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In vitro Germination and Establishment of *Encyclia Vitellina*

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Abstract: *Encyclia vitellina* is a Mexican endemic orchid. It is also known as *Prosthechea vitellina* or *Epidendrum vitellinum.* Like many other orchid species, *E. vitellina* is a threatened epiphytic orchid. The research objective was to evaluate the effects of three different media components on the *in vitro* germination time of *E. vitellina*. Seeds were germinated on Murashige and Skoog media (30%) with kinetin (1 mg l^{-1}) and indoleacetic acid (0.5 mg l^{-1}) (T1), MS (30%) (T2), and a medium with sucrose (T3). Analysis of variance was used for data analysis, and the Tukey test was performed to compare means. The shorter time to germination was found in the T1 medium, where it occurred on day 28, while in T2 and T3, it took place on day 41 and 62, respectively (statistical significance 5%). The frequency of germinated seedlings was high in the three treatments; however, several replicants of T3 had 0% germinated seeds. According to research reported previously, the time to germination was 110 days; in this research, the time to reach the germination state in all media was reduced even by two times or less. The fact that MS media at 30% and a medium without salts plus sucrose allowed the germination of *E. vitellina*, proved that the seeds need only a source of energy to germinate. Thus, the inexpensive medium under study is enough for the initial *in vitro* propagation phase of *E. vitellina*. This finding will contribute to the development of a propagation and conservation program of the endemic *E. vitellina.*

Key words: orchidaceous, in vitro culture, kinetin, germination, *Encyclia vitellina*

1. Introduction

Mexican orchids are a group of plants that have ignited interest in horticulture at national and international levels. Among the over 30,000 species reported worldwide, Mexico has around 1,200; regrettably, recent data report 200 species at some level of extinction risk [1, 2]. Many wild species are distributed in nine states of Mexico, including tropical, subtropical, temperate, and even arid climates, sharing habitats with cacti [3, 4]. According to their growth habits, orchids can be terrestrial, lithophytic, subterranean, and epiphytic. The latter establish on tree branches and trunks in a commensal association; this group is the most abundant, comprising 75% of the four groups [5]. The aesthetic and medicinal use of orchids dates from pre-Hispanic times, while their cultivation in America has been mentioned since Aztec times [4]. Their religious importance has also been cited [6]. Currently, few genera of orchids are cultivated commercially. In our country, wild species are traditionally exploited and harvested without a sustainability program. This is one reason why many wild species are on the brink of extinction: Genera *Laelia*, *Stanhopea*, and *Prosthechea* are threatened, among others [7]. The *Encyclia vitellina* orchid is endemic to Mexico; it is also known as *Prosthechea vitellina*, or *Epidendrum vitellinum*, and its common name is “Manuelitos”. It is the only species with red-orange sepals and petals and yellow lip and column [4, 6]. This species is epiphytic and, due to the destruction of its habitat, the wild overexploitation, illegal trafficking, climate change, and its intricate biotic dependence, it is placed as a species subject to special protection (Pr) according to NOM 059

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SEMARNAT 2010. That is why building strategies is crucial for their conservation, so one of the most viable alternatives is through *in vitro* culture techniques [8, 9], generating practical and low-cost protocols. Firstly, this research aims to improve our understanding of the germination phase to establish seedlings *in vitro* conditions and, later, to start its cultivation and sustainable exploitation through this method.

2. Materials and Methods

Immature seed capsules were collected from 7-year-old *Encyclia vitellina* plants, provided by Professor Abel Bonfil Campos, head of the Botanical Garden of the Faculty of Higher Studies Cuautitlan (FESC) of the National Autonomous University of Mexico (UNAM) for this research. The experimental work was carried out in the Plant Biochemistry and Physiology Laboratory (FESC).

The capsules were washed with running water for 2 minutes to remove the remains of dust and soil. Capsule disinfection was carried out in a clean laminar flow hood, previously sterilized with UV light for 20 minutes.

The capsules were placed in 96% ethanol in the laminar flow hood, totally covering them for 20 minutes. Subsequently, they were extracted with tweezers and passed through a burner flame until the alcohol on the surface was consumed; they were again immersed in clean alcohol and passed through the burner. The same procedure was repeated twice again to ensure capsule disinfection [10]. Next, they were placed in a Petri dish and allowed to cool. A longitudinal incision was made along the dehiscence suture with a sterile scalpel to extract the seeds. They were then spread in flasks containing 25 ml of culture medium and distributed in a thin layer over the surface of the medium [10].

Analysis of variance was used for data analysis, and the Tukey test ($\alpha = 0.05$) was applied for means comparison. There were three treatments with ten replicates each. The experimental unit was a glass flask medium. Time to germination was the variable evaluated, and contamination of the media was also investigated. The treatment seeds were germinated on Murashige and Skoog media (30%) with kinetin (1 mg l^{-1}) and indoleacetic acid (0.5 mg l^{-1}) (T1), MS (30%) (T2), and a media with sucrose (T3). The treatments consisted of different culture media (Table 1). The base medium used was MS [11]. Treatment 1 consisted of MS salts supplemented with Kin and IAA; Treatment 2 consisted of MS salts without regulators, and Treatment 3 was a medium without salts, containing water added with sugar and gelling agent. Activated charcoal 0.5 g l^{-1} was added to all treatments.

The jars containing the seeds were randomly placed in an incubation chamber under light and temperature-controlled conditions. The incubation conditions were: 26°C±2°C temperature, 16-hour photoperiod, and 1900-2300 lux light intensity emitted by 24 watts LED lamps at a distance of 30 cm from the flasks. Periodic observations were made to record germination in the culture media.

