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# Seed Viability and Morphology Evaluation of Three Mexican Orchid Species

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**Abstract** Here we studied some aspects of morphology and seed viability of three Mexican orchid species with different life forms, two epiphytes *Cuitlauzina pendula* and *Laelia anceps* and one terrestrial *Epidendrum radicans,* evaluating its viability with a modification of triphenyl tetrazolium chloride (TTC) test, applying pretreatment of 10% sucrose. Initial red staining was observed on viable seeds *Laelia anceps* 88.89%, *Cuitlauzina pendula* 44.44% and *Epidendrum radicans* 24.44%, which diminished after 30-, 60-days storage. Orchid seed morphology was measured in length, width and volume, with Toupview software. Seeds from the two epiphytes showed similar size: testa 0.48- and 0.45-mm length, 0.18; 0.11mm width, its embryo 0.26, 0.23mm length 0.16; 0.09-mm width, embryo volume 0.856; 0.684 $mm^3$. The terrestrial *Epidendrum radicans* had the largest size testa 0.24 length 0.48 mm width: small embryo 0.60 mm. The use of viability bioassays might help on orchid conservation and on the *in vitro* germination procedures.

**Keywords** Orchidaceae, Cuitlauzina pendula, Laelia anceps, Epidendrum radicans, orchid seeds, seed viability, morphology

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## 1. Introduction

Orchidaceae is one of the largest flowering plant families in the world with 27 801 species (Chase et al., 2015; García-Suárez et al., 2023). Almost 1300 species of 170 genera have been found growing wild in Mexico; up to 40% are endemic species (Gutiérrez-Rodríguez et al., 2022, Laguna-Cerda et al., 2022). Orchid seeds are inside a capsule or seed pod. They are extremely tiny and light in weight, produced in high amounts and are wind dispersed. Inside, the embryo is protected by a membranous testa surrounded by space air, and they usually have no endosperm (Arditti & Ghana, 2000; Yeung, 2017; Yung & Yeung, 2023). Seeds measure from 0.05 up to 6.0 mm in length and morphologically variable form filiform, fusiform, ellipsoidal, scobiform or clavatea (Arditti et al, 1979; Molvray & Kores, 1995; Gallo et al 2016; Diantina et al., 2020). Beside these basic forms, variations can be found among populations, due to genetic differences and plant history (Arditti *et al*., 1979, 1980; Healey et al., 1980; Barbour et al., 1999; Arditti & Ghana, 2000; Barthlott et al., 2014). Due to the human extraction, natural populations are endangered, and it has been urged that investigations of its propagation through germination and plantlet survival must be intensified, as native species are considered in high risk of extinction (Ruíz et. al., 2008; Barthlott et al., 2014; García-Suárez et al., 2023).

One of the first steps to contribute to understand orchid seed biology is to evaluate their germinative capability and seed viability after storage (Pritchard, 1985; Pritchard & Nadarajan, 2008; Diantina et al., 2022). The lack of endosperm causes a complex seed germination, and sometimes seeds might not be viable. It is therefore necessary to determine the embryo viability to evaluate seeds and improve their *in vitro* germination to contribute to develop a strategy for orchid propagation, plant research and conservation (Harrison & Arditti, 1972; Arditti & Ernst, 1993; Arditti & Ghana, 2000; Ruíz et al., 2008; Pradhan et al., 2022; García Suárez et al., 2023).

Various methods have been developed to assess seed viability including chemical staining with indigo carmine (Salazar & Gélvez, 2015), fluoresceine diacetate (FDA) (Pritchard, 1985; Wood et al., 2003) and triphenyl tetrazolium chloride (TTC) (Lakon, 1949; Vujanovic et al., 2000; Salazar & Gélvez, 2015). The TTC method was initially developed by Lakon (1949) and is based on the reduction of the colorless 2,3,5 triphenyl tetrazolium chloride into a red color formazan derivative produced by live cells. The TTC reduction and formazan production are associated to the activity of mitochondrial dehydrogenase activity during cellular respiration (Canuto, 2012). This test has been applied to different orchid seeds (Singh, 1981; Hosomi et al., 2012) with highly variable results. Here we evaluated some aspects of seed morphology and applied a modified TTC test to evaluate seed viability of three ornamental Mexican orchid seeds: the epiphytes *Cuitlauzina pendula* and *Laelia anceps* and the terrestrial *Epidendrum radicans*, contributing to orchid seed research and native orchid conservation.

