Physiology / Fisiología

Asymbiotic germination, effect of plant growth regulators, and chitosan on the mass propagation of *Stanhopea hernandezii* (Orchidaceae)

Germinación asimbiótica, efecto de los reguladores de crecimiento vegetal y el quitosano en la propagación masiva de *Stanhopea hernandezii* (Orchidaceae)

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Abstract

Background: *Stanhopea hernandezii* was collected from natural habitat in Mexico for its beautiful fragrant flowers. Biotechnological strategies of propagation may satisfy the market demand and are useful for conservation programs.
Hypothesis: Vigorous seedlings of *S. hernandezii* can be produced *in vitro* by asymbiotic seed germination techniques and the addition of chitosan to the culture medium in the temporary immersion system (RITA®) and in semi-solid medium systems.
Methods: The first step was the *in vitro* germination of seeds obtained from a mature capsule of wild plants, followed by multiplication via adventitious protocorm induction known as protocorm-like bodies, using plant growth regulators. For this purpose, we utilized Murashige and Skoog (MS) basal medium amended with 0.5 mg/L α-Naphthaleneacetic acid, combined with different concentrations of 6-Benzylaminepurine (1, 3, and 5 mg/L). The following step comprised the growth and development of protocorms to obtain plantlets in RITA® flasks containing 250 mL of liquid MS medium combined or not with different chitosan concentrations (5, 10, 15, 20, and 25 mg/L).
Results: The results showed that media supplemented with 5, 10, and 15 mg/L chitosan concentrations enabled the obtaining of a larger biomass with a range of 40-48 seedlings/RITA® and an average height of 13 mm. The last step was the development from seedlings into plantlets, the latter being, vigorous and achieving up to 100 % survival after 12 weeks of *ex vitro* cultivation.
Conclusion: This paper describes an efficient process of asymbiotic germination and mass propagation of *S. hernandezii*, a vulnerable orchid species endemic to Mexico.
Keywords: Micropropagation, orchid, protocorm-like bodies, temporary immersion system.

Resumen

Antecedentes: *Stanhopea hernandezii* es colectada de su hábitat natural en México por sus bellas y fragantes flores. Las estrategias biotecnológicas de propagación pueden satisfacer las demandas del mercado y ser de utilidad en los programas de conservación.
Hipótesis: Plántulas vigorosas de *S. hernandezii* pueden producirse *in vitro* mediante la germinación asimbiótica de semillas y adición de quitosano al medio de cultivo en sistemas de inmersión temporal (RITA®) y medio semisólido.
Método: El primer paso fue la germinación *in vitro* de semillas obtenidas de cápsulas maduras procedentes de plantas silvestres, seguido por la multiplicación vía inducción de protocormos adventicios conocidos como cuerpos parecidos a protocormos, usando reguladores de crecimiento vegetal. Nosotros usamos medio basal Murashige y Skoog (MS) adicionado con 0.5 mg/L de ácido α-naftalenacético combinado con diferentes concentraciones de 6-bencilaminopurina (1, 3 y 5 mg/L). El siguiente paso fue el desarrollo y crecimiento de protocormos para la obtención de plántulas en contenedores RITA® con 250 mL de medio MS líquido combinado o no con 5, 10, 15, 20 y 25 mg/L de quitosano.
Resultados: El medio suplementado con quitosano a concentraciones de 5, 10 y 15 mg/L permitió obtener la mayor cantidad de biomasa, entre 40 a 48 plántulas por RITA® de 13 mm de altura promedio. Después de 12 semanas de cultivo *ex vitro* el desarrollo de las plántulas fue vigoroso y se alcanzó un 100 % de supervivencia.
Conclusión: Este artículo describe un proceso eficaz de germinación asimbiótica y propagación masiva de una especie vulnerable y endémica de México.
Palabras claves: Cuerpos parecidos a protocormos, micropropagación, orquídea, sistema de inmersión temporal.

Stanhopea hernandezii (Kunth) Schltr. (Orchidaceae) is a plant species endemic to Mexico, considered as threatened at the vulnerable level (Khoshbakht & Hammer 2007, Castillo-Pérez *et al.* 2019). The genus *Stanhopea* comprises 61 species distributed from Northern Mexico to Northwestern Argentina (Gerlach 2010, Zotz 2013). Fifteen species of this genus grow in Mexico, represent 24.6 % of the biological diversity of *Stanhopea* species (Soto *et al.* 2007). The flowers of several *Stanhopea* species have diverse forms, sizes, and colors, which makes renders them very attractive for orchid lovers, giving rise to their excessive overexploitation in their natural environments and large-scale illegal trade in local markets (Emeterio-Lara *et al.* 2016, Ticktin *et al.* 2020).

