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Seed analysis of *Dichromanthus aurantiacus*, terrestrial orchid from Toluca valley, México

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ABSTRACT

Objective: The objective of this work was to analyze the viability and germination of *Dichromanthus aurantiacus* seeds, a terrestrial orchid from Toluca valley, México.

Design/methodology/approach: The size and color were evaluated. Two methods determined the viability: 1) the tetrazolium test (imbibition for 24 hours in the water, 2 hours in calcium hypochlorite ($CaCOCl_2$), and drops of Tween-80). 2) the asymbiotic seed germination by *in vitro* culture (imbibition for 24 hours in the water, and the concentration of MS medium plus natural extracts).

Results: The seeds of this specie showed approximately 0.2 mm long and 0.05 mm wide; they possess an embryo and a brownish testa. There were significant differences ($P<0.05$) between the treatments finding a positive effect with the tetrazolium test, achieving up to 91.4% viability. In the *in vitro* germination, the imbibition of the seeds favored contamination. The concentration of MS and the addition of natural extract presented significant differences ($P<0.05$), the 50% MS plus 10% of coconut water showed up to 92.8% of germination at 60 days.

Study limitations/implications: The results are preliminary of a long-term experiment.

Findings/Conclusions: The seeds of *Dichromanthus aurantiacus* showed brown testa and an oval embryo with dimensions of 0.2 mm long and 0.05 mm wide. The tetrazolium test's viability showed 91.4% viability when they were soaked in sodium hypochlorite solution ($CaCOCl_2$) for two hours, 24 hours soaking in tetrazolium solution (1%) plus two drops of Tween-80. The asymbiotic *in vitro* culture showed up to 92.8% germination in 60 days using MS medium at 50% enriched with 10% coconut water.

Keywords: *Dichromanthus aurantiacus*, tetrazolium, asymbiotic germination, *in vitro* culture, natural extracts.

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INTRODUCTION

There are around 35,000 orchids worldwide, of which approximately 60% are epiphytic, and the rest are terrestrial. In Mexico, about 1,400 species are registered, and grouped into 164 genera, around 40% are endemic (Hágsater *et al*., 2015) in the national territory. The states of Chiapas, Oaxaca, Jalisco, Guerrero, Veracruz, Mexico, and Michoacán have the highest number of species recorded (Szeszko, 2011; Castañeda-Zarate *et al*., 2012; CONABIO, 2013; Carvajal, 2014; Hassler and Rheinheimer, 2016). According to (Szeszko, 2011), there are 251 species grouped into 71 genera in the state of Mexico, of which 55% are terrestrial, and the rest epiphytic or lithophytic species. One of these terrestrial orchids is *Dichromanthus aurantiacus*; the botanical characteristics of *Dichromanthus aurantiacus* are: thick and tuberous roots, it has 6 or 7 leaves organized throughout the plant, it has an inflorescence of orange color with 7 to 15 successive flowers up to 2.5 cm long and arranged horizontally; the blooming occurs in from June to August, and it loses the leaves at the beginning of winter. In Mexico, it grows between 1,500 to 3,200 meters, and is located in grasslands, open bushes, and next to the highways (Bertolini *et al*., 2012; García-Martínez and Rodríguez, 2018; Martínez-De la Cruz *et al*., 2018). In the state of Mexico, there are reports of sightings in Zacualpan, San Simón de Guerrero, Zumpahuacán, Tenancingo, Coatepec Harinas, Amatepec, Texcaltitlán, Donato Guerra, Valle de Bravo, Ixtapan del Oro, Almoloya de Alquisiras, Amanalco, Luvianos, Santo Tomás, Villa de Allende, Ocuilan, Temascaltepec, Ixtapan de la Sal, Tejupilco Sultepec, Toluca, Malinalco (Szeszko, 2011; Martínez-De la Cruz *et al*., 2018).

The evolution of this orchid is surprising; its structure has evolved to be pollinated by hummingbirds; however, bumblebees interferes with the pollination process because they are related to the fungus of the genus *Cantharellales*, and fungal genus *Ceratobasidium* and *Thanatephorus* (Rasmussen, *et al*., 2015). In symbiotic germination, the fungi provide the embryo with the necessary nutrients for its development (Rasmussen *et al*., 2015); however, in asymbiotic germination, it is essential to add nutrients to fulfill the same function as the symbiotic fungus germination. These nutrients required for growth are supplied utilizing a culture medium either in solid (agar) or liquid state (De Lucas, 2004; Levitus *et al*., 2010).

