

FRANÇA-NETO, J. B.; KRZYZANOWSKI, F. C. Use of the tetrazolium test for estimating the physiological quality of seeds. *Seed Science and Technology*, v. 50, n. 1, p. 31-44, 2022.

FRANCISQUETI, A. M.; MARIN, R. R.; HENGLING, M. M.; HOSOMI, S. T.; PRITCHARD, H. W.; CUSTODIO, C. C.; MACHADO-NETO, N. B. Orchid seeds are not always short lived in a conventional seed bank! *Annals of Botany*, v. 133, n. 7, p. 941-952, 2024.

HOSOMI, S. T.; SOUZA, T. B.; CUSTÓDIO, C. C.; MACHADO-NETO, N. B. Refining the tetrazolium test for evaluation of *Cattleya labiata* and *C. tigrina* seeds viability. *Australian Journal of Crop Science*, v. 11, n. 10, p. 1320-1326, 2017.

HWANG, J. E.; PARK, H. B.; JEON, D. Y.; PARK, H. J.; KIM, S.; LEE, C. W.; KIM, Y. J.; YOON, Y. J. Effect of different basal media and organic supplements on *in vitro* seedling development of the endangered orchid species *Dendrobium moniliforme* (L.) Swartz. *Plants*, v. 13, n. 19, e2721, 2024.

INTERNATIONAL UNION FOR CONSERVATION OF NATURE (IUCN). *The red list of threatened species*. 2025. Available at: https://www.iucnredlist.org/search?query=orchids&searchType. Access on: Jan. 16, 2026.

JOLMAN, D.; BATALLA, M. I.; HUNGERFORD, A.; NORWOOD, P.; TAIT, N.; WALLACE, L. E. The challenges of growing orchids from seeds for conservation: an assessment of asymbiotic techniques. *Applications in Plant Sciences*, v. 10, n. 5, e11496, 2022.

LEE, Y.; YEUNG, E. C. The orchid seed coat: a developmental and functional perspective. *Botanical Studies*, v. 64, e27, 2023.

LÓPEZ, Y. O. B.; CORCHADO, J. Á. L.; SERRANO, H.; GARCÍA-SUÁREZ, M. D. Seed viability and morphology evaluation of three Mexican orchid species. *Journal of Agriculture and Life Sciences*, v. 12, n. 1, p. 10-17, 2025.

MERCADO, S. A. S.; CALEÑO, J. D. Q.; ROZO, L. Y. M. Improvement of the methodology of the tetrazolium test using different pretreatments in seeds of the genus *Epidendrum* (Orchidaceae). *Journal of Seed Science*, v. 42, n. 1, e202042013, 2020.

MERCADO, S. A. S.; DELGADO, Y. A. P. Management of different pretreatments to optimize seed viability of orchids. *Acta Agronómica*, v. 72, n. 4, p. 346-352, 2023.

NAKUM, P.; SINGH, B.; SOLANKI, H. The significance of seed banks: a review of techniques and technologies. *World Journal of Advanced Research and Reviews*, v. 25, n. 2, p. 2401-2407, 2025.

NONGDAM, P.; BELESKI, D. G.; TIKENDRA, L.; DEY, A.; VARTE, V.; EL MERZOUGUI, S.; PEREIRA, V. M.; BARROS, P. R.; VENDRAME, W. A. Micropropagation

of orchids using semiconventional solid and temporary immersion systems: a review. *Plants*, v. 12, n. 5, e1136, 2023.

OIKONOMIDIS, S.; THANOS, C. A. Orchid embryo-to-seed (E:S) ratio as an indicator of germination behaviour and its ecological implications. *Seed Science Research*, v. 34, n. 4, p. 218-227, 2024.

PRADHAN, N.; FAN, X.; MARTINI, F.; CHEN, H.; LIU, H.; GAO, J.; GOODALE, U. M. Seed viability testing for research and conservation of epiphytic and terrestrial orchids. *Botanical Studies*, v. 63, e3, 2022.

RIBEIRO, L. M.; SORGATO, J. C.; SOARES, J. S.; RAMOS, J. C. M. Methodology of the tetrazolium test for identifying viable seeds of orchids of the genus *Cattleya* native to the Brazilian Cerrado. *Semina: Ciências Agrárias*, v. 42, n. 3, p. 1351-1360, 2021.

SHUKLA, V.; BARBERON, M. Building and breaking of a barrier: suberin plasticity and function in the endodermis. *Current Opinion in Plant Biology*, v. 64, n. 1, e102153, 2021.

SILVA, J. C.; SILVA, J. J.; GOMES, R. A.; CRUZ, C. R. P.; DANTAS, B. F. Seed viability of *Commiphora leptophloeos* (Mart.) J. B. Gillet (Burseraceae) assessed by the tetrazolium test. *International Journal of Plant Biology*, v. 16, n. 4, e139, 2025.

SMIDERLE, O. J.; OLIVEIRA, J. M. F.; SOUZA, A. G. Protocolo para conservação de longo prazo para sementes de orquídeas: uma revisão de literatura e proposta metodológica. *Revista de Gestão e Secretariado*, v. 16, n. 8, e5138, 2025.

SOARES, J. S.; RAMOS, J. C. M.; SORGATO, J. C.; RIBEIRO, L. M.; REIS, L. C. *Brassavola tuberculata* Hook.: *in vitro* growth and *ex vitro* establishment as a function of the micropropagation system and sucrose. *Brazilian Journal of Biology*, v. 83, e270892, 2023.

SOARES, J. S.; SORGATO, J. C.; RIBEIRO, L. M.; RAMOS, J. M. C. Seed viability test of orchids native to the Brazilian Savanna. *Pesquisa Agropecuária Tropical*, v. 51, e67069, 2021.

WHITEHOUSE, K.; HAY, F. R.; LUSTY, C. Why seed physiology is important for genebanking. *Plants*, v. 9, n. 5, e584, 2020.

WORLD FLORA ONLINE (WFO). *Ionopsis utricularioides (Sw.) Lindl. snapshots of the taxonomy*. 2025. Available at: www.worldfloraonline.org/. Access on: Jan. 15, 2026.