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Pre-treatments effect on the tetrazolium test on *Epidendrum barbaricum* Hágsater & Dodson seeds

Efecto de pretatamientos en la prueba de tetrazolio en semillas de *Epidendrum barbaricum* Hágsater & Dodson

Seir Antonio Salazar Mercado[1]; Edison Alexander Botello Delgado[2]; y Jesús David Quintero Caleño[2]*

1.Universidad Francisco de Paula Santander, Departamento de Biología. Avenue Gran Colombia # 12E-96B Colsag. San José de Cúcuta, Colombia. Postal Code: 540003., 2.Universidad Francisco de Paula Santander, Departmento de Ciencias Agrarias. Avenue Gran Colombia # 12E-96B Colsag. San José de Cúcuta, Colombia. Postal Code: 540003. *Author for correspondence: salazar663@hotmail.com

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Abstract

Orchids are affected by several factors that impair their spreading, which is necessary to know the viability of their seeds. The aim of this research was to determine the most suitable preconditioning treatment to potentiate the tetrazolium test in *Epidendrum barbaricum* seeds. Initially, the mature capsules were collected near the city of Pasto in the Department of Nariño (Colombia), and seeds were obtained. Subsequently, the seeds were submitted to four pretreatments: immersion in distilled water, 1% hypochlorite, 10% alcohol and 10% sucrose. Seeds were then rinsed with distilled water and exposed to two concentrations of 2,3,5-trifenyl tetrazolium chloride (0.25%, 1%) and different exposure times (6, 12, 24, and 48 hours). To perform the tests, the 5 ml syringe with cloth filter method was used. The viability test results were corroborated with the *in vitro* germination test, using the MS (Murashige and Skoog) culture medium. The most reliable viability findings (93%) were obtained by using preconditioning with sodium hypochlorite, a value that has a high correlation with the percentage of germination (93%), independent of the tetrazolium concentration at an exposure time of 24 hours.

Key words: Capsules, Germinative capacity, Orchids, Seeds quality, Sodium hypochlorite.

Resumen

Las orquídeas son afectadas por varios factores que perjudican su propagación. En este estudio se evaluaron diferentes métodos de pre-acondicionamento para potenciar la prueba de tetrazolio en semillas de *Epidendrum barbaricum*. Inicialmente las cápsulas maduras fueron recolectadas en la ciudad de Pasto, departamento de Nariño (Colombia), las cuales fueron sometidas a los pretratamientos: inmersión en agua destilada, hipoclorito al 1%, alcohol al 10% y sacarosa al 10%, antes de ser lavadas con agua destilada y tratadas con concentraciones de 2,3,5- cloruro trifenil tetrazolio (0.25%, y 1%) durante tiempos de exposición de 6, 12, 24 y 48 horas. Para las aplicaciones fueron utilizadas jeringas de 5 ml con filtro de tela. Los resultados del test de viabilidad fueron validados con la prueba de germinación *in vitro*, utilizando el medio de cultivo MS (Murashige and Skoog). Los mejores porcentajes de viabilidad (93%) se encontraron con la aplicación de hipoclorito de sodio, con una alta correlación con el porcentaje de germinación (93%), independiente de la concentración de tetrazolio y un tiempo de exposición de 24 horas.

Palabras claves: Capacidad germinativa; Calidad de semillas; hipoclorito de sodio; Orquídeas; pre-acondicionamiento.

Introduction

Epidendrum barbaricum Hágsater & Dodson is an epiphytic plant belonging to the Orchidaceae family. This group of plants has a wide variety and has more than 28,000 accepted species with 763 genus (Zhang et al., 2018), representing about 10% of the diversity of the plant kingdom. Orchids are very successful regarding the adaptation mechanisms to a wide variety of environments (Alghamdi 2017), occupying almost all the habitats on the Earth (Ramya et al., 2017) between tropical and subtropical forests, where they are found mainly as epiphytes plants (Srivastava et al., 2018), which represents 70% of the species of this family and approximately 30% of the known species are terrestrial (Fracchia et al., 2016). The commercial production of orchids is one of the most important and profitable economic activities in the nursery industry worldwide as they are very popular plants because of their beautiful flowers, their long floral longevity, their medicinal and horticultural importance (Deka et al., 2017).