The use of modern biological tools for the trade and conservative purposes of wild species has been successfully applied to orchid species, mainly via asymbiotic seed germination, since seeds permit the preservation of genetic diversity and maintain a more variable gene pool than clonal propagation (Bembemcha *et al.* 2016). The seed germination process in orchids begins with the use of reserve substances and embryo swelling, followed by seed coat rupture, and protocorm (PC) protrusion (Yeung 2017). The PC is considered by some authors as the seedling of orchids, but it is also considered as the embryo proper by others. Batygina *et al.* (2003) discuss this concept widely and describe the whole embryogenesis process in Orchidaceae and the further development of the PC after seed germination as well. From the works of Batygina, we assume that the PC is the structure that protrudes from the seed envelope during seed germination, while embryo proper is present in the seed.

It is noteworthy that, depending on the species, orchids are able to form somatic embryos and polyembryony. Somatic embryos of orchids are also known as protocorm-like bodies (PLB), as denominated by Lee (2013). PLB can be induced from *in vitro*-cultured PC by the addition of plant growth regulators (PGR) to the culture medium, but, in some orchid species, they are also produced spontaneously during seed development when the capsule is still maturing (Yeung 2017). In this regard, it could be assumed that, during *in vitro* asymbiotic germination of orchid species, PC and PLB could be present simultaneously and could be indistinguishable structures.

At present, orchids are propagated by asymbiotic germination and the tissue culture of vegetative plant material. Both methods allow the production of a large number of disease-free plants and plants that are genetically identical in origin, thus providing useful tools for the *ex situ* conservation of plants with aesthetic, medicinal, or horticultural value that have become threatened at levels of being critically endangered, endangered, or vulnerable species (Kunakhonnuruk *et al.* 2018, Shen & Hsu 2018).

Temporary immersion systems (TIS), such as the RITA® (Récipient d'Immersion Temporaire Automatique) system, have been employed to produce or multiply several plants species. TIS are automated systems that resemble the characteristics of liquid medium cultures (Ramírez-Mosqueda & Iglesias-Andreu 2016). The principal advantages of this type of system include the overcoming of problems related to the traditional procedures of tissue culture, such as plant-tissue hyperhydricity, continued contact between the explant and the culture medium, reduced contamination by low manipulation, adequate oxygen transfer inside the culture vessel, and reduced costs (Pérez *et al.* 2013, Murthy *et al.* 2018, Leyva-Ovalle *et al.* 2020). The RITA® system has been widely employed for propagating of *Phalaenopsis* hybrids, *Vanilla planifolia*, and *Cattleya forbesii* (Ramos-Castellá *et al.* 2014, Spinoso-Castillo *et al.* 2017, Ekmekçigil *et al.* 2019).

The growth and development of the *in vitro* cultivated plants depends on factors such as macro- and microelement composition, organic elements, carbon sources, and plant growth regulators (Shahzad *et al.* 2017). The utilization of complex organic additives such as: coconut water, banana or potato extract, several fruit juices, and other additives in the culture medium, has enhanced the developmental responses of *in vitro* grown plantlets. Chitosan is a biopolymer composed of glucosamine and N-acetyl-glucosamine, with the deacetylated form of chitin present in the exoskeleton of crustaceans, insects, and in the cell wall of fungi and some algae (Philibert *et al.* 2017). Recently, several orchids have been tested with chitosan, revealing various morphogenetic and physiological effects, such as increased growth in young plants, increased seed germination, enhanced growth of PLB, accelerated development of meristematic tissue, generation of a maximal number of orchid plants, reduced plant transpiration, and early flowering (Kananont *et al.* 2010, Pornpienpakdee *et al.* 2010, Rahmah *et al.* 2015, Acemi 2020), due its effect as growth stimulator and its abilities to improve the biochemical and physiological attributes of the plants. Combining TIS with chitosan treatments could enhance the success in obtaining seedlings and plantlets with a higher capacity to acclimatize from the *in vitro* into the *ex vitro* culture.

