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Management of different pretreatments to optimize seed viability of orchids

Manejo de diferentes pretratamientos para optimizar la viabilidad de semillas de orquídeas

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Abstract

Orchids are one of the most difficult plant families to propagate, so it is important to determine the viability of their seeds. In this sense, the tetrazolium test and *in vitro* germination are the best alternatives to evaluate their germination capacity in a fast, accurate, and effective way. Considering the above, the aim of this research was to evaluate the viability of *Pleurothallis* sp, *Spathoglottis plicata*, and *Stelis* sp. seeds, using different pretreatments to optimize the tetrazolium test. For this, the efficacy of five pretreatments was compared in which the seeds were subjected to different solutions: chlorine 0.1 %, chlorine 0.2 %, deionized water (H_2Od), 10 % (w/v) sucrose solution, and a control (no pretreatment), for a period of 8 minutes. Then, the seeds were submerged in two doses of tetrazolium (0.25 % and 0.5 %) during three exposure times (6, 12, and 24 h), in complete darkness. Subsequently, the basal MS culture medium was used for 55 days to evaluate *in vitro* germination. According to the obtained results, it was observed that the pretreatments with deionized water (H_2Od) and the 10 % sucrose solution presented the best viability indexes for each of the studied species, which correlated with the *in vitro* germination percentages obtained. It should be emphasized that *Stelis* sp. was the species with the best index, with 99 % using the pretreatment of deionized water at a dose of 0.25 % and for a exposure time of 24 hours, and with 100 % viability using the pretreatment of 10 % sucrose solution at a dose of 0.25 % for 24 hours.

Keywords: germination, *Pleurothallis* sp, *Spathoglotti plicata*, *Stelis* sp, tetrazolium.

Resumen

Las orquídeas son una de las familias de plantas con más inconvenientes para su propagación, por lo cual es importante conocer la viabilidad de sus semillas. En este sentido, la prueba de tetrazolio y la germinación *in vitro* son la mejor alternativa para evaluar de forma rápida, exacta y efectiva su capacidad germinativa. Teniendo en cuenta lo anterior, el objetivo de esta investigación fue evaluar la viabilidad de semillas de *Pleurothallis* sp, *Spathoglottis plicata* y *Stelis* sp, utilizando diferentes pretratamientos para optimizar la prueba de tetrazolio. Para esto, se comparó la eficacia de cinco pretratamientos en los cuales las semillas fueron sometidas a diferentes soluciones: cloro 0.1 %, cloro 0.2 %, agua desionizada (H_2Od), solución con sacarosa 10 % (p/v) y un control (sin pretratamiento) durante un lapso de 8 minutos. Posteriormente, las semillas se sumergieron en dos dosis de tetrazolio (0.25 % y 0.5 %) durante tres tiempos de exposición (6, 12 y 24 h), en completa oscuridad. Seguidamente, se empleó el medio de cultivo basal MS durante 55 días para evaluar la germinación *in vitro*. De acuerdo con los resultados obtenidos, fue posible observar que los pretratamientos con agua desionizada (H_2Od) y la solución con sacarosa al 10 % presentaron los mejores índices de viabilidad para cada una de las especies estudiadas, los cuales se correlacionaron con los porcentajes de germinación *in vitro* obtenidos. Conviene subrayar que *Stelis* sp. fue la especie con el mejor índice, con 99 % usando el pretratamiento de agua desionizada a una dosis de 0.25 % y tiempo de exposición de 24 horas, y con un 100 % de viabilidad utilizando el pretratamiento de solución de sacarosa al 10 % con una dosis de 0.25 % durante 24 horas.

Palabras clave: germinación, *Pleurothallis* sp, *Spathoglotti plicata*, *Stelis* sp, tetrazolio.

Introduction

Orchids are regarded as the group of flowering plants with the highest diversity of species (Chase *et al*., 2015). Colombia is one of the countries with the greatest diversity of orchids in the world, it is estimated that there are more than 4275 species distributed in 274 genera across the different regions of the country (Gil Clavijo *et al*., 2022). Of these,1572 are endemic species.