Orchids are highly vulnerable to habitat disturbance compared to other plants (Salazar and Gelvez 2015), and they are being threatened due to several factors such as intensive collection, forest fragmentation, overexploitation and also due to the fact that they largely depend on biotic interactions such as pollinators and mycorrhizae (Segovia-Rivas et al., 2018). In addition, they produce small seeds, which lack of endosperm so that under natural conditions they depend on mycorrhizal fungi for symbiotic germination which are necessary until adulthood for their survival (Chaves et al., 2015), accomplishing an important role in the nutrition of orchids. Moreover, The seeds present relative low germination rates of 5% (Vudala et al., 2019). The complicated propagation of the seeds plus the threats of extinction harms diverse species of orchids, in such a way that they tend to decline their population, for which; generating information about the quality of the seed is important both for conservation in germplasm banks and for crop production (Ramya et al., 2017). Due to the value of this parameter, several tests have been developed to know the germinative capacity of seeds, such as germination test and tetrazolium staining (Salazar et al., 2018; Salazar et al., 2020a). The last one stands out for offering a fast and reliable alternative for the determination of the seeds viability (Carvalho et al., 2017), and in comparison with the in vitro germination test, it is not affected by microorganisms (Schultz et al., 2014). Likewise, it has been proven to be effective in determining the germinative capacity of several orchid species (Salazar et al., 2013; Salazar and Gelvez 2015; Salazar and Vega, 2017), based on the activity of the dehydrogenated enzymes involved in the respiration process, especially malic acid dehydrogenase, which reduces the tetrazolium salt upon contact with living tissues, forming triphenyl formazan, a stable, non-diffusible red colored compound (Salazar and Botello 2018), which it is possible to indicate the viability of the seeds based on the embryo staining.

The tetrazolium test effectiveness is mediated by the development of preconditioning procedures, which facilitate the entry of the tetrazolium solution to the seed (Hosomi et al., 2017). It has been shown that pre-treatment with 10% sucrose in species of the *Cattleya* genus increases the accuracy of the tetrazolium test when compared with the in vitro germination rate (Hosomi et al., 2011). On the other hand, some orchid seeds have a strong coat that difficult wetting (Duarte 2017). According to Salazar (2012) the use of 1% sodium hypochlorite scarifies the tegument facilitating the entry of the tetrazolium salt into *Cattleya mendelli* seeds. According to the above, it is important to look for different pretreatments that optimize the seeds viability and germination. This study aims to evaluate the influence of preconditioning on the tetrazolium test effectiveness to determine the viability in *E. barbaricum* seeds.

Materials and methods

Vegetal material

Epidendrum barbaricum Hágsater & Dodson seeds were obtained from mature, naturally pollinated, indehiscent capsules that were collected near the city of Pasto (1° 10' 07" N and 77° 09' 09" W) at 2800 m.a.s.l. in the department of Nariño, Colombia. Afterwards, the capsules were stored at room temperature in Kraft paper envelopes (Salazar and Gelvez 2015) in a glass bottle with silica gel, in order to dehydrate them and avoid deterioration due to excess of moisture, during a period of 48 hours until the natural dehiscence of the capsules. The study was carried out at the Biology Laboratory, located in the Faculty of Basic Sciences, at the Francisco de Paula Santander University.