The final step in a micropropagation protocol is the transfer of plant material from the *in vitro* culture to the *ex vitro* culture, the latter frequently considered as a bottleneck due to the high percentage of plants that are lost or damaged during acclimation to *ex vitro* conditions (Sherif *et al.* 2018, Shah *et al.* 2019). *In vitro* culture conditions generate plants with morphological, anatomical, and physiological alterations that make them susceptible to pathogen attack and render difficult their capacity to confront environmental conditions (Chandra *et al.* 2010). Yusof (2019) reported a

new role for thiamine as an activator of systemic acquired resistance (SAR) in several plant species. SAR enables the plant to successfully resist the attack of pathogenic fungi, bacteria, and viruses. In this respect, the use of a thiamine pre-treatment in the process of acclimatization could be useful.

The objectives of the present work were as follows: (1) document the *in vitro* asymbiotic seed-germination process and the morphological development of the PC and seedlings of *S. hernandezii*; (2) evaluate the effect of the plant growth regulator α-Naphthaleneacetic acid combined or not with different concentrations of 6-Benzylaminepurine on PC development and the induction of PLB, and (3) study the effect of chitosan at different concentrations on PC growth and development using two culture systems: RITA® and semisolid culture medium; (4) carry out the acclimatization of *S. hernandezii* plantlets, from *in vitro* to *ex vitro* culture, using a thiamine pre-treatment. This is, to our knowledge, the first paper that describes an efficient process of asymbiotic germination and the mass propagation of the vulnerable and attractive orchid species *S. hernandezii* endemic to Mexico.

Materials and methods

Seed source and sterilization procedure. Seeds were obtained from two naturally pollinated mature capsules collected in a forest of pine-oak trees located at an altitude of 1,926 m and 18° 57' 54'' N, 99° 00' 49.9'' W at Tlayacapán, Morelos State, Mexico. The voucher specimen (No. 20544) was deposited at the HUMO Herbarium, Universidad Autónoma del Estado de Morelos. The capsules were washed with liquid detergent and then rinsed in running tap water for 15 min. The cleaned capsules were surface-sterilized with 70 % (v/v) ethanol during 3 min. followed by three rinses in sterile distilled water. Subsequently, the capsules were dipped into a water solution containing 15 % sodium hypochlorite for 15 min, followed by three rinses in sterile distilled water. Then the capsules were opened by cutting them with a sterile scalpel, and the seeds were removed and transferred onto Petri dishes containing half-strength MS basal medium (Murashige & Skoog 1962) supplemented with 1 g/L activated charcoal, 2.5 g/L Phytagel® (Sigma-Aldrich Co., USA), and pH adjusted to 5.7 and autoclaved at 121 °C (1.05 kg/cm² pressure) during 20 min. The seeds were sown and evenly spread using a wire inoculation loop. All of these operations were undertaken under sterile conditions in a laminar airflow cabinet. Then, the Petri dishes containing the sown seeds were incubated at 25 ± 2 °C and at a16/8-h light/darkness cycle under cool fluorescent white light at a 30 μmol m⁻²s⁻¹ photon flow.

Asymbiotic seed germination and early plant development. Evaluation of the asymbiotic germination of *S. hernandezii* seeds was carried out in 10 of the previously sown Petri dishes. Each Petri dish was considered an experimental unit and was divided into 20 squares measuring 10 × 10 mm. The percentage of germinated seeds was recorded from 10 squares of each plate per day (time zero). Germination and seedling growth and development were scored on a scale of 0-9 (Table 1). The seeds were allowed to germinate and differentiate until they formed protocorms and early seedlings in the same half-strength MS basal medium with activated charcoal, and later until they achieved full development, which was scored weekly. From these Petri dishes, a part of the PC/PLB were taken and employed in other experiments. When early PC were formed, they were allowed to develop for 2 additional weeks in Petri dishes to stages 5-6 before being transferred into glass jars containing 50 mL of the same half-strength MS basal medium with activated charcoal. All of these were subcultured monthly until mature plantlets were observed after 5 months of culture. All of the developmental steps were observed and photographed with a stereomicroscope equipped with a camera (Nikon, SMZ 1500).