Due to their distinctive characteristics, orchids are highly sought-after plants for decorative purposes by collectors, botanists, and the general public (Bello-Castañeda *et al*., 2023). This makes the commercial production of orchids one of the most significant and profitable economic activities in the global plant nursery industry (Mantovani *et al*., 2018).

The diversity of shapes and colors exhibited by their flowers is a key factor in this commercial success. In addition, they are of great relevance in medicine and horticulture (Kumar, 2022). However, despite all these characteristics, orchids have been subjected to high pressures caused by humans, altering soil composition, increasing nitrogen deposition, and thus contributing to climate change (Díaz-Álvarez *et al*., 2019), which negatively affects their development and reproduction. However, there are not many studies on these plant populations at present, and many of the factors that determine reproductive success in different ecosystems are unknown (Sánchez *et al*., 2017).

One of the most significant reproductive challenges is the low germination rate of their seeds, averaging only 5 % (Vudala *et al*., 2019). Therefore, it is necessary to use tests that help to determine the germination capacity of the seeds, being the tetrazolium test and the germination test the most used (Salazar and Vega, 2017; Buendía *et al*., 2022).

The tetrazolium test is a biochemical assay that relies on the oxidation-reduction of salts to form triphenylformazan, a red pigment that indicates respiratory activity in the seed embryo (Flores *et al*., 2019). Numerous studies have demonstrated the efficacy of this technique in assessing the germination capacity and viability of orchid species (Salazar and Osorio, 2022). This is achieved through the interaction of enzymes involved in the cellular respiration process (Portuguez-García *et al*., 2021). In particular, malic acid dehydrogenase plays a role in this process, reducing the tetrazolium salt upon contact with living tissues and forming formazan, a stable, red, non-diffusible compound (Salazar and Osorio, 2022).

It is therefore evident that the propagation of orchids in their natural habitat is challenging, and the threat of extinction is significant. Furthermore, the decline in the number of specimens is a matter of grave concern (Koene *et al*., 2020). In light of these considerations, it is imperative to employ reliable and effective methods to assess the viability of asymbiotic seed germination.

The tetrazolium test is a valuable tool in this regard, as it offers a reliable indicator of seed viability. However, the test's efficacy may be limited by various factors, such as the dosage and exposure time. Therefore, it is essential to optimize the test parameters to ensure accurate and reliable results, making it an effective marker for determining seed viability (Salazar *et al*., 2020). It is essential to establish methods that provide information about seed viability and physiological potential, ensuring rapid and uniform germination (Salazar *et al*., 2020a). Consequently, this study assesses the viability of asymbiotic germination of seeds from three Colombian orchid species using the tetrazolium test with varying doses and exposure times.

Material and methods

Plant material

For this research, ripe fruits of *S. plicata, Pleurotalis* sp, and *Stelis* sp. (Figure 1) were collected from the municipality of Pamplona, Norte de Santander, Colombia. This municipality has an average temperature of 14.4 °C and an annual rainfall of 921 mm (IDEAM, 2022). After harvesting the ripe fruits, they were stored in Kraft paper bags for 24 hours at room temperature (the time at which the fruit opens naturally). The fruits were labeled with the species name, the geographic coordinates, and the altitude at which they were found, to identify them correctly and avoid confusion with other collected material.

Tetrazolium test

To evaluate the viability of the collected seeds, the syringe method was used (Salazar *et al*., 2020a), where a small amount of seeds was introduced in a sterile 6 mL syringe with a cloth filter, adding each of the pretreatments (deionized water, sucrose 10 %, chlorine 0.1 %, chlorine 0.2 %, and a control without pretreatment) in a span of 8 minutes for each one. This was followed by 4 washes with distilled water. The seeds were then subjected to two doses of tetrazolium (0.25 % and 0.5 %) in complete darkness for the time corresponding to each treatment (6, 12, and 24 h). At the end, the seeds were observed using an Olympus SZ61/SZ51 stereo microscope. Seeds that acquired a red coloration were considered viable, indicating respiratory activity (Salazar *et al*., 2020; Salazar *et al*., 2020b). On the other hand, seeds that showed no coloration were considered non-viable (Figure 2). Finally, the viability results were presented in percentages.