Pre-conditioning and seeds Viability

In order to increase the effectiveness of the tetrazolium test when determining the seeds viability, four pretreatments were evaluated, such as: 10 minutes immersion in distilled water (Duarte et al., 2017), 10% sucrose solution (Hosomi et al., 2011), 1% sodium hypochlorite (Salazar 2012), and 10% alcohol. By using a 5 ml syringe, which consisted on placing a small portion of seeds in a sterile 5 ml syringe with a cloth filter. Subsequently, pretreatments were carried out (Salazar 2012). The seeds without previous

conditioning were taken as control. Once the immersion time had elapsed, the solutions were extracted and the surpluses were eliminated with three rinses, using distilled water. Subsequently, for the determination of viability, 5 ml of the tetrazolium solution (2, 3, 5 triphenyl tetrazolium) was suctioned, exposing the seeds to different concentrations (0.25% and 1%) and exposure times (6, 12, 24 and 48 hour), under conditions of darkness at 25 ºC. The seeds viability was classified according to the coloration of the embryo with the help of a LEICA EZ4 stereomicroscope. Viable seeds showed red staining in the embryo, due to the reduction of tetrazolium by cellular respiration, whereas dead tissues maintained their original color (Iossi et al., 2016).

Seeds disinfection and germination test

The disinfection and sowing of the seeds was carried out by implementing the syringe method (Salazar, 2012). Seeds were superficially disinfected with 70% ethanol for one minute, then immersed in sodium hypochlorite (NaOCl) at 0.75% plus 0.1% Tween-20, for a period of 5 minutes in constant agitation. At the end of the immersion time, 5 rinses were done with sterile deionized water (Vudala et al., 2019), then the filter was removed from the syringe and 100 seeds were planted in Petri dishes containing 25 ml of basal MS medium with a composition of 100% macro and micronutrients (Murashige and Skoog, 1962), supplemented with 3000 mg/L of sucrose, 8000 mg/L of agar, and 1000 mg/L of activated carbon. The pH was adjusted to 5.8 using NaOH, before autoclaving at 15 pounds of pressure (Psi) at 121 °C for 25 minutes. The cultures were kept in a growth room at 18 ± 2 °C (nigh/ day) and 64% relative humidity, under a photoperiod of 16 hours of light and 8 hours of darkness, provided by fluorescent tubes with 28 µmol m^{-2} s^{-1} intensity, for a period of 70 days. In order to determine the germination percentage, 100 seeds were examined per treatment with a LEICA EZ4 stereomicroscope, the germinated seed was considered to be the seed that presented a rupture of the seed coat due to the enlargement of the embryo according.

Statistical analysis

In the tetrazolium test and germination test, the data were randomly distributed with 5 repetitions and 100 seeds per repetition. The data were analyzed by means of a variance analysis (ANOVA), followed by Tukey's multiple range HSD test (Honest Significant Difference), in order to compare the averages and to determine the significant differences at a level of $P \leq 0.05$ using statistical software Statgrafic Centurion XVII version.

Results and discussion

The results obtained (Table 1 and 2) show that the coloration in the seeds tissues responds differently to the pre-conditioning treatment, the concentration and exposure time to tetrazolium (TZ). The staining in the *E. barbaricum* seeds after using a pre-conditioning treatment with both sodium hypochlorite (NaOCl) and 10% sucrose obtained the highest viability percentages, when compared with the other pre-treatments, although they are statistically heterogeneous with each other.

However, when the concentration of TZ increased to 1%, the viability percentages tended to be statistically homogeneous (Table 2), using pre-conditioning treatments with sodium hypochlorite (1%) and sucrose (10%). This proves that by increasing the TZ salt concentration, the detection of the seeds viability optimized due to the greater interaction of the solution with the living tissues, which increases the intensity of the coloration (Espitia et al. 2017), taking control group as reference. On the other hand, the 24 hours of exposure time, using immersion in sodium hypochlorite (1%), both for the 0.25% and 1% TZ concentrations gave the most optimal viability results (93%) compared with the germinated seeds percentage (93%). The data are in agreement with Hosomi et al. (2017) where they found good results, when using the 1% tetrazolium solution during 24 hours of exposure.

Table 1. *Epidendrum barbaricum* seeds viability subjected to five pretreatments and evaluated by the tetrazolium test (0.25%) at different exposure times.