Effect of PGR on PC development and the obtaining of PLB. Four weeks from soaking, protocorms derived from germinated seeds approximately 4.0 mm in width, were transferred to different treatments containing full-strength MS basal medium amended with plant growth regulators (PGR) and without activated charcoal to induce seedling development and PLB formation. To accomplish this, we utilized 0.5 mg/L α-Naphthaleneacetic acid (NAA) combined or not with 6-Benzylaminepurine (BAP) at different concentrations: 0, 1, 3, and 5 mg/L. Each treatment was identified according to the PGR and their concentration; for example: the N0B0 treatment did not contain any PGR, the N0B1 treatment did not contain NAA but did contain 1.0 mg/L of BAP, and so on. For each treatment, 50 PC (4.0 mm in width) were planted and distributed in five 250-mL flasks containing 50 mL of the corresponding culture medium, with 10 PC per flask. To test whether the effect of time of exposure to PGR could play a role in the morphogenetic response, each treatment was incubated during three different times: 15, 30, and 45 days at 25 ± 2 °C under cool fluorescent white light at a 30 μmol m⁻²s⁻¹ photon flow and a 16/8-h light/darkness cycle. When each incubation time was finished, the plant material was transferred back into the full-strength MS basal medium without PGR and the effect of the exposure time for each treatment was recorded periodically every 15 days until cultures reached 120 days as the average number of PC surviving, PLB formation and the height of the plantlets formed.

Table 1. *Stanhopea hernandezii* developmental stages from seed to complete seedling.

Stage	Description
0	Un-germinated seed. Hyaline embryo, seed coat intact. (Not shown)
1	Seed imbibition: Embryo proper swells surrounded by seed coat.
2	Pre-germination: Late embryo, some rhizoids present.
3	Germination: Early protocorm with rhizoids, seed coat broken.
4	Primary protocorm: The shoot apical meristem is visible.
5	Medium protocorm: Increase in size, foliar primordia is present.
6	Late protocorm: Early leaf development.
7	Early seedling: Small leaves are present.
8	Primary seedling: Leaves and roots are present.
9	Complete seedling: Leaves, roots and pseudo-bulb are present.

PC development in semi-solid culture media and the chitosan effect. To evaluate the chitosan effect on PC development when these were cultivated in semi-solid medium, PC of an approximate diameter of 4.0 mm were transferred into full-strength MS basal medium amended with different concentrations of chitosan, including 0, 5, 10, 15, 20, and 25 mg/L. For each treatment, 0.4 g of PC per flask were planted in five 250-mL flasks containing 50 mL of full-strength MS basal medium with the chitosan concentrations mentioned previously. Then, the plant material was incubated under the same conditions: $25 \pm 2°C$ under cool fluorescent white light at a 30 µmol m^{-2}s^{-1} photon flow and a 16/8-h light/darkness cycle and subcultured at 30-day intervals in fresh medium. After 2 months of culture, number of plantlets, average height, and fresh weight were determined. Fresh weight of the total biomass was recorded and expressed as Relative Growth Rate (RGR) and calculated according to Sopalun *et al.* (2010) on the basis of the fresh weight of the cultured tissue as follows:

$$RGR\ (\%) = \left(\frac{Wf - Wi}{Wi}\right) \times 100$$

Where
Wf - Final weight
Wi - Initial weight

PC development on the RITA® system, and the chitosan effect. For the temporary immersion RITA® system (VITROPIC, Saint Mathieu-de-Tréviers, France), PC cultures were inoculated and grown in 250 mL of full strength MS basal liquid medium and immersed for 1 min. every 6 h. Several chitosan concentrations, including 0, 5, 10, 15, 20, and 25 mg/L, were prepared, and 2 g of PC approximately 4.0 mm in diameter were subjected to these chitosan conditions in three RITA® containers per each concentration. The airflow in each RITA® was sterilized by passage through 0.2 µm hydrophobic filters to permit that air pressure from an air compressor to push the medium into the RITA® container. The frequency and length of the immersion period was controlled with an electronic timer and a solenoid valve provided with an on/off operation. The plant material was incubated at 25 ± 2 °C under cool fluorescent white light at a 30 µmol m^{-2}s^{-1} photon flow and a 16/8-h light/darkness cycle and subcultured at 30-day intervals in fresh medium under the same conditions. After 2 months of culture, the number of seedlings and their height and fresh weight were determined and the RGR was calculated as mentioned previously.