Figure 1. Flowers of the investigated species: (A) *Pleurotalis* sp., (B) *Spathoglottis plicata*, (C) *Stelis* sp.

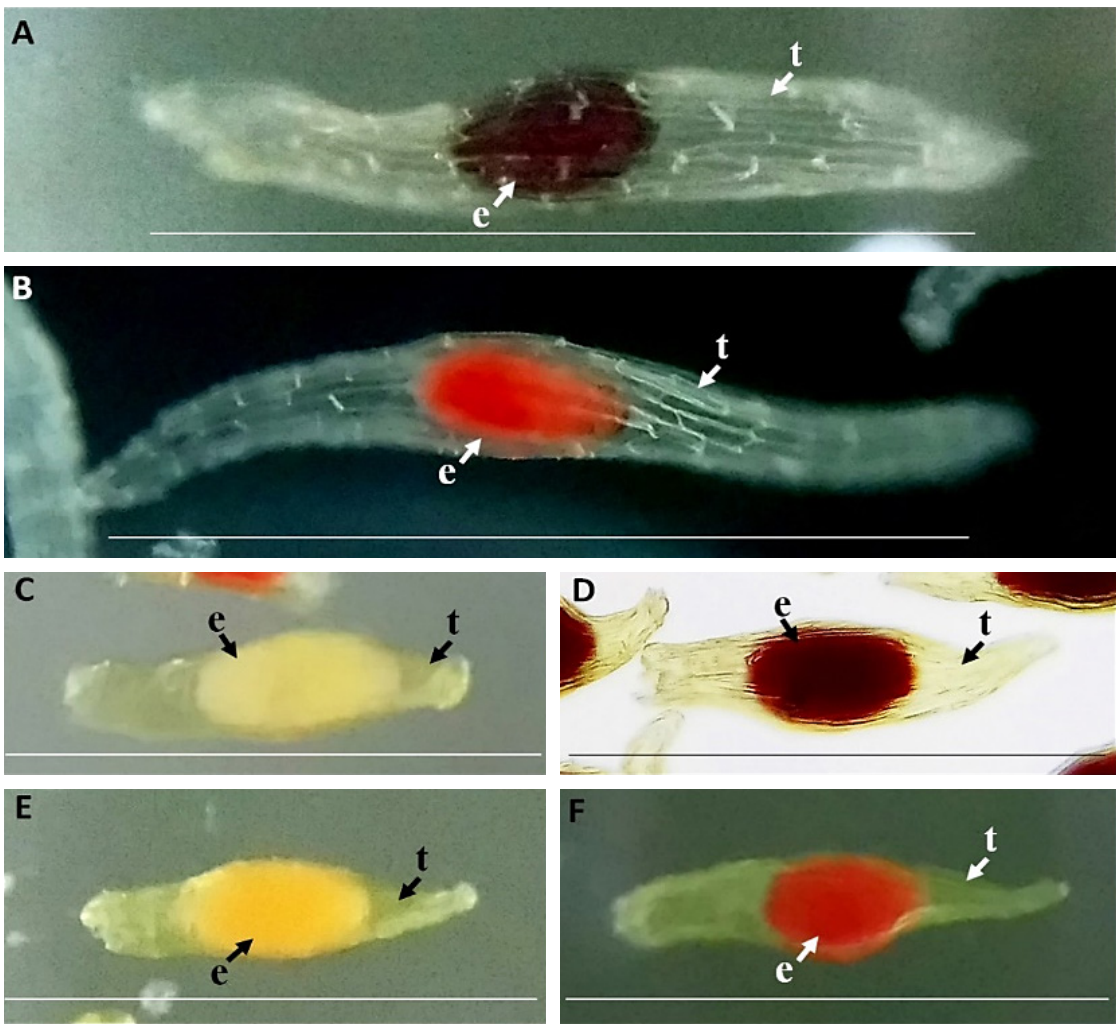

Figure 2. Viability assay using the tetrazolium test. (A and B) Non-viable and viable seed of *S. plicata*. (C and D) Non-viable and viable seed of *Pleurotalis* sp. (E and F) Non-viable and viable seed of *Stelis* sp. Scale bar = 1 mm. t: testa; e: embryo.