3. Results and Discussion

The technique used to disinfect immature *Encyvlia vitellina* capsules effectively eliminated 100% contaminants in all treatments, as per Ruiz et al. [10]. This technique is more practical and economical in contrast to those used by other authors that include sodium hypochlorite solution, Tween soap or detergent, hydrogen peroxide, and even fungicide to treat immature capsules [3, 12-16].

Table 1 Culture media treatments.

Medium	T1	T2	T3
MS	30 %	30%	0
Sucrose	30 g l^{-1}	30 g l^{-1}	30 g l^{-1}
Kinase	1 mg l^{-1}	0	0
Indoleacetic acid	0.5 mg l^{-1}	0	0
Gellan gum	2.8 g l^{-1}	2.8 g l^{-1}	2.8 g l^{-1}
pH	5.7	5.7	5.7
Activated charcoal	0.5 g l^{-1}	0.5 g l^{-1}	0.5 g l^{-1}

A germinated seed is considered to be one that presents a faint green coloration that indicates the appearance of the initial protocorm, as described by Cruz [17]. He points out that the embryo, due to the increase in volume, tears the *testa* allowing its release and marking the beginning of the protocorm stage. This faint green coloration later acquires a more intense tone, visible in all treatments, matching the characteristics observed by Aguilar-Morales et al. [13].

The days to germination within each treatment were synchronous, but they were asynchronous among treatments considering the results show significant differences (Table 2).

In Treatment 1 (MS + Kin + IAA) the germination speed was lower compared to Treatments 2 (MS alone) and 3 (Water + sucrose). The MS medium added with growth regulators had the best effect on germination induction speed, significantly reducing the days to germination to 28. In Treatment 2, the average number of days to germination was 41. In comparison, in Treatment 3, germination was observed after 62 days besides, this phase did not occur in certain repetitions. The means for all treatments were different ($T_{\alpha=0.05}$ statistical significance).

The difference among the days to germination in the various treatments can be explained by two factors: 1) species is determinant in germination response and 2) the medium and the added organic supplements. Edy and Supcito [18] observed this behaviour in a recent analysis performed on the organic components of the asymbiotic orchid seed germination, finding that the reported days to germination oscillated between 14 and 90 days in 30 different species and different culture media formulations. Ávila-Díaz & Salgado-Garciglia [14] reported 30 and 45 days to germination in 9 species of Mexican orchids. Ávila-Díaz et al. [15] reported, for *Laelia speciosa*, 90 days to germination. Remarkably, the conditions used for this species allowed the germination time to be reduced by half. T3 showed the longest time (62 days) and T1 only 28 days, in contrast to more than 100 days reported by Cruz [17].

Table 2 Analysis of variance.

F.V.	G.L.	S.C.	C.M.	F
Among treatments	2	4360.62	2180.31	659.83**
Within treatments	23	76	3.30	
Total	25	4436.62		

$^{**}F_{t\ (\acute{\alpha}=0.05)} = 3.42$

Undoubtedly, the addition of growth regulators to the culture medium stimulates and accelerates germination and seedling development.

In other studies, they used MS medium containing 100% salts on induction of orchid germination Edy & Supcito [18]. Ávila-Díaz & Salgado-Garciglia [14] achieved the maximum germination percentage on MS medium at 100% concentration. On the other hand, Aguilar-Morales & López-Escamilla [12] observed that the best treatment for *Laelia* germination was MS (50%) medium. In the present investigation, the 30% concentration MS medium was used in Treatments 1 and 2, demonstrating that *E. vitellina* seeds, despite lacking endosperm and having an embryo with heterotrophic nutrition, do not demand excessive amounts of nutrients to stimulate their germination. Therefore, the MS salts concentration is sufficient, as it was possible to verify with the germination and the development of complete seedlings.

On the other hand, it is essential to mention that the induction of germination was also possible in Treatment 3, which consisted of a medium without MS salts but only sucrose. It is feasible because the embryo, once hydrated (first phase of germination), requires carbohydrates as an energy source to activate its cellular metabolism and initiate the second phase of germination, as described by Grajales [9]. Likewise, Velásquez [20] agrees with this explanation when he refers to the importance of carbohydrates during the change from protocorm to seedling. In concentrations of 30% DM, germination in culture media, even with only sucrose, reduced the initial *in vitro* propagation phase costs.

The seedlings had good development in all treatments and presented a profitable growth of up to 1

cm on average, a vigorous appearance, and a good root development 60 days after starting the experiment (Fig. 1). The seedlings were transferred to fresh medium without regulators to promote their growth and development.

This work will be used to initiate micropropagation experiments and standardize this protocol. Germplasm of this species will be available at the germplasm bank of the Biochemistry and Plant Physiology area of the FESC-UNAM for future studies of its morphogenetic behaviour under these conditions, as well as for genetic analysis and improvement. These actions will enable its conservation and make a strong case to establish a sustainable exploitation program.

4. Conclusion

The technique used to disinfect immature *E. vitellina* capsules effectively eliminated 100% contaminants in all treatments. One hundred percent seed germination of *Encyclia vitellina* was obtained. The time to germination was only 28 days in 30% MS medium supplemented with Kin-AIA, notably reducing the days of this phase. Moreover, this protocol yielded significant cost savings for the germination stage, using only a sucrose source and gelling agent.

Plants established *in vitro* will form part of the laboratory's germplasm bank. The *in vitro* material will lay the foundation for future biochemistry, physiology, and genetics studies of *E. vitellina*. At the same time, the information obtained in this research will contribute to developing programs for the propagation and conservation of this endangered species.

Fig. 1 ***Encyclia vitellina*** **seedlings, 60 days after sowing.**

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