Greenhouse acclimatization. An acclimatization procedure was established to enhance the survival rate from *in vitro* to *ex vitro* regenerated orchids. Prior to acclimatization, seedlings > 10 cm in height and well-rooted were removed from the media and washed carefully under running tap water to remove traces of media. Then the roots were dipped into a 0.5-g/L solution of thiamine during 20 min. and transferred onto $27 \times 53 \times 6$ cm trays filled with pine bark, pumice, and wood charcoal (3:1:1) or pine bark, coconut fiber, and peat moss (3:1:1). A control without thiamine treatment was performed for each chitosan treatment according to the number of seedlings obtained (approximately 10 % of the total number from each treatment, when possible). The seedlings were irrigated with tap water once every other day and fertilized with Peters® (N:P:K) at 8-day intervals. The seedlings were maintained for 8 weeks in a well-ventilated greenhouse with an approximate 200-µmol m^{-2}s^{-1} photon flow and at a relative humidity of 70 ± 10 %. The photoperiod during acclimatization was of approximately 12 h and the growth and survival rates of the plantlets were recorded.

Experimental design and data analysis. We performed an analysis of variance (ANOVA) to analyze the effect of PGR on growth and development. Also, an ANOVA analysis was conducted to ascertain the chitosan effect on RGR, the number of seedlings, and the length of the seedlings, for both the RITA® system and semisolid culture media system. To determine the statistical difference between treatments, a post-hoc Duncan test was performed. All statistical analyses were carried out with STATISTICA software (Stat Soft, Tulsa, OK, USA).

Results

Asymbiotic seed germination, early seedling development, and full seedling formation. Seed germination and seedling development was monitored during 20 weeks of *in vitro* culture on a scale from 0-9 ontogenetic steps (Table 1 and Figure 1). Phenology stages were recorded from stage 0 when the seeds were sown (around 0.5-1.0 mm in length) to the following stages: stage 1 (imbibition), during which the embryo proper swells, being surrounded by the seed coat being evident; stage 2 (pre-germination), the clear visual evidence that germination has been initiated takes the form of a marked increase in size (5-fold) and a color change from translucent white to pale green; stage 3 (germination), 3 weeks from sowing, seed germination was 97.53 %, PC

formed rhizoids on their surface, and the seed coat is broken. It is noteworthy that 1.03 % of the seeds were empty (without embryo) and that 1.45 % did not germinate. At stage 4 (primary PC), after 28 days, the color of the PC changed from pale green at the beginning of germination to bright green, and the young apical meristem became evident as a small protuberance; at stage 5 (medium PC), the PC increased in size as a result of cell division and enlargement; during stage 6 (late PC), the PC exhibited the emergence of first leaf primordia, observed between weeks 6 and 7; during stage 7 (early seedling), the form of the PC changed in appearance and size, the leaves were clearly developed; during stage 8 (primary seedling), the roots started to develop around week 8, the first roots arising from the base of the leaves. These seedlings that, when they are 10 weeks old and are 1-2 cm in height, reveal well-developed leaves and velamentous roots, and during stage 9 (seedling or full seedling), at the end of the week 20, each seedling had their leaves, roots, and a pseudo-bulb, and also, new leaves or pseudobulbs began to form from these seedlings.

Effect of PGR on PC growth and development. The results of the effect of the PGR on the growth and development of PC are presented in Table 2. It is noteworthy that there were no differences in the growth and development of PC nor in
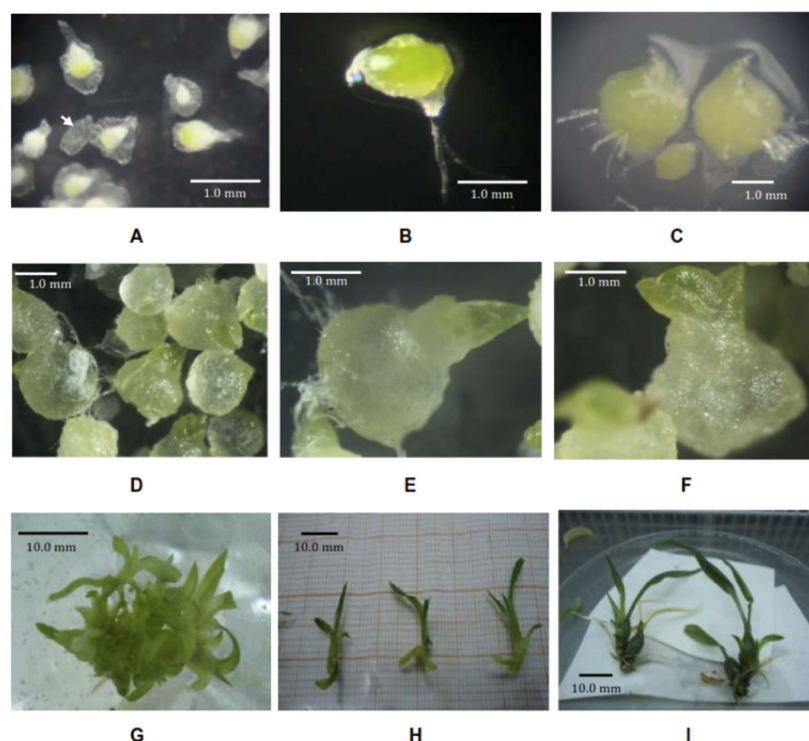