Germination test

A total of 100 seeds were sown in Petri dishes (10 replicates per treatment) with MS basal medium, which provides the necessary macro- and micronutrients for plant growth (Murashige and Skoog, 1962). The latter is an essential component for in vitro germination in orchids, as it positively impacts germination rates (Adhikari and Pant, 2019). The germination percentage was determined by examining the seeds under a stereoscopic microscope (SZ61/SZ51), classifying them as germinated when exhibiting embryo expansion and testa coat rupture, following the methodology proposed by Salazar *et al.* (2020a).

Statistical analysis

A randomized experimental design was used for the tetrazolium and germination tests, with 10 replications of 100 seeds each. The results were expressed in percentages. The obtained data were analyzed using an analysis of variance (ANOVA) in order to establish the effect of each treatment on the number of seeds considered viable. Then, Tukey's Honest Significant Difference (HSD) multiple range test was used to determine the means with significant differences at a level of $p < 0.05$. Next, the viability values were compared with the germination percentage to determine differences between the two factors. Finally, statistical analysis was performed using InfoStat Student Version software.

Results and discussion

Viability of *Spathoglottis plicata*

In the results presented in Table 1, the treatment with 0.25 % tetrazolium for 6 h showed maximum viability when sucrose pretreatment was used, with no significant differences compared to the values obtained with the 0.1 % chlorine pretreatment. The lowest viability index was observed with the control treatment. It was also observed that using 0.25 % tetrazolium for 12 h resulted in a 96 % viability rate with sucrose, showing the highest viability indexes for both tetrazolium doses (0.25 % and 0.5 %) across different exposure times (6, 12 and 24 h), except for the treatment with 0.5 % tetrazolium for 6 h with the pretreatment of deionized water ($H_2$0d), which had a value of 25 %, being statistically similar (22.6 %). In contrast, with the 0.5 % tetrazolium treatment for 6 h, the control treatment showed a minimum viability of 2.6 %, followed by the results obtained with the 0.1% chlorine pretreatment, which produced the lowermost viability values across all three exposure times (6, 12 and 24 h) at 13 %, 24 %, and 2.6 % respectively.

These results are similar to those obtained by Salazar *et al.* (2019) in seeds of *Epidendrum barbaricum Hágsater and Dodson*, which showed the highest viability values when using 1 % chlorine and 10 % sucrose at the same dose of tetrazolium and exposure time, with respect to the control treatment.

Sucrose is an effective alternative for increasing seed germination capacity, as it provides a carbohydrate source for seedling development and activates embryo metabolism (Vegas *et al.*, 2019), showing great results.

Viability of *Pleurothallis* sp.

An increase in viability was observed with sucrose and deionized water pretreatments, both showing five maximum viability values that were similar to each other (Table 2). That is, pretreatments with sucrose and H_2Od improved viability in all treatments except for tetrazolium exposure at 0.25 % for 6 h, where the pretreatment with 0.1 % chlorine presented the highest viability value.

These results can be explained by the fact that by hydrating the seeds, the chemical inhibitors found in the testa are removed and softened at the same time, promoting germination (Campos-Hermosillo *et al.*, 2022).

Similarly, the pretreatment with 0.2 % chlorine showed a tendency to decrease viability, presenting the lowest values in all treatments except for the treatment with 0.25 % tetrazolium for 6 h. These results are similar to those obtained by Salazar *et al.*

Table 1. Viability of seeds of *S. plicata*

	Seed viability at different concentrations and exposure periods with tetrazolium					
Pretreatments	**0.25 % - 6 h**	**0.25 % - 12 h**	**0.25 % - 24 h**	**0.5 % -6 h**	**0.5 % - 12 h**	**0.5 % - 24 h**
Control	2.6a	16a	20a	2.6a	16a	22a
Chlorine 0.5 %	91.3c	89c	58a	6.6a	13a	21a
Chlorine 1 %	42b	48b	25a	13a,b	24a,b	2.6b
H_2Od	16a	21a	58a	25c	30a,b	96c
Sucrose	92c	96c	58a	22.6b,c	34b	96c

Different letters represent significant differences ($p < 0.05$).