## 2. Materials and Methods

To set up and evaluate seed viability, two ripe mature capsules before dehiscence from *C. pendula, L. anceps* and *E radicans* individuals obtained from a particular collection were used. Seeds from dehiscent capsules were kept on paper bags and stored at room temperature until use.

We used a modification of the classical TTC viability tests described by Lakon (1949) by soaking seeds in a 10% (w/v) sucrose solution (Hosomi et al., 2011) added with 0.1% (v/v) tween 20 at room temperature and complete darkness overnight. Seeds were stained by imbibition in a 1% (w/v) TTC (Sigma Chem Co. St. Louis, Mo) aqueous solution. maintained at room temperature for a 24 h period before microscopic evaluation. To see the effect of storage and the usefulness of the method, three replicates with 100 seeds of each capsule and species, were stored for 0, 30, 60 days. Seed viability determinations were made in a Zeiss light microscope at 400 and 1000 diameters. Viable seeds show the red stain embryos differing from the dark brown non-viable or embryo-lacking seeds.

Morphological characteristics of *C. pendula*, *L. anceps* and *E. radicans* seeds were obtained by measuring 100 seeds from each species. Longitudinal and transversal axis of either the seed or embryo were obtained by direct measurement with the ToupView Software (ToupTec, v 3.7), excluding the suspensor cells. Data were used to obtain both seed and embryo volume length and width by using the formula reported by Arditti et al., (1980)

$$V_s = 2\left[\left(\frac{A}{2}\right)^2\left(\frac{1}{2}L\right)\left(\frac{\pi}{3}\right)\right]$$

Where

$V_s$ = seed volumen ($mm^3$)

$V_e$ = embryo volumen ($mm^3$)

$L$ = seed or embryo length ($mm$)

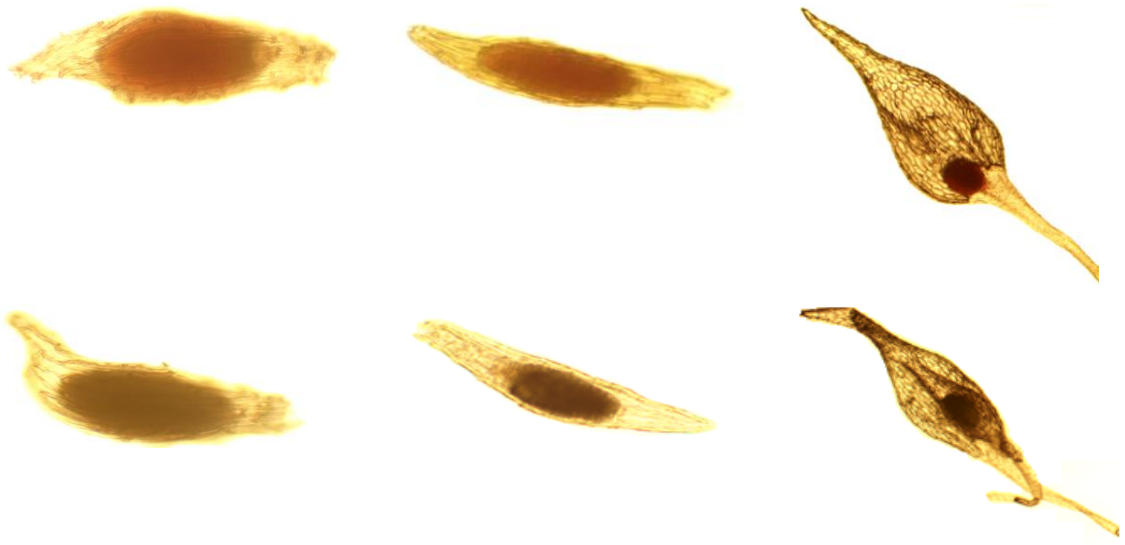
$$V_e = \left(\frac{1}{2}A\right)^2\left(\frac{1}{2}L\right)\left(\frac{4\pi}{3}\right)$$