Figure 1. Stages of the process of asymbiotic seed germination in *Stanhopea hernandezii*: A) Seed imbibition, arrow showing an empty seed, B) Pre-germination, embryo swells C) Germination, seed coat broken D) Primary protocorm (PC), apical meristem visible (E) Medium PC, foliar primordia present (F) Late PC, early leaf development (G) Early seedling, small leaves present (H) Primary seedling, leaves and roots present (I) Seedling, pseudo-bulb present.

Table 2. Effect of PGR on PC survival, seedling developed from PC and growth registered as height of seedlings.

Treatments	NAA (mg/L)	BAP (mg/L)	*PC surviving after 45 days in PGR induction: (% ± SD)	*Seedlings from PC after 120 days on culture: (% ± SD)	**Height of seedlings after 120 days: cm (Mean ± SD)
N0B0	0.0	0.0	3.0 ± 2.94	2.0 ± 1.97	5.07 ± 0.02[b]
N0B1	0.0	1.0	6.0 ± 5.76	6.0 ± 5.76	4.98 ± 0.04[b]
N0B3	0.0	3.0	6.0 ± 5.76	6.0 ± 5.76	5.15 ± 0.02[b]
N0B5	0.0	5.0	6.0 ± 5.76	6.0 ± 5.76	5.73 ± 0.22[a]
N05B0	0.5	0.0	12.0 ±11.04	3.0 ± 2.94	5.61 ± 0.02[a]
N05B1	0.5	1.0	7.0 ± 6.67	9.0 ± 8.46	4.50 ± 0.03[c]
N05B3	0.5	3.0	2.0 ± 1.97	5.0 ± 4.83	4.10 ± 0.03[c]
N05B5	0.5	5.0	6.0 ± 5.76	2.0 ± 1.97	4.74 ± 0.02[b]

*Mean of data obtained from protocorm (PC) cultured 45 days on plant growth regulators (PGR), seedlings formed from PC at 120 days on culture and **height of the seedlings formed. There were no statistical differences between treatments of PGR on PC surviving and seedlings formed ($F_{(7, 16)} = 0.68$, $P = 0.5769$). There are significant differences regarding height ** ($F_{(7, 16)} = 126.4$, $P < 0.0001$).

the induction of PLB formation with regard to incubation time, *i.e.*, 15, 30, and 45 days in PGR; in this regard, we only present the data corresponding to 45 days of incubation time, since the main differences were observed with regard to the PGR concentration. Also important is that the majority of the treatments, without and with different concentrations and combinations of PGR, exerted a harmful effect on the growth and development of the PC and on their capacity to induce the formation of PLB. In this respect, we evaluated survival rather than PLB formation. The higher values of each column are noted. There were no significant differences among all of the PGR treatments in terms of the number of PC surviving at 45 days and in the number of the seedlings formed by such PC at 120 days in culture. Nonetheless, there were differences in growth and development, registered as the height of seedlings derived from PC.

Chitosan effect on PC development in semisolid media culture and RITA® systems. Results on the effect of chitosan at different concentrations on the growth and development of PC under both conditions, that is, the semi-solid medium culture and the RITA® systems, are depicted in the Tables 3 and 4, respectively. When RGR was evaluated for the semisolid culture system, the highest value was obtained with 5.0 mg/L chitosan (1,319.0 ± 63.4), with statistically significant differences among several of the chitosan treatments. Regarding the number of seedlings obtained, the highest value was 2.6 ± 1.3 in 10 mg/L chitosan, and the highest value of seedlings length was 17.06 ± 2.5 with 5.0 mg/L chitosan (Table 3). On the other hand, when RGR was evaluated for the RITA® system, the highest RGR was obtained with 15.0 mg/L chitosan (1,920.1 ± 494.7); however, statistically significant differences among all of the chitosan treatments were not observed. Regarding the

total number of seedlings developed in each chitosan treatment, the best result was observed using 10 mg/L of chitosan with 48.0 ± 23 seedlings and in the evaluation of growth and development measured as the length of the seedlings; the highest values was 13.5 ± 0.9 with 15 mg/L chitosan.