In the natural state of orchids, germination occurs in 5 to 10% (Morales, 2018); for this reason, seeds are inoculated with fungi to stimulate plant growth (symbiotic seed germination), or by mechanical scarification, and nutrients added (asymbiotic seed germination). According to CITES (SEMARNAT, 2010), *Dichromanthus aurantiacus* could be threatened because the urban area is expanding. Therefore, this study's objective was to evaluate the viability and germination of *Dichromanthus aurantiacus* seeds for conservation purposes.

MATERIALS AND METHODS

The seeds were donated in December 2019 by the Orchid Seed Germplasm Bank of the State of Mexico (Faculty of Agricultural Sciences of the Autonomous University of the State of Mexico). The seeds were collected in San Cayetano de Morelos Toluca, Mexico, in 2018. They were stored in an amber glass bottle and refrigerated at 10 °C. The shape, size, and weight of the seeds were evaluated (Aguilar-Morales, 2016); for this analysis, 0.2 grams were weighed in triplicate. The length, width, and number of seeds per gram were recorded.

Seed viability

Two tests were carried out; the tetrazolium test (Bohm, 1996; Aguilar-Morales *et al*., 2016) and asymbiotic germination by *in vitro* culture. Briefly, tetrazolium solution 1% (2,3,5-triphenyl-2-H tetrazolium chloride) diluted with distilled water (pH 6±0.1) was stored in an amber bottle and refrigerated until later use. Six treatments were evaluated in triplicate; the seeds were placed in filter paper envelopes by repetition. Treatment 1 (T1): seed soaking for 24 hours in distilled water, then soaking in calcium hypochlorite ($CaCOCl_2$) for two hours to finish with a 24-hour soak in tetrazolium. Treatment 2 (T2): seed soaking for 24 hours in distilled water and subsequently soaking for 24 hours in tetrazolium. Treatment 3 (T3): two hours soaking in $CaCOCl_2$ + 24 hours soaking in tetrazolium + 2 drops of Tween-80. Treatment 4 (T4): 24 hours soaking in tetrazolium + 2 drops of Tween-80. Treatment (T5): 24 hours soaking in tetrazolium. Treatment (T6): 24 hours soaking in distilled water + two hours soaking in $CaCOCl_2$ + 24 hours in tetrazolium + 2 drops of Tween-80. The seeds viability (%) was obtained, dividing the number of embryos stained by the total number of seeds and multiplying by 100. An optical microscope (Leica Microsystems Ltd. Leica Application Suite V4) was used for this purpose, and ten fields were recorded per repetition of each treatment.

Asymbiotic germination

The Murashige and Skoog (1962) culture medium (MS, 100% and 50%) enriched with natural extracts were evaluated: 0% extracts (T), 10% banana, 10% coconut water, and activated carbon 1 g L^{-1} during four germination periods (15, 30, 45, 60 days). All treatments were supplemented with 30 g L^{-1} of sucrose and 7 g L^{-1} of bacteriological agar. The pH was adjusted to 5.7±0.1, and 25 ml of the medium were added to Gerber-type flasks and sterilized for 15 minutes at 120 degrees °C. The seeds were weighed (0.2 g), placed in filter paper envelopes, and disinfected in a 0.5% sodium hypochlorite solution plus a drop of Tween-80 for 10 minutes and rinsed with sterile water until the foam despair. In the laminar flow hood, the seeds were rinsed with sterilized distilled water three times, and the sowing was carried out in the culture media of each treatment. The stage identified as late protocorm was considered as seed germination (Shimura and Koda, 2004).