(2020c) in their research on three species of orchids present in the Andean forest, where chlorine at doses of 0.5 % and 1 % significantly decreased the viability of seeds as the time of exposure to tetrazolium increased, specifically of *Lephantes* sp.

Viability of *Stelis* sp.

According to the results presented in Table 3, a 100 % viability rate was achieved with 0.25 % tetrazolium for 24 h using sucrose pretreatment, producing the highest viability values compared to the other pretreatments. The only exception was in the 0.25 % tetrazolium pretreatment for 6 h, where the control treatment showed the highest viability value. In addition, the use of chlorine at both doses (0.1 % - 0.2 %) produced the lowest viability values across all treatments.

This is likely because chlorine easily penetrates the seed, causing damage to the embryo and potentially leading to chromosomal abnormalities, according to the results obtained by Salazar *et al.* (2020a), where the use of 0.5 % – 1 % chlorine on seeds of *Epidendrum elongatum, E. fimbriatum* and *E. microtum,* reduced the reliability of the tetrazolium test. In addition, it is a highly oxidizing compound (Silva *et al.*, 2019). Overall, the sucrose pretreatment used proved to work well for these orchid species, representing a useful and basic protocol for many plant species.

In vitro germination

A germination test was conducted as an alternative method to evaluate the viability of the three orchid species studied (*S. plicata*, *Pleurotalis* sp., and *Stelis* sp.). This approach serves as an alternative for the propagation of various threatened plant species, helping to preserve their genetic variability (Utami and Hariyanto, 2019).

For *S. plicata*, a 96 % *in vitro* germination rate was achieved. This value correlates directly with the viability rate achieved with $H_2$0d and sucrose pretreatments upon exposure to 0.5 % tetrazolium for 24 h (Table 1). Likewise, viability values where similar when using 0.25 % sucrose pretreatment for 6 and 12 h (96 % and 92 %, respectively), and to a lesser degree with the 0.1 % chlorine pretreatment (91.3 % and 89 %, respectively), under the same tetrazolium concentration and exposure times (0.25 % for 6 and12 h).

For *Pleurotalis* sp. seeds, a germination percentage of 94 % was achieved (Figure 3). Table 2 shows statistically similar values when H_2Od and sucrose were used as pretreatments in both concentrations of tetrazolium (0.25 % - 0.5 %) at the three exposure times (6, 12, and 24 h). Similarly, a comparison of all viability values obtained shows that chlorine at both concentrations (0.1 % and 0.2 %) does not approach the germination percentage, indicating that it reduces viability in the three orchid species studied. However, despite the fact that the results obtained when using chlorine (0.1 % - 0.2 %) were not optimal, this pretreatment can be very useful, since it allows the exchange of water with the external environment by eliminating the seed coat (Catraro *et al.*, 2022).

Finally, *Stelis* sp. seeds achieved a 96 % germination rate. According to the values presented in Table 3, pretreatments with H_2Od and sucrose have a positive

Table 2. Viability of seeds of *Pleurotalis* sp.

	Seed viability at different concentrations and exposure periods with tetrazolium					
Pretreatments	**0.25 % - 6 h**	**0.25 % - 12 h**	**0.25 % - 24 h**	**0.5 % - 6 h**	**0.5 % - 12 h**	**0.5 % - 24 h**
Control	59a	76a	81a	61a	46a	80a
Chlorine 0.5 %	76b	49b	29b	56a	43a	21b
Chlorine 1 %	44a	44b	16c	22b	39a	1.3c
H_2Od	4c	93a	98d	96c	81b	96d
Sucrose	6.7c	87a	98d	94c	81b	97d

Different letters represent significant differences ($p < 0.05$).

Table 3. Viability of seeds of *Stelis* sp

	Seed viability at different concentrations and exposure periods with tetrazolium					
Pretreatments	**0.25 % - 6 h**	**0.25 % - 12 h**	**0.25 % - 24 h**	**0.5 % - 6 h**	**0.5 % - 12 h**	**0.5 % - 24 h**
Control	81a	85a	91a	83a	85a,c	88a
Chlorine 0.5 %	33b,c	11b	6.6b	63b	17b	4b
Chlorine 1 %	44b	36b	28c	59b	54.6a	11b
H_2Od	66c	91a	99d	93a	83a	81a
Sucrose	62c	97a	100d	93a	97c	81a

Different letters represent significant differences ($p < 0.05$).