Greenhouse acclimatization. Around 100 % of the seedlings survived the acclimatization process and there were no differences between thiamine-treated and the non-treated control, nor among the substrates used (Figure 2). The seedlings demonstrated no sign of desiccation under constant humidity for 8 weeks. The regenerated plants exhibited normal growth and did not exhibit any noticeable sign of abnormal phenotypic variation that might compromise further culture in the greenhouse.

Discussion

Asymbiotic seed germination, early seedling development, and complete seedling formation. During the first 3 weeks, we realized that the germination process was asynchronic; in this regard, determination of each stage was performed when we observed that the predominant form corresponded to the described stage. Polyembryony was not observed during the germination process, but the spontaneous formation of somatic embryos (PLB) could take place in the same medium used for germination, in such a way that we could have secondary protocorms, as described by Batygina *et al.* (2003) by the time at which we considered the germination process complete. All of the processes from seed to complete seedling were registered following the steps observed under the microscope and describing each step according to the observed structures (Table 1), as in a previous study of Yamazaki & Miyoshi (2006).

Table 3. Effect of chitosan on the growth and development of protocorms under the semi-solid media system.

Culture system	Chitosan (mg/L)	*Relative growth rate (% ± SE)	*Number of seedlings (Mean ± SE)	*Length of seedlings: cm (Mean ± SE)
Semisolid system	0.00	1,126.0 ± 86.10[b]	2.4 ± 0.8[a]	12.10 ± 1.0[b]
	5.00	1,319.0 ± 63.40[a]	2.0 ± 0.7[a]	17.06 ± 2.5[a]
	10.00	837.5 ± 12.60[c]	2.6 ± 1.3[a]	10.36 ± 0.4[c]
	15.00	596.0 ± 97.30[d]	2.2 ± 0.7[a]	12.82 ± 1.3[b]
	20.00	296.5 ± 29.50[e]	2.0 ± 0.2[b]	9.00 ± 0.0[d]
	25.00	285.0 ± 34.10[e]	2.2 ± 1.5[a]	12.88 ± 1.0[b]

Different letters within a column indicate significant differences.

Effect of PGR on PC growth and development and PLB induction. Regarding the effect of PGR on PC growth and development and PLB induction, we found that the effect of both NAA and BAP at the concentrations used was harmful because, instead of inducing PLB formation, we observed that the majority of the PC employed in this experiment died during the time of induction or later during the next step in the medium without PGR. The main effect of the majority of treatments on PC development was phenolization, which induced browning and death. It is probable that not only the addition of PGR, but also the manipulation of the PC, their age, the use of full-strength instead half-strength MS basal medium, and the lack of activated charcoal in the culture medium. Caused this unwanted effect, because the treatment without PGR also demonstrated this adverse effect. Contrary to our results, Sopalun *et al*. (2010) obtained 100 % survival of the PLB of *Grammatophyllum speciosum* growing in half-strength MS medium without PGR. The treatment with the fewest adverse effects for the survival of PC at 45 induction days was N05B0, but N05B1 was less harmful 75 days later, at day120 after the beginning of the experiment. The best treatment for PC growth and development was N0B5 with an induction time of 45 days and 75 days in medium without PGR; this treatment revealed the greatest height of the seedlings formed. There were no significant differences among treatments in terms of PC survival; however, we found statistically significant differences in growth and development evaluated as the height of the seedlings after 120 days in culture.

Chitosan effect on PLB development in semisolid media culture and RITA® systems. With regard to the effect of different concentrations of chitosan on biomass increase, when the semi-solid media culture system was utilized, at low concentration of chitosan, the RGR increased and decreased as chitosan concentration is increased (Table 3). These observations are in good agreement with the "hormesis effect", which could be interpreted as a trend of induction in low concentration and inhibition in high concentration (Xia *et al*. 2019)

The effect of the different concentrations of chitosan on biomass increase and the growth and development of PC on the RITA® culture system revealed positive effects in biomass increase, the number of seedlings formed, and on the growth and development of PC as compared with the control without chitosan, as can be observed in Table 4. Highest RGR were induced from 5.0-20.0 mg/L of chitosan; however, there was high variability in these measurements and there was no statistically significant difference among all of the treatments. Also, no particular statistical trend was observed in the number of plants and their length. Increasing the concentration of chitosan from 20 to 25 mg/L resulted in a decrease in the RGR and a lower amount in the number of seedlings obtained. Previous studies of the chitosan effect on *in vitro* cultured PC in the orchid genus *Dendrobium* demonstrated that the addition of higher doses of > 20 mg/L chitosan resulted in negative growth effects (Nge *et al*. 2006, Kananont *et al*. 2010, Pornpienpakdee *et al*. 2010).