Statistical analysis

A complete randomized design was used to evaluate the viability of the seeds. The general linear model was:

$$Y_i = \mu + T_i + e_i$$

where μ = is the overall mean, A_i = is the *i*-th treatment effect (*i* = T1, T2, T3, T4, T5, T6), and e_{ijk} = is the *i*-th random error effect. A bifactorial design with completely randomized blocks was used to evaluate the germination percentage in four periods. The general linear model was:

$$Y_{ijk} = \mu + A_i + B_j + C_k + A_i * B_j + e_{ijk}$$

where μ = is the overall mean, A_i = is the *i*-th factor *A* effect (*i* = MS-50%; MS-100%), B_j = is the *j*-th factor effect (*j* = T, P10, C10, CA1), C_k = is the *k*-th random block effect (*k* = 15, 30, 45, 60 days), $A_i * B_j$ = is the i_j-th interaction of the Factor *A* and *B*, and e_{ijk} = is the *ijk*-th random error effect. When the ANOVA identified statistical differences (P<0.05) among the treatments, we applied the Tukey test for multiple means comparisons. The SAS System for Windows 9.0 was used for the statistical analysis.

RESULTS AND DISCUSSION

The seeds of *Dichromanthus aurantiacus* have two basic structures: testa and embryo. This species' seed coat poses brown color that surrounds the embryo; the shape of its cells is hexagonal, forming a network—the analyzed seeds presented 0.2 mm in length and 0.05 mm wide approximately (Figure 1).

These results are in line with a study of four terrestrial species from Argentina (Lallana and Di Persia, 2018); the seeds showed a transparent testa with brownish to dark brown cell walls with dimensions ranging from 0.395 mm to 0.805 mm in length and 0.091 to 0.226 mm wide. The seeds of *Phaius tankervilleae*, a terrestrial orchid (Ranquel *et al.*, 2017), presented a transparent testa with cells forming a network and an oval embryo of 0.469 to 0.5597 mm length. For *Prosthechea*, measurements of 0.7 mm in length and 0.03 mm wide (Banda-Sánchez *et al.*, 2017). Lallana *et al.* (2016) reported dimensions in ten species of native orchids ranging from 0.202 to 0.805 mm in length by 0.056 to 0.226 in wide.

The tetrazolium test showed no significant differences (P>0.05), suggesting that a similar number of embryonic seeds were under evaluation in the tetrazolium test. There were highly significant differences in the percentage of seeds viability (P<0.0001) among the treatments. The highest percentage of seeds viability was observed in T3 with 91.4%, followed by T4 with 90.5%, and the lowest percentage was observed in T2 with 23.2%.

The embryos' staining presented significant differences (P<0.0001). The imbibition of the seeds 24 hours in distilled water affected the embryos' staining; when they were soaked in water, the viability was 55.5%; on the other hand, if the seeds were not soaked, the viability was 75.6%. It was observed a positive effect of the $CaCOCl_2$ solution, obtaining up to 78% viability compared to not using the solution (52.9%). Similarly, the addition of Tween-80 increased the embryos' staining from 43.6% to 87.5%. For *Prosthechea* sp., an epiphytic orchid (Banda-Sánchez *et al.*, 2017), the tetrazolium concentrations and immersion time were evaluated, showing the highest

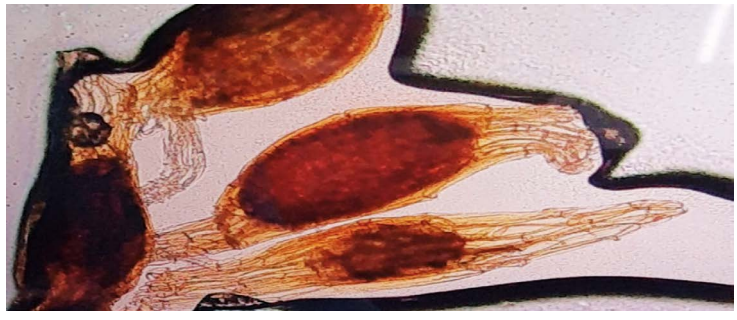

Figure 1. *Dichromanthus aurantiacus* seeds.