$A =$ seed or embryo width ($mm$)

### 2.2 Viability Evaluation of Stored Seeds.

It is well documented that seed viability is affected by storage. To ensure precise and reliable evaluation, highly reproducible and controlled technique should be used. The classical TTC technique developed by Lakon (1949) has been modified and optimized for several seeds indicating that the characteristic of each species is not uniform and should be modified in both components as well as in incubation times to give reproducible results (Hosomi et al., 2011; Salazar et al., 2020). Some major additions to the basic TTC stain are the use of detergent that increase the TTC diffusion into the embryo where the formazan formation indicate the presence of living cells (Hosomi et al., 2017). Also, to maintain the cell viability and increase the metabolic activity, one major modification is the addition of sucrose or glucose that promote the increase in mitochondrial activity for a formazan production (Hosomi et al., 2012; Salazar et al., 2020).

As we have shown for orchid seeds, the incubation period in an aqueous TTC solution added with Triton-X100 and sucrose for a 24 h period gives a reproducible and stable stain. As can be seen in Figure 1, in both epiphytic as well as in terrestrial orchids, viability ranges in freshly obtained seeds are rather homogeneous. Viability index from *E. radicans* has the lowest viability index whereas seeds from *L. anceps* are mostly viable. The uniformity of the data obtained rise the possibility to use a homogeneous tool to evaluate the effect of storage on the viability of orchid seeds.



**A** B C

**Figure 1.** Morphological characteristic of viable and no viable seeds of *C. pendula*, L. anceps and *E. radicans* obtained with the modified TTC method. Viable (upper) and nonviable (lower panel) seeds of *C. pendula (A, 400X)) L. anceps,* (B, 1000X) and *E. radicans (C, 400X)*. Orchid seeds consist of a seed coat with an embryo and an internal air space volume between the seed coat and embryo. As is characteristic of orchids, the lack of endosperm is evident. The bigger air space was found in the terrestrial orchid *E. radicans.*

As has been reported for several species, there is a decrease in seed viability during storage (Machado-Neto & Castillo Custodio, 2005). By using this standardized test, we found that a there was a differential seed viability decrease in the terrestrial *E. radicans* seeds when compared to those obtained for the epiphytic orchids (Table 1).

### 2.3 Seed Size

Seed and embryo dimension of *C. pendula*, *L. anceps* and E. *radicans* were obtained by direct microscopic measurements of processed seeds with ToupView software previously calibrated for accuracy. Primary length and width of both testa and embryo data were then used to obtain the embryo volume and testa ratio. The largest seed is the terrestrial orchid

*E radicans* and the smallest one is the epiphytic *L. anceps*. C. pendula have the largest embryo to testa ratio whereas *E. radicans* embryo has the smallest (Table 2).

### 2.4 Statistical Analysis

Data were statistically evaluated by using the ANOVA facility of the GraphPad Prism software (V.10.1.2). Statistical differences were taken at $p<0.005$.

## 3. Discussion

Seed size is quite diverse as has been reported by Arditti & Ghana (2000). The size determined in this study is quite homogeneous. Whereas Arditti and Ghana (2000) show a 1.31 ±1.41 mm for the *Epidendrum* genus, we determined a more homogeneous 3.24 ± 0.55 seed size for *Epidendrum radicans*. Although the study of Arditti and Ghana is quite meticulous, additional data on seed width or embryo characteristics are lacking for this genus. We have used their formula to obtain the complementary characteristics and extended to two other orchids. The embryo to seed ratio provides information about the space between the testa and the embryo that has been identified as "air" by Arditti & Ghana (2000) and other authors. As orchids lack endosperm or storage-containing cells, one possibility is that orchid seed embryos have proteins or lipids that could help in their symbiotic germination process (Zhao et al., 2025).