Pre-treatments	6h	12h	24h	48h
Control	72a*	71a	73a	75a
Chlorine 1%	82a	91b	93b	95b
Sucrose	80a	88b	91b,c	92b
Alcohol 10%	57b	69a	72a	80a
H_2Od	72a	80a,b	77a,c	79a

*The values of the averages with different letter of each column indicate statistically significant differences, according to the Tukey HSD test (P≤0.05).

Table 2. *Epidendrum barbaricum* seeds viability subjected to five pretreatments and evaluated by the tetrazolium test (1%) at different exposure times.

Pre-treatments	6h	12h	24h	48h
Control	81a*	87a	84a,b	84a
Chlorine 1%	83a	88a	93b	91a
Sucrose	80a	83a,b	80a,b	85a
Alcohol 10%	57b	72b	75a	80a,b
H_2Od	72a	80a,b	73a	69b

*The values of the averages with different letter of each column indicate statistically significant differences, according to the Tukey HSD test (P≤0.05).

The seeds viability was determined by the ability to reduce the TZ to formazan, so the presence of the red color indicated that the seed was viable and the absence of this, the embryo´s death (Figure 1). The TZ biochemical test has been used in various species of orchids, such as: *Dendrobium bigibbum* var. *Compactum, Dendrobium formosum* (Kananont et al., 2010), *Cymbidium pendulum* (Sw.) Roxb (Aggarwal and Nirmala 2012), *Cypripedium lentiginosum* PJCribb and SCChen (Jiang et al., 2017), *Elleanthus arauntiacum, Epidendrm* sp., *Maxillaria* sp., *Odontoglossum lindenii, Prosthechea* sp., *Telipogon dubios, Stelis* sp., *Elleanthus* sp., *Epidendrum elongatum, Cyrtochilum aemulun* (Salazar et al., 2020b; Salazar and Cancino, 2012; Salazar and Gélvez 2015), *Cattleya mendelii* (Salazar, 2012), *Phalaenopsis* (Carmela's Wild Thing x Taipei Pearl) (Salazar et al., 2013), among others.

Germination percentage

In the in vitro germination test, a 93% of the seeds from *E. barbaricum* germinated at in vitro conditions, breaking the seed coat by the expansion of the embryo (Figure 2). This value correlates directly with the viability percentage obtained by exposing the seeds to a scarification with 1% NaOCl which is statistically homogeneous with the 10% sucrose pretreatment, independent of the concentration (0.25% and 1%) at 24-hour exposure time (Table 1).

When comparing the Viability results from the TZ concentrations applied (0.25% and 1% W/V) demonstrate the pretreatment with NaOCl (1% W/V, 10 min) positively influenced effectively the seeds germination capacity, providing a profitable alternative to evaluate the seeds germinative capacity since, viable results are obtained independent of the concentration, in this way, the concentration to be used could be reduced by 0.75%, using an exposure time of 24 hours. Being these viability results (93%) those of greater correlation with the in vitro germination percentage (93%). The present observation would imply to improve the application of the laboratories financial resources and likewise a larger number of samples could be evaluated with a lower cost (Carvalho et al., 2017).

Although, orchid seeds present a simple structure, to moist them is complicated, due to the fact that the cell walls of the outer tegument are lignified and covered with a lipid cuticle (Jevšnik and Luthar 2015), the presence of these compounds of hydrophobic nature contribute to the impermeability of the seed. However, this characteristic depends on the seed state of development, since it is at immature state, the testa may not be lignified so it would be more permeable, when compared to a mature seed which has a fully formed testa, some pretreatments made on seeds such as immersion in NaOCl solutions can improve their permeability (Zhang et al., 2013). The NaOCl is usually used to degrade the wood pulp lignin, as it is a strongly oxidizing agent (Jiang et al., 2017), therefore, the importance of this chemical compound relies mainly in the scarification of the seed (Bae et al., 2014) thus improving the effectiveness of the TZ test. It is most likely to degrade some hydrophobic compounds of the testa, such as lignin, thus breaking the obstacle from the seed coat cell walls increasing its hydrophilic character, allowing the entry of TZ salt in a uniform way and generating a stronger stain. Previous treatment with NaOCl also stimulates seed germination by degrading the seed coat and facilitating the absorption of water and oxygen (Vasudevan and Staden, 2010) and accelerating the inhibitors washing, such as endogenous abscisic acid (ABA) from seeds (Jiang et al. al., 2017, Bae et al., 2014). However, chlorine causes a cytotoxic effect by generating chromosomal anomalies (Salazar and Maldonado; 2020; Salazar et al., 2019).