percentage of viability (92%) using 1% tetrazolium solution for 120 minutes. In *Encyclia adenocaula*, an epiphyte orchid (Aguilar-Morales *et al*., 2016), the $CaCOCl_2$ solution affected the seeds' viability negatively, decreasing from 50 to 24%, and the Tween-80 did not affect the viability significantly (P>0.05). In some species, better precision in the viability has been reported using the tetrazolium test. Mercado *et al*. (2015) performed a study in ten species of orchids (epiphytes and terrestrial) using tetrazolium and indigo carmine tests; his results suggested that tetrazolium test is more efficient in determining seed viability. Karol *et al*. (2015) reported 70% viability in *Comparettia falcata* (epiphyte) employing the 1% tetrazolium. Imbibition for 24 hours in water increased the seeds' viability in *Trichocentrum jonesianum* (Liliana and Garcia, 2013). Ossenhach *et al*. (2007) reported that $CaCOCl_2$ considerably affected the viability of five orchids seeds (all epiphytes); these findings contradict our results in the present study. It cannot be generalized that $CaCOCl_2$ has a positive effect on the viability of terrestrial orchids; however, several studies can be carried out with a considerable diversity of terrestrial orchid seeds due to there are no specific studies regarding this pretreatment for terrestrial orchids.

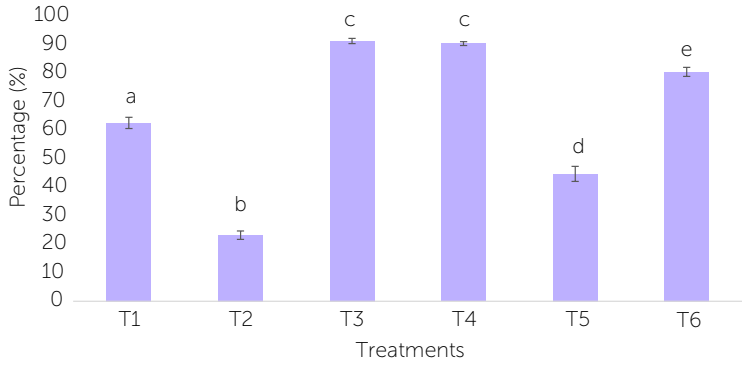

Figure 2. Percentage of seeds viability (*Dichromanthus aurantiacus*) in the tetrazolium test 1% (2,3,5-triphenyl-2-H tetrazolium chloride). T1: 24 hours soaking in distilled water + two hours soaking in calcium hypochlorite ($CaCOCl_2$) + 24 hours soaking in tetrazolium. T2: 24 hours soaking in distilled water + 24 hours soaking in tetrazolium. T3: two hours soaking in $CaCOCl_2$ + 24 hours soaking in tetrazolium + 2 drops of Tween-80. T4: 24 hours soaking in tetrazolium + 2 drops of Tween-80. T5: 24 hours soaking in tetrazolium. T6: 24 hours soaking in distilled water + two hours soaking in $CaCOCl_2$ + 24 hours soaking in tetrazolium + 2 drops of Tween-80.

Undoubtedly, the best orchid seed germination technique is the *in vitro* culture; this could be as asymbiotic or symbiotic approach; the asymbiotic method has shown promising results in several studies (Flores-Escobar *et al*., 2011; Menchaca *et al*., 2012; Salazar and Cancino, 2012; Dalzoth and Lalllana, 2013; Billard *et al*., 2014; Karol *et al*., 2015; Aguilar-Morales *et al*., 2016; Lallana *et al*., 2016; Banda-Sánchez *et al*., 2017). The asymbiotic germination by *in vitro* culture of *D. aurantiacus*, presented significant differences (P<0.0001) due to the concentration of the base medium (factor A), MS50% showed up to 44.9% and MS100% up to 22.8% germination at 60 days. Regarding the addition of natural extracts (factor B), there were significant differences (P<0.0001) in the four periods; coconut 10% water extract showed up to 78.8% germination at 60 days, and the banana extract considerably affected the asymbiotic germination. There was a highly significant difference among periods (Table 1), MS50-C10 showed similar germination as viability in the tetrazolium tests (90 to 92%); on the other hand, MS100-P10 presented the lowest germination in the four periods (Table 1).

It is essential to mention that the seeds' germination is asynchronous because it was possible to observe all the stages (Figure 3) for this specie from green seeds to complete seedlings, as mentioned in Aguilar-Morales *et al*. (2016).