**Table 1. Changes in viability percentages of seed orchids used in this study.**

| Days | 0 | 30 | 60 |
|---|---|---|---|
| C. pendula | 44.44 ± 1.92 | 15.36 ± 1.56 | 13.89 ± 3.11 |
| L. anceps | 88.89 ± 5.09 | 42.5 ± 3.50 | 32.53 ± 4.87 |
| E. radicans | 24.44 ± 1.92 | 8.38 ± 0.30 | 4.37 ± 2.25 |

Seeds from mature capsules were maintained under the conditions described in the text. At least 100 individual seeds were evaluated with the modified Lakon viability test at selected times. Data are mean ± mean standard deviation of 3 independent experiments.

**Table 2. Dimensions and proportions of the seed orchids analyzed**

| | Length (mm) | Width (mm) | Testa volume (Vt) | Length (mm) | Width (mm) | Embryo volume (Ve) | Ratio (Ve/Vt) |
|---|---|---|---|---|---|---|---|
| C. pendula | 0.48 ±0.06 | 0.18 ± 0.02 | 0.0041[a] | 0.26 ±0.03 | 0.16 ± 0.02 | 0.0035[a] | 0.856[a] |
| L. anceps | 0.45 ±0.02 | 0.11 ± 0.01 | 0.0014[b] | 0.23 ± 0.02 | 0.09 ± 0.01 | 0.0010[b] | 0.684[b] |
| E. radicans | 3.24 ± 0.55 | 0.48 ± 0.07 | 0.1954[c] | 0.39 ± 0.08 | 0.24 ±0.04 | 0.0118[c] | 0.06[c] |

Seeds were measured under the light microscope after the modified Lakon viability test by a calibrated Top View software. Testa and embryo volumes were calculated according to the formulas described in the main text. Data are from at least 100 seeds and 3 independent experiments. Different superscripts in each column indicate statistically different by ANOVA test ($p<0.05$) obtained by using Prisma software.

As shown in Table 2, even though *E. radicans* has the largest seed, the embryo to seed ratio is the lowest indicating some fragility that it correlates to the higher loss in viability (Table 1), this could also be associated to the terrestrial life form of *E. radicans*. Both *C. pendula* and *L. anceps*, the epiphytic orchids have the mean size and embryo dimensions. Their embryo to seed ratio is more than 60% and the time-associated loss of viability. In both species, the more dramatic loss of viability in the first 30 days where viability decreases more than 50% but this decrease is lower for the second 30 to 60 days period being 9.57% for *C. pendula* and 23.46% for *L. anceps* that has the smallest embryo (Table 2).

As orchid seeds embryo lack endosperm, the 10% sucrose pretreatment to seeds before TTC test enhanced the activity of embryo metabolism (Arditti et al., 1980; Hosomi et al., 2011; Hosomi et al., 2012; Hosomi et al., 2017). A practical derivative of this study is that some equivalent pretreatment might as well be applied before the *in vitro* germination bioassays can be carried out. The modified TTC test clearly distinguished viable embryos from not viable but most importantly, it can detect embryo-lacking seeds. The lack of embryo has been reported for several orchid species, Santos et al., (2006) attributed embryo absence in *Laelia albida* to eco-physiological aspects or genetic variability; Ávila and co-workers (2009) indicate that in *Laelia speciosa* embryo absence is due to gamete incompatibility when mixed crossing occurs. Low embryo viability is often frequent in Orchidaceae family, besides it needs a special in *vitro* germination which is a complex method, where the physiological quality of seeds might allow the success in propagation programs (Hosomi, et al., 2011), and contribute to promote its conservation (Thornhill & Koopowitz, 1992; Vujanovic et al., 2000; Wood et al., 2003; Salazar & Gélvez, 2015.). The use of a simple and highly reproducible viability tests avoids the seed culture of empty or vain seeds like in *Laelia speciosa* (Aguilar-Morales & López Escamilla, 2013).