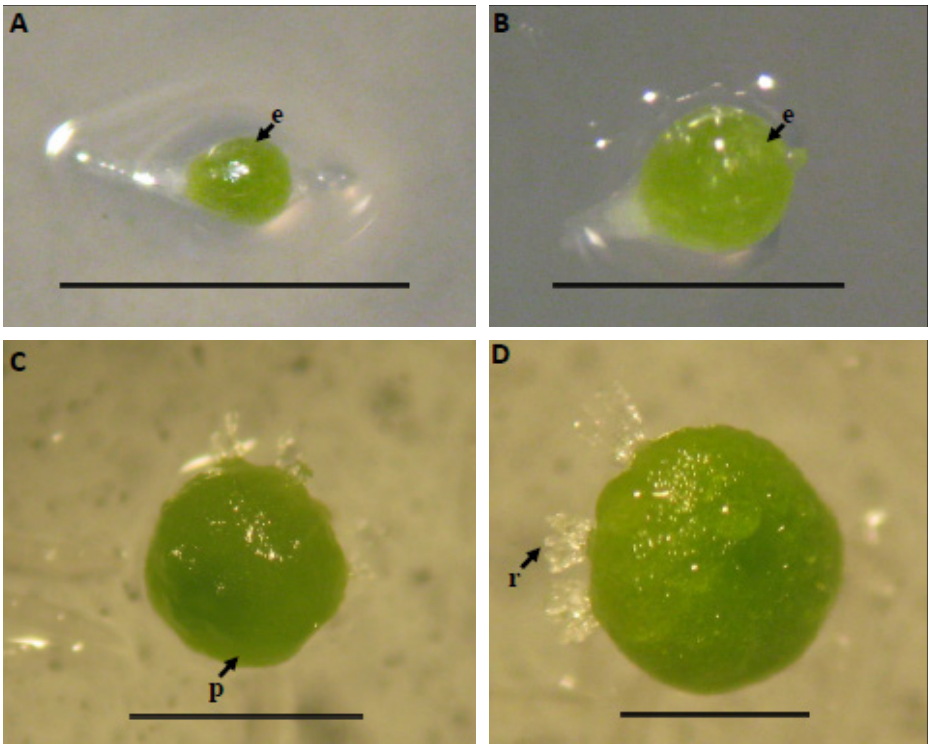

Figure 3. Asymbiotic germination of *Pleurotalis* sp. (A and B) Expanded embryo. (C) Protocorm formation. (D) Rhizoid production. (D) Scale of the bar = 1 mm. e: embryo; p: protocorm; ra: root; r: rhizoid t: testa.

influence on seed germination, making them the best alternative for evaluation, as they yield the highest correlation with *in vitro* germination. When using tetrazolium 0.25 % at 24 h, a viability of 100 % was obtained using sucrose pretreatment, and 99 % when using H_2Od pretreatment, with the germination percentage presented above (96 %).

Thus, it is demonstrated that sucrose and deionized water are the best options among the evaluated pretreatments to increase the viability of these orchid seeds regardless of the concentration and time of exposure to tetrazolium. It is recommended to perform the germination test (*in vitro*) as a propagation alternative, since it ensures their growth. It also reports favorable results in orchid development, promoting their rapid and effective propagation (Maharjan *et al.*, 2020), with germination rates between 80 % and100 %, suitable for large-scale production (Kang *et al.*, 2020).

Conclusion

Based on the results, pretreatment with 10 % sucrose is the best alternative to enhance the viability testing of *Spathoglottis plicata* seeds using tetrazolium, regardless of the dose. For *Pleurothallis* sp. seeds, H_2Od and sucrose were the best options to improve viability. However, since both pretreatments showed similar results, H_2Od is recommended due to its cost-effectiveness. Similarly, for *Stelis* sp., sucrose and H_2Od were the most effective pretreatments for improving germination capacity. Chlorine pretreatments (0.1 % and 0.2 %) are not recommended for these orchid species, since it reduced the viability in the tetrazolium test for most of the treatments.

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