Regarding the effect of chitosan at different concentrations on RGR, number of seedlings, and length of seedlings of PC cultivated in the semi-solid culture system, it was harmful in the majority of the treatments. The higher increase of biomass evaluated by RGR was obtained with the 5.0-mg/L concentration of chitosan (1,319.0 ± 63.40), but the number of developed seedlings was lower than the number obtained with 10-mg/L chitosan (2.6 ± 1.3) and of the control without chitosan (2.4 ± 0.8). The statistical analysis revealed that there were significant differences among different chitosan treatments.

Although both the RITA® system and the semisolid culture system were not statistically comparable in terms of their differences regarding the amount of PC and the number of experimental units employed, the RITA system permitted us to obtain a higher number of seedlings; for example, when the number of seedlings is compared in both systems with 5.0 mg/L chitosan, the RITA system produced between 41.2 ± 17.5 seedlings, while the semisolid system produced 2.0 ± 0.7 seedlings.

Figure 2. General appearance of plants of *Stanhopea hernandezii*. A) Micropropagated plant prior to acclimation, B) Growth and development of plantlets after two months in the greenhouse.

Greenhouse acclimatization. Around 100 % of the plantlets survived the acclimatization process and exhibited no sign of desiccation under constant humidity for 8 weeks, nor later when humidity was reduced for up to 12 weeks. This result could be due to the combined action of chitosan treatments and the thiamine pre-treatment during the acclimatization process: the transfer from *in vitro* to *ex vitro* culture. Another possibility could be that to the thiamine pre-treatment did not induce a significant effect on the acclimatization process, since the control without thiamine pre-treatment did not reveal any difference with regard to thiamine-treated seedlings. In this regard, it appears that the effect could be mostly due to chitosan. All of these plantlets demonstrated a very healthy appearance in the greenhouse on both of the substrates utilized (Figure 2) and exhibited normal growth without any noticeable sign of abnormal phenotypic variation that might compromise further culture in the greenhouse.

In the present study, we showed that *S. hernandezii* seedlings can be produced *in vitro* by asymbiotic seed-germination techniques, the addition of chitosan to the culture medium, and RITA® and semi-solid medium systems. Combining the use of chitosan and RITA® could be more advantageous on to orchids propagation for inducing PC growth, and seedling development. The use of PGR could be harmful and further assays should be conducted to test their effect under different conditions. Resistance to further acclimatization under *ex vitro* conditions appears to be good, combining, as it does, chitosan with TIS. Further work will be necessary to improve the development of PC and the induction of somatic embryos (PLB) for the mass propagation of this orchid species. In this regard, changes to this protocol could include modification of strength of the culture medium, the concentration and nature of the PGR used, as well as the age of the explant, and the addition of activated charcoal. This report describes an efficient and simple procedure for seed germination and PC development that could be employed for large-scale propagation. This system may contribute to alleviate the diminishing population of *Stanhopea hernandezii*.

Table 4. Effect of chitosan on the growth and development of protocorms under the RITA® system.

Culture system	Chitosan (mg/L)	*Relative growth rate (% ± SE)	*Number of seedlings (Mean ± SE)	*Length of seedlings: cm (Mean ± SE)
RITA® System	0.00	1,106.5 ± 208.00[a]	44.4 ± 17.6[a]	11.02 ± 0.34[b]
	5.00	1,797.0 ± 384.90[a]	41.2 ± 17.5[a]	13.4 ± 2.20[b]
	10.00	1,878.1 ± 568.20[a]	48.0 ± 23.6[a]	13.2 ± 0.50[b]
	15.00	1,920.1 ± 494.70[a]	40.4 ± 14.2[a]	13.5 ± 0.90[b]
	20.00	1,860.5 ± 430.80[a]	18.0 ± 7.8[a]	12.8 ± 1.40[b]
	25.00	1,310.7 ± 289.50[a]	19.8 ± 9.1[a]	15.4 ± 1.00[a]

*Mean of data obtained. Different letters within a column indicate significant differences ($F_{(5, 6)} = 5.17$, $P < 0.05$).

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