Hosomi et al., (2012) suggested that sucrose pretreatment helps to activate seed metabolism varying this activity between species. The epiphytic *Laelia anceps* had the highest viability percentage among the studied species. Although the seed viability is low compared to that determined by *Cattleya tigrina* (97%) and 98% in Cattleya *walkeriana* (Hosomi et al., 2011), 89.9% for *Bletilla formosana* (Rung et al., 2013), or 72.8% of *Comparettia falcata* (Karol et al., 2015), the modified method gives additional information about structural seed and embryo characteristics.

Orchid seeds present a similar behavior to other orthodox seeds. Embryo physiology is affected by storage conditions (Pritchard & Prendergast, 1989; Pritchard & Seaton, 1993). which contribute to seed banking and *ex situ* conservation efforts (Schofield, et al., 2018). As mentioned by Thornhill & Koopowiths (1992), temperature control and water content have a determinant effect on seed preservation of different orchid seeds (Hay et al., 2008). A strict control and maintenance of storage conditions should be implemented for a successful preservation of different orchid species. Yeung (2017) considers that mature orchid seeds tolerate desiccation due to high levels of abscisic acid, lipid, and protein deposits. Seed viability testing is an important characteristic to consider for orchid propagation and conservation programs, especially for seeds that need special conditions for germination.

Besides storage conditions, ageing is another factor (Stanley & Butler, 1961; Álvarez & Gui, 2006; Hay et al., 2010). *Maxillaria picta* and *Cattleya labiata*, after two and a half years almost lose completely its seed viability (Álvarez and Gui, 2006). Seed storage conditions need to be optimized to ensure seeds remain viable (Hay & Whitehouse, 2017; Magrini et al., 2019; Pradhan et al., 2022).

Diantina et al., (2022) indicate a relationship between embryo size, and the embryo-to-seed volume ratio in relation to the dispersal and germination success where terrestrial orchid seed species have larger internal spaces as an adaptation like in *Epidendrum* or the larger embryos contained in smaller seeds of some epiphytic species like *Dendrobium* or as we have determined with *L. anceps* and *C. pendula*.

Orchid seed banking studies are scarce particularly in Mexico, and standardized data should be based on comparative studies related to storage characteristics (Magrini et al., 2019). Seed storage conservation and longevity vary within species and are not consistent; the use of accurate seed viability tests enhances success for *ex situ* conservation programs and any aspect that demonstrate how to handle orchid seeds, contribute to protect endemic and endangered orchid species and give important data in storage behavior, seed quality, viability, longevity and possible germination. The use of metabolically stimulated technique for seed germination and evaluation like sucrose might improve germination efficiency (Fileti et al., 2021; Francisqueti et al 2024).

## 4. Conclusions

Seed biology studies are needed for orchid conservation efforts to enable a successful asymbiotic or symbiotic seed germination, for their maintenance and propagation. The pretreatment applied before triphenyl tetrazolium on the three orchid seed species tested easily allowed to distinguish viable and unviable seeds and evaluated the viability of seeds during storage. Orchid seed micro-morphometry has been evaluated considering its Importance to species biology, ecology, and conservation as (Diantina et al., 2022).

Orchid seeds of the two epiphytes *Laelia anceps* and *Cuitlauzinia pendula* had similarities in size, testa and embryo; they are smaller than the terrestrial *Epidendrum radicans* orchid seeds. The modified tetrazolium tests prove to be a very reliable technique to evaluate seed viability, and the use of similar solutions could have a preconditioning effect to increase the in vitro germination success. Orchid species need to be propagated as today are considered very

threatened species, due to their habitat degradation and climate change. This work contributes to their conservation and seed banking possibilities (Pradhan et al., 2022)

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