The germination was lower in our study than Suárez-Quijada (2010) for the same specie; he observed 74% of germination at 28 days (stage C). Dalzotto and Lallana

Table 1. Asymbiotic germination (%) *in vitro* culture of *Dichromanthus aurantiacus* through four germination periods.

Treatment	Germination periods (days)			
	15	30	45	60
MS50-T	33.80 ^{a}	33.80 ^{a}	40.00 ^{a}	67.60 ^{b}
MS50-P10	2.40 ^{f}	4.80 ^{d}	6.00 ^{d}	9.60 ^{d}
MS50-C10	19.60 ^{b}	27.20 ^{b}	34.80 ^{b}	92.80 ^{a}
MS50-CA1	4.80 ^{d}	4.80 ^{d}	5.20 ^{e}	9.60 ^{e}
MS100-T	0.40 ^{g}	4.80 ^{d}	13.20 ^{c}	18.80 ^{d}
MS100-P10	2.40 ^{f}	2.40 ^{f}	2.40 ^{g}	2.40 ^{g}
MS100-C10	6.40 ^{c}	20.00 ^{c}	40.00 ^{a}	64.80 ^{c}
MS100-CA1	2.60 ^{e}	2.60 ^{e}	2.60 ^{f}	5.20 ^{f}
M.S.E.	4.4	5.3	6.9	11.4
P-value				
Factor A	< 0001	< 0001	< 001	< 0001
Factor B	< 0001	< 0001	< 0001	< 0001
Interaction A*B	< 0001	< 0001	< 0001	< 0001
Blocks	NS	NS	< 001	< 001

Mean values between treatments with different superscript differ significantly (P<0.05). M.S.E.: mean squared error.

(2013) achieved 40%, 98%, and 85% germination in 48 days in *Isochilus linnearis*, *Oceoclades maculate*, and *Oncidium viperium*; respectively. Karol *et al*. (2015) established asymbiotic *in vitro* propagation of *Comparettia falcata*, achieving up to 56.6% in MS medium. Lallana *et al*. (2016) recorded asymbiotic germination *in vitro* culture greater than 50% in MS at 50% in ten species of orchids. Banda-Sanchez *et al*. (2017) obtained 29% germination in *Prosthechea* in 90 days of *in vitro* culture using MS medium enriched with 0.003 mg L^{-1} of auxins and gibberellins.

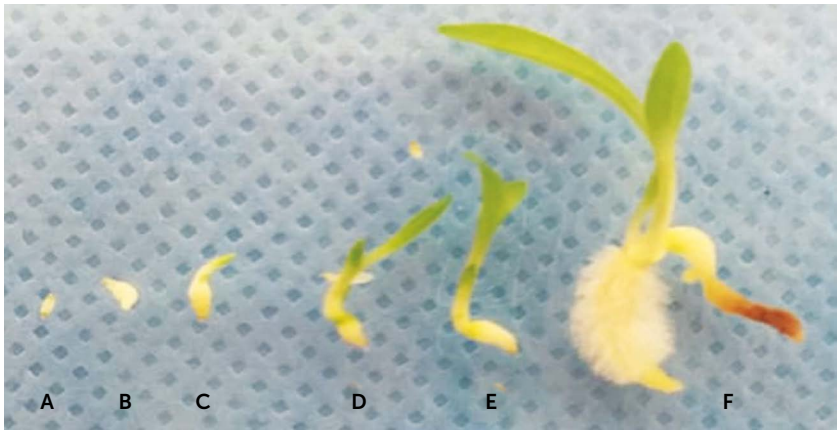

Figure 3. Germination stages of *Dichromanthus aurantiacus in vitro* culture. A: Green seeds; B: Initial protocorm; C: Late Protocorm; D: First leaves; E: Root development; F: Complete plant.

CONCLUSIONS

The seeds of *Dichromanthus aurantiacus* showed brown testa and an oval embryo with dimensions of 0.2 mm long and 0.05 mm wide, approximately. The tetrazolium test's viability showed 91.4% viability when they were soaked in sodium hypochlorite solution ($CaCOCl_2$) for two hours, 24 hours soaking in tetrazolium solution (1%) plus two drops of Tween-80. The asymbiotic *in vitro* culture showed up to 92.8% germination in 60 days using MS medium at 50% enriched with 10% coconut water.

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