Preconditioning with 10% sucrose improved the coloration proving to be adequate for a better identification of viable seeds. These results are similar to those obtained in *Cattleya* species by Hosomi et al. (2011, 2017). This is due to the

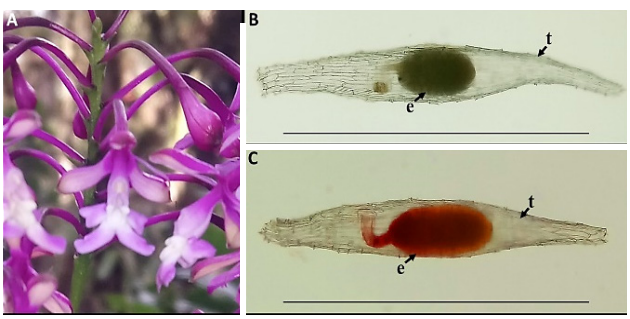

Figure 1. *Epidendrum barbaricum* flower and seeds. *(A) E. barbaricum* flower. *(B) E. barbaricum* not viable seeds. *(C) E. barbaricum* viable seeds. Bar scale = 1mm. t: seed coat; e: embryo.

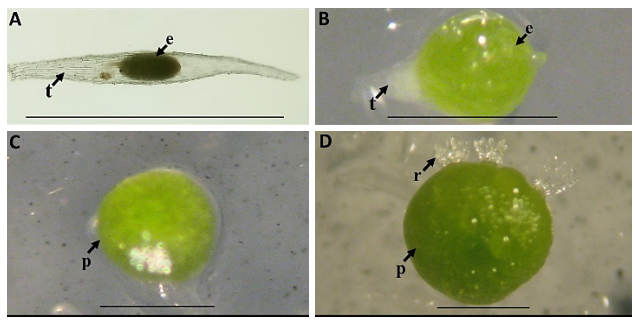

Figure 2. *Epidendrum barbaricum* asymbiotic germination in MS culture medium. (A) No germinated seed (B) Expanded embryo. (C) Protocorms formation. (D) Rhizoids production. (D) Bar scale= 1mm. e: embryo; p: protocorm; r: rhizoid; ra: root; t: testa/seed coat.

fact that the sucrose solution offers a benefit by maintaining a balance between the seeds and their external environment, thus avoiding an imbibitional injury, which could cause damage to the embryo cells when immersed in the TZ solution, also sucrose immersion may be important to activate the embryo´s metabolism, mainly the dehydrogenase enzymes (Hosomi et al., 2011), ensuring a sharper coloration and more reliable results.

Hydration facilitates the TZ absorption and provides the enzymatic metabolite´s activation (Carvalho et al., 2017). The results obtained with the control group and direct imbibition in water, were statistically homogeneous with each other for the two TZ concentrations evaluated, except for exposure to a concentration of 1% TZ for 48 hours where it was surpassed by the control group, with significant differences (Table 2). One of the reasons is that the water absorption by the seed is limited by the presence and permeability of the seed coat, on the other hand, submerging the seeds directly in water can cause damage by imbibition, thus affecting the effectiveness of the test and influencing the differences comparing with the germination test.

Conclusion

The pre-treatment with 1% sodium hypochlorite for 10 minutes allows to enhance the viability reading using the tetrazolium test in *E. barbaricum* seeds, when using a concentration of 0.25 or 1% during 24 hours of exposure. This preconditioning treatment not only improves embryo staining by generating a scarification of the seed coat, but also decreases the concentration of tetrazolium solution to be used, which translates into a cost reduction at the laboratory level to determine the seeds germination